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Neuroprotective effects of poly (ADP-ribose) polymerase inhibitors in transient focal cerebral ischemia of rats[1]

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KEY WORDS cerebral ischemia; reperfusion injury; NAD^+ ADP-ribosyltransferase; 3-aminobenzamide; niacinamide; $NAD(P)^+$-arginine ADP-ribosyltransferase; phytonadione

AIM: To explore the role of poly (ADP-ribose) polymerase (PARP) in focal cerebral ischemia with reperfusion injury. **METHODS**: Male Wistar rats underwent 3.5-h of temporary middle cerebral artery occlusion by intraluminal suture. Infarction volume was showed with 2, 3, 5-triphenyltetrazolium chloride (TTC) staining and quantitated by image analysis system, neurologic scores were determined with a 0 - 5 grading scale. **RESULTS**: 3-Aminobenzamide (3-AB) 10 mg · kg^{-1} or nicotinamide (Nic) at 20 mg·kg^{-1} showed potent neuroprotective effects within 0 - 6 h, neurologic deficits were attenuated. With the increasing dose of PARP inhibitors, beneficial effects were compromised, particularly, administration of Nic 60 mg · kg^{-1} at the onset of reperfusion drastically accelerated brain damage. Phytomenadione, a selective inhibitor of mono(ADP-ribosyl) transferase, had little effect on infarction volume. **CONCLUSION**: Transient incomplete inhibition of PARP provides a neuroprotective effects against cerebral ischemia-reperfusion injury, with a relatively wide therapeutic window, whereas severe inhibition of this enzyme, especially in reperfusion phase, is detrimental.

Poly(ADP-ribose) polymerase (PARP, EC2.4.2.30) is an abundant nuclear enzyme that catalyzes the transfer of ADP-ribose moiety of NAD^+ to form a polymer of ADP-ribose chain on nuclear acceptor proteins such as histone, topoisomerase, and predominantly on itself. PARP can influence cell survival in a number of ways. Unmodified PARP bind tightly to DNA breaks, once activated by extensive DNA damage, auto-poly(ADP-ribosyl)ation of protein causes its release and allows access for DNA repair enzymes[1], thus may play a role in maintenance of DNA integrity. On the other hand, excessive PARP activation following DNA damage under many oxidative stress conditions causes rapid depletion of NAD, ATP, and thiol pools, which could lead to metabolic cell death. The recent discovery of proteolytic cleavage of PARP by caspase indicates that it could be involved in cell apoptosis[2].

In vitro system, inhibition of PARP had various effects on different cell types. PARP inhibitors had been showed to reduce cell death caused by oxidative stress in lymphocytes, endothelial cells, fibroblasts, PC12 cell, and intestinal epithelial cell line[3], but not in hepatocytes or Chinese hamster ovary cells[4]. In isolated heart and skeletal muscle, inhibition of PARP reduced ischemia-reperfusion injury[5]. PARP activation had also been implicated as a mediator of the neurotoxicity of nitric oxide (NO) in a neuron culture system[6]. As excessive production of NO has been demonstrated to be an important mechanism of brain injury following ischemic stroke, the above studies raise the possibility of developing PARP inhibitors as neuroprotective agents. In this study, we explored the roles of PARP activity in ischemic brain injury *in vivo*.

MATERIALS AND METHODS

Focal cerebral ischemia model Adult ♂ Wistar rats (clean grade, certificate № 02-22-2) weighing 287 ± *s* 21 g were anesthetized with 8 % chloral hydrate 350 mg · kg^{-1} ip. During and after surgical procedures, room temperature was controlled under 25 - 28 ℃, and rat body temperature was maintained at 36.5 - 37.5 ℃ with a heating lamp during anesthesia. The left common carotid artery (CCA) was exposed, two branches of the left external carotid artery (ECA), the terminal lingual artery and maxillary artery, were transacted. The extracranial branch of internal carotid artery (ICA), the

