Expression patterns of ClC-3 mRNA and protein in aortic smooth muscle, kidney and brain in diabetic rats

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Abstract

ClC-3, a member of the ClC family of voltage-gated chloride channels, regulates cell proliferation of cultured rat aortic vascular smooth muscle cells, pathogenesis of allergic rhinitis and tumor cell migration. However, its role in diabetic animals is still unknown. To address this issue, we investigated the expression patterns of ClC-3 in diabetic rats. Five-week-old Sprague-Dawley rats were divided into two groups, 50 non-diabetic control rats (non-DM) and 50 diabetic model rats (DM). ClC-3 mRNA and protein expression in aortic smooth muscle, kidney and brain tissues were examined by fluorimeter-based quantitative RT-PCR assay and Western blot analysis, respectively. ClC-3 mRNA and protein were endogenously expressed in aortic smooth muscle, kidney (cortex and medulla) and brain tissues of both control and streptozotocin-induced diabetic rats. ClC-3 mRNA and protein expression levels were significantly higher in aortic smooth muscle and brain tissues of diabetic rats, but significantly decreased in kidney medulla tissue, relative to non-DM controls. There were no significant differences in ClC-3 mRNA and protein expression in kidney cortex between non-diabetic control and diabetic rats. Furthermore, the altered ClC-3 expression patterns in diabetic rat aortic smooth muscle, brain, and kidney medulla tissues all correlated with the changes in blood glucose levels (p<0.05). In conclusion, our data show for the first time that diabetes alters both the gene and protein expression of ClC-3 channels. These changes may contribute to the impaired vascular, brain and kidney functions observed in diabetes.

Diabetes is the fastest growing metabolic disorder worldwide. It has been predicted that the developing world will be hit the hardest by the escalating diabetes epidemic. Chronic hyperglycemia due to uncontrolled diabetes causes a number of secondary complications including cardiovascular, renal, neurological, and ocular disorders, which are the main causes of morbidity and mortality. A more thorough understanding of the biochemical changes that contribute to the occurrence of these secondary complications during the progression of diabetes is necessary in order to improve the efficacy of therapeutic treatments.

The maintenance of a constant cell volume, despite fluctuating intra- and extra-cellular osmolarity, is essential for normal cell function. In animal cells, cell volume is maintained by the activation of channels and transporters in the plasma membrane. Chloride channels may play a role in this process: the loss of K+ and Cl− ions and organic osmolytes, followed by concomitant loss of water, leads to a decrease in cell...
Recently, it has been shown that the chloride channels are active in the cortex under both isotonic and hypotonic conditions. In addition, the chloride channels may have a role in the regulation of lens hydration during diabetes. ClC-3, as a member of the ClC superfamily of chloride channels and Cl-/H+ exchangers, is found predominantly in intracellular membranes. Several seemingly incompatible functions have been attributed to ClC-3: it may mediate swelling-activated Cl currents or Ca2+-activated Cl current in plasma membranes, and it may play a role in neutrophil and smooth muscle reactive oxygen species generation and in β-cell insulin secretion. ClC-3 has been considered as the molecular candidate for volume-sensitive outwardly rectifying anion channels in certain mammalian cell types, including cardiac myocytes and vascular smooth muscle cells. ClC-3 also has been found in different cancer cell types, including prostate cancer epithelial cells, PC12 cells, and glioma cells.

In light of these observations, it was deemed important to study the expression pattern of ClC-3 in a diabetes model. In this report, we studied the ClC-3 mRNA and protein expression in aortic smooth muscle, kidney and brain tissues of streptozotocin-induced diabetic rats.

**Materials and Methods**

**Animals**

Animals were housed in facilities according to International Guidelines, and studies were approved by and conducted in accordance with the Institutional Animal Care and Use Committee.

Female Sprague-Dawley (SD) rats (100 rats, five weeks old, 220–250g, Experimental Animal Center of Fourth Military Medical University (P.R.China) were housed 5 per cage with free access to food and water, and were kept in a constant environment (22 ± 2°C, 50 ± 5% humidity, 12 h light/dark cycle). Thee rats were divided into two groups: 50 non-diabetic control rats (non-DM) and 50 diabetic model rats (DM).

