Redundant role of the cytochrome c-mediated intrinsic apoptotic pathway in pancreatic β-cells

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Abstract

Cytochrome c is one of the central mediators of the mitochondrial or the intrinsic apoptotic pathway. Mice harboring a ‘knock-in’ mutation of cytochrome c, impairing only its apoptotic function, have permitted studies on the essential role of cytochrome c-mediated apoptosis in various tissue homeostasis. To this end, we examined the role of cytochrome c in pancreatic β-cells under homeostatic conditions and in diabetes models, including those induced by streptozotocin (STZ) and c-Myc. Previous studies have shown that both STZ- and c-Myc-induced β-cell apoptosis is mediated through caspase-3 activation; however, the precise mechanism in these modes of cell death was not characterized.

The results of our study show that lack of functional cytochrome c does not affect glucose homeostasis or pancreatic β-cell mass under basal conditions. Moreover, the cytochrome c-mediated intrinsic apoptotic pathway is required for neither STZ- nor c-Myc-induced β-cell death. We also observed that the extrinsic apoptotic pathway mediated through caspase-8 was not essential in c-Myc-induced β-cell destruction. These findings suggest that cytochrome c is not required for STZ-induced β-cell apoptosis and, together with the caspase-8-mediated extrinsic pathway, plays a redundant role in c-Myc-induced β-cell apoptosis.


Introduction

Apoptosis is a genetically regulated cell suicide program, which is essential for multicellular organisms. It is necessary for embryonic development and adult tissue homeostasis by mediating elimination of unwanted cells, which can be potentially harmful (Thompson 1995, Jacobson et al. 1997). The process of apoptosis is tightly regulated in an orderly series of signal cascades under specific circumstances. The caspase cascade system plays a vital role in the induction, transduction, and amplification of intracellular apoptotic signals through two main pathways: the extrinsic and the intrinsic apoptotic pathways. In the extrinsic pathway (also known as death receptor pathway), apoptosis is triggered by ligand-induced activation of death receptors at the cell surface. On receptor ligation, procaspase-8 is recruited to interact with its death domain to form the death–inducing signal complex, where it gets activated (Muzio et al. 1996). In the intrinsic pathway (also called mitochondrial pathway), apoptosis is activated in response to cellular stressors and results in an intracellular cascade of events in which mitochondrial permeabilization is perturbed and cytochrome c is released to initiate the apoptotic signaling (Kuwana et al. 2002, Lakhani et al. 2006). Both pathways activate the apoptosis ‘executioners’ including caspases-3, -6, and/or -7, leading to the terminal phase of apoptosis (Woo et al. 2000, Zimmermann et al. 2001).

Our previous studies have shown the integral role of caspases-3 and -8 in pancreatic β-cell apoptosis in experimental models of diabetes. Mice lacking caspase-3 are protected from β-cell apoptosis and diabetes induced by both streptozotocin (STZ; Liadis et al. 2003) and c-Myc activation (Radziszewska et al. 2009). In addition, mice with β-cell-specific caspase-8 deletion are protected against both STZ- and high-fat diet-induced models of diabetes (Liadis et al. 2007). Furthermore, neither caspase-3 nor caspase-8 appeared to be essential for β-cell homeostasis under physiological conditions. Collectively, these studies demonstrate that caspases-3 and -8 are essential in β-cell apoptosis leading to diabetes development.

Cytochrome c has been primarily known for its function in the mitochondria as a key factor in the respiratory chain for ATP synthesis. However, on apoptotic stimulus, such as DNA damage, metabolic stress, or the presence of excess unfolded proteins, the intrinsic apoptotic pathway is triggered and the mitochondrial cytochrome c is released into the cytosol (Liu et al. 1996, Kluck et al. 1997a,b, Yang et al. 1997). Cytoplasmic cytochrome c engages the apoptotic protease-activating factor (APAF-1) to form the apotosome (Li et al. 1997),
which recruits procaspase-9 and promotes its efficient activation (Rodriguez & Lazebnik 1999, Zou et al. 1999). Activated caspase-9 in turn activates the downstream effectors caspases-3, -6, and/or -7, which rapidly cleave intracellular substrates. As well as its role in the canonical intrinsic apoptotic pathway, cytochrome c release can also be activated through Bid, which is cleaved by caspase-8, which is activated by the extrinsic pathway (Li et al. 1998), thereby amplifying the apoptotic signaling (Kleffstrom et al. 2002). The importance of cytochrome c in apoptosis has been assessed using a ‘knock-in’ approach in transgenic mice (Hao et al. 2005), whereby the expression encodes for a cytochrome c protein that retains normal respiratory function but lacks apoptotic function due to failure in oligomerizing APAF-1 and forming the apoptosome, which is required for procaspase-9 activation. Elegant experiments using this mutant mouse model demonstrated that loss of cytochrome c-mediated apoptosis disturbs normal brain development and lymphocyte homeostasis (Hao et al. 2005). However, the role of this apoptotic pathway in pancreatic β-cells was not assessed.

