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Pannexin-2-deficiency sensitizes pancreatic β-cells to cytokine-induced apoptosis *in vitro* and impairs glucose tolerance *in vivo*

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ABSTRACT

Pannexins (Panx's) are membrane proteins involved in a variety of biological processes, including cell death signaling and immune functions. The role and functions of Panx's in pancreatic β-cells remain to be clarified. Here, we show *Panx1* and *Panx2* expression in isolated islets, primary β-cells, and β-cell lines. The expression of *Panx2*, but not *Panx1*, was downregulated by interleukin-1β (IL-1β) plus interferon-γ (IFNγ), two pro-inflammatory cytokines suggested to contribute to β-cell demise in type 1 diabetes (T1D). siRNA-mediated knockdown (KD) of Panx2 aggravated cytokine-induced apoptosis in rat INS-1E cells and primary rat β-cells, suggesting anti-apoptotic properties of Panx2. An anti-apoptotic function of Panx2 was confirmed in isolated islets from *Panx2*$^{-/-}$ mice and in human EndoC-βH1 cells. Panx2 KD was associated with increased cytokine-induced activation of STAT3 and higher expression of inducible nitric oxide synthase (iNOS). Glucose-stimulated insulin release was impaired in *Panx2*$^{-/-}$ islets, and *Panx2*$^{-/-}$ mice subjected to multiple low-dose Streptozotocin (MLDS) treatment, a model of T1D, developed more severe diabetes compared to wild type mice. These data suggest that Panx2 is an important regulator of the insulin secretory capacity and apoptosis in pancreatic β-cells.

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1. Introduction

Pannexins (Panx1-3) are a family of integral membrane proteins sharing topological similarities with the connexin gap junction proteins (Penuela et al., 2013; Sosinsky et al., 2011). Whereas connexins form intercellular junctions between adjacent cells, pannexins assemble into single membrane channels, allowing for the passage of small molecules such as ATP and glutamate, thereby eliciting biological effects different from gap junctions (Sosinsky et al., 2011; Locovei et al., 2006a). Panx1 and Panx3 are ubiquitously expressed at the mRNA and protein levels, and have been detected in the central nervous system (CNS), skeletal muscle, pancreas, spleen, skin, osteoblasts, and chondrocytes (Baranova et al., 2004; Penuela et al., 2007; Ishikawa et al., 2011; Bond et al., 2011). The evaluation of Panx2 tissue expression at the protein level has been hampered by the lack of adequate antibodies (Cigliola et al., 2015). Initial gene expression studies suggested that Panx2 transcriptional activity is largely restricted to the CNS (Baranova et al., 2004; Bruzzone et al., 2003), while a more recent report indicated ubiquitous expression of Panx2 (Le et al., 2014).

Numerous physiological roles have been ascribed to pannexins which may be implicated in several human diseases (Penuela et al., 2014). Panx1 is the most widely studied Pannexin member and is involved in variety of biological functions including Ca^{2+} wave initiation (Locovei et al., 2006b; Scemes et al., 2009), apoptotic signalling via the release of ATP (Chekeni et al., 2010), regulation of vasoconstriction (Billaud et al., 2011), and in facilitating HIV-1 viral

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infections (Seror et al., 2011). Panx1 may also be involved in the activation of the inflammasome and IL-1β release from macrophages *in vitro* (Pelegrin and Surprenant, 2006), although this has not been proven *in vivo* (Bargiotas et al., 2012; Wang et al., 2013). Recently, whole exome sequencing identified a patient with a homozygous missense and loss-of-function *PANX1* variant, who suffers from multiple organ pathologies thereby providing the first direct link between pannexin function and human disease (Shao et al., 2016).

Panx2 is involved in neuronal differentiation (Swayne et al., 2010) and growth regulation in glioma cells (Lai et al., 2009), whereas Panx3 is important for osteoblast, keratinocyte, and chondrocyte differentiation (Ishikawa et al., 2011; Iwamoto et al., 2010; Celetti et al., 2010). Functional studies utilizing *Panx1* knockout (*Panx1*$^{-/-}$), *Panx2* knockout (*Panx2*$^{-/-}$) and double knockout (*Panx1*$^{-/-}$*Panx2*$^{-/-}$) mice in a model of ischemia-induced neurodegeneration, showed that while *Panx2*$^{-/-}$ mice are partially protected from neurodegeneration, double knockout mice (*Panx1*$^{-/-}$*Panx2*$^{-/-}$) exhibit the most pronounced protection against ischemic neurological deficits (Bargiotas et al., 2012).

The role and functions of pannexins in pancreatic islets and β-cells remain unexplored. Recently, however, we identified Panx2 as an interaction partner in a type 1 diabetes (T1D) network generated from genetic data and protein-protein interactions (Bergholdt et al., 2012). Interestingly, we also found that *Panx2* transcripts were upregulated in human islets in response to treatment with pro-inflammatory cytokines (IL-1β + IFNγ + TNFα) (Bergholdt et al., 2012), suggesting that Panx2 might be involved in mediating the detrimental effects of the cytokines which are early mediators of β-cell apoptosis in T1D (Eizirik et al., 2009). Based on these findings, we set out to examine the expression and potential functional roles of pannexins in β-cells and islets in relation to immune-mediated β-cell apoptosis. We document the presence of *Panx1* and *Panx2* transcripts in islets and β-cells, and show that Panx2-deficiency leads to impaired insulin secretion, increased apoptosis and more severe diabetes in a mouse model of T1D.

