Regular Article

Cinepazide Maleate Improves Cognitive Function and Protects Hippocampal Neurons in Diabetic Rats with Chronic Cerebral Hypoperfusion

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To determine the combined effect of type 2 diabetes (T2D) and chronic cerebral hypoperfusion (CCH) on learning and spatial memory, we developed a rat model of CCH by permanent occlusion of bilateral common carotid arteries (2-vessel occlusion (2VO)) in high-fat diet (HFD)-fed rats injected with low-dose streptozotocin (STZ). Furthermore, we examined the effect of cinepazide maleate (CM) on cognitive deficits and brain damage in this rat model. Rats were maintained on HFD for 6 weeks and then injected with 35 mg/kg STZ to induce T2D. Sham or 2VO surgery was performed in non-diabetic or diabetic (DM) rats to obtain four groups: blank, DM, CCH, and DM-CCH groups. Cognitive function was tested by the Morris water maze (MWM) test. To determine the effects of the vasodilator cinepazide maleate (CM) on cognitive deficits and brain damage, DM-CCH rats were administered with 10 mg/kg CM or saline daily for 14 d. Neuronal damage in DM-CCH rats was associated with increased expression of glial fibrillary acidic protein (GFAP) and β-secretase 1 (BACE1), but decreased expression of choline acetyltransferase (ChAT). Moreover, the levels of all these proteins were significantly alleviated by CM treatment. These results suggest that T2D exacerbated CCH-induced brain damage and cognitive impairment, and CM ameliorated these effects.

Key words diabetes; chronic cerebral hypoperfusion; cognitive impairment; cinepazide maleate

Chronic cerebral hypoperfusion (CCH) is thought to be an important factor contributing to cognitive decline, both in aging and in age-related neurological disorders.1–3 The severity of memory dysfunction and the decline in cerebral blood flow are strongly correlated in disorders, such as Alzheimer’s disease (AD), vascular dementia, and stroke.4,5 Diabetes is one of the fastest growing epidemics worldwide and diabetic patients are at a high risk for development of cognitive impairment or dementia.6,7 Moreover, diabetes is often associated with CCH, and can further aggravate neurodegeneration.

Permanent bilateral occlusion of both common carotid arteries (or 2-vessel occlusion, 2VO) is a widely used rodent model of CCH. As the model mimics several features of human AD and vascular dementia, it is used to investigate the consequences of hypoperfusion on cerebral blood flow, cognitive ability, and histopathology in these neurological disorders.2,7 Progressive cognitive impairment has been reported after 2VO in rats,2,8–11 and behavioral changes resulting from cognitive impairment are associated with delayed loss of neurons in the hippocampal CA1 region, a feature commonly observed in human aging and dementia.9 CA1 cell loss is characterized by gliosis, cholinergic dysfunction,12 and activation of β-secretase 1 (BACE1).13 Consistent memory impairment after 2VO in rats occurs in the chronic phase of the ischemic event, i.e., up to 3 months after surgery, and not within 7 d, as assessed by water maze and object recognition tasks.14

Cinepazide maleate (CM) is a calcium-channel blocker with vasodilator action that can increase blood flow to the brain and peripheral organs, such as cardiac muscle, skeletal muscle, and kidneys.15 CM is known to potentiate the effects of adenosine by preventing both its degradation by deaminase and its accumulation by atrial tissue.16 Recent studies suggest that CM can provide neuronal protection in humans and is effective in the treatment of acute cerebral ischemic stroke.17 However, the mechanism by which CM elicits its neuroprotective effect is largely unknown.

In order to determine the combined effect of diabetes on CCH-induced brain damage and cognitive deficits in the hippocampus of rats in the acute phase of CCH, we developed a rat model of diabetes and 2VO. Using this model, we explored the effects of CM on neuroprotection and its possible mechanism of action.