**Chemicals and Reagents**

All chemicals were of analytical or reagent grade. Before the experiment, all of the vessels and tips for pipetting were dipped in HNO3 for 24 h and then washed with ultrapure water. The water used was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

**Modeling and tissue sampling**

Rats were rendered diabetic by a single intravenous injection of streptozotocin (65 mg/kg body weight; Sigma-Aldrich, St Louis, MO, USA) in citrate buffer (pH=4.5) as described. Body weights and blood glucose values were monitored at three and seven days. Ten rats in non-DM and DM groups were anaesthetised and killed at two, four, six, eight, and 10 weeks after diabetes induction. Samples of thoracic aorta, brain and kidney tissues were removed. The kidney tissues were placed on glass (chilled on ice), sliced, and dissected into cortex and medulla (medulla contained outer and inner strip) under a stereomicroscope. The separated tissues were frozen in liquid nitrogen and stored at -80°C.

**Metabolic data analysis**

At two, four, six, eight, and 10 weeks after diabetes induction, body weight, kidney weight, blood pressure, blood glucose level, 24 h urinary albumin excretion (UAE) and creatinine clearance was measured for 10 animals in each group. Blood pressure was measured using the tail-cuff method. Rats were placed individually in metabolism cages and 24 h urine samples were collected in metal-free propylene tubes. UAE was determined using a kit (Rat Albumin Enzyme Immunoassay; SPI-BIO, QC, Canada). Urinary albumin:creatinine ratio (UACR) was calculated from these two measurements. Creatinine clearance was
calculated on the basis of urinary creatinine, serum creatinine, urine volume and body weight using the following equation: 

\[ C_{cr}(\text{ml} \cdot \text{s}^{-1} \cdot [\text{kg} \cdot \text{body weight}]^{-1}) = \frac{[\text{urinary Cr (\mu mol/l)} \times 24\text{h urine volume (ml)}]}{\text{serum Cr(\mu mol/l)} \times [1000/\text{body weight (g)}] \times [1/86, 400 (s)]}, \]

where \( C_{cr} \) is creatinine clearance and \( \text{Cr} \) is creatinine.

Fluorimeter-based quantitative RT-PCR assay

The mRNA expression level of ClC-3 in thoracic aorta smooth muscle, brain and kidney tissues of rats in each group were detected by fluorescein-based quantitative RT-PCR. Total RNA was extracted from the tissues with a phenol/guanidine isothiocyanate based reagent (TrizolTM, Gibco BRL, Scotland). Briefly, 1 ml of Trizol and 200µl of chloroform were added to each tube. The RNA was then precipitated with 500µl of isopropanol (v/v) and washed with 75% ethanol and air dried. The purified RNA was then dissolved in 10 µl of diethyl pyrocarbonate (DEPC) treated water, the optical density measured at 260/280 wavelength and stored at -80°C prior to being used in the synthesis of cDNA.

RNA was reverse transcribed using the Super scripte™ Preamplification System (Life Technology, Guangzhou, Guangdong, China). Total RNA was mixed with oligo (dT) primers and incubated at 70°C for 10 min. The mixture was then chilled on ice and incubated with a 1× reverse transcriptase buffer (50 mM Tris-HCl, pH 8.4, 75 mM KCl), 3 mM MgCl₂, 500 µM of each deoxynucleotide, 10 mM dithiothreitol and 200U of Superscript II RT reverse transcriptase at 42°C for 50 min. The 20 µl reactions were further incubated at 70°C for 15 min and 2 U RNase H was added to each tube. Following a final incubation at 37°C for 20 min, the cDNAs were stored at -80°C.

PCR was performed in MJ Opticon Monitor 2.0 (MJ Ltd, Atlantic Avenue Alameda, CA, USA) using SYBR Green I (Biogene, Valencia, CA, USA) as fluorescent. The following pairs of primers were used: β-actin (258bp): 5’ GTC ACG CAC GAT TTC CCT CTC 3’ (sense), 5’ CTC-3 (206bp): 5’ TTG CCT ACT ATC ACC ACG AC 3’(sense), 5’ GCA TCT CCA ACC CAT TTA CT 3’(antisense). All primers were synthesized by Sangon Co. (Shanghai, P.R.China). Reverse transcription and amplification was performed using a thermocycler (Biometra, Göttingen, Germany) that was programmed with the following parameters: (1) reverse transcription at 50°C for 30 min; (2) PCR activation at 95°C for 15 min; (3) 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min; and (4) final extension at 72°C for 10 min.

Sample quantification was carried out by the software of Sequence Detection System (SDS2.0) by constructing a standard calibration curve using serial dilutions of β-actin DNA of known concentration, from which the concentration of an unknown sample could be determined. In order to analyze inter-assay variation, a least square curve fitting test was used to construct a fit, and the standard error of the data points to the curve was estimated by plotting the log of the sample concentration against the number of cycles the PCR machine undertook to yield a set of fluorescent data. The standard error was estimated to be 15% on the log and was within the values described for β-actin quantification using competitive PCR.

Western blot analysis

Thoracic aorta, brain and kidney tissues in six groups were homogenized in lysis buffer (PBS, 1 % nonidet P-40 (NP-40), 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 100 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate) at 4°C throughout all procedures, and sonicated for 70 s. Then 300 µg PMSF per gram of tissue was added and samples were incubated on ice for 30 min, followed by centrifugation at 15,000 rpm for 20 min at 4°C. The protein content was determined according to Bradford’s method, with bovine serum albumin used as a standard.© 2010 CIM Fu et al. ClC- 3 in diabetic rats Clin Invest Med • Vol 33, no 3, June 2010 E148
TABLE 1. Metabolic data of diabetic and non-diabetic rats (\(\bar{x} \pm s\), \(n=10\))

<table>
<thead>
<tr>
<th>Groups</th>
<th>Non-DM-2</th>
<th>DM-2</th>
<th>Non-DM-4</th>
<th>DM-4</th>
<th>Non-DM-6</th>
<th>DM-6</th>
<th>Non-DM-8</th>
<th>DM-8</th>
<th>Non-DM-10</th>
<th>DM-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>388.6 ±10.8</td>
<td>362.6 ±10.3</td>
<td>382.7 ±10.1</td>
<td>328.1 ±10.9**</td>
<td>378.9 ±11.2</td>
<td>309.6 ±10.9*</td>
<td>376.9 ±10.9</td>
<td>262.1 ±10.2**</td>
<td>378.5 ±11.0</td>
<td>227.3 ±9.8**</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.5 ±0.1</td>
<td>1.7 ±0.2</td>
<td>1.6 ±0.1</td>
<td>1.8 ±0.1*</td>
<td>1.4 ±0.2</td>
<td>2.0 ±0.1**</td>
<td>1.6 ±0.1</td>
<td>2.3 ±0.1**</td>
<td>1.5 ±0.2</td>
<td>2.7 ±0.2**</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>113.1 ±2.2</td>
<td>115.3 ±3.6</td>
<td>114.5 ±1.9</td>
<td>116.9 ±3.2</td>
<td>112.1 ±1.9</td>
<td>115.7 ±2.4</td>
<td>115.7 ±1.2</td>
<td>114.2 ±2.3</td>
<td>113.8 ±2.0</td>
<td>114.3 ±2.1</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>142.2 ±18.3</td>
<td>225.2 ±20.4*</td>
<td>145.2 ±18.6</td>
<td>289.6 ±22.1**</td>
<td>143.5 ±19.8</td>
<td>352.6 ±19.5**</td>
<td>140.1 ±20.2</td>
<td>422.6 ±20.3**</td>
<td>146.3 ±19.6</td>
<td>528.6 ±23.9**</td>
</tr>
<tr>
<td>UACR (µg/mg)</td>
<td>112.4 ±31.8</td>
<td>204.5 ±35.7*</td>
<td>111.2 ±30.5</td>
<td>268.3 ±38.8**</td>
<td>112.8 ±31.9</td>
<td>311.5 ±40.6**</td>
<td>113.0 ±29.6</td>
<td>366.2 ±45.2**</td>
<td>114.2 ±30.1</td>
<td>498.1 ±58.3**</td>
</tr>
</tbody>
</table>

*p<0.05 vs. non-DM; **p<0.001 vs. non-DM

were boiled with 2 × sample buffer containing 5 % β-mercaptoethanol for 5 min, separated by size on 15 % polyacrylamide gel under SDS denaturing conditions, and transferred to a nitrocellulose membrane at 90 V for 2h. The nitrocellulose membranes were stained with ponceau S to assess the efficiency of transfer. Non-specific binding was blocked by incubation in block buffer (5 % non-fat dry milk, 0.05 % Tween-20, 1 × tris-Cl-buffered saline) overnight at 4°C. The membranes were hybridized with a 1:1000 dilution of polyclonal rabbit anti-rat ClC-3 (Santa Cruz Biotechnology, CA, USA), then incubated with a horseradish peroxidase-labeled goat anti-rabbit IgG (1:500). The bound secondary antibody was detected by enhanced chemiluminescence (Amersham Life Science, Little Chalfont, UK). Housekeeping protein β-actin was used as a loading control. Positive immunoreactive bands were quantified densitometrically (Leica Q500IW image analysis system) and expressed as ratio of ClC-3 to β-actin in optical density units.