2. Results

2.1. Panx2 expression is regulated by pro-inflammatory cytokines

We first investigated if transcripts of the three pannexin members are present in isolated whole mouse pancreatic islets and in FACS-purified β-, α-, and δ-cells. Brain and liver tissues were included as positive controls. Following mRNA extraction and preparation of cDNA, PCR was performed and the products visualized by agarose gel electrophoresis. As seen in Fig. 1A, *Panx1* and *Panx2* transcripts were present in both whole islets and β-cells, whereas only *Panx2* was present in α- and δ-cells. *Panx3* was only weakly detected, and appeared exclusively in β-cells. PCR analyses of insulin (*Ins1/2*), glucagon (*Gcg*), and somatostatin (*Sst*) confirmed the purity of the individual islet cell types (Fig. 1A). We next analyzed the presence of *Panx* transcripts in the β-cell lines INS-1E and MIN6, and in rat, mouse, and human islets. The results obtained further established expression of *Panx1* and *Panx2* in all samples analyzed, including human islets from three different donors, whereas the expression of *Panx3* was minimal and spurious (Fig. 1B).

Next, we examined if pro-inflammatory cytokines (IL-1β and IFNγ), known to contribute to β-cell impairment in T1D, affected the mRNA expression of *Panx1* and *Panx2* in INS-1E cells and rat islets. Quantitative real-time PCR showed that exposure of both INS-1E cells and rat islets to IL-1β plus IFNγ for 24 h caused a reduction in *Panx2* mRNA whereas *Panx1* transcripts were not regulated by cytokines (Fig. 1C and D). A more detailed time-course analysis in INS-1E cells revealed that the suppressive effect of cytokines on *Panx2* expression was already evident after 4 h of cytokine treatment (Fig. 1E). These findings indicate that *Panx1* and *Panx2* are expressed in islets and β-cells, and that *Panx2* mRNA expression is downregulated by pro-inflammatory cytokines in rat islets and INS-1E cells. Based on the observed suppressive effect of cytokines on *Panx2* expression we concentrated on Panx2 in all subsequent experiments.

2.2. Panx2 has anti-apoptotic properties in INS-1E cells

A recent study provided evidence for Panx2 in ischemia-induced neuronal damage (Bargiotas et al., 2011). Based on this and other accumulating evidence suggesting that Panx1 also plays a role in the regulation of cell death (Furlow et al., 2015; Zhao et al., 2015; Gulbransen et al., 2012) we evaluated if Panx2 could regulate cytokine-induced β-cell death. siRNA was used to knockdown the expression of Panx2 in INS-1E cells (Fig. 2A). Knockdown of Panx2 did not significantly affect the basal INS-1E cell death rate (Fig. 2B), but augmented IL-1β + IFNγ-induced cell death, as compared to that observed in cells transfected with a non-targeting negative control siRNA (siCTRL). A cytotoxicity assay confirmed that knockdown of Panx2 aggravated cytokine-mediated death (Fig. 2C), which correlated with increased caspase 3/7 activity (Fig. 2D) indicative of increased apoptotic cell death. To examine if knockdown of Panx2 also augmented cell death induced by other stimuli, we used the endoplasmic reticulum (ER) stress inducer thapsigargin. As opposed to cytokine-induced cell death, Panx2 knockdown did not potentiate thapsigargin-mediated cell death (Suppl Fig. 1).

Next, we examined if overexpression of Panx2 could counteract cytokine-induced apoptosis. INS-1E cells were transiently transfected with an expression plasmid encoding rat Panx2. Control experiments with a GFP-encoding plasmid revealed a transfection rate of ~50% (Fig. 2E). Overexpression of Panx2 partially inhibited the IL-1β + IFNγ-induced apoptosis, as revealed by decreased caspase 3/7 activity and reduced cleavage of caspase 3, as compared to the levels observed in cells transfected with an empty pcDNA plasmid (Fig. 2F and G). Collectively, these results suggest that Panx2 has anti-apoptotic properties in INS-1E cells.

2.3. Panx2-deficieny augments cytokine-induced cell death in primary β-cells, mouse islets, and human EndoC-βH1 cells

To confirm an anti-apoptotic role of Panx2 in primary β-cells, we next examined if Panx2 knockdown also modulated cytokine-mediated apoptosis in FACS-purified primary rat β-cells. β-cell preparations (95.4± 1.4% purity) were transfected with either siPanx2 or siCTRL. Knockdown of Panx2 caused a modest, but significant increase in cytokine-induced apoptosis, as compared to that observed in control-transfected cells (Fig. 3A and B). To further demonstrate an anti-apoptotic function of Panx2, we isolated islets from C57BL/6 wild type (WT) and Panx2-deficient (*Panx2*$^{-/-}$) mice and examined cell death. We found increased basal and cytokine-mediated cell death, as well as increased caspase 3/7 activity in *Panx2*$^{-/-}$ islets, as compared to WT islets (Fig. 3C-E). Cytokines also suppressed Panx2 expression in both purified primary β-cells transfected with siCTRL (Fig. 3A) and in WT islets (Fig. 3C) as seen in INS-1E cells and rat islets (Fig. 1C and D). Finally, we investigated the effects of knocking down Panx2 in the human β-cell line EndoC-βH1. In line with the observations in the rodent models, knockdown of Panx2 in EndoC-βH1 cells was associated with increased basal- and cytokine-induced apoptosis (Fig. 3F and G). Collectively, these findings confirm that Panx2 has anti-apoptotic properties in rodent and human β-cells.

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