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pterygopalatine artery, was ligated close to its origin with 4 – 0 silk suture. A 4 – 0 nylon suture of 4 cm in length with a slightly enlarged and rounded tip was immersed in heparin solution (6000 kU·L^{-1}), then placed at room temperature until dry. The treated suture was introduced into the ECA after temporarily clipping CCA and ICA, and the silk suture around the ECA stump was tightened to prevent bleeding. The nylon suture was advanced from ECA into ICA until slight resistance was felt. The distance from the bifurcation of CCA to the tip of suture was about 22 mm. Pilot postmortem study indicated that the tip of suture reached the proximal segment of the anterior cerebral artery (ACA), thus blocked the origin of middle cerebral artery (MCA), with occluding all sources of blood flow from ICA, ACA, and posterior cerebral artery. After 3.5 h of MCA occlusion, the suture was slowly withdrawn for reperfusion.

Neurologic deficit evaluation The neurologic deficit status of each rat was evaluated at 24 h after surgery by observers blinded to the treatments. Grade 0, no observable neurologic deficit; Grade 1, failure to extend the left forepaw fully; Grade 2, intermittent circling; Grade 3, sustained circling without moving forward; Grade 4, unable to walk spontaneously with a depressed level of consciousness (usually indicates diffuse cortical infarction existed); Grade 5, death.

Morphometric measurement of infarction volume Forty-eight hours after ischemia, rats were decapitated, and their brain were removed and washed with saline, the reperfusion of MCA was visually confirmed. Coronal sections of 2 mm thick were dissected with a brain slicer. Then, the slices were immersed in saline containing 2 % 2,3,5-triphenyltetrazolium chloride (TTC) and kept at 37 ℃ in the dark for 30 min. The stained slices were fixed with 10 % formalin and photographed. The infarction area of each slice was traced and calculated using an image analysis system (T-90), with both direct and indirect measurements. Indirect methods followed a formula to minimize the influence of edema: right hemispheric area – noninfarcted area of left hemisphere ipsilateral to ischemia. Total infarction volume for each brain was calculated by summation of infarction areas of the subsequent slices (total area × thickness).

Experimental protocol Each rat was randomly assigned to one of the following groups: Group 1, ischemia control treated with saline; Group 2 and 3, treated with ip 3-aminobenzamide (3-AB) 10 mg·kg^{-1}, 30 mg·kg^{-1} respectively, just before ischemia; Group 4 and 5, treated with ip nicotinamide 20 and 60 mg·kg^{-1} respectively, just before ischemia. In the second part of experiment, 3-AB 10 mg·kg^{-1} was injected 3, 6, and 12 h after ischemia. In the third part of experiment, cerebral ischemia rats were treated with 3-AB 30 mg·kg^{-1}, nicotinamide 60 mg·kg^{-1} or phytomenadione 20 mg·kg^{-1}, just after reperfusion.

Chemicals 3-AB was purchased from Sigma, and dissolved in saline 15 min before use. Nicotinamide was the product of Shanghai 2nd Reagent Factory. TTC was the product of E Merck. All these agents were protected from light. Phytomenadione was the product of Shanghai 1st Pharmaceutical Factory.

Statistics ANOVA was used to test the differences between control and PARP inhibitor treatment groups. For differences between PARP inhibitors injected during ischemia or reperfusion phase, t-test was used. The ratios of positive TTC staining between groups was analyzed by exact probabilities.

RESULTS

Effects of PARP inhibitors on focal cerebral ischemia All control rats after 3.5-h ischemia with reperfusion developed visible infarction under TTC-staining. The majority had a striatal infarction, and 2 rats also had a cortical infarction. Most of rats in this group exhibited circling (Tab 1).

Tab 1. Neurologic outcome in rats 24 h after middle cerebral artery occlusion (3.5 h) and reperfusion in control, 3-aminobenzamide (3-AB, ip) or nicotinamide (Nic, ip) groups.
$^{a}P > 0.05$, $^{b}P < 0.05$, $^{c}P < 0.01$ *vs* control.

Dose/ mg·kg^{-1}	Grade of neurologic deficit 0	1	2	3	4	5	Rats	Score $\bar{x} \pm s$
Control		1	6	4	1		12	2.4±0.8
3-AB 10	1	3	5	1			10	1.6±0.8^{b}
30			1	3	2		6	3.2±0.8^{a}
Nic 20		4	4	1			9	1.7±0.7^{b}
60				2	3	1	6	3.8±0.8^{c}

After treatment with 3-AB 10 mg·kg^{-1}, the infarction volumes were drastically reduced, 4/10 rats in this group had no visible infarct, and the areas at risk had a pink TTC staining, suggesting that selective neuronal death replaced the full infarction (Tab 2).

In more mild ischemia model (ischemia for 3 h), the neuroprotective effects of 3-AB were more prominent. Treatment with 3-AB 30 mg·kg^{-1} showed a trend of aggravating infarction. Similarly, treatment with Nic 20 mg·kg^{-1} showed a neuroprotective effect, whereas 60 mg·kg^{-1} showed detrimental effects, worsened the neurologic deficit and enlarged the infarction volume, even death in 1 rat.

Therapeutic window of single dose of 3-AB When 3-AB 10 mg·kg^{-1} was injected 3 h after MCA occlusion, similar neuroprotection was found, 2/7 rats

Tab 2. Effects of 3-AB, ip and Nic, ip on infarction volumes of TTC-staining brain slices. Rats underwent 3.5 h of temporary middle cerebral artery occlusion followed with reperfusion, the beginning of ischemia was used as a reference point for injection time.
$n = 5-12$, $\bar{x} \pm s$.
[a] $P > 0.05$, [b] $P < 0.05$, [c] $P < 0.01$ *vs* control.

Dose/ mg·kg^{-1}	Injection time/h	Infarction volume/mm^3	Ratio of visible infarction
Control	0	68 ± 27	12/12
3-AB 10	0	25 ± 18[c]	6/10[b]
10	3	20 ± 12[c]	5/7[a]
10	6	43 ± 20[a]	5/5[a]
10	12	59 ± 18[a]	5/5[a]
30	0	108 ± 32[b]	6/6[a]
Nic 20	0	31 ± 19[c]	6/9[b]
60	0	136 ± 44[c]	6/6[a]

showed no mature infarction; when given 6 h after MCA occlusion, neuroprotection was reduced in some rats, but still significant in some other rats. 3-AB given 12 h after ischemia showed no neuroprotection (Tab 2).

High dose of nicotinamide drastically accelerated the brain damage following reperfusion Injection of Nic 60 mg·kg^{-1} (ip) at the beginning of reperfusion was extremely detrimental, rats usually exhibited hemiplegia and depressed consciousness 1 – 2 h after Nic treatment. 4/7 rats died within 12 h. Besides striatal infarction, diffuse "mature" infarction in ipsilateral cortex occurred early as revealed by immediate postmortem TTC staining. The infarction volume of this group was 217 ± 69 mm^3 ($n = 7$, $P < 0.01$). Injection of 3-AB 30 mg·kg^{-1} at the beginning of reperfusion also enlarged the infarction volume (166 ± 33 mm^3, $n = 5$, $P < 0.01$), but to less degree as compared with Nic.

No effect on infarction volume by selective inhibitor of mono(ADP-ribosyl) transferase 3-AB and Nic inhibited mono(ADP-ribosyl) transferase nonspecifically, particularly over 20 mg·kg^{-1}. Phytomenadione, a selective inhibitor of mono(ADP-ribosyl) transferase, was injected (20 mg·kg^{-1}, ip) just after reperfusion, the infarction volume is 60 ± 16 mm^3 ($n = 6$, $P > 0.05$). Thus, phytomenadione had little effect on the infarction volume. Neurologic examination showed the results consistent with morphometric measurement.

