Research report

Reduction of brain injury by antithrombotic agent acutobin after middle cerebral artery ischemia/reperfusion in the hyperglycemic rat

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Abstract

In vivo magnetic resonance imaging (MRI) was used to observe the effect of acutobin, a purified thrombin-like enzyme (TLE), isolated from the snake venom of \textit{Deinagkistrodon acutus}, on MRI-detected brain lesion volume and tissue perfusion deficit in a hyperglycemic rat right middle cerebral artery occlusion/reperfusion (MCAO/R) model. Acutobin (0.75 U/ml) was intravenously injected with a dosage of 2.5 U/kg body weight 30 min after MCAO (MCAO duration=60 min) and again 24 h after reperfusion. Multislice diffusion weighted imaging (DWI) and single-slice dynamic bolus tracking gradient echo (GE) imaging were sequentially acquired before and after MCAO/R. DWI-detected lesion volume was significantly ($p<0.05$) reduced by 24–31% from 350\textsuperscript{F}45, 369\textsuperscript{F}45 and 374\textsuperscript{F}36 mm\textsuperscript{3} in the saline-treated group to 239\textsuperscript{F}17, 282\textsuperscript{F}26 and 259\textsuperscript{F}32 mm\textsuperscript{3} at 3, 4 and 24 h after reperfusion in the acutobin-treated group, respectively. Residual cerebral blood flow (CBF) in the right hemisphere recovered and remained at ~80% of normal perfusion over the measurement period in the acutobin-treated group, compared to ~40% in the saline-treated group. Mortality at 1 week after MCAO/R in the acutobin-treated group was significantly lower (25% mortality) than the saline control group (85% mortality). Our results indicate that acutobin improves brain tissue perfusion and reduces infarct volume and mortality in the hyperglycemic rat MCAO/R model.

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Keywords: Cerebral ischemia; Acutobin; Thrombolytic; Defibrinogenating agent; Magnetic resonance imaging; Hyperglycemia

1. Introduction

It has been widely recognized that pre-existing hyperglycemia can lead to an exacerbation of post-ischemic brain tissue injury \cite{8,40,53,63}. Following a mismatch between the regional cerebral blood flow (CBF) and regional cerebral metabolic demands, a series of pathophysiologic processes occur at the cellular level. Animal studies have shown that hyperglycemia exaggerates the following damaging processes: intracellular acidosis \cite{52}, accumulation of extracellular glutamate \cite{58}, free radical formation \cite{59}, brain edema formation \cite{49}, blood brain barrier disruption and tendency of hemorrhagic transformation \cite{21}. One hallmark of hyperglycemic exacerbation of post-ischemic brain tissue is a significant reduction in perfusion of the tissue once reflow is established to the tissue \cite{18,37,39,49}. The degree to which compromised tissue perfusion contributes to hyperglycemic exacerbation of post-ischemic brain tissue injury is debatable \cite{21,27}. In this study we examine whether an experimental antithrombotic therapy can improve microcirculatory perfusion in hyperglycemic post-ischemic brain, thereby sparing brain tissue from necrosis.
Acutobin (MW 26,000 Da), a purified thrombin-like enzyme (TLE) isolated from the snake venom of *Deinagkistrodon acutus* [29] (similar to ancrod from the venom of Malayan pit viper [13]), shows defibrinogenating activity and has been investigated as an anticoagulant for various cerebrovascular disorders [65,66]. The most important pharmacologic action in both ancrod and acutobin is hydrolysis of the arginine16–glycine17 peptide bond of fibrinogen, thereby releasing “A” class fibrinopeptides (FpA, AY, AP) but not B-fibrinopeptides [5,19,33]. What remains is a fibrin-like soluble aggregate that is unable to undergo covalent cross-linking. Because the plasma fibrinogen level correlates with whole-blood viscosity, defibrinogenating agents, such as ancrod and acutobin, might improve the microcirculation around the edges of a focal area of cerebral ischemia by improving blood rheology.

In the present study, we monitor the extent of focal cerebral ischemia and the degree of brain tissue edema formation using magnetic resonance imaging (MRI) techniques, which have proven to be powerful tools in the characterization of cerebral ischemia models in our previous studies [18,48,49,58,59]. Diffusion weighted imaging (DWI) is useful for identifying regions with significant increase in intracellular water fraction and/or cytotoxicity [26,35,36]. Plasma volume imaging (PVI), carried out using intravascular paramagnetic and superparamagnetic tracers, detected by dynamic bolus tracking gradient echo (GE) [56] and spin echo (SE) MRI methods [23], document the perfusion deficit in the ischemic region. In the present study, we test the hypothesis that antithrombotic therapy with acutobin decreases infarct size and perfusion deficit in a hyperglycemic rat right middle cerebral artery occlusion/reperfusion (MCAO/R) model.

2. Materials and methods

2.1. Animal model

All animal procedures were approved by the University of Texas Medical Branch Animal Care and Use Committee. Male Sprague–Dawley rats (300–330 g body weight) were initially anesthetized with 3% isoflurane in balanced breathing air (30% O2/70% N2), then intubated and mechanically ventilated with isoflurane maintained at 1.0–1.5% during the surgery and MRI procedures. The femoral artery was ventilated with isoflurane maintained at 1.0–1.5% during the period of baseline, MCAO and 1 h into reperfusion. Blood glucose concentration was measured during the period of baseline, MCAO and 1 h into reperfusion. MCAO was induced using the intraluminal suture insertion method with some modifications [18,32,38]. Following an oblique incision on the right ventral lateral side of the neck, the internal carotid artery (ICA) at the base of the common carotid artery bifurcation and the pterygo- palatine artery (which is the only branch of the extracranial ICA) were ligated. An incision was made in the ICA and a 15-mm length of 3–0 monofilament nylon suture coated with a thin layer of silicone was introduced into the lumen of the ICA and passed through to the origin of the ipsilateral MCA thereby blocking the blood supply to it. Reperfusion was performed at 60 min after MCAO by the removal of the suture. Animals were divided into two experimental groups: (1) hyperglycemic rats treated with acutobin (n=8). Acutobin (0.75 U/ml, Sanming Pharmaceutical Factory, Fujian, P.R. of China) was intravenously injected with a dosage of 2.5 U/kg body weight at 30 min into MCAO and 24 h after reperfusion. (2) Hyperglycemic rats treated with saline (n=7); this group received 0.6-ml saline only. All animals were allowed to recover from anesthesia after MRI procedures (~4.5 h after reperfusion) and returned to their cages. They were re-anesthetized for further MRI study at 24 h and/or later (5–7 days) after MCAO/R. Because of the high rate of death in the saline-treated hyperglycemic group and unexpected technical problems of the MRI scanner, the MRI data at 24 h or 1 week was not acquired completely in all cases.

2.2. Magnetic resonance imaging

Proton MRI was performed using a 4.7-T, 330-mm horizontal bore system (Varian, USA). Excitation and signal detection were achieved with a 50-mm surface coil. MRI was acquired before and 30 min after MCAO/R and repeated every 60 min up to 4.5 h and again at 24 h and 5–7 days. The protocol consisted of (1) multislice DWI, (2) T2-weighted spin-echo imaging (T2WI), (3) PVI, (4) single-slice dynamic bolus tracking GE imaging. DWI, T2WI and PVI were acquired as 12 contiguous coronal slices covering a 20-mm length from the cerebellum to the olfactory lobe using the following parameters: field of view (FOV)=60×60 mm, matrix=256×256, repetition time (TR)=3.0 s, TE=65 ms, slice thickness=1.6 mm and 128 phase encode steps. For the single-slice dynamic MRI in the area of the caudate putamen, a T2* sensitive low-flip angle (FLASH) pulse sequence [15] with FOV=60×60 mm was used. Each of the 40 frames was acquired with 64 phase encode steps, a TE of 3.0 ms, a TR of 8 ms and one acquisition per phase encode step. A bolus of 0.50 mmol/kg body weight gadopentate-dimeglumine (Magnevist®, Berlex Lab. Wayne, NJ 07470, USA) was injected followed by a 0.6-ml saline flush into...
the femoral vein after the sixth frame. Magnevist® was administered to all animals at baseline, 1 h into MCAO, 5 min, 1 h, 2 h and 3 h after reperfusion. The PVI was acquired only at 4 h, 24 h and 1 week after MCAO/R, and required the T2 shortening effect of a superparamagnetic iron oxide (SPIO) nondiffusible intravascular tracer (Advanced Magnetics, Cambridge, MA 02138, USA). The bolus of SPIO (2.0 mg Fe/kg body weight) was the same as the method of Magnevist® injection.