Statistical Analysis

The software of SPSS version13.0 for Windows (SPSS Inc, IL, USA) was used for statistical analysis. Data obtained were expressed as mean±S.D. and analyzed by one-way ANOVA with the post-hoc Tukey’s test applied for paired comparisons. The correlation of CLC-3 alterations with blood glucose levels of diabetic rats was analyzed by Spearman Rank Correla-

tion. A difference between means was considered significant if p<0.05.

Results

Effects of streptozotocin on metabolic data of rats

At ten weeks after injection with streptozotocin, the blood glucose levels of the streptozotocin-induced diabetic groups were significantly higher than those of the non-DM groups (p<0.05, Table 1). The body weights of DM groups at four, six, eight and 10 weeks post streptozotocin injection were significantly lower than those in the corresponding control groups (p<0.05, Table 1); however, the kidney weights of the DM animals were significantly higher than those in the control group (p<0.05, Table 1). In additional, from two to 10 weeks after induction of diabetes, diabetic rats exhibited a time-dependent significant increase in UACR in comparison with the corresponding non-diabetic controls (p<0.05, Table 1). Thus, streptozotocin promoted the development of albuminuria in diabetic rats. There was no significant difference in the systolic blood pressure of the ten groups.

CIC-3 mRNA expression in aortic smooth muscle, kidney and brain tissues of diabetic rats

In comparison with the mRNA concentration of control rats, the relative expression levels of CIC-3 mRNA in aortic smooth muscle and brain tissues of
Diabetic rats were significantly up-regulated from four weeks post-streptozotocin injection (p<0.05, Figure 1A and 1B), and down-regulated in kidney medulla tissues from six weeks post streptozotocin injection (p<0.05, Figure 1D). ClC-3 mRNA showed no obvious changes in kidney cortex of streptozotocin-induced diabetic rats relative to non-diabetic rats (p>0.05, Figure 1C).

**ClC-3 protein expression in aortic smooth muscle, kidney and brain tissues of diabetic rats**

Similar to the results of fluorimeter-based quantitative RT-PCR assay, the Western blotting analysis confirmed that ClC-3 protein expression increased in a time-dependent manner in both aortic smooth muscle and brain tissues of diabetic rats from four weeks post-streptozotocin injection (p<0.05 in comparison with the corresponding non-DM groups, Figure 2A and 2B, respectively). In contrast, ClC-3 protein expression was markedly reduced in kidney medulla tissues of diabetic rats from six weeks post-streptozotocin injection, in comparison with the non-DM group (p<0.05, Figure 2D). Moreover, there was no significant difference in ClC-3 protein expression in kidney cortex between non-diabetic control and diabetic rats.
Correlation of CLC-3 alterations with blood glucose levels of diabetic rats

The mRNA levels of ClC-3 in aortic smooth muscle (r=0.56, p=0.01) and brain tissues (r=0.53, p=0.01) showed a positive correlation with blood glucose levels of the diabetic rats, which was consistent with the altered trend in its protein levels. In contrast, ClC-3 expression in kidney medulla tissues showed an inverse correlation (ClC-3 expression decreased as blood glucose levels increased) (r=-0.32, p=0.02). There was no correlation between ClC-3 expression patterns in kidney cortex tissues and blood glucose levels of diabetic rats (p>0.05).

Discussion

Diabetes exerts profound adverse effects on cardiovascular, endothelial, neurological, and renal functions. The results of this study provide compelling evidence that ClC-3 chloride channels are critically linked to the progression of diabetes. Fluorimeter-based quantitative RT-PCR assay and Western blot analysis demonstrated that ClC-3 is endogenously expressed in rat aortic smooth muscle, brain, and kidney tissues. ClC-3 protein expression in aortic smooth muscle and brain tissues of diabetic rats increased in a time-dependent manner, while ClC-3 mRNA and protein levels from kidney medulla were lower in diabetic rats.
than in non-diabetic rats. Furthermore, the altered CIC-3 expression patterns in rat aortic smooth muscle, brain, and kidney tissues were all found to be correlated with the changing of blood glucose levels of diabetic rats.

