Cysteamine prevents vascular leakage through inhibiting transglutaminase in diabetic retina

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Abstract

Cysteamine (an aminothiol), which is derived from coenzyme A degradation and metabolized into taurine, has beneficial effects against cystinosis and neurodegenerative diseases; however, its role in diabetic complications is unknown. Thus, we sought to determine the preventive effect of cysteamine against hyperglycemia-induced vascular leakage in the retinas of diabetic mice. Cysteamine and ethanolamine, the sulfhydryl group-free cysteamine analogue, inhibited vascular endothelial growth factor (VEGF)-induced stress fiber formation and vascular endothelial (VE)-cadherin disruption in endothelial cells, which play a critical role in modulating endothelial permeability. Intravitreal injection of the amine compounds prevented hyperglycemia-induced vascular leakage in the retinas of streptozotocin-induced diabetic mice. We then investigated the potential roles of reactive oxygen species (ROS) and transglutaminase (TGase) in the cysteamine prevention of VEGF-induced vascular leakage. Cysteamine, but not ethanolamine, inhibited VEGF-induced ROS generation in endothelial cells and diabetic retinas. In contrast, VEGF-induced TGase activation was prevented by both cysteamine and ethanolamine. Our findings suggest that cysteamine protects against vascular leakage through inhibiting VEGF-induced TGase activation rather than ROS generation in diabetic retinas.

Introduction

Cysteamine (β-mercaptoethylamine), an aminothiol with a primary amine group and a sulphhydryl group, is derived from coenzyme A degradation and metabolized into taurine (Pinto et al. 2005, Besouw et al. 2013). Cysteamine depletes cystine through reacting with its disulfide bridge, changes the enzymatic activity of several proteins as a result of binding to their thiol groups and enhances brain-derived neurotrophic factor secretion (Besouw et al. 2013).
Due to cysteamine’s varied effects, it has been proposed as a treatment for many diseases, including cystinosis and neurodegenerative and neuropsychiatric disorders (Besouw et al. 2013). Cysteamine was initially utilized to protect against X-rays and as a therapy for radiation sickness (Bacq et al. 1953). This compound was introduced as a potential treatment for cystinosis and is currently used to treat corneal cystine crystal accumulation in patients with cystinosis (Kaiser-Kupfer et al. 1987, Tsilou et al. 2003). Cysteamine has been evaluated as a treatment for neurodegenerative diseases, including Huntington’s disease (Gibrat & Cicchetti 2011, Shannon & Fraint 2015) and Parkinson’s disease (Gibrat & Cicchetti 2011, Cisbani et al. 2015) and has been proposed to treat schizophrenia (Buckley et al. 2014), cystic fibrosis (De Stefano et al. 2014) and nonalcoholic fatty liver disease (Dohil et al. 2011). However, its beneficial effects against diabetic complications, including diabetic retinopathy, remain unknown.

Hyperglycemia and the resulting subsequent physiological changes develop into, among others, microvascular and macrovascular diabetic complications (Bhatt et al. 2014). One of the major microvascular complications, diabetic retinopathy, is the leading cause of blindness in adults (Wang et al. 2012, Gupta et al. 2013, Bhatt et al. 2014), which progresses from nonproliferative abnormalities, characterized by vascular leakage, to severe proliferative diabetic retinopathy (Fong et al. 2004). In nonproliferative diabetic retinopathy, retinal blood vessels are damaged by pericyte loss, microaneurysm and vascular leakage (Caldwell et al. 2003, Hammes 2005). Retinal vascular leakage in the early stages of diabetic retinopathy is predominantly caused by vascular endothelial growth factor (VEGF)-mediated stress fiber formation and vascular endothelial (VE)-cadherin disruption (Spindler et al. 2010, Lim et al. 2014). Recently, we demonstrated that reactive oxygen species (ROS)-mediated activation of transglutaminase (TGase) 2 plays a key role in VEGF-induced retinal vascular leakage in diabetic mice (Lee et al. 2016). Though various drugs against VEGF, oxidative stress and inflammation have been evaluated against VEGF-induced molecular events in the diabetic retina, long-lasting therapies with minimal complications are required to treat diabetic retinopathy (Stewart 2016, Tolentino et al. 2016).