MATERIALS AND METHODS

Animals and Experimental Protocol Male Sprague–Dawley rats (180–200 g) were group-housed at 23±1°C, with a 12-h light/dark cycle, and were allowed free access to food and water. All experiments were performed according to the guidelines of the Medical Experimental Animal Administrative Committee of Shanghai and in accordance with the principles outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Firstly, the rats were divided into four groups: blank group (normal diet+sham), diabetic (DM) group (high-fat diet (HFD)+streptozotocin (STZ)+sham), CCH group (normal diet+2VO), and DM-CCH group (HFD+STZ+2VO). Normal diet consisted of commercially available normal pellet diet (NPD) (5% fat, 53% carbohydrate, and 23% protein with a total calorific value of 25kJ/kg). HFD was prepared in-house and consisted of 22% fat, 48% carbohydrate, and 20% protein, with a total calorific value of 44.3kJ/kg. All rats were maintained on their respective diet until the end of the study period. Six weeks after the start of NPD or HFD feeding, ani-

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mals were intraperitoneally injected with either a low dose of STZ (35 mg/kg) or vehicle citrate buffer.

One month after the STZ injection, 1.0 mL of blood each was collected from the caudal vein of the rats under anesthesia with 10% chloral hydrate (400 mg/kg intraperitoneally (i.p.)). Plasma glucose, triglycerides, and cholesterol were measured in a Hitachi Model 7600 Series Automatic Analyzer, and insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) Kit (Shibayagi, Japan) according to the manufacturer’s protocol.

Two weeks after STZ injection, animals were subjected to either sham treatment or 2VO surgery according to the procedure described by Ni et al.8 After surgery, rats were randomly divided into three groups (n=6): control group (blank rats with saline), model group (DM-CCH rats with saline), and treatment group (DM-CCH rats with CM). The Morris Water Maze (MWM) test was performed to determine cognitive function values at baseline 1 week after surgery. Using this DM-CCH model, we further explored the effects of CM.

Animals were then administered with either CM (10 mg/kg; i.p.) or vehicle (saline; i.p.) daily for 14 consecutive days and this DM-CCH model, we further explored the effects of CM. After the drug treatment, the MWM test was repeated. All rats were fed their original diets until the end of the study.

**MWM Test** The MWM test was performed to evaluate cognitive function in each rat before and after drug treatment (DM-CCH rats with CM). In brief, the maze consisted of a circular platform (diameter, 10 cm) was submerged 2 cm below the surface of the water and hidden from the rat’s view. Reference objects around the pool remained unchanged.

The protocol consisted of four trials/d for five consecutive days, starting randomly from one of the four quadrants each time. A trial began by placing the rat into the water facing the wall of the pool; the rat was allowed to swim until it reached the target quadrant that was set as an assessment of spatial memory. The swimming paths and escape latencies were recorded by a video camera. If it failed to escape within 90 s, the rat was guided to the platform and allowed to stay on it for 30 s before being subjected to the next trial. On day 6, a probe trial was conducted in which the platform was removed from the pool and the rat was allowed to swim freely for 90 s. The test began with the rat in the quadrant opposite to the quadrant in which the platform was located. The percentage of time spent in the target quadrant was recorded as an assessment of spatial memory.

**Tissue Preparation** At the end of the study period, rats were anesthetized with 10% chloral hydrate and intracardially perfused with 0.9% saline. The brains were dissected along the midline and divided into two hemibrains. One hemibrain was fixed in 4% paraformaldehyde overnight at 4°C; the fixed brains were then serially dehydrated, embedded in paraffin blocks, and 4-μm thick coronal sections were stained for glial fibrillary acidic protein (GFAP) or choline acetyltransferase (ChAT). Sections were first incubating with rabbit polyclonal anti-GFAP antibody (1:200 dilution; Abcam, U.S.A.) or rabbit polyclonal anti-ChAT antibody (1:300 dilution; Millipore, England) overnight at 4°C, followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:200 dilution; Vector Laboratories, Inc., Burlingame, CA, U.S.A.) for 45 min at 37°C, and finally with avidin–biotin–peroxidase (1:200 dilution; Vectastain Elite ABC kit, Vector Laboratories) for 30 min at 37°C. Immunoreactivity was visualized with 0.05% diaminobenzidine (DAB) (Sigma, U.S.A.) as the chromagen. To determine non-specific staining, negative control sections were treated in the same manner, except that primary antibodies were omitted.