2.3. Data analysis

Ischemic lesions, estimated from DWI, were calculated based on intensity threshold segmentation using image analysis software developed in the Medical Imaging Laboratory at UTMB [46]. Regions of restricted water diffusion measured in DWI were considered to represent cytotoxic edema [25,35,55], while regions of prolonged T2 relaxation time in T2WI represented vasogenic edema [25]. The lesion area in each slice was multiplied by the slice thickness and summed to yield the total lesion volume in cubic millimeters. This technique, using threshold values, was used in previous studies in our laboratory [18,46,48,49,58,59].

The region of the no-reflow zone was estimated in a similar fashion from the signal-void region in PVI, which represented the area where no detectable intravascular tracer had perfused into the tissue [22,23,47,49]. Parametric images were constructed based on a pixel-by-pixel calculation of hemodynamic parameters. Time–concentration curves representing the passage of tracer were constructed based on the observed signal intensity changes during the dynamic GE acquisition.

Single-slice digital image analysis was performed using indicator dilution methods [2,49,67] to estimate CBF from cerebral blood volume (CBV) and mean transit time (MTT). A region of interest (ROI) analysis of relative flow indices was applied to the corresponding DWI and T2WI at the level of caudate putamen (one slice per animal at sequential time points) in six regions per hemisphere (Fig. 1) [49]. Referring to stereotaxic coordinates [44], the six regions correspond to (1) cingulate, frontal, hindlimb and forelimb cortices (FCC), (2) parietal cortex (PC), (3) piriform and insular cortex (PIC), (4) preoptic area (PA), (5) caudate putamen and septal nuclei (CP), and (6) globus pallidus and basal forebrain area including bed nucleus (GP). (A) T2WI, (B) DWI, (C) CBV, (D) six ROI.

3. Results

The physiological variables are shown in Table 1. There were no significant differences in physiological variables (blood glucose concentration, pH, PCO2, PO2 and rectal temperature) between the two experimental groups. Seven of the eight acutobin-treated rats survived 24 h and six of the eight survived 1 week after MCAO/R. In contrast, three of
the seven saline-treated rats survived for 24 h and only one rat lasted the full week protocol.

Fig. 2 shows a representative coronal DWI series for the two experimental groups. The area of DWI hyperintensity, indicative of cytotoxic edema, increased from 20% of the total hemisphere before reperfusion to about 62% at 24 h after reperfusion in the saline-treated group. In contrast, the area of DWI hyperintensity increased from 10% of the total hemisphere before reperfusion to 43% at 24 h after reperfusion in the acutobin-treated rats. The hemispheric DWI-detected lesion volume was also significantly (p<0.05) reduced by 24–31% from 350±46, 370±46 and 374±37 mm³ in the saline-treated group to 240±17, 282±27 and 260±33 mm³ at 3, 4 and 24 h after reperfusion in the acutobin-treated group, respectively. The time course of changes in DWI measured ischemic lesion size during the first 6 h period (2 h MCAO followed by 4 h reperfusion) are summarized in Fig. 3. The DWI measured lesion volume at 24 h after MCAO/R also showed a decreased area of hyperintensity in the acutobin-treated group (259±33 mm³) compared to the saline-treated group (374±37 mm³). Our previous studies [18,48] demonstrated that the result of volumetric assessments of high signal intensity in DWI measured within 24 h after MCAO/R accurately depicts the area of acute infarction, as confirmed by the 2,3,5-triphenyltetrazolium chloride (TTC) stain which delineates infarcted tissue [3]. We also note that low intensity spots within the DWI and T₂WI area of hyperintensity appeared during the later reperfusion phase in the saline-treated hyperglycemic rats. This suggests the formation of microthrombi or microhemorrhage in the MCA territory. The
volume of ischemic damage detected in the T₂WI was also significantly reduced ($p<0.05$) in the acutobin-treated group 24 h after MCAO/R ($242 \pm 28 \text{ mm}^3$) compared to the saline-treated group ($346 \pm 33 \text{ mm}^3$).