Therefore, we aimed to determine the preventive effect of cysteamine on hyperglycemia-induced vascular leakage in the retina of diabetic mice. We hypothesized that cysteamine prevents vascular leakage by inhibiting hyperglycemia-induced TGase activation rather than ROS generation in the diabetic retina. We found that the primary amine group of cysteamine was implicated in the prevention of VEGF-induced endothelial cell permeability and hyperglycemia-induced vascular leakage in the retinas of streptozotocin-induced diabetic mice. The primary amine was also involved in hyperglycemia-induced TGase activation in diabetic retinas. However, the sulphhydryl group of cysteamine was required for inhibition of ROS generation, but not essential for preventing TGase activation and vascular leakage. Our findings suggest that cysteamine prevents vascular leakage by inhibiting hyperglycemia-induced TGase activation in the retinas of diabetic mice, and thus, has therapeutic potential for preventing diabetic retinopathy and vascular leakage-associated diseases.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from the human umbilical cord vein according to the Declaration of Helsinki as previously described (Lim et al. 2014, Lee et al. 2016). Written informed consent was obtained from all umbilical cord donors. Cells from several batches were grown at 37°C in a humidified 5% CO₂ incubator in M199 culture media supplemented with 20% FBS, 3 ng/mL basic fibroblastic growth factor (Millipore), 5 U/mL heparin (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells in passages three to six were incubated for 6 h in low-serum medium supplemented with 1% FBS and antibiotics. This study was approved by the Institutional Review Board of Kangwon National University Hospital.

Measurement of intracellular ROS generation

Intracellular ROS generation was determined using 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA, Invitrogen, Molecular Probes) staining as previously described (Bhatt et al. 2016). Cells were treated with amine compounds for 30 min and then incubated with 10 ng/mL VEGF (Millipore) and 10 µM H₂DCFDA in low-serum media (phenol red-free) for 10 min. Labeled cells were immediately analyzed by confocal microscopy (Fluoview-300; Olympus), and intracellular ROS levels (fold) were determined by measuring
Measurement of in situ TGase activity

In situ TGase transamidating activity was determined by confocal microscopic assay as previously described (Lee et al. 2016). Briefly, cells were incubated with 1 mM 5-(biotinamido)pentylamine for 1 h at 37°C, fixed with 3.7% formaldehyde in PBS for 30 min and permeabilized with 0.2% Triton X-100 in PBS for 30 min. After incubation with blocking solution (2% BSA in 20 mM Tris (pH 7.6), 138 mM NaCl, and 0.1% Tween-20) for 30 min, cells were treated with FITC-conjugated streptavidin (1:200, v/v) in blocking solution for 1 h. Single-cell fluorescence intensities of stained cells were determined for 10 randomly selected cells per experiment.

Immunofluorescence and VE-cadherin internalization

Actin filaments and VE-cadherin were visualized as previously described (Lim et al. 2014). In order to visualize actin filaments, cells were treated with 10 ng/mL VEGF for 1 h at 37°C, fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Cells were incubated with Alexa Fluor 488 phalloidin (1:200, Invitrogen, Molecular Probes) for 1 h at room temperature, and actin filaments were observed with a confocal microscope.

VE-cadherin was visualized in cells preincubated with TGase inhibitors for 30 min and treated with 10 ng/mL VEGF for 90 min at 37°C. Following fixation and permeabilization, cells were incubated overnight with a monoclonal VE-cadherin antibody (1:200, Santa Cruz Biotechnology, Cat. No. sc-9989) at 4°C. Cells were then probed with a FITC-conjugated goat anti-mouse antibody (1:200, Sigma, Cat. No. F0257) in PBS for 2 h, and VE-cadherin was visualized using confocal microscopy. VE-cadherins were represented by line profiles displaying the distribution of relative fluorescence intensities (RFIs) obtained using the Fluoview software (FV-300), indicated by dotted lines crossing two cell–cell contacts.

