Opposite effects of the gap junction blocker octanol on focal cerebral ischemia occluded for different durations

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Abstract. Protectants and executioners have been demonstrated to be used by gap junctions in focal cerebral ischemia. Certain researchers hypothesized that the opposite role of gap junctions may be associated with the injury extent, which has been demonstrated to be highly correlated with occlusion duration. In order to examine this hypothesis directly, the effects of octanol, a frequently used drug, were examined to investigate the role of gap junctions, in rats following middle cerebral artery occlusion (MCAO) for 30 min/2 h and 24 h reperfusion, respectively. Octanol significantly reduced the infarct volume following 2 h of occlusion concomitant with lower neurological deficits, whereas it enlarged the infarct volume following 30 min of occlusion. Consistently, octanol attenuated the number of transferase dUTP nick-end labeling (TUNEL) positive neurons in the hippocampal CA1 region following 2 h of occlusion, while opposite effects were observed for 30 min of occlusion. Further immunohistochemical studies demonstrated that the expression of B-cell leukemia-2 (Bcl-2, anti-apoptotic protein) was upregulated and that Bcl-2-associated X (Bax, proapoptotic protein) was downregulated following 2 h of occlusion in the octanol group compared with the ischemic group. Conversely, octanol downregulated the expression of the Bcl-2 protein concomitant with increased Bax protein following 30 min of occlusion. These results indicated that the gap junction blocker octanol can protect against ischemic injury following long-term occlusion, however, can aggravate ischemic injury following short-term occlusion.

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Abbreviations: MCAO, middle cerebral artery occlusion; OGD, oxygen-glucose deprivation; TUNEL, transferase dUTP nick-end labeling; IR, immunoreactive; Bcl-2, B-cell leukemia-2; Bax, Bcl-2-associated X

Key words: gap junctions, focal cerebral ischemia, octanol, occlusion time, astrocyte

Introduction

Gap junctions connect the interior of cells and are important in the maintenance of tissue homeostasis. Certain deleterious metabolites involved in cerebral ischemia and apoptotic associated molecules, including Ca^{2+}, IP_3, ATP and cAMP, may be small enough to pass through gap junctions and modulate cell death of the neighboring cells, enhancing the spreading of injury (1,2). Alternatively, the network of gap junctions could prevent the death of injured cells by the buffering of toxic metabolites to healthy surrounding cells (3). Thus, gap junctions could have damaging and protective effects (4).

There is increasing evidence that suppressing gap junctions is able to reduce the spread of damage following ischemia (5). For example, the gap junction blocker octanol restricted the flow of undesirable neurotoxins and significantly decreased the spread of cell death (6). Similarly, connexin 43 (the predominant connexin in gap junctions) heterozygous mice demonstrated reduced shrinkage and metabolite abnormality in the ipsilateral hippocampus following middle cerebral artery occlusion (MCAO) (7). Lin *et al* demonstrated *in vitro* that the resistance of C6 cells to calcium overload and oxidative stress was compromised when they formed gap junctions with more vulnerable cells (8). Despite the detrimental role of gap junctions, it is noteworthy that gap junctions may mediate survival or be protective for adjacent cells. In mixed astrocyte neuron cultures, the inhibition of astrocyte coupling with gap junction blockers increased neuronal vulnerability to oxidative stress or glutamic acid toxicity (9,10). Consistently, connexin 43 overexpression substantially suppressed zinc (released during ischemia) toxicity whereas its knockdown caused a significant enhancement of the toxicity (11).

Certain investigators proposed that the opposite role of gap junctions may be associated with the extent of neuronal death (12), which was highly correlated with the duration of ischemia (13,14). Therefore, the present study examined whether the opposite role of gap junctions was associated with occlusion time. In order to examine this issue, a gap junction

blocker octanol was used, which has been administered to investigate the role of gap junctions in numerous studies (6,15). The effects of octanol in MCAO was evaluated using neurological deficits, infarct volume and transferase dUTP nick-end labeling (TUNEL) staining. To further investigate the mechanism of the opposite roles of octanol, the expression of Bcl-2 and Bax was studied, which play opposite roles in apoptosis (16). The present study provides evidence that may be used to further elucidate the opposite role of gap junctions in ischemia.