**Immunofluorescence** For immunofluorescence staining with BACE1, brain sections were first incubated with rabbit polyclonal anti-BACE1 antibody (1:200 dilution; Millipore, U.S.A.) overnight at 4°C, then with anti-rabbit immunoglobulin G (IgG) Alexa Fluor 488 (1:100 dilution; Life Technologies, U.S.A.) at 37°C for 2 h, and finally with 4'-6-diamidino-2-phenylindole (DAPI) for 8 min. A laser scanning confocal microscope ( Olympus, Japan) was used to visualize the fluorescent intensity (excitation wavelength, 488 nm) within the CA1 subfield of the hippocampus. Nuclear staining of control sections was performed using 4',6-diamidino-2-phenylindole (DAPI) to determine non-specific staining, negative control sections were treated in the same manner, except that primary antibodies were omitted.

**Western Blotting** The hippocampal tissues were homogenized in lysis buffer (Beyotime, China) and centrifuged (12000 rpm) at 4°C for 15 min. Forty microgram of protein lysates were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and then transferred to polyvinylidifluoride (PVDF) membranes (Millipore, U.S.A.). The membranes were blocked with 50 g/L nonfat milk at room temperature for 1 h, and then incubated overnight at 4°C with rabbit polyclonal anti-GFAP antibody (1:4000 dilution; Abcam, U.S.A.), rabbit polyclonal anti-ChAT (1:1000 dilution; Millipore), or rabbit polyclonal anti-BACE1 antibody (1:1000 dilution; Millipore). Thereafter, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution; Jackson Immunoresearch Laboratories, U.S.A.) for 60 min at room temperature. The peroxidase activity was visualized using Western Lightning Plus-ECL (Thermo Fisher Scientific, U.S.A.). Band densities were quantified and normalized with those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control (1:3000 dilution; ComWin, China).

**Statistical Analysis** Data are expressed as the mean±standard error of the mean (S.E.M.). Comparisons of multiple groups were done by one-way ANOVA followed by Student’s t-test. Comparison between two experimental groups was made using the unpaired Student’s t-test. A p-value of <0.05 was considered statistically significant.

**RESULTS**

**Development of Rat Model of DM and CCH** After chronic feeding with normal diet (NPD) or high-fat diet (HFD) for 6 weeks, a single dose of streptozotocin (STZ) was injected. One month after STZ injection, metabolic parameters were evaluated. Compared to normal diet (NPD)-fed vehicle-treated rats, HFD-STZ rats had significantly higher fasting
plasma glucose (FPG), triglycerides (TG), total cholesterol (TC), and fasting serum insulin (FSI) and significantly lower body weight ($p<0.05$; Table 1). These results demonstrate that HFD-STZ treated rats showed clinical characteristics of type 2 diabetes (T2D) as described in previous reports.$^{19}$ Using these animals, we further investigated the effects of diabetes on CCH-induced cognitive deficits and damage in the rat hippocampus.

**Diabetes-Associated CCH-Induced Learning and Memory Impairment Improved by CM**

Normal non-diabetic and HFD-STZ rats underwent sham or 2VO surgery and four groups of rats were obtained, namely, blank, DM, CCH, and DM-CCH. MWM test was performed 2 weeks after surgery to obtain the baseline values for decline in cognitive function. Rats were then treated with CM or saline daily for 14 d, after which the MWM test was repeated. Either DM or CCH alone prolonged escape latency compared to the blank group in the trials with invisible platform, but without any significant difference. However, DM combined with CCH could significantly prolong escape latency relative to the blank group, especially on days 2–5 ($p<0.01$; Fig. 1A). Thus, diabetes combined with hypoperfusion could induce significant learning deficit in our rat model. When these rats, were treated with CM (10 mg/kg) for 14 d, the escape latency was significantly shortened compared with the vehicle-treated animals, especially on days 3–5 ($p<0.05$; Fig. 1C). In the probe trials, the percentage of time spent in the probe quadrant was used to evaluate spatial memory. DM-CCH group rats spent significantly less time in the correct quadrant compared to blank ($p<0.01$), DM ($p<0.05$), or CCH ($p<0.05$) group rats (Fig. 1B). Moreover, CM treatment significantly improved this deficit ($p<0.05$; Fig. 1D).