Internalization of VE-cadherin was visualized as previously described (Li et al. 2012). Briefly, cells were incubated with a monoclonal antibody against VE-cadherin extracellular domain (1:200, Millipore, clone BV6) for 1 h and treated with 10 ng/mL VEGF for 90 min in the presence of 100 µM chloroquine.

Cells were acid washed for 30 min using PBS (pH 2.7) containing 25 mM glycine and 3% BSA and then fixed and permeabilized as described previously. Cells were incubated with a FITC-conjugated goat anti-mouse antibody (1:200, Sigma, Cat. No. F0257) for 2 h and with 1 µg/mL DAPI in PBS for 5 min. VE-cadherin and nuclei were visualized using confocal microscopy (K1-Fluo, Nanoscope Systems, Taejon, Korea).

Generation of diabetic mouse model

Six-week-old male C57BL/6 mice were obtained from DBL (EumSeong, Korea). Diabetic mice were generated by a single intraperitoneal injection of streptozotocin (150 mg/kg body weight, Sigma) freshly prepared in 100 mM citrate buffer (pH 4.5) as previously described (Bhatt et al. 2013). Mice with non-fasting blood glucose levels greater than 19 mM, polyuria and glucosuria were considered diabetic. Two weeks after streptozotocin injection, diabetic mice were subjected to the measurements of ROS levels, in vivo TGase activity and vascular leakage in retinas. No mice were treated with insulin. All animal experiments conformed to the NIH guidelines (Guide for the Care and Use of Laboratory Animals) and were approved by the Kangwon Institutional Animal Care and Use Ethics Committee.

Measurement of vascular leakage in retinas

Microvascular leakage in mouse retinas was investigated by fluorescein angiography as previously described (Lim et al. 2014). Briefly, 24 h after intravitreal injection of 2 µL 500 mM cysteamine or 100 mM ethanolamine, mice were deeply anesthetized and 1.25 mg 500 kDa FITC-dextran (Sigma) was injected into the left ventricle. After 5 min, mice were killed by cervical dislocation. The eyes were enucleated, and immediately fixed with 4% paraformaldehyde for 45 min. The retinas were dissected in the Maltese cross configuration and flat-mounted onto glass slides. The superficial vessels of the retinas (n = 6 per group) were observed by confocal microscopy, and vascular leakage was quantitatively analyzed using Fluoview software (FV-300).

Measurement of ROS levels in mouse retinas

ROS levels in mouse retinas were determined by confocal microscopy as previously described (He et al. 2014). Briefly, mice were killed by cervical dislocation and the eyes

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Cysteamine and ethanolamine inhibit VEGF-induced stress fiber formation and VE-cadherin disruption in HUVECs

In order to investigate whether cysteamine can prevent hyperglycemia-induced vascular leakage, we examined the effect of cysteamine on VEGF-mediated stress fiber formation and VE-cadherin disruption in HUVECs. Vascular leakage in the retinas of diabetic mice is predominantly caused by hyperglycemia-induced stress fiber formation and adherens junction disruption (Lim et al. 2014, Lee et al. 2016). We found that VEGF activated the formation of stress fibers, which was prevented by cysteamine (Fig. 1B). Furthermore, through visualization of VE-cadherin to determine adherens junction integrity, we found that cysteamine inhibited VEGF-induced adherens junction disassembly (Fig. 1C). The changes in VE-cadherin stability are represented by line profiles displaying the distribution of relative fluorescence intensities (RFIs), indicated by dotted lines crossing two cell–cell contacts (Fig. 1C). VE-cadherin disassembly was further investigated using VE-cadherin internalization assay. VEGF significantly increased intracellular VE-cadherin, which was reversed by cysteamine ($P < 0.001$, Fig. 1D). Thus, it is likely that cysteamine prevents VEGF-induced endothelial cell permeability by inhibiting stress fiber formation and adherens junction disruption.

We then treated endothelial cells with ethanolamine, the sulfhydryl group-free cysteamine analog (Fig. 1A), to study the function of the primary amine group of cysteamine in the prevention of VEGF-induced endothelial cell permeability. Ethanolamine prevented VEGF-mediated stress fiber formation, VE-cadherin disruption and VE-cadherin internalization (Fig. 1B, C and D). These results indicate that the primary amine of cysteamine is implicated in the prevention of VEGF-induced endothelial cell permeability.
Cysteamine and ethanolamine inhibit hyperglycemia-induced vascular leakage in the retinas of diabetic mice

In order to validate our in vitro findings, we investigated the preventive role of cysteamine against hyperglycemia-induced vascular leakage in the retinas of streptozotocin-induced diabetic mice. Diabetic mice were intravitreally injected with cysteamine and then vascular leakage was investigated using fluorescence angiography (Fig. 2A). High levels of extravasation of FITC-dextran were observed in the retina of diabetic mice (n=6); this vascular leakage was blocked in the retinas of cysteamine-injected contralateral eyes (n=6). We quantitatively analyzed vascular leakage by determining the fluorescence intensity of FITC-dextran in mouse retinas (Fig. 2B) and found that intravitreal injection of cysteamine significantly inhibited this leakage (n=6, P<0.001).
We then intravitreally injected diabetic mice with ethanolamine and examined vascular leakage using fluorescence angiography. Intravitreal injection of ethanolamine significantly inhibited hyperglycemia-induced extravasation of FITC-dextran in the retinas of diabetic mice (Fig. 2A and B, n = 6, P < 0.001). These results suggest that the primary amine of cysteamine is involved in the prevention of hyperglycemia-induced vascular leakage in the retinas of diabetic mice.

Cysteamine, but not ethanolamine, inhibits VEGF-induced ROS generation in endothelial cells and hyperglycemia-induced ROS generation in the retinas of diabetic mice

In order to study the mechanism by which cysteamine prevents vascular leakage, initially, we determined the effects of cysteamine and ethanolamine on VEGF-induced generation of intracellular ROS in HUVECs (Fig. 3). We found that cysteamine inhibited VEGF-induced ROS generation in a concentration-dependent manner, with maximum effect at 50 µM (P < 0.001). The ROS scavengers N-acetylcysteine and Trolox prevented VEGF-induced ROS generation (data not shown). However, the sulfhydryl group-free ethanolamine had no effect on VEGF-induced ROS generation at any concentration (up to 500 µM), demonstrating that the sulfhydryl group is essential for antioxidant activity of cysteamine. Consistently, ethanolamine had no DPPH free radical scavenging activity (data not shown).

We further investigated the effect of cysteamine on hyperglycemia-induced ROS generation in the retina of diabetic mice (Fig. 4A). We found that ROS levels were elevated in diabetic compared to normal mouse retinas. Cysteamine prevented this hyperglycemia-induced ROS generation. The changes in retinal ROS levels are represented by line profiles displaying the distribution of DHE RFIs, as shown by dotted lines crossing the mouse retina. We then quantitatively analyzed ROS generation by measuring the RFIs of DHE in retinal tissues (Fig. 4B). The average ROS level in the diabetic retina was approximately...
2.8-fold higher than that in normal mice (P<0.001, n=6). Cysteamine reversed this hyperglycemia-induced increase of ROS levels in diabetic retinas (P<0.001, n=6). Consistent with in vitro findings, ethanolamine had no inhibitory effect on the hyperglycemia-induced ROS generation in the retinas of diabetic mice (Fig. 4A and B). Thus, our results suggest that the sulfhydryl group of cysteamine is required for inhibiting ROS generation, but not essential for preventing vascular leakage in diabetic mouse retinas.

