

# Increased membrane localization of pannexin1 in human corneal synaptosomes causes enhanced stimulated ATP release in chronic diabetes mellitus

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## Abstract

In the present study, we investigated the potential changes in the corneal nerve terminals in non-insulin-dependent diabetes mellitus of moderate duration. The dissected corneas were subjected to a protocol of ultracentrifugation to obtain synaptosomes of sensory nerve terminals. Within these nerve varicosities, 2 major mechanisms were examined, viz., alterations of the mechanosensitive channel pannexin1 and ATP release on stimulation of these terminals. We hypothesized that altered cellular location and function of the pannexin channel may contribute to altered mechanosensitivity of the cornea, which in turn may affect wound healing and primary visual function of the cornea. The chief rationale for focusing on examining the pannexin channel is due to its role in mechanosensitivity, as well as its glycosylation property. Pannexin1 remains unchanged between diabetic subjects in comparison to nondiabetic controls. However, lectin immunoassay showed that pannexin1 is significantly more glycosylated in diabetic corneal synaptosomes. Membrane biotinylation assay showed that membrane localization of pannexin1 is significantly enhanced in diabetic samples. Furthermore, S-nitrosylation of the glyco-pannexin1 is significantly decreased in comparison to pannexin1 obtained from corneal varicosities of normoglycemic subjects. The diabetic corneal synaptosomes show enhanced ATP release after potassium chloride stimulation, when compared to controls. Furthermore, we have shown that S-nitrosylation of pannexin1 actually diminishes the ability of pannexin1 to release ATP. Thus, much like the peripheral nerves, the corneal nerves also show increased hypersensitivity in diabetes of chronic duration. All of these pathological changes may cumulatively alter corneal function in diabetes.

**Abbreviations:** CBX = carbenoxolone, NO = nitric oxide, PBS = phosphate-buffered saline.

**Keywords:** ATP, cornea, glycosylation, mechanosensitive, synaptosomes

## 1. Introduction

Chronic diabetes mellitus causes a wide variety of subtle and frank pathological changes in the eye.<sup>[1–5]</sup> These changes affect organs both in the anterior chamber and in the posterior chamber of the eye. The pathological changes may occur in a highly stochastic fashion. Also, some of these changes are potentially irreversible, including impaired epithelial healing, the formation of cataracts, wet exudates, and retinal ischemia.<sup>[6–13]</sup> A characteristic neurological complication of moderate long-standing type II diabetes mellitus and with inadequate therapeutic efficiency involves neuropathy of fine unmyelinated fibers.<sup>[14–16]</sup> Patients often have complaints of increased somatic hypersensi-

tivity, including feelings of grit or foreign body in the eye, increased eye rubbing, and increased watering. These changes often lead to numerous clinic visits, primarily because of potential refractive errors. However, these neuropathic changes involving the corneal nerves may also be a surrogate measure of progressive pathophysiologic changes due to ineffective control of circulating blood glucose.

In the present study, we wanted to perform a basic investigation of the potential changes in the corneal nerve terminals in non-insulin-dependent diabetes mellitus of moderate duration and with less than optimal medical management. The dissected corneas were subjected to a protocol of ultracentrifugation to obtain synaptosomes of sensory nerve terminals. Within these nerve varicosities, 2 major mechanisms were examined, viz., alterations of the mechanosensitive channel pannexin1 and ATP release on stimulation of these terminals. We hypothesized that altered cellular location and function of the pannexin channel may contribute to altered mechanosensitivity of the cornea, which in turn may affect wound healing and primary visual function of the cornea. The chief rationale for focusing on examining the pannexin channel is due to its role in mechanosensitivity in multiple cells, including the retina and cornea,<sup>[17–21]</sup> as well as its propensity to get biochemical modification by glycosylation on cysteine residues.<sup>[22,23]</sup>

## 2. Materials and methods

### 2.1. Human cornea samples

Explicit permission was obtained from Institutional Review Board of Chinese PLA General Hospital, China, and all experiments were performed according to Helsinki guidelines. Men between the ages of 50 and 70 years were included in the

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present investigation. Age-matched samples were used for control studies. A total of 60 freshly obtained whole-thickness cornea samples from the eye bank (30 controls and 30 documented type II diabetes mellitus of >3-year duration) were used for obtaining corneal synaptosome samples. Experiments were performed by pooling samples and performing the studies in triplicates for each condition. Written consent was obtained from the subjects' families postmortem to conduct the study.

## 2.2. Antibodies and chemicals

All antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA), and chemicals from either Sigma-Aldrich (Shanghai, China) or Tocris Bioscience (Avonmouth, Bristol, UK).

## 2.3. Corneal synaptosome preparation

Corneal tissues were homogenized in a Brinkmann homogenizer. This was thereafter cold centrifuged at increasing speeds of 1000 to 10,000 g to obtain a clear supernatant and a largely epithelial pellet. The supernatant was decanted, and overlaid on a 0.3/0.8-M sucrose gradient and centrifuged at  $15,000 \times g$  for 30 minutes. A scum-like ring was formed at the interface, which was carefully aspirated. This layer tested positive for synaptophysin, neuro-beta-tubulin III, and transient receptor potential vanilloid 4 in Western blotting (data not shown), confirming that the synaptosomal layer was isolated. This was aliquoted in microfuge tubes and stored at  $-20^{\circ}\text{C}$  until further experiments. For obtaining membranes of varicosities, the synaptosomal rich layer was ultracentrifuged at  $100,000 \times g$  for 1 hour in the cold in the presence of a  $\text{Mg}^{2+}$ -supplemented isotonic buffer. Membrane varicosities were stored for further experiments related to membrane localization of pannexin channels.

## 2.4. Lectin immunosorbent assays to identify glyco-pannexin

Lectin immunosorbent assay is a method akin to enzyme-linked immunosorbent assays, using lectins as probes to identify glycan structures.<sup>[24]</sup> Buffer composition was 10 mM phosphate-buffered saline (PBS) containing 150 mM sodium chloride, pH 7.2, at  $4^{\circ}\text{C}$ . The wells containing the synaptosomal lysates were washed several times with PBS containing 0.05% (w/v) Tween 20 and blocked with bovine serum albumin. A total of 100  $\mu\text{L}$  of biotinylated *Ulex europaeus* agglutinin I lectin at 0.6  $\mu\text{g}/\text{mL}$  (Vector Laboratories, Burlingame, CA) in PBS was added to each well and allowed to incubate for 1 hour. A total of 100  $\mu\text{L}$  of 1:3000 dilution of 1 mg/mL streptavidin conjugated to alkaline phosphatase (Vector Laboratories) was added and signal was developed by incubation for 30 minutes with 100  $\mu\text{L}$  of 1 mg/mL *para*-nitrophenyl phosphate substrate (Sigma-Aldrich). The reaction was stopped using 50  $\mu\text{L}$  of 3 M sodium bicarbonate. Negative and positive controls were used for all of the experiments.

## 2.5. Biotin switch assay to assess pannexin S-nitrosylation

Basal S-nitrosylation signals of pannexin1 of control as well as diabetic corneal varicosities were examined by biotin switch assay, based on protocols described earlier.<sup>[25]</sup> Briefly, varicosities were incubated with the nitric oxide (NO) donors, 100  $\mu\text{M}$  S-nitrosoglutathione, 50  $\mu\text{M}$  diethylamine NONOate, or untreated (diabetic/control) for 15 minutes in an incubator at  $37^{\circ}\text{C}$ . The varicosities were subsequently washed with PBS, and further incubated with radioimmune precipitation assay buffer with added protease inhibitors. Protein quantity was estimated using

the Bradford technique. Proteins were pelleted by acetone and centrifugation. The unbound cysteine thiols were inhibited by N-ethylmaleimide for 30 minutes. Proteins were again precipitated to decant excess N-ethylmaleimide. Thereafter, S-nitrosylated cysteines were altered of their oxidizing potential with 1 mM ascorbate and 1 mM  $\text{Cu}^{2+}$  and subsequently biotinylated with N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide for 1 hour. Biotinylated proteins were immobilized with streptavidin-agarose beads. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed for detection of pannexin that was S-nitrosylated. Ascorbate was not reduced to assay the specificity of the reducing controls.

## 2.6. Cell surface biotinylation to identify cell membrane-bound pannexin

Cell surface biotinylation was performed to assess the membrane distribution of pannexin1 channels in synaptosomes, and whether they were altered in diabetic varicosities, using protocols described earlier.<sup>[26]</sup> Corneal varicosities from control (normoglycemic) and diabetic patients, with and without glycosidase, were washed with cold PBS and then treated with Dulbecco's Modified Eagle Medium and 50  $\mu\text{M}$  carbenoxolone (CBX) over ice for an hour. CBX toxin prevents biotin from percolating through Panx1 channels and prevents labeling intracellular proteins. The varicosities were further incubated in cold PBS containing EZ-Link sulfosuccinimidyl-6-[biotinamido]hexanoate (1 mg/mL), including CBX (50  $\mu\text{M}$ ). Proteins were eluted and equal amounts of protein were incubated with streptavidin-agarose beads overnight in the cold room to pull down biotinylated proteins. Membrane-bound pannexin1 was detected by Western blotting.

## 2.7. In vitro ATP release assay of corneal synaptosomes

Synaptosomes from control and diabetic patients were incubated in isotonic 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid media in an incubator at  $37^{\circ}\text{C}$  for an hour, and stimulated with 100 mM potassium chloride. A total of 200  $\mu\text{M}$  ARL 67156 ectonucleotidase was present in the medium. Glycosidase inhibitors, as well as NO donors, were added to the medium to assess their effects on ATP release. The medium was aspirated and cold stored. A total of 30  $\mu\text{L}$  of luciferin-luciferase reagent (ATP luminescence assay, Perkin Elmers, Waltham, MA) was added to a well, followed by detection of the optical density of luminescence (Luminoskan Ascent Microplate Luminometer, Thermo Scientific, Waltham, MA, USA). This was utilized to plot the standard curve for estimation of ATP concentration. The percentage changes of ATP were used to compare ATP release between nondiabetic and diabetic corneal nerve varicosities.

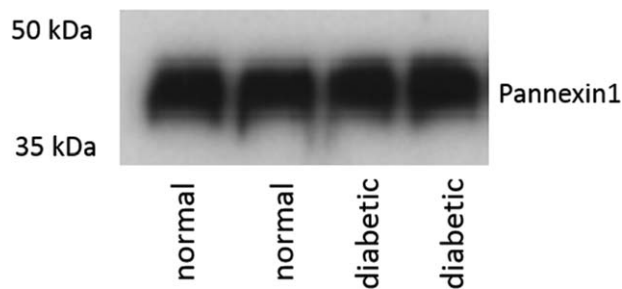
## 2.8. Statistics

Results are presented as means  $\pm$  standard error. Statistical significance was determined by  $P < 0.05$  using analyses of variance.

## 3. Results

### 3.1. Total pannexin1 levels unchanged in diabetic as well as control, nondiabetic synaptosomes

Western blotting revealed that the total pannexin levels of type 1 isoform remained unchanged between diabetic and nondiabetic



**Figure 1.** Total pannexin1 levels unchanged in diabetic as well as control, nondiabetic synaptosomes. Western blot showing unchanged total pannexin1 between diabetic and nondiabetic controls. Two representative lanes are shown for each group, which are pooled samples from 10 cornea samples in each lane.

controls (Fig. 1). The band intensities were estimated from 3 independent pooled samples in each group, and did not show any significant difference (data not shown). Representative images from 2 pooled samples in each group are demonstrated in Fig. 1.

### 3.2. Lectin immunoassays reveal increased glycosylation of pannexin1 in diabetic corneal synaptosomes

Quantitative lectin immunoassays revealed increased glycosylated pannexin1 levels in corneal nerves obtained from patients with long-standing diabetes, when compared to age-matched controls. When the synaptosomal samples of diabetic synaptosomes were incubated in glycosidase, these increased glycosylated pannexin1 levels decreased significantly. The distribution of these values, performed in triplicates to reveal the quantitative data, is shown in Fig. 2.

### 3.3. S-Nitroso-pannexin1 in diabetic corneal synaptosomes

Figure 3 shows the reduction of S-nitroso-pannexin1 in synaptosomes obtained from diabetic nerve terminal in the biotin switch assay. Normal control varicosities showed significantly increased S-nitrosylation signals, which were also

enhanced by external stimulation with the NO donors. Synaptosomal treatment with the reducing agent dithiothreitol (1 mM) immediately after S-nitrosoglutathione or diethylamine NONOate treatment to reduce S-nitrosothiols inhibited biotinylation. In addition, S-nitrosylation of Panx1 was not seen when ascorbic acid was not added to prevent the availability of free S-nitrosylated cysteines for subsequent biotinylation.

### 3.4. Increase in membrane pannexin1 in diabetes

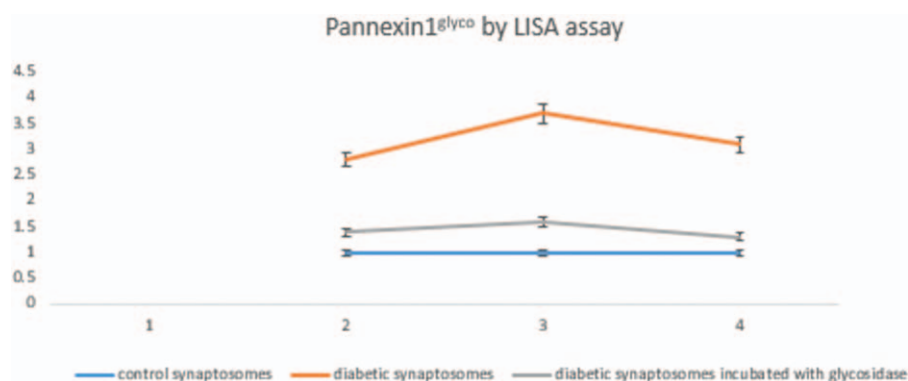
Membrane biotinylation assay revealed signals of enhanced intensity in diabetic synaptosomes, indicating that glycosylated pannexin1 is located at the membranes in comparison to controls (Fig. 4A). When diabetic synaptosomes were incubated with a glycosidase, these membrane-bound signals were decreased. Representative blots of the western blotting signals of the membrane-bound pannexins are also shown in Fig. 4B.

### 3.5. Increase in ATP release in diabetic corneal synaptosomes

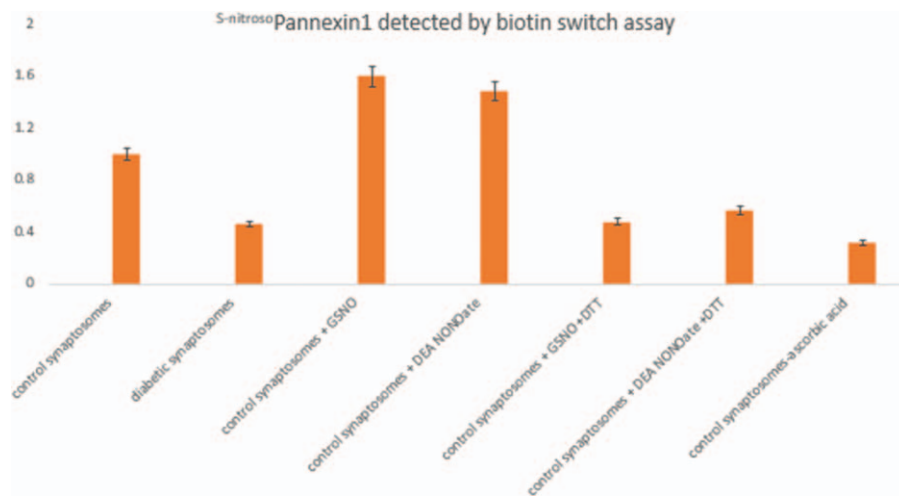
Potassium stimulation enhanced ATP release of diabetic synaptosomes, in comparison to controls (Fig. 5). NO-donor--incubated control synaptosomes, as well as glycosidase-treated diabetic synaptosomes, showed minimum ATP release on nerve stimulation by potassium chloride.

## 4. Discussion

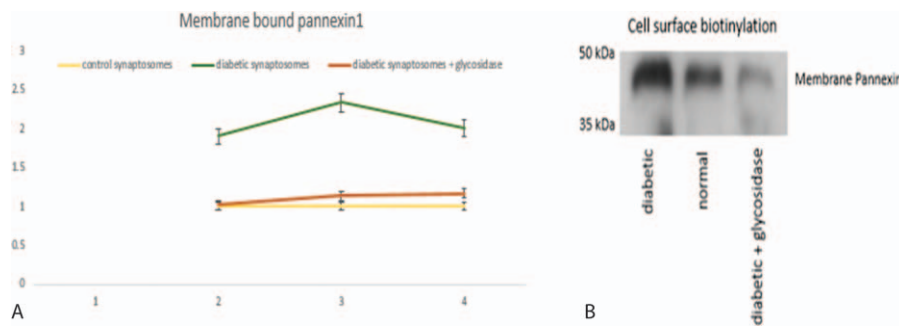
The results of the present study reveal unique mechanistic changes in the mechanosensitive pannexin1 channel in human corneal synaptosomes in subjects with long-standing type II diabetes mellitus. Namely, using a combination of relevant molecular techniques to track intracytologic trafficking, evidence has been provided that although the levels of pannexin1 remain unchanged between diabetic subjects in comparison to nondiabetic controls, pannexin1 is significantly more glycosylated in diabetic corneal synaptosomes, and its membrane localization is significantly enhanced in comparison to controls. Furthermore, we have demonstrated that S-nitrosylation of the glyco-pannexin1 is significantly decreased in comparison to pannexin1 obtained from corneal varicosities of normoglycemic subjects.