Fig. 3 shows the time course of changes in DWI lesion size (A) and residual CBF (B) in the right (ischemic) hemisphere. There was no difference in the residual CBF between acutobin- and saline-treated groups during the period of baseline and MCAO (49.6 ± 12.5% of normal in acutobin-treated rats and 52.4 ± 12.8% of normal in saline-treated rats). Following release of the MCAO, right hemispheric CBF recovered and remained at ~80% of normal perfusion over the measurement period in the acutobin-treated group. In contrast, recovered tissue reperfusion started to decline at 3 h after reperfusion onset in saline cohorts to a level of ~40% of pre-ischemia. Fig. 4 shows a representative series of calculated (bolus tracking) CBF images at the level of the caudate putamen from the two groups.

Table 2 shows the perfusion levels in 6 ROI within the ischemic hemisphere. During the MCAO period, prior to acutobin administration, ipsilateral CBF dropped in four of the six brain areas measured. Areas 1 (FCC) and 6 (GP), which are in the dorsomedial areas of this slice, did not show significant decline in CBF during MCAO. Upon reperfusion, all brain areas initially showed increased perfusion. Acutobin-treated rats showed higher residual CBF during the reperfusion period in areas 1 (FCC), 2 (PC) and 4 (PA) ROIs, which are generally considered as ischemic penumbra. Reduced CBF in area 1 (FCC) was not significant during MCAO in either group compared to the baseline. CBF remained normal in the acutobin-treated rats but declined gradually in saline-treated rats during the reperfusion period. In area 3 (PIC), significantly higher residual CBF was recorded after reperfusion in the acutobin-treated group compared to saline controls, although residual CBF did not reach baseline levels in either group.

Fig. 5 shows the T₂WI and relative PVI from a representative rat in each of the two experimental groups, acquired at 4 and 24 h after MCAO/R. In PVI images, signal intensity is proportional to the circulating blood volume, therefore, areas with a perfusion deficit are represented by low signal intensity. Hyperglycemic rats treated with saline show very low intensity in the right hemisphere by PVI, indicating an extensive no-flow zone. In contrast, there is a relative hyper- and normal-intensity area of PVI in the right hemisphere (except in the ischemic core) in the acutobin-treated group. This confirms improved tissue reperfusion through out most of the previously ischemic tissue, including the ischemic penumbra. Likewise, development of cerebral lesions in acutobin-treated rats was mainly confined to subcortical brain regions, as indicated by high signal intensity in T₂WI.

4. Discussion

In the present study, we used MRI as a tool to evaluate the therapeutic effects of acutobin (0.75 U/ml) when administered at 2.5 U/kg (450 μg/kg) 30 min after a 60-min MCAO in hyperglycemic rat. This dose was chosen based on previous studies in animals (dog, rabbit, rat and mouse) in our laboratories [28,30,31,57] where target level fibrinogen depletion was achieved in a dosage range between 300 and 600 μg/kg. Acutobin doses over 600 μg/kg were noted to potentially cause bleeding problems [28,31]. Noninvasive MRI provides a dynamic view of the development of cerebral blood flow change, brain damage and perfusion deficit. Single-slice perfusion imaging was performed using indicator dilution methods [2,49,67] to...
Table 2
Residual cerebral blood flow of right hemisphere and six ROI