Cysteamine and ethanolamine inhibits VEGF-induced TGase activation in endothelial cells and hyperglycemia-induced TGase activation in the retinas of diabetic mice

Because the antioxidant activity of cysteamine is not essential for preventing vascular leakage, we examined the effect of cysteamine on VEGF-induced TGase activation in HUVECs (Fig. 5). VEGF increased in situ TGase activity in a time-dependent manner, with maximal activation at 2h (P<0.001, Fig. 5A), and cysteamine inhibited VEGF-induced TGase activation in a concentration-dependent manner, with maximal effect at 500 µM (P<0.001, Fig. 5B). Ethanolamine also inhibited VEGF-induced TGase activation in a concentration-dependent manner. The TGase inhibitors cystamine and monodansylcadaverine (MDC) prevented VEGF-induced elevation of TGase activity (data not shown). These results demonstrate that the primary amine of cysteamine and ethanolamine is involved in preventing VEGF-induced TGase activation in endothelial cells.

We then investigated the effect of cysteamine on hyperglycemia-induced TGase activation in the diabetic retinas (Fig. 6A). In vivo TGase activity was highly elevated in the blood vessels and ganglion cells of the diabetic compared to the normal mouse retina (n=6); this hyperglycemia-induced TGase activation was suppressed by intravitreal injection of cysteamine. We quantitatively analyzed in vivo TGase activity by determining the fluorescence intensities of FITC-conjugated streptavidin in retinal tissues (Fig. 6B). The average TGase activity in the diabetic retina was approximately 2.5-fold higher than that of normal mice (P<0.001, n=6), and cysteamine prevented this increase (P<0.001, n=6). Ethanolamine also prevented hyperglycemia-induced TGase activation in diabetic retinas (Fig. 6A and B). Consistent with a previous report (Lee et al. 2016), the TGase inhibitors cystamine and MDC inhibited hyperglycemia-induced vascular leakage in the retinas of diabetic mice (data not shown), demonstrating the important role of TGase in hyperglycemia-induced vascular permeability. Taken together, our results demonstrate that the primary amine of cysteamine plays a key role in the inhibition of hyperglycemia-induced TGase activation and subsequent vascular leakage in the retinas of diabetic mice.
Discussion

It has been suggested that cysteamine has beneficial effects against diseases such as cystinosis, Huntington’s disease and Parkinson’s disease. The various functions of cysteamine include: depletion of lysosomal cysteine in cystinosis, antioxidative effects through increasing intracellular glutathione levels and regulating enzyme activity (e.g., caspase 3 and transglutaminases) (Shin et al. 2011, Wilmer et al. 2011, Besouw et al. 2013). In addition, cysteamine affects the gene expression of brain-derived neurotrophic factor and heat shock proteins, among others (Besouw et al. 2013). Here, we describe a new function of cysteamine for preventing hyperglycemia-induced vascular leakage in the retinas of diabetic mice. Hyperglycemia significantly elevated the levels of VEGF in the retinas of diabetic mice ($P<0.01$; Table 1). Cysteamine inhibited VEGF-induced TGase activation in HUVECs, and intravitreal injection of cysteamine into the eyes of streptozotocin-induced diabetic mice prevented vascular leakage by inhibiting hyperglycemia-induced TGase activation in diabetic mouse retinas.

TGase activation is essential for VEGF-induced vascular leakage in the retinas of diabetic mice (Lee et al. 2016), and we found that cysteamine prevented this leakage by inhibiting hyperglycemia-induced TGase activation. Due to the key role of TGase in vascular leakage in the diabetic retina, it is important to investigate TGase inhibitors to identify potential drugs for prevention and treatment of diabetic retinopathy. We demonstrated the inhibitory effect of cysteamine on VEGF-induced TGase activation using an in situ TGase activity assay in HUVECs. Furthermore, using an in vivo TGase activity assay, we found that intravitreal injection of cysteamine inhibited hyperglycemia-induced TGase activation in diabetic mouse retinas. Thus, the sequential application of confocal microscopy-based in situ and in vivo TGase activity assays is a useful approach for identifying potential drugs to prevent TGase-associated diseases, including diabetic retinopathy, and for investigating physiological functions of TGase in cells and tissues.

We demonstrated that cysteamine is a bifunctional aminothiol with ROS scavenging and TGase-inhibiting activities. Here, we found that the antioxidant activity of cysteamine was attributed by the sulfhydryl group. Ethanolamine, a sulfhydryl group-free cysteamine analog, had no DPPH scavenging activity and no effect on VEGF-induced ROS generation, while cysteamine had a ROS scavenging effect in vitro and in situ. The primary amine group of cysteamine played an essential role in the inhibition of VEGF-induced TGase activation in endothelial cells and diabetic retinas. Intracellular ROS generation and/or TGase activation have been implicated in the disease pathogenesis of diabetic complications including diabetic retinopathy and cardiovascular diseases, as well as neurodegenerative diseases including Huntington’s disease and Parkinson’s disease (Park et al. 2010, Besouw et al. 2013, Bhatt et al. 2013, 2016, Lee et al. 2016).

Table 1 Clinical data from age-matched non-diabetic control and two-week diabetic mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice (n)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Non-fasting blood sugar (mmol/L)</td>
<td>$8.1\pm0.9$</td>
<td>$29.3\pm3.7$</td>
</tr>
<tr>
<td>Retinal VEGF (pg/mg)</td>
<td>$161.5\pm9.8$</td>
<td>$250.9\pm16.2^{**}$</td>
</tr>
</tbody>
</table>

Data are means ± s.d. $^{**}P<0.01$ vs control mice.
Thus, cysteamine may be used as a multifunctional drug for preventing diseases involving ROS generation and/or TGase activation.

Amine compounds are potential competitive TGase inhibitors by competing with natural amine substrates in the transamidation reaction. We showed that cysteamine and ethanolamine inhibited TGase activation in endothelial cells. Polyamines, including cystamine, putrescine, spermine and spermidine, are effective substrates for TGase and can be used as competitive TGase inhibitors (Lentini et al. 2004, Lee et al. 2016); the monoamine MDC is a well-known TGase inhibitor (Lee et al. 2016, Bhatt et al. 2013). The transamidation of monoamines, including serotonin, dopamine and norepinephrine, is mediated by TGase (Hummerich et al. 2012). Thus, amine compounds, including monoamine and polyamines, might have potential as drugs for treating TGase-associated diseases.

In this study, intravitreal injection of cysteamine prevented hyperglycemia-induced TGase activation and vascular leakage in the retinas of diabetic mice. Low-molecular-weight inhibitors prevent TGase transamidating activity, and the most widely used TGase inhibitor is cystamine, the dimer form of cysteamine (Basso & Ratan 2013). Previous clinical and preclinical trials using cystamine for treatment of neurodegenerative diseases and cystic fibrosis suggest that cystamine could be used for prevention of diabetic vascular leakage. Consistent with a previous report (Lee et al. 2016), we demonstrated that intravitreal injection of cysteamine prevents vascular leakage in diabetic mouse retinas. Thus, TGase inhibitors, including cysteamine and cystamine and TGase 2-specific siRNA, may have the potential for topical ocular application to prevent diabetic retinopathy or other TGase2-associated ocular diseases.

In conclusion, we found that cysteamine prevents vascular leakage by inhibiting hyperglycemia-induced TGase activation rather than ROS generation in the retinas of diabetic mice. The primary amine group of cysteamine was involved in VEGF-induced endothelial permeability and vascular leakage. The sulfhydryl group of cysteamine was important for inhibiting ROS generation, but not essential for TGase activation and vascular leakage. Thus, cysteamine has a potential for treating diabetic retinopathy and TGase-associated diseases.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Y-J L and S-H J designed and performed experiments, analyzed data and wrote the manuscript. J Y H contributed to samples and analyzed data. S J, E-T H, W-S P, S-H H and Y-M K designed experiments and analyzed data. K-S H conceptualized the study, designed experiments, analyzed and interpreted the data and wrote the manuscript. All authors have approved the final version of the manuscript.
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Cysteamine prevention of vascular leakage


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