**Figure 2.** Lectin immunoassays reveal increased glycosylation of pannexin1 in diabetic corneal synaptosomes. Quantitative lectin immunoassays revealed increased glycosylated pannexin1 levels in corneal nerves obtained from patients with long-standing diabetes, when compared to age-matched controls. When the synaptosomal samples of diabetic synaptosomes were incubated with glycosidase, the increased levels of glycosylated pannexin1 levels decreased significantly. The distribution of these values from pooled samples is shown. The Y-axis represents arbitrary units of normalized values with respect to controls. LISA = lectin immunosorbent assay.



**Figure 3.** S-Nitroso-pannexin1 in diabetic corneal synaptosomes. Biotin switch assay revealed diminution of S-nitroso-pannexin1 in synaptosomes obtained from diabetic nerve terminals. Normal control varicosities showed significantly increased S-nitrosylation signals, which were also enhanced by external stimulation with the nitric oxide donors GSNO and DEA NONOate. Synaptosomal treatment with the reducing agent DTT (1 mM) immediately after GSNO or DEA NONOate treatment to reduce S-nitrosothiols prevented the biotinylation reaction. In addition, S-nitrosylation of Panx1 was not seen when ascorbic acid was not added to prevent the availability of free S-nitrosylated cysteines for subsequent biotinylation. DEA NONOate = diethylamine NONOate, DTT = dithiothreitol, GSNO = S-nitrosoglutathione.

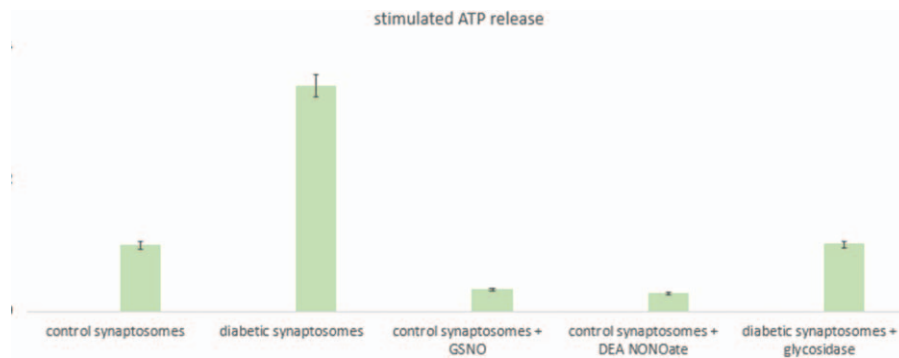


**Figure 4.** Increase in pannexin1 in corneal synaptosomal membrane in diabetes. Membrane biotinylation assay revealed signals of enhanced intensity in diabetic synaptosomes, indicating that glycosylated pannexin1 is located at the membranes in comparison to controls (A). When diabetic synaptosomes were incubated with a glycosidase, these membrane-bound signals were decreased, as shown in “B.”

The S-nitrosylation of pannexin1 may be a significant mechanism in gating the channel for its mechanochemical activity.

Namely, pannexin1 plays a significant role in ATP release in response to external mechanical cues.<sup>[19–21]</sup> We have also

demonstrated in the present study that diabetic corneal synaptosomes show enhanced ATP release, when compared to controls. Furthermore, we have shown that S-nitrosylation of pannexin1 actually diminishes the ability of pannexin1 to release



**Figure 5.** Increase in ATP release in diabetic corneal synaptosomes. Potassium chloride stimulation enhanced ATP release of diabetic synaptosomes, in comparison to controls. NO-donor-incubated control synaptosomes, as well as glycosidase-treated diabetic synaptosomes, showed minimum ATP release on nerve stimulation by potassium chloride. DEA NONOate = diethylamine NONOate, GSNO = S-nitrosoglutathione, NO = nitric oxide.

ATP. Thus, much like the peripheral nerves, the corneal nerves also show increased hypersensitivity in diabetes of chronic duration. This altered mechanosensitivity of the corneal nerves may affect visual refraction of the cornea and epithelial healing, as well as affect aqueous humor flow, functions that are directly affected by mechanosensitive pannexin channels in the cornea.<sup>[6–8,27–30]</sup> The reduction in S-nitrosylation may have resulted from alterations of neuronal nitric oxide synthase in diabetes. All of these pathological changes may cumulatively alter corneal function in diabetes. These functional aspects remain our future scope of investigation.

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