DISCUSSION

Our results suggest that PARP inhibitors, only at low dose, had neuroprotective effects. On the contrary, at relatively higher doses, they enlarge or accelerate ischemia-reperfusion brain injury. The latter detrimental effect was not due to the cytotoxicity of the drug, as Nic at the same high dose did not cause brain infarction in the normal subjects. Thus, minimal PARP activity is required for brain to limit ischemia-reperfusion injury, and the therapeutic index of PARP inhibitors for cerebral ischemia might be quite low.

PARP can influence cell survival in different ways, both enhancement of survival or mediation of cell death had been reported in cell cultures or isolated organs, and its physiological role is still a subject of much debate. Early DNA damage following cerebral ischemia-reperfusion was found[7,8], then was expected to cause excessive activation of PARP, the latter can rapidly deplete energy stores. Transient inhibition of PARP showed potent neuroprotective effects, which can be compromised by multiple consecutive doses at intervals of 3 h. 3-AB 10 mg·kg^{-1} is estimated to produce a plasmic concentration of about 1 mmol·L^{-1}. On the other hand, 3-AB only have low penetration of blood brain barrier in normal subjects, and its brain concentration is about 1/10 – 1/30 of peripheral tissues[9], although its permeability might be enhanced in ischemia-reperfusion brain. So, the estimated effective concentration of 3-AB in this *in vivo* study is close to or below that in neuron culture system (100 μmol·L^{-1})[6]. This concentration may cause incomplete inhibition of augmented PARP activity in ischemic brain tissue, as 3-AB has an IC_{50} of 33 μmol·L^{-1} for PARP in fibroblasts[10]. Our stroke model of 3.5 h ischemia only showed moderate brain injury, with infarction volumes much smaller than those reported in literature, indicating that MCA occlusion by suture technique is incomplete, the model under our conditions might be more close to "penumbra" model, thus allows neuroprotective agents to show greater effects.

Two chemically distinct inhibitors of PARP reduce ischemic brain injury at low dose, suggesting that the

neuroprotective effects observed are mainly due to inhibition of PARP, and *in vivo* activation of PARP contributes to neuronal damage following transient cerebral ischemia, though we have not measured *in vivo* PARP activity directly. In addition, possible stimulation of DNA repair by low dose 3-AB might be another mechanism of neuroprotection. High dose of PARP inhibitors lose beneficial effects, even aggravated ischemic brain injury, whereas phytomenadione, a selective inhibitor of mono (ADP-ribosyl) transferase[10], had little effect on infarction volume, suggesting that the detrimental effect of PARP inhibitors at high dose might be related to excessive inhibition of PARP activity, but not due to their nonspecific inhibition of mono (ADP-ribosyl) transferase. Thus, even small residual activity of PARP is important for neuronal recovery from reperfusion insult. On the other hand, above results also imply that even excellent selective PARP inhibitor developed in future seem unable to devoid of this unwanted shortcoming. Combination of very low dose of PARP inhibitor with other neuroprotective agents (such as free radical scavengers) will become an alternative strategy. In consistent with our *in vivo* findings, *in vitro* study showed that 3-AB (1 mmol·L^{-1}) could cause a rapid death of HL-60 cells in serum-free culture[11], and another potent PARP inhibitor 1, 5-dihydroxyisoquinoline at high concentration (1 mmol·L^{-1}) prevented the recovery of C3H10T1/2 cells from oxidative stress[12]. Nic at high dose is more detrimental than 3-AB, which can be explained by additional effects for an elevation of intracellular NAD level and aggravated oxidative injury by Nic[13]. It was reported that different basal NAD levels determine beneficial or detrimental effects of PARP inhibitors on H_2O_2-induced cell death[14]. It is interesting to see whether PARP activity is involved in the neuroprotection of neurotrophins expressed following focal cerebral ischemia.