The mitochondria are central to the maintenance of β-cell function (Silva et al. 2000, Lowell & Shulman 2005) and are also an important mediator of β-cell apoptosis (Bruin et al. 2008, Holohan et al. 2008, Chen et al. 2009). However, the essential role of cytochrome c-mediated apoptosis in pancreatic β-cells under basal and diabetic conditions in vivo was not known. Basal β-cell apoptosis is known to play a role in the remodeling and development of the normal endocrine pancreas (Finegood et al. 1995). Excessive β-cell apoptosis is also a well-appreciated mode of cell death that contributes to both type 1 and type 2 diabetes (O’Brien et al. 1997, Donath et al. 2005). Mechanistic insights into the control of pancreatic β-cell apoptosis both during homeostatic conditions and during the course of diabetes development are therefore important for the prevention and treatment of diabetes.

Gene targeting strategies can provide valuable tools to study the physiological function and pathophysiological role of individual apoptotic mediators. In this study, the ‘knock-in’ mice expressing the mutant cytochrome c (KA allele; Hao et al. 2005), which retains normal electron transfer function but fails to participate in the apoptosome formation, were used to assess the role of cytochrome c-mediated apoptotic pathway in pancreatic β-cells.

Materials and Methods

Mouse protocol

Cytochrome c mutant mice (Cytc<sup>KA/KA</sup>) were generated previously (Hao et al. 2005) and contain the mutation of lysine 72 to alanine (K72A), which specifically abolishes the apoptotic function. c-Myc-ER (TAM) (Myc<sup>+</sup>) transgenic mice were generated as reported (Pelengaris et al. 2002), in which the c-Myc transgene in pancreatic β-cells is activated on tamoxifen injection. Cytc<sup>KA/KA</sup> mice were crossed to M-my<sup>-</sup> mice to obtain M-my<sup>-</sup>, M-my<sup>+</sup> Cytc<sup>+/+</sup>, M-my<sup>+</sup> Cytc<sup>+/KA</sup>, and M-my<sup>+</sup> Cytc<sup>KA/KA</sup>. M-my<sup>+</sup> Cytc<sup>+/KA</sup> and M-my<sup>+</sup> Cytc<sup>+/+</sup> littermates were used as controls for M-my<sup>+</sup> Cytc<sup>KA/KA</sup> mice in all experiments. RIP<sup>+</sup>Casp8<sup>+/</sup>/KA mice were generated as previously described (Liadis et al. 2007), in which the tissue-specific deletion of caspase-8 was under the control of the rat insulin promoter (RIP). M-my<sup>+</sup> mice were bred to RIP<sup>+</sup>Casp8<sup>+/</sup>/KA mice to generate Myc<sup>+</sup>RIP<sup>+</sup>Casp8<sup>+/</sup>/KA mice, and these mice were then intercrossed to generate Myc<sup>+</sup>RIP<sup>+</sup>Casp8<sup>+/</sup> M-my<sup>-</sup>RIP<sup>+</sup>Casp8<sup>+/</sup>/KA, and Myc<sup>+</sup>RIP<sup>+</sup>Casp8<sup>+/</sup>/KA littermates. Mice were maintained on a C57BL/6 background. Genotyping was performed by PCR with genomic DNA from ear or tail tissue as described previously (Salmena et al. 2003, Hao et al. 2005). The activity of the mice was not restricted, and they were maintained on a 12 h light:12 h darkness cycle. All mice were fed regular chow (Harlan–Teklad, Mississauga, Ontario, Canada). Mice used in experiments were all 8–12 weeks of age unless indicated. All protocols were approved by the Ontario Cancer Institute, Animal Resource Colony.

Induction of diabetes by STZ and glucose monitoring

The mice were injected i.p. with multiple low doses of STZ (40 mