INCREASED CONNEXIN43 EXPRESSION CORRELATES WITH THE LOSS OF BLOOD VESSEL INTEGRITY IN DIABETIC RETINOPATHY

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Purpose

Diabetic retinopathy (DR) is a chronic disease that develops due to hyperglycaemia and inflammation-induced vascular disruptions in the retina. Previous studies suggest a decrease in Cx43 expression in hyperglycaemia-only models that do not take the role of inflammation into account. Therefore, this study investigated the interplay between hyperglycaemia and inflammation, and also evaluated the pattern of Cx43 expression using endothelial cells, mouse and human donor retinas.

Methods

Primary human retinal microvascular endothelial cells (hRMECs) were exposed to high glucose (25 mM) or pro-inflammatory cytokines IL-1β and TNF-α (10 ng/mL each) or both. The lactate dehydrogenase (LDH) assay was used as a measure of cell death and Cx43 expression was determined using immunohistochemistry. Cx43, glial fibrillary acidic protein (GFAP), and plasmalemma vesicular associated protein (PLVAP) were labelled in retinal sections of 24 week old wild-type (C57BL/6), Akita (diabetic) and Akimba (diabetic retinopathy) mice. Cx43 and GFAP expression was also studied in human donor retinas with confirmed DR and compared to normal tissues. All statistical comparisons were performed using one-way ANOVA with Tukey's test.

Results

Co-application of high glucose and pro-inflammatory cytokines **increased** LDH release in hRMECs relative to high glucose or pro-inflammatory cytokines alone. There was **increased** expression of Cx43 and GFAP in the ganglion cell layer in Akimba mice compared to wild-type but not Akita mice. On PLVAP-positive vessels, Cx43 was **higher** in Akimba mice but unchanged in Akita compared to wild-type. Cx43 expression was **elevated** in human donor retinas with confirmed DR compared to normal, correlating with the *in vitro* and mouse tissue results.

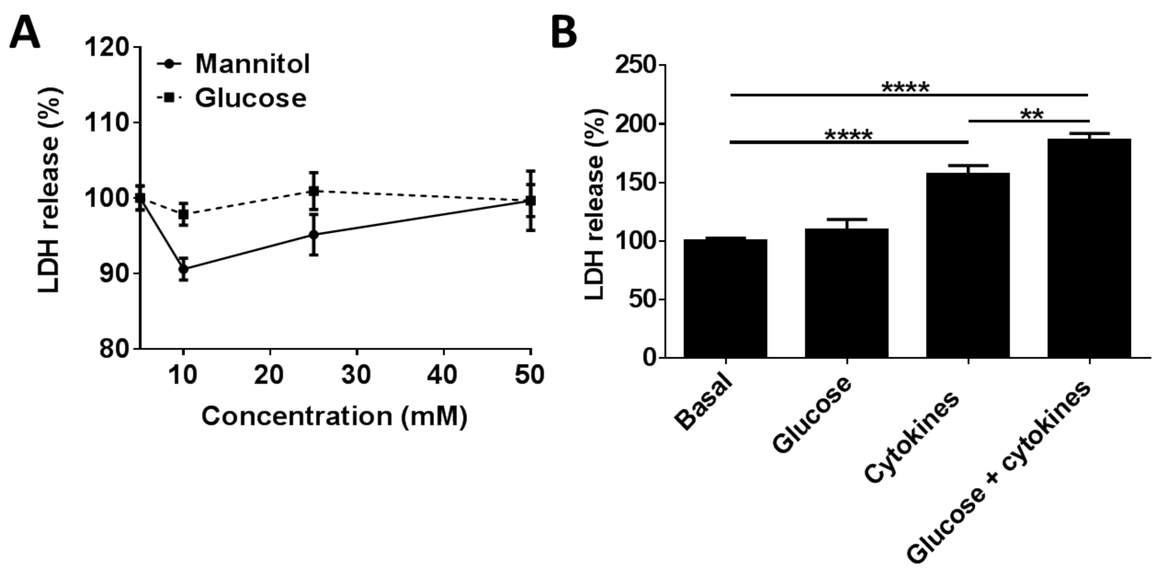

Figure 1: Effect of high glucose and pro-inflammatory cytokines on LDH release by hRMECs.
(A) Increasing concentrations of glucose (or mannitol – osmolarity control) did not affect LDH release by hRMECs. (B) High glucose (25 mM) alone did not increase LDH release but exacerbated pro-inflammatory cytokine (IL-1β and TNF-α)-mediated LDH release in hRMECs compared to high glucose ($p < 0.0001$), cytokines only ($p = 0.0003$) and basal ($p < 0.0001$) conditions. Data presented as mean ± SEM; n = 6; t = 24 h; **p ≤ 0.01; ****p ≤ 0.0001.

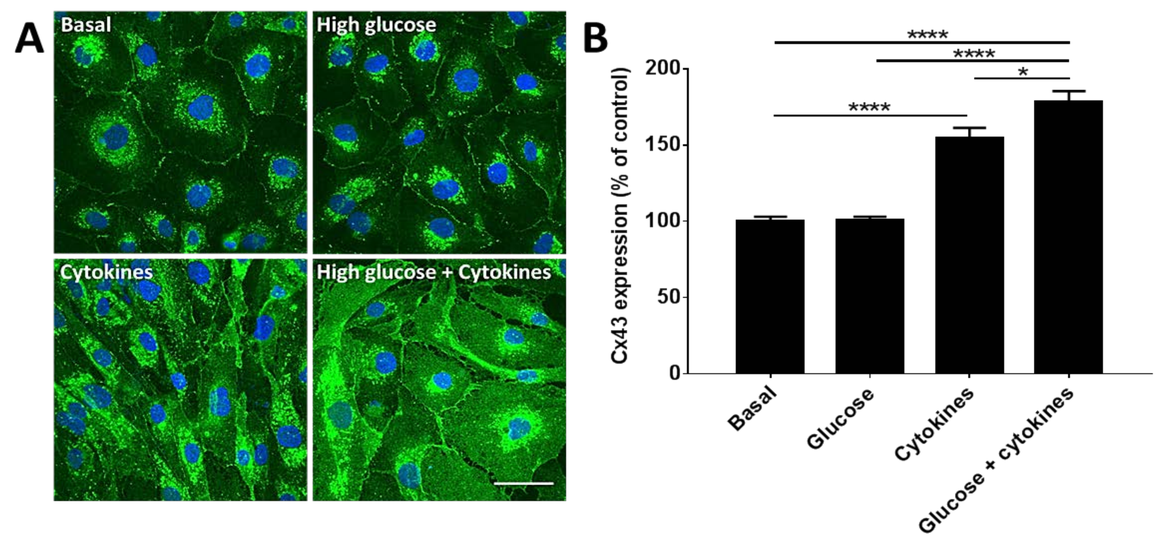

Figure 2: Effect of high glucose and pro-inflammatory cytokines on Cx43 expression in hRMECs.
(A) Cx43 expression in basal, high glucose, pro-inflammatory cytokine (IL-1β and TNF-α), and a combination of treated cells. (B) Quantification of Cx43 expression relative to basal levels. High glucose did not affect Cx43 expression while the cytokines increased Cx43 expression ($p > 0.0001$). Co-application of high glucose and pro-inflammatory cytokines further increased Cx43 levels relative to basal ($p < 0.0001$), high glucose ($p < 0.0001$) and pro-inflammatory cytokines alone conditions ($p = 0.0241$). Scale bar = 5 nm; Data presented as mean + SEM; n = 6; t = 24 h; *p ≤ 0.05; ****p ≤ 0.0001

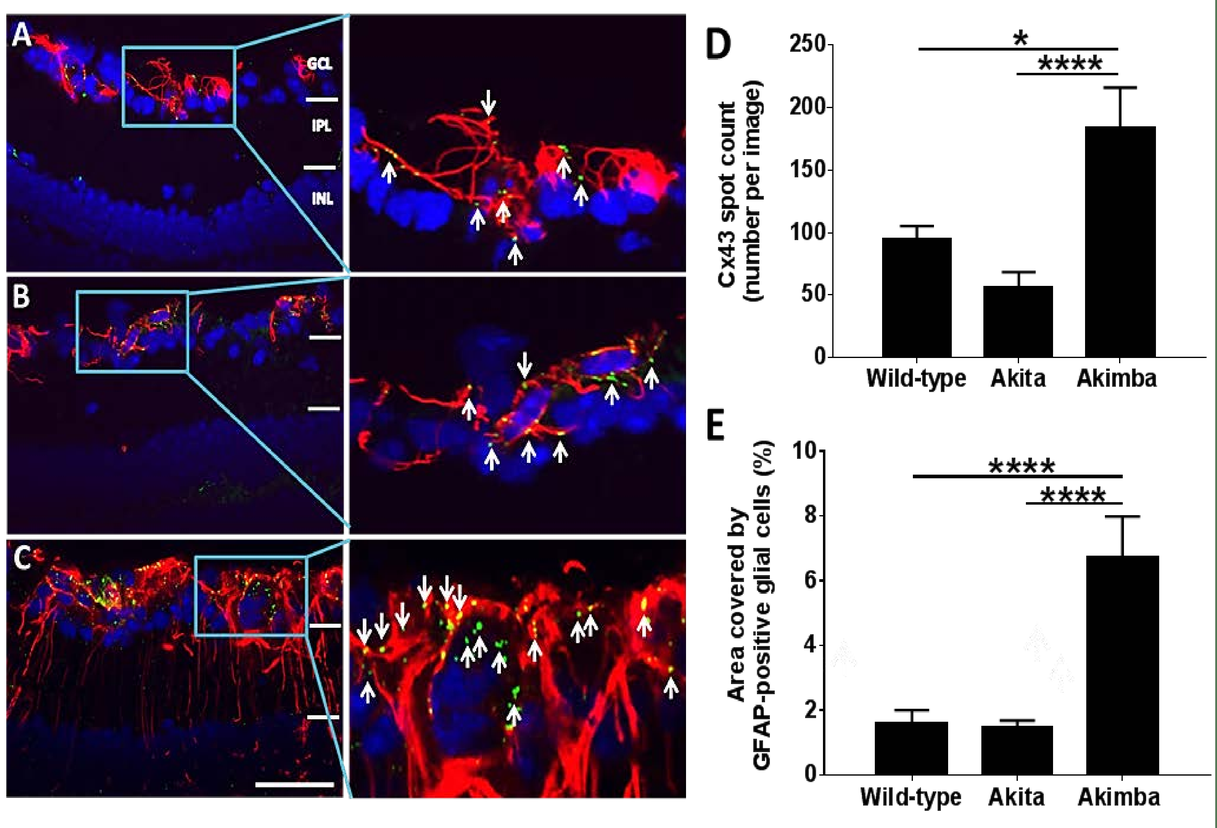

Figure 3: Cx43 and GFAP expression in retinas of wild-type, Akita and Akimba mice.
Cx43 (green) and GFAP (red) expression in (A) wild-type, (B) Akita and (C) Akimba mice. (D) Quantification of Cx43 (spots per image) and (E) quantification of the % area covered by GFAP labelling in wild-type, Akita and Akimba mice. There was no statistically significant difference in Cx43 spot counts between Akita and wild-type mice ($p = 0.3047$). However, the Cx43 spot count was higher in Akimba compared to wild-type ($p = 0.0159$) and Akita ($p < 0.0001$) mice. GFAP labelling was evident within the GCL only in wild-type and Akita but spanned all retinal layers in Akimba mice. Cx43 spots co-localized with GFAP in all mice strains. There was no statistically significant difference in GFAP labelling in Akita compared to wild-type mice ($p = 0.9878$). However, GFAP expression was significantly higher in Akimba compared to wild-type ($p < 0.0001$) and Akita ($p < 0.0001$) mice. GCL = Ganglion cell layer; IPL = Inner plexiform layer; INL = Inner nuclear layer. Scale bar: 50 µm; mean + SEM; n = 4 animals/group; * $p = 0.05$; ****$p < 0.0001$.