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多聚 ADP-核糖多聚酶抑制剂对大鼠暂时性局灶脑缺血具有神经保护作用[1]

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关键词 脑缺血；再灌注损伤；多聚 ADP-核糖多聚酶；3-氨基苯甲酰胺；尼克酰胺；单 ADP-核糖酰转移酶；维生素 K_1 抑制剂

目的：研究多聚 ADP-核糖多聚酶（PARP）在局灶性脑缺血再灌注损伤中的作用. **方法**：雄性 Wistar 大鼠用插丝法阻塞大脑中动脉 3.5 h 后再灌注，梗塞灶用 TTC 染色显示，图象分析测量；神经功能缺损采用 0－5 级评分. **结果**：低剂量 PARP 抑制剂 3-氨基苯甲酰胺（10 mg·kg^{-1}）或尼克酰胺（20 mg·kg^{-1}）具有明显的神经保护作用，治疗窗近 6 h；高剂量反而加重脑损伤，特别是尼克酰胺在再灌注起始给药. 选择性单 ADP-核糖酰转移酶抑制剂对脑梗塞无明显作用. **结论**：暂时非完全性抑制 PARP 对脑缺血再灌注损伤产生神经保护作用，然而完全抑制该酶（尤其是在再灌注期）则产生损害作用.

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Enhancing effects of β-endorphin on glutamate neurotoxicity[1]

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KEY WORDS beta-endorphin; endorphins; calcium; sodium glutamate; arcuate nucleus

AIM: To study the effects of β-endorphin (β-End) on monosodium glutamate (MSG)-induced neurotoxicity (GNT). **METHODS**: Image analysis of neuronal areas and determination of mitochondrial membrane protein-bound Ca^{2+} and intracellular free Ca^{2+} ($[Ca^{2+}]_i$) were used. **RESULTS**: β-End aggravated MSG-induced neuronal injury in arcuate nucleus of hypothalamus in a dose-dependent manner in the range from 0.5 to 5.0 mg·kg^{-1}. MSG-induced increase in mitochondrial membrane protein-bound Ca^{2+} was enhanced when treated with β-End 2 g·L^{-1}. MSG-induced elevation in $[Ca^{2+}]_i$ in single neuron was also augmented from 320 ± 84 to 589 ± 78 nmol·L^{-1} by the treatment with β-End 2 g·L^{-1}. **CONCLUSION**: β-End enhanced GNT via aggravating the disruption of intracellular Ca^{2+} homeostasis induced by MSG.

Excessive activation of glutamate receptors results in neuronal injury and death, associated with acute brain injury and chronic neurodegenerative disorders, eg, hypoxic-ischemic injury, eclamptic seizures, Alzheimer's disease, and Huntington's diseases[1,2]. Our previous work showed that morphine enhanced the glutamate neurotoxicity (GNT)[3,4], suggested that morphine-like substances might produce neuronal damage in CNS via enhancing GNT.

β-Endorphin (β-End) exerts a wide spectrum of actions, such as analgesic effect and stress-induced reproductive dysfunction[5]. The purpose of this study was to investigate whether β-End might has the same effect as morphine on GNT.

MATERIALS AND METHODS

Morphological observation and image analysis of neuronal areas Neonatal mice ($n = 64$) of either sex of 7－9 d, weighing 5.8 ± 0.5 g were divided into 8 groups at random. The mice in each group were daily injected sc with saline, MSG (1.0 g·kg^{-1}) or MSG + β-End (0.5, 1.5, 2.0, 3.0, 5.0 mg·kg^{-1}) for 7 d. β-End was injected 15 min after MSG. Mice were killed 1 wk after the final treatment and 6 μm thick serial coronal sections in hypothalamus were stained with cresyl violet[3]. Morphological changes of neurons were photographed, and neuronal areas of arcuate nucleus (AN) were measured by 540-Biological Medicine Color Image Analyser.

Detection of mitochondrial membrane protein-bound Ca^{2+} The 0.2 mm thick brain slices of mice[6] were

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