**Table 1. Metabolic Parameters of Non-diabetic and Diabetic Rats**

<table>
<thead>
<tr>
<th>Item</th>
<th>Blank group</th>
<th>DM group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>403.90±3.77</td>
<td>314.71±6.05*</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>5.16±0.52</td>
<td>19.68±0.28*</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.80±0.26</td>
<td>2.52±0.49*</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>1.34±0.38</td>
<td>1.99±0.29*</td>
</tr>
<tr>
<td>Fasting serum insulin (ng/mL)</td>
<td>0.52±0.17</td>
<td>0.89±0.15*</td>
</tr>
</tbody>
</table>

Values are expressed as the mean±S.E.M. ($n=12$). Blank group = normal diet+two-vessel occlusion. DM group = High-fat diet + streptozotocin injection + two-vessel occlusion. $^*p<0.05$ relative to the blank group.

**Diabetes Promoted and CM Attenuated CCH-Induced Astrocyte Activation**

Astrocytes in the hippocampal regions of all rats in the blank group showed moderate expression of GFAP and ionized calcium-binding adapter molecule 1 (Iba-1). In diabetic rats, there was a significant higher expression of GFAP and Iba-1 (Fig. 1C). Treatment with CM significantly decreased the expression of GFAP and Iba-1 compared to saline-treated diabetic rats (Fig. 1D).

**Table 2. Swimming Speeds of Different Groups Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Swimming speeds (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>6</td>
<td>15.86±3.28</td>
</tr>
<tr>
<td>DM</td>
<td>6</td>
<td>16.13±3.55</td>
</tr>
<tr>
<td>CCH</td>
<td>6</td>
<td>13.67±2.17</td>
</tr>
<tr>
<td>DM-CCH</td>
<td>12</td>
<td>15.84±1.98</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>17.27±2.33</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>14.95±1.69</td>
</tr>
<tr>
<td>Treatment</td>
<td>6</td>
<td>15.64±2.08</td>
</tr>
</tbody>
</table>

Values are expressed as the mean±S.E.M.
sion of the gliosis marker GFAP. The DM or CCH groups did not show any significant GFAP staining compared to the blank group. However, GFAP staining was significantly higher in the DM-CCH group compared to the remaining three groups (Fig. 2A). Western blotting analysis confirmed that the relative expression of GFAP was markedly increased in the hippocampus of DM-CCH rats compared to that of the blank ($p<0.01$), DM ($p<0.05$), or CCH group rats ($p<0.01$; Fig. 2B). Chronic administration of CM substantially reduced the relative expression of GFAP in DM-CCH rats compared with saline-treated normal rats ($p<0.001$) or DM-CCH rats treated with the vehicle ($p<0.05$; Fig. 2C).

**Diabetes Worsened and CM Reduced the CCH-Induced**

Fig. 2. Diabetes Promoted and Cinepazide Attenuated CCH-Induced Astrocyte Activation

(A) Representative images of hippocampal tissue sections showing the CA1 area immunostained with glial fibrillary acidic protein (GFAP) antibody (magnification: 400×). (B) Western blots showing relative expression of GFAP in the hippocampus of rats belonging to the blank, DM, CCH, and DM-CCH groups. (C) Western blots showing GFAP protein levels in rats belonging to blank, DM-CCH+saline, and DM-CCH+cinepazide groups. Values are expressed as the mean±S.E.M. ($n=6$). **$p<0.01$ and ***$p<0.001$ relative to CCH+saline and blank groups in B and control group in C.

Fig. 3. Diabetes Worsened and CM Improved the CCH-Induced Decrease in Choline Acetyltransferase Expression

(A) Representative images of hippocampal tissue sections showing the CA1 area immunostained with choline acetyltransferase (ChAT) antibody (magnification: 400×). (B) Western blots showing relative expression of ChAT in the hippocampus of rats belonging to the blank, DM, CCH, and DM-CCH groups. (C) Western blots showing ChAT protein levels in rats belonging to blank, DM-CCH+saline, and DM-CCH+cinepazide groups. Values are expressed as the mean±S.E.M. ($n=6$). **$p<0.01$ relative to CCH+saline and blank groups in B and model (DM-CCH+saline) group in C.
Decrease in Choline Acetyltransferase Expression  Immuno-histochemical staining revealed that expression of the cholinergic neuronal marker ChAT was significantly decreased in the hippocampus of DM-CCH group rats compared to the blank group rats. Furthermore, DM and CCH groups alone had significantly lower expression of ChAT compared to the control group (Fig. 3A). Western blotting confirmed that the relative expression of ChAT was markedly decreased in the hippocampus of DM-CCH rats compared to that of rats in the blank (p<0.01), DM (p<0.05), or CCH (p<0.05) groups (Fig. 3B). Chronic administration of CM significantly attenuated the decrease in expression of ChAT observed in the DM-CCH rats relative to the normal rats treated with saline (p<0.01), DM (p<0.05), or CCH (p<0.05) groups (Fig. 3C), suggesting that CM has neuroprotective effects.

Diabetes Worsened and CM Ameliorated the CCH-Induced Increase in BACE1 Expression  BACE1 expression was significantly increased in the CA1 region of DM, CCH, and DM-CCH group rats compared to the control rats (Fig. 4A). Moreover, Western blotting confirmed that the relative expression of BACE1 was markedly increased in the hippocampus of DM-CCH rats compared with that of blank (p<0.01), DM (p<0.05), or CCH (p<0.05) group rats (Fig. 4B). After treatment with CM at 10mg/kg, BACE1 protein was markedly reduced in the hippocampus of DM-CCH rats compared to normal rats treated with saline (p<0.05) or DM-CCH rats treated with vehicle (p<0.05; Fig. 4C).

DISCUSSION

Our results demonstrated that HFD and STZ significantly exacerbated CCH-induced brain damage and cognitive deficits. In addition, 14d treatment with CM significantly improved cognitive deficits by reducing the activation of astrocytes, preventing damage to cholinergic neurons, and reducing BACE1 expression.

Behavioral dysfunction, especially memory loss, is a prominent and early symptom of AD. The MWM test is commonly used to test hippocampal function in spatial learning and memory. A previous study showed that CCH resulting from 2VO causes progressive cognitive impairment and long-term memory deficit 3 to 6 months after surgery. As previously described, many patients with diabetes are at an increased risk of cognitive dysfunction and dementia. Diabetes alone could cause deficits in spatial learning in a rat model of STZ-induced diabetes 3 months after STZ treatment. In rats, DM could have caused the loss of choline acetyltransferase (ChAT) activity in the hippocampus. This sign of astroglial activation has been observed in the brains of subjects with Alzheimer’s disease and indicates the presence of neuronal damage. Furthermore, DM synergistically exacerbates post-stroke dementia by amyloid β (Aβ) formation via the activation of β-secretase (BACE) in the hippocampus. In the present study, we maintained young adult rats on HFD for 6 weeks and then treated them with low-dose STZ injection to develop a model of T2D. Consistent with previous reports, these rats exhibit clinically relevant characteristics of T2D. However, in the early period of DM, our study did not find there are changes in these aspects. Next we induced CCH in these diabetic rats via 2VO. CCH and diabetes were found to impair cognitive function, as determined by escape latency and percentage of time spent by rats in the target quadrant. These results suggest that there are deleterious interactions between chronic cerebral hypoperfusion and T2D.

GFAP is an astroglial cell marker commonly used to detect...
changes in astrocyte function during brain development and injury. Indeed, injury of the central nervous system because of trauma, disease, genetic disorders, or chemical insult causes astrocyte activation, a condition characterized by an increase in GFAP expression. Our results demonstrate that astrogliosis occurs in the acute phase after occlusion and diabetes exacerbates the activation process.

Neurodegenerative diseases are also associated with neuroinflammation. CM probably elicits its neuroprotective effect via an interrelated mechanism that elicits its anti-inflammatory effect and inhibits astrocyte activation, thereby reducing astrocyte proliferation in the hippocampus. Considering the distribution pattern and relative expression profile of GFAP in activated astrocytes, it is possible that inhibition of astrocyte activation caused by CM could have partially protected neurons in the damaged brain lesions. These results are consistent with the notion that histological abnormalities are indicative of impairment in cognitive function parameters, such as learning and spatial discrimination.

As the enzyme responsible for acetylcholine synthesis, ChAT is a characteristic marker of cholinergic neurons. Determination of ChAT levels is considered a reliable neurochemical assay to test cholinergic function, and it has been proposed with the notion that histological abnormalities are indicative of activation caused by CM could have partially protected neurons from CM prevents BACE1 expression in the hippocampus. Considering the inhibitory effect and inhibits astrocyte activation, thereby reducing astrocyte activation, a condition characterized by an increase in spatial discrimination.

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Conflict of Interest The authors declare no conflict of interest.

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