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>H</th>
<th>1 FCC</th>
<th>2 PC</th>
<th>3 PIC</th>
<th>4 PA</th>
<th>5 CP</th>
<th>6 GP</th>
</tr>
</thead>
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<tr>
<td>Baseline</td>
<td>A (n=8)</td>
<td>97±4</td>
<td>102±3</td>
<td>98±4</td>
<td>101±8</td>
<td>99±4</td>
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<td>101±3</td>
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<tr>
<td></td>
<td>S (n=7)</td>
<td>100±7</td>
<td>101±2</td>
<td>99±6</td>
<td>100±3</td>
<td>102±7</td>
<td>103±5</td>
<td>102±2</td>
</tr>
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<td>MCAO</td>
<td>A (n=8)</td>
<td>50±13%</td>
<td>82±23</td>
<td>39±15%</td>
<td>33±17%</td>
<td>41±21%</td>
<td>40±15%</td>
<td>92±20</td>
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<tr>
<td></td>
<td>S (n=7)</td>
<td>52±15%</td>
<td>85±11</td>
<td>26±9%</td>
<td>23±18%</td>
<td>49±17%</td>
<td>32±14%</td>
<td>95±24</td>
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<tr>
<td>Reperfusion 5 min</td>
<td>A (n=8)</td>
<td>96±12</td>
<td>143±16</td>
<td>112±12*</td>
<td>87±38</td>
<td>88±22</td>
<td>110±23*</td>
<td>105±16</td>
</tr>
<tr>
<td></td>
<td>S (n=7)</td>
<td>83±15</td>
<td>123±34</td>
<td>72±15</td>
<td>63±46</td>
<td>88±42</td>
<td>65±27%</td>
<td>94±19</td>
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<td>Reperfusion 1 h</td>
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<td>80±15</td>
<td>105±28</td>
<td>81±14*</td>
<td>65±37%</td>
<td>87±24*</td>
<td>74±16%</td>
<td>93±32</td>
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<td>84±37</td>
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<td>Reperfusion 2 h</td>
<td>A (n=8)</td>
<td>87±21</td>
<td>107±41</td>
<td>79±20*</td>
<td>68±24%</td>
<td>96±30</td>
<td>82±26</td>
<td>86±21</td>
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<tr>
<td></td>
<td>S (n=7)</td>
<td>81±13</td>
<td>113±20</td>
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<td>67±35%</td>
<td>65±29%</td>
<td>68±32%</td>
<td>97±33</td>
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<tr>
<td>Reperfusion 3 h</td>
<td>A (n=8)</td>
<td>84±16*</td>
<td>114±28</td>
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<td>78±24%</td>
<td>91±16</td>
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<td>91±13</td>
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<tr>
<td>Reperfusion 4 h</td>
<td>A (n=8)</td>
<td>82±16*</td>
<td>113±15*</td>
<td>86±19*</td>
<td>73±36</td>
<td>102±26*</td>
<td>75±25%</td>
<td>96±30</td>
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<td>79±21%</td>
<td>42±32%</td>
<td>43±11%</td>
<td>61±17%</td>
<td>48±30%</td>
<td>86±25</td>
</tr>
<tr>
<td>Reperfusion 24 h</td>
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<td>90±15%</td>
<td>115±31</td>
<td>99±37</td>
<td>81±22*</td>
<td>116±28</td>
<td>82±29</td>
<td>96±30</td>
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<td></td>
<td>S (n=3)</td>
<td>62±7%</td>
<td>89±13</td>
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<td>47±11%</td>
<td>70±21</td>
<td>42±36%</td>
<td>66±20</td>
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<tr>
<td>Reperfusion 1 week</td>
<td>A (n=6)</td>
<td>97±8</td>
<td>102±9</td>
<td>95±9</td>
<td>95±9</td>
<td>110±22</td>
<td>81±15</td>
<td>87±21</td>
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<td>S (n=1)</td>
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The data is tested by ANOVA adjusted for multiple comparison. There is no statistical analysis of 1-week data because of the high mortality in saline treated rats.

Values are presented mean±S.D. percent changes in residual CBF of right hemisphere and six brain regions, measured in single-slice dynamic MRI in the area of the caudate putamen detects the passage of tracer through the cerebral vasculature. The six brain regions correspond to: (1) cingulate, frontal, hindlimb and forelimb cortices (FCC), (2) parietal cortex (PC), (3) piriform and insular cortex (PIC), (4) preoptic area (PA), (5) caudate putamen and dorsal hypothalamus (CP), (6) globus pallidus and basal forebrain area including septum, and medial bed nucleus (GP). H: hemisphere, A: acutobin-treated group, S: saline-treated group.

* p<0.05 different from saline-treated group.

* p<0.05 different from the baseline.

estimate CBF from CBV and MTT. This bolus tracking MRI technique allows repeated measurements of blood flow reduction in the right hemisphere during MCAO and reperfusion. Our data indicate that acutobin improved blood flow to the ischemic area, and reduced the extent of cytotoxic and vasogenic edema at 3, 4 and 24 h after reperfusion. Regions of restricted water diffusion measured in DWI were considered to represent cytotoxic edema [25,35,55], while regions of prolonged T2 relaxation time in T2WI represented vasogenic edema [25]. The sharp increase in T2WI signal intensity seen in the saline control group strongly suggests that the development of vasogenic edema quickly follows MCAO/R. The cause of the extensive damage in the hyperglycemic condition may be due to vascular damage with subsequent shutdown of the cerebral vasculature in the ischemic region, potentially leading to the formation of microthrombi or microhemorrhage [9,10,21]. The data from our PVI technique provide

Fig. 5. Consecutive coronal forebrain T2WI (top two rows) and relative plasma volume images (PVI, bottom two rows) at 4 and 24 h after MCAO/R from one representative rat of each experimental group. (A) Acutobin-treated rat shows a T2WI hyperintensity area and PVI relative hyper and/or normo-intensity area in the right MCA territory ischemic core. (B) Saline-treated hyperglycemic rat shows a T2WI hyperintensity area and significant PVI no-reflow zone (low intensity) in the right MCA territory including the ischemic core and penumbral area.
images of the distribution of circulating blood plasma. Hypointensity in PVI images corresponds to areas of low perfusion [22,23,47,49]. Our data indicate that the extent of the no-reflow zone was reduced at 4 h, 24 h and 1 week after ischemia in the acutobin-treated group. These observed data suggest that acutobin improves brain ischemic outcome in this animal model by amelioration of local cerebral blood flow resulting in a minimization in the extent of brain damage and prevention of further brain injury secondary to the cascading vascular insults.

We chose to test acutobin’s effectiveness on a hyperglycemic MCAO model with reperfusion instigated at 60 min post MCAO. This model was chosen based on our previous studies where we observe accelerated and larger brain regions of edema and necrosis, accompanied by a more severe perfusion deficit in hyperglycemic models compared to normoglycemic counterparts [49]. While the 60-min occlusion period would be considered relatively short in normoglycemic rats, due to the rapidity in growth of the lesion in the hyperglycemic model, a 60-min occlusion time is more severe [58–60]. Longer periods of ischemia result in even higher mortality and may preclude 24-h measurements. Following reperfusion of the MCA, hyperglycemic rats show limited reperfusion into the post-ischemic tissue and the perfusion declines further over the next several hours. The aim of this study was to determine if acutobin could improve and sustain higher levels of post-ischemic tissue perfusion and ultimately salvage brain tissue. Our published [48,58,59] and unpublished observations indicate that a 1-h MCAO in hyperglycemic rats results in a large ischemic lesion. With insight to these data from previous findings, acutobin was used 30 min after ischemia onset in the present study to achieve therapeutic effect.

High mortality in saline-treated hyperglycemic rats subjected to 60–120-min MCAO followed by reperfusion within 24 h was also observed in our previous reports [48,60]. Recently published data [60] showed that only two of nine saline-treated hyperglycemic rats survived over 24 h after 60-min MCAO/R and 24 h DWI-measured lesion volume (408±89 mm³) was very close to the present study. In order to minimize high death rate during the first 24-h experimental period in the present study, we limited the time of ischemia to 60 min. According to the observation of long-term survival evaluation by MRI, the acutobin-treated group had better outcome after MCAO/R and 1 week mortality was significantly lower (25% mortality) than the saline control group (85% mortality).

Occlusion of MCA results in progressive impairment of downstream cerebral microvascular plasma perfusion. Our previous data [18,49,60] have shown a significant reduction of cerebral plasma perfusion with concomitant cerebral injury in the ischemic core after MCA occlusion followed by reperfusion. Endothelial cellular swelling, cerebrovascular plugging [9,10,41], platelet aggregation in downstream cerebral microvessels, and loss both of perfusion and vascular integrity of these microvessels in response to ischemia and reperfusion have been reported [4,10,26]. Immunohistochemistry data has emerged to suggest that intravascular fibrin deposition contributes to microvascular obstruction [51,64]. One of the current medical treatments being tested for acute stroke is fibrinolysis using purified fractions of TLEs, which are derived from the snake venom members such as ancrod (from Malayan pit viper, Agkistrodon rhostoma), Batroxobin (from Bothrops atrox moojent), Crotalase (from Crotalus adamanteus) and acutobin (from Agkistrodon acutus). Within minutes of intravenous administration of these TLEs, there is a significant reduction in plasma fibrinogen levels [57,61]. Although their primary mechanism of action is a proteolytic effect on circulating fibrinogen, the release of fibrinopeptide A but not B results in the conversion of fibrinogen to a soluble fibrinopolymer. This takes place in the absence of calcium, prothrombin or thrombin. The resultant polymer is characterized by only end-to-end anastomotic bonding [5,19,33,61]. Soluble non-crosslinked ‘ancrod-fibrin’ appears to stimulate the release of t-PA of vessel wall [14,17,57]. The protective mechanism of acutobin, assumed to be similar to the well studied ancrod [5,6,12,13,16,17,19,33,45,50,54], is likely to be initiated by proteolysis of fibrinogen. We chose to administer acutobin 30 min prior to reperfusion so that fibrinogen levels would be reduced at the time of reperfusion, thereby lowering blood viscosity and limiting potential fibrin deposition in post-ischemic brain.

Elevated plasma fibrinogen levels have been observed in stroke patients [7] and in the rat MCAO models [12]. Reduction of plasma fibrinogen leads to diminished blood viscosity and increased cerebral blood flow [20]. Our previous study [57] on rabbits showed that 4 h after intravenously injecting acutobin (4.5 U/kg body weight) the plasma fibrinogen level was lowered from 510±167 mg/dl in the saline-treated group to 243±116 mg/dl in an acutobin-treated group. The resulting circulation of soluble, noncrosslinked “ancrod-fibrin”, may also stimulate the release of endogenous t-PA from the endothelium of vessel walls [17]. We have previously reported [57] in rat experiments that plasma t-PA activity was significantly increased 5 min after intravenous injection of acutobin (2.25–4.5 U/kg body weight) and remained elevated over 45 min. Elger et al. [12] reported that post-treatment with the TLE ancrod reduced total lesion volume by 30% on a permanent MCAO model of a spontaneously hypertensive rat. Our present study shows the same reduction of MRI-measured infarct volume and perfusion deficit in acutobin-treated hyperglycemic rats.

In the present and previous studies [48,49,58], we have noted that, in saline-treated hyperglycemic rats, there are low-intensity spots contained within the hyperintense lesion in both T²,WI and DWI by 4 h into reperfusion. This is likely due to the formation of microthrombi or
microhemorrhage. Previous studies involving defibrinogenating agents in occlusive ischemia indicate that actubrin [65,66] and ancrod [45,50,54] are associated with a low incidence of hemorrhage. Therefore TLEs may offer a useful alternative to tPA treatment owing to a lower rate of intracranial hemorrhage [11,50]. In the present study, in addition to lesion size reduction and ameliorated reperfusion in rats treated with actubrin, we did not detect hemorrhagic transformation (as would be indicated by hypointense regions in T2WI), which are prevalent in this hyperglycemic MCAO model [49,58,59].

Other studies [1,34,42,62] have demonstrated the effectiveness of the thrombolytic agent tPA in normoglycemic animals using the thrombotic occlusion model where a coagulated blood clot is injected into the internal carotid artery/MCA system. Kilic et al. [24] reported post-treatment with 10 mg/kg recombinant tPA in normoglycemic mice at 15 min after a nonthrombotic MCAO (suture insertion model). Subsequent measurement of blood flow, infarct volume, brain swelling and neurological performance revealed faster recirculation and a significant reduction of ischemic injury. This finding indicated that tPA protects the brain even after non-thrombotic vascular occlusion.

In the present study, like in that of Kilic et al. [24], the antithrombotic therapy is effective even in a non-thrombotic (suture insertion) occlusion model. Our findings in hyperglycemic rats suggest that a significant problem following reperfusion of the MCA is microvascular plugging that appears to occur after initial reperfusion. Endothelial cell swelling and cerebral vascular plugging in response to ischemia and reperfusion under hyperglycemic conditions have been reported [41]. Thus, in addition to dissolving a clot, these types of agents may also function by preventing the formation of microthrombi following reperfusion. In future work we plan to compare actubrin with those compounds which are directed towards potentiating reperfusion and result in alterations in blood viscosity, and will provide more information of therapeutic opportunities in hyperglycemic ischemia.

In conclusion, post-treatment with the defibrinogenating agent actubrin leads to a reduction of MRI-measured ischemic lesion and perfusion deficit in a hyperglycemic MCAO/R non-thrombotic rat model. The MRI data suggest that actubrin improves post-ischemic perfusion, resulting in minimizing the extent of brain lesion and preventing further brain injury secondary to the cascading vascular insults.

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References