The CIC family, which includes nine proteins, is the largest family of mammalian chloride channels and its members play a surprisingly diverse set of biological roles. CLC-1 and CLC-2 chloride channels reside in plasma membranes, whereas CLC-3, CLC-4, CLC-5, CLC-6, and CLC-7 are thought to reside predominantly in the membranes of intracellular organelles. Oshima et al. reported that CLC-3 is expressed in sub-mucosal nasal gland cells in chronic sinusitis, but this has not been fully investigated. Miller et al. have demonstrated that CIC-3 functions as a chloride-proton exchanger and is required for charge neutralization of the electron flow generated by NADPH oxidase in endosomes of non-phagocytes. In cardiac and vascular smooth myocytes and many other cell types, CIC-3 has been confirmed to be a volume-regulated chloride channel. In human blood vessels, CIC-3 is the most abundantly expressed chloride channel and may underlie the swelling-activated Cl⁻ current found in these tissues. In addition, antisense oligonucleotide against CIC-3 has been shown to significantly inhibit the swelling-activated Cl⁻ current and the functional regulatory volume decrease in HeLa cells, Xenopus laevis oocytes, and nonpigmented ciliary epithelial cells. Wang et al. showed that CIC-3 channels are important regulators of the cell cycle and may play a crucial role in such pathogenic processes as hypertension and arteriosclerosis. Moreover, CIC-3 has been cloned from lens epithelium and other ocular epithelia and experiments using antisense indicate that CIC-3 may be partially responsible for regulating cell volume in nonpigmented ciliary epithelial cells. Ramana et al. observed the upregulation of CIC-3 protein in the lens cortex of diabetic rats and its prevention by antioxidants, which suggested that it regulates chloridion transport in the development of diabetic cataracts. Therefore, the expression patterns of the CIC-3 channel in diabetic animals are intriguing.

In the present study, diabetes was induced in rats by a single injection of streptozotocin. The diabetic rats showed a significant elevation in blood glucose levels throughout the experimental period (p<0.05). At the same time, the diabetic rats appeared sick and polyuric, and showed loss of body weight despite hyperphagia. Higher levels of CIC-3 mRNA and protein were found in aortic smooth muscle and brain tissues of diabetic rats than in these tissues of normal controls, which may be caused by oxidative stress, inflammatory factors and ion channel reconstruction. Supportive evidence for this includes the following: (1) it has been demonstrated that cataracts of the eye occur during the process of diabetes mellitus and CIC-3 protein and mRNA expression in lens cortex fiber cells is elevated. After the intervention with antioxidants, CIC-3 levels were reduced. In addition, the increased expression of CIC-3 induced by oxidative stress could inhibit the cell apoptosis. (2) Recent studies have shown that inflammatory factors could stimulate the expression of CIC-3. In diabetes mellitus, high blood glucose levels could stimulate the expression of many vasoactive substances and cytokines, including AngII, ET-1, NO, NOS, VEGF and TNF-α. (3) It has been shown that, in an animal model of abdominal aortic stenosis, CIC-3 expression levels are elevated at the early stage of postoperative stenosis, and the decreased blood pressure could inhibit the reconstruction of cardiac ion channel without changes in CIC-3 expression. Furthermore, it has been demonstrated that hyperfiltration is a leading hallmark of early diabetes resulting in progressive diabetic nephropathy. The hemodynamic phenotype of early diabetes has been attributed to abnormalities of renal vessels and glomerular vasculature. Several factors, including altered function of calcium and potassium channels, lack of insulin action, elevated levels of atrial natriuretic peptide and locally produced renal angiotensin, have been implicated in the afferent arteriolar dilation and efferent arteriolar constriction.
observed in the early state of diabetes, contributing to glomerular hyperfiltration. Our data displayed the decreased expression of ClC-3 mRNA and protein in the medulla of diabetic kidney, implying that the function of chloride channel may be also altered in the progression of diabetes.

In conclusion, our results show for the first time that diabetes alters gene and protein expression of the ClC-3 Cl channel, which may contribute to the impaired vascular, brain and kidney functions observed in diabetes. Recently, Ramana et al. observed that the preventive effect of anti-oxidants on diabetic cataractogenesis are due to changes in the ClC-3 channel in the lens cortex. It appears that a variety effects of ClC-3 channels are involved in the mechanism of diabetic complications. Further study will be necessary to assess these hypotheses.

References


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