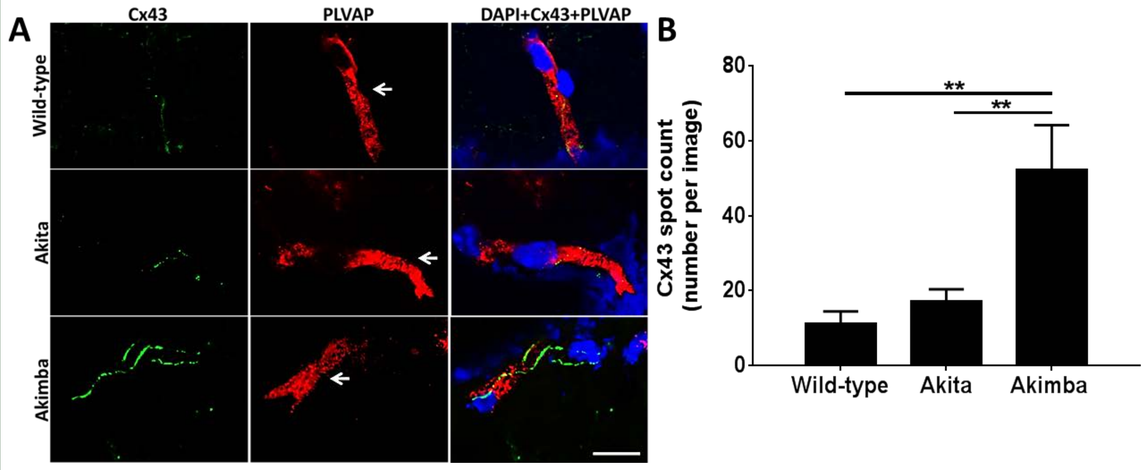

Figure 4: Cx43 expression in PLVAP-positive blood vessels in the IPL layer of wild-type, Akita and Akimba retinas.
(A) Cx43 expression (green) in blood vessels (PLVAP, red) in the IPL of wild-type, Akita and Akimba mouse retinas. Blood vessels are indicated by white arrows. (B) Quantification of the number of Cx43 spots per blood vessel shows that there is no significant difference in number of Cx43 spots in blood vessels of Akita compared to wild-type mice while Akimba mice showed significantly higher Cx43 spot count number compared to the wild-type ($p = 0.0011$) and Akita ($p = 0.0011$) retinas. IPL = Inner plexiform layer; Scale bar: 10 µm; n = 4 animals/group; mean + SEM; **p ≤ 0.01.

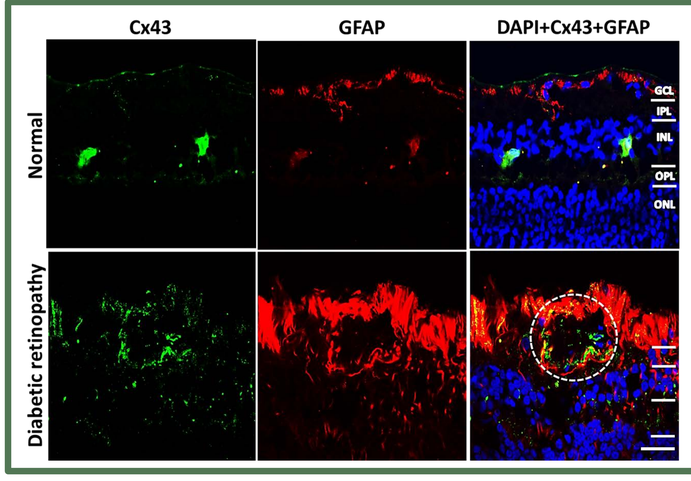

Figure 5: Cx43 and GFAP expression in normal and DR human donor retinas.
Cx43 (green) and GFAP (red) expression in normal and DR human donor retinal sections in regions of extensive vascular damage. Cx43 expression was higher in the GCL of DR donors compared to age-matched controls, and also strongly expressed throughout all retinal layers. GFAP labelling was also markedly higher in DR compared to normal donor eyes. Cx43 was increased in regions identified as blood vessels and was correlated with increased GFAP labelling at these sites, indicating glial cell activation (white circle). GCL = Ganglion cell layer; IPL = Inner plexiform layer; INL = Inner nuclear layer; OPL = Outer plexiform layer and ONL = Outer nuclear layer. Scale bar: 100 µm.

Conclusion

The pathology of diabetic retinopathy seems to require the concerted action of hyperglycaemia and inflammation and is associated with increased Cx43 expression in areas of astrocytosis and neovascularization. These findings support a causal role of Cx43 channels in disease progression. Therefore, targeting Cx43 channels may be an effective strategy for the treatment of diabetic retinopathy.