Materials and methods

Animal model of cerebral ischemia. Male adult Sprague-Dawley rats weighing 250-280 g were maintained in a temperature- and light-controlled environment with a 12 h light/dark cycle. All experiments were performed in accordance with the NIH guidelines and approval by the Animal Care and Use Committee of the Guangzhou University of Chinese Medicine (Guangzhou, Guangdong, China) and all efforts were made to minimize animal suffering.

A focal cerebral ischemic rat model was induced by MCAO as previously described (17). In brief, the rats were anesthetized with 350 mg/kg of chloral hydrate via intraperitoneal injection. The right carotid bifurcation was exposed and the external carotid artery was coagulated distal to the bifurcation. A 4-0 nylon monofilament suture with a rounded tip was introduced into the internal carotid artery through the stump of the external carotid artery and gently advanced for ~20 mm. Following 30 min/2 h of occlusion, the filament was gently withdrawn and the incision was closed (18). During the whole process, the rat rectal temperature was maintained at 37˚C by placing the animals on a heating bed. In the sham-operated rats, the common carotid was exposed without ligation.

Drug treatment. Octanol purchased from Sigma (St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; 0.005% v/v) and a final concentration of 5 mmol/kg was administered intraperitoneally 30 min prior to ischemia (6). The vehicle-treated rats were administered equal volumes of DMSO.

Measurement of neurological deficits. An independent observer performed the assessment of the neurological deficit score: 0, no neurological deficit; 1, failure to fully extend left forepaw; 2, circling to the left; 3, falling to the left; 4, loss of spontaneous walking with a depressed level of consciousness and 5, dead (17).

Measurement of infarct volume. Following reperfusion for 24 h, rats were overdosed with sodium pentobarbital (100 mg/kg). The brains were quickly removed and cut into coronal slices of 2 mm in thickness. The sections were immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Mym Biological Technology, Andhra Pradesh, India) for 20 min at 37˚C and then fixed with 4% paraformaldehyde. The infarct area was measured by each slice using a computerized image analysis system (Image-Pro-Plus; Media Cybernetics, Silver Spring, MD, USA). Infarct areas of all sections were added to derive the total infarct area, which was multiplied by the thickness. The infarct volume was expressed as a percentage of the ipsilateral hemispheric volume (%).

Tissue preparation and TUNEL assay. Rats were deeply anesthetized with sodium pentobarbital and perfused through the ascending aorta with cold saline, followed by 4% paraformaldehyde in 0.1 M of phosphate-buffered saline (PBS; pH 7.2-7.4, 4˚C). The brains were cytoprotected by 15 and 30% sucrose sequentially. Coronal sections were cut on a freezing microtome at a thickness of 15 μm starting at +1.60 mm to -4.80 mm from the bregma and then were mounted onto polylysine-coated slides. Intervening sections were air dried, then stored at -80˚C until future use. TUNEL staining was processed using an *in situ* cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Images were captured using an Olympus microscope (Olympus, Tokyo, Japan) and the number of TUNEL-positive neurons within the CA1 subfield was counted.

Immunohistochemistry. Brain sections (15 μm thickness) were mounted with 2% goat serum in 0.1 M of Tris buffered saline (TBS)/0.3% Triton X-100 for 1 h at room temperature and incubated overnight at 4˚C with the primary antibody for Bcl-2/Bax (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following washing in TBS, sections were incubated with biotinylated goat anti-rabbit IgG and further processed with the ABC method (Vector, San Diego, CA, USA). The stained sections were captured with a CCD spot camera. Non-specific staining was determined by omitting the primary antibodies.

To quantify Bcl-2 and Bax immunoreactivity (IR), the measurement was performed with a computerized image analysis system (Image-Pro-Plus, Media Cybernetics) according to the previously described method (19). Briefly, a density threshold was set above the background level firstly to identify a positively stained structure and the area occupied by these structures was measured as positive area. An average percentage of Bcl-2-IR or Bax-IR area to the total outlined area was obtained. Five to six animals were included in each group for the quantification of immunohistochemistry.

Quantification and statistical analysis. Observers blinded to the experimental conditions, evaluated the outcome measures. All data are presented as the mean ± SEM. The Student's t-test was used to compare the difference between the vehicle group and the octanol group. All analysis was performed using the Statistical Package for Social Sciences (SPSS, version 13.0 for Windows). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Neurological deficits. The effect of octanol was evaluated by the Zea Longa test. As shown in Fig. 1, octanol significantly lowered the neurological deficits compared with the vehicle group following 2 h of ischemia and 24 h of reperfusion ($P=0.028$). As for the 30 min ischemia group, although the mean of the neurological deficit scores was higher in the octanol group, the difference between the two groups was not statistically significant ($P=0.181$).

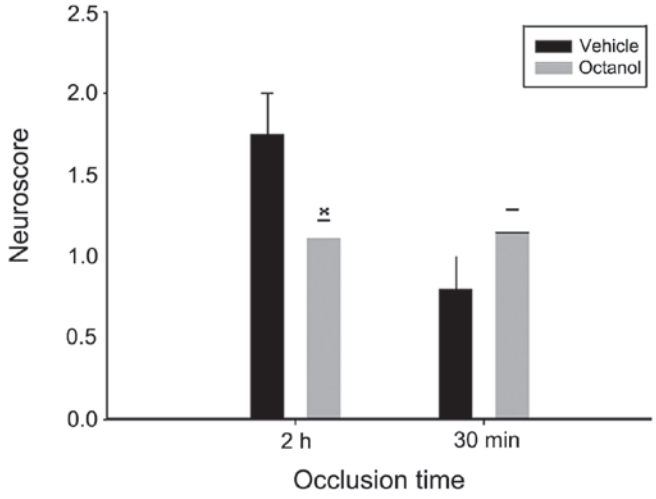

Figure 1. Evaluation of neurological impairment following treatment. Octanol pretreatment significantly decreased the neurological deficit score in ischemia for 2 h. *P<0.05 vs. the vehicle group for 2 h.

Infarct volume evaluation. Representative images of TTC staining were shown in Fig. 2. The infarct volume following 2 h of occlusion was reduced by octanol pretreatment compared with the vehicle group (P=0.001). Conversely, a larger stroke volume was observed in the octanol group following 30 min of occlusion compared with that in the vehicle group (P=0.025).

TUNEL assay. A moderate number of markedly TUNEL-labeled CA1 pyramidal neurons were identified in the CA1 region in the vehicle group following ischemia for 2 h and reperfusion for 24 h. However, the number of TUNEL-positive neurons in the CA1 region of rats pretreated with octanol was markedly decreased (t=7.812, P<0.001; Fig. 3). Notably, in the 30 min ischemia group, octanol significantly increased the number of TUNEL-positive cells compared with the vehicle group (t=4.595, P=0.002; Fig. 3).

Expression of Bcl-2 and Bax. Apoptosis contributes to delayed neuronal death causing expansion of the stroke lesion, therefore we investigated the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax expression. In the 2 h ischemia group, compared with the vehicle group, octanol significantly increased the expression of Bcl-2 (t=6.151, P<0.001), however decreased the expression of Bax (t=2.768, P=0.016; Figs. 4 and 5). However, in the 30 min ischemia group, the rats treated with octanol demonstrated that, accompanied by a decrease in Bcl-2 expression (t=2.907, P=0.012), Bax expression was markedly increased (t=4.149, P=0.001; Figs. 4 and 5).

Discussion

The present study demonstrated and compared for the first time, to the best of our knowledge, the effect of the gap junction blocker octanol in MCAO for 2 h and 30 min, respectively. Compared with the vehicle-treated rats, octanol attenuated the ischemia injury induced by 2 h of occlusion demonstrated by the neurological deficit, infarct volume and TUNEL staining, whereas it aggravated the ischemia injury induced by 30 min of occlusion, indicating that octanol was protective following 2 h of occlusion but harmful following 30 min of occlusion. Octanol, an eight-carbon aliphatic alcohol, is a relatively specific inhibitor of gap junction permeability and is frequently used to study the role of gap junctions. Although certain studies have demonstrated that octanol can interfere with synaptic transmission (20), it is hard to interpret these two opposite effects with gap junction-independent mechanisms. The opposite role of octanol appears to be closely associated with the function of gap junctions (21). For example, gap junctions could be neuroprotective since adjacent healthy cells may act as an effective spatial buffer against the extracellular accumulation of neurotoxic substances (22,23). By contrast, they could also be harmful when neighboring cells are unable to aid in the clearance of the neurotoxic substances, which could depolarize a large number of neurons/glia promoting the release of glutamate and therefore causing aggravated injury (24).

Gap junctions have been revealed to be protective in the model of hypoxic preconditioning (subthreshold insults) (25), MCAO above and below the rhinal fissure (26-29) and MCAO for 30 min (30). By contrast, previous investigations also demonstrated that gap junctions were harmful in the model of four vessels occlusion (31), clamping the common carotid arteries and lowering the mean arterial blood pressure to 40 mmHg (6), MCAO for 16 h (32) and oxygen-glucose deprivation (OGD) for 6 to 10 days (33). As the intensity and/or the duration of the ischemic episode increases, the predominant function of gap junctions may be different. For example, when the buffering effect is stronger than the harmful factors' propagation in the condition that injury is mild, gap junctions may be neuroprotective; otherwise gap junctions may be harmful. In the present study, the infarct volume following 30 min of occlusion was only 6.4%, thus gap junctions were efficient in buffering harmful factors. By contrast, 2 h of occlusion induced a much larger infarct volume, therefore gap junctions could not buffer harmful factors efficiently and more neurotoxic substances propagated to neighbouring cells aggravating the injury instead.

Notably, while octanol significantly increased the infarct volume and cell death following 30 min of occlusion, no statistical differences in neurological deficits were identified between the octanol group and the vehicle group. Similarly, Longa *et al* demonstrated that the infarct area following 2 h of temporary MCAO were 15.7% smaller than that following permanent MCAO, however the neurological deficit was not significantly reduced (17). In light of these findings, the neurological score was not closely associated with the infarct volume as occasionally the changes in infarct volume may not be enough to create differences in the neurological test.

To explore the possible mechanisms, we detected Bcl-2 and Bax immunoreactivity, respectively, which are two opposite apoptotic factors. Bcl-2 can help neuronal survival and protect brain tissue from ischemic injury (34,35). It acted upstream to prevent the activation of caspases, inhibited free radical formation and regulated calcium sequestration (36). However, Bax inhibition reduced apoptotic neuronal injury in the hippocampal CA1 region and behavioral deficits following global ischemia (37,38). In the present study, we

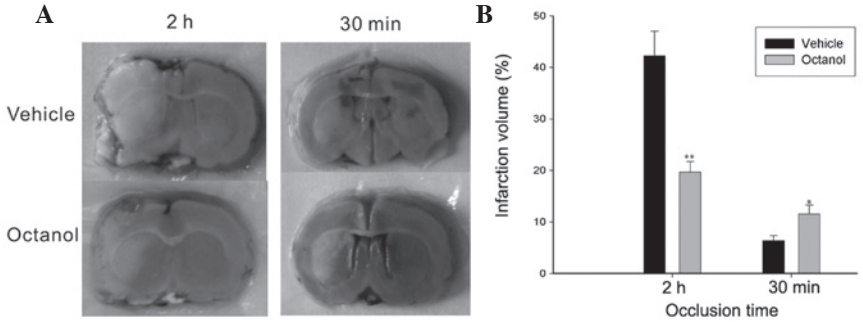

Figure 2. Effect of octanol on infarct volume following ischemia for 2 h/30 min and 24 h reperfusion. (A) Infarct volumes of the octanol- and vehicle-treated groups. (B) Changes between the vehicle group and the octanol group were compared using the percentage of infarct area of the ipsilateral hemisphere. **P<0.01, vs. the vehicle group for 2 h; *P<0.05 vs. the vehicle group for 30 min.

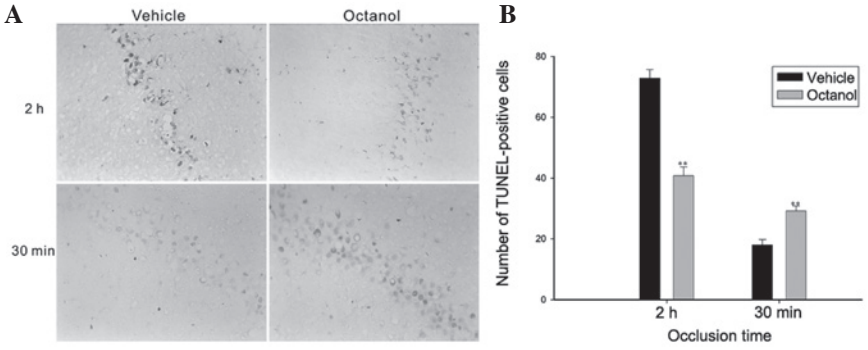

Figure 3. Effect of octanol on DNA fragmentation in CA1 neurons. (A) In the 2 h ischemia group, octanol decreased TUNEL- positive cells; however, in the 30 min ischemia group, octanol increased TUNEL- positive cells (magnification, x400). (B) Statistical results are shown. **P<0.01, respectively vs. the vehicle group. TUNEL, transferase dUTP nick-end labeling.

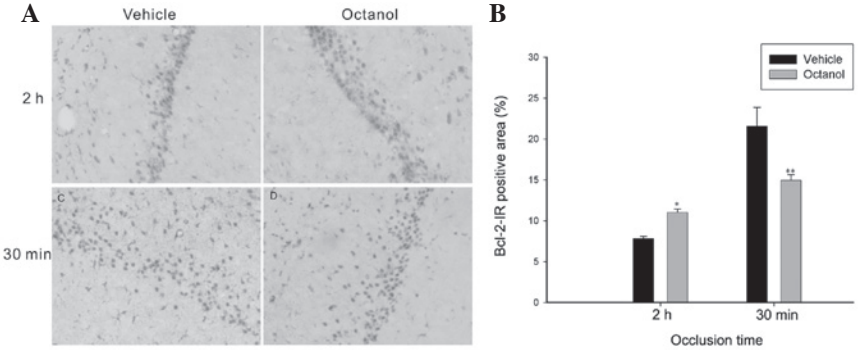

Figure 4. Expression of anti-apoptotic Bcl-2 in different groups. (A) Expression of Bcl-2 in the CA1 area following ischemia for 2 h/30 min and 24 h reperfusion (magnification, x400). (B) There were significant differences between the vehicle group and the octanol group, respectively. *P<0.05, vs. the vehicle group for 2 h; **P<0.01 vs. the vehicle group for 30 min. Bcl-2, B-cell leukemia-2.

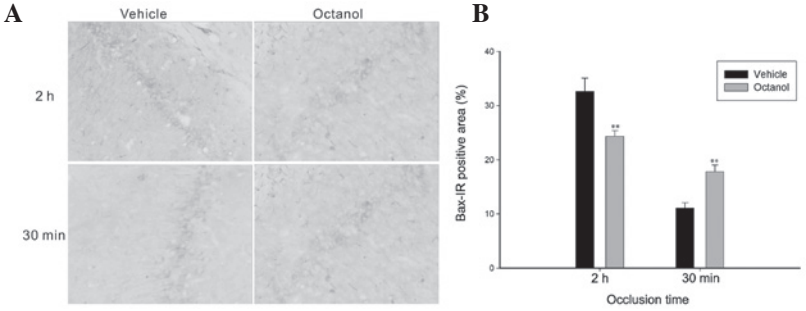

Figure 5. Expression of pro-apoptotic Bax in different groups. (A) Expression of Bax in the CA1 area following ischemia for 2 h/30 min and 24 h reperfusion (magnification, x400). (B) There were significant differences between the vehicle group and the octanol group, respectively. **P<0.01 respectively vs. the vehicle group. Bax, Bcl-2-associated X.

revealed that octanol induced Bcl-2 expression and attenuated Bax expression in MCAO for 2 h. By contrast, it inhibited Bcl-2 expression and increased Bax staining in MCAO for 30 min. These results demonstrated that the protective role of octanol in ischemia for 2 h may be associated with its ability to prevent apoptosis via facilitation of the anti-apoptotic factor Bcl-2 or inhibition of the apoptotic biochemical factor Bax. Conversly, the decreased Bcl-2 and increased Bax contributed to apoptosis, thus resulting in a harmful role of octanol in ischemia for 30 min. We are not completely certain that the opposite roles of octanol on Bcl-2 and Bax is directly mediated by gap junction activity. However, the consistency between ischemia injury and the corresponding Bcl-2/Bax expression in ischemia for different durations, to a certain extent, verifies that the present results are mainly attributed to the effect of octanol on gap junctions.

In summary, the present study investigated and compared the effects of octanol in MCAO for the first time, to the best of our knowledge. Our results demonstrated that the gap junction blocker octanol played opposite roles in MCAO for different durations, which was demonstrated by the neurological outcome, infarct size and TUNEL staining. In addition, the function of octanol was at least in part mediated through the counter-regulation of Bcl-2 and Bax. These data provide a novel perspective for the role of gap junctions in cerebral ischemia and may be beneficial for the potential clinical treatment of various ischemic conditions. However, more studies are required to examine this, including using other blockers of gap junctions or connexin 43 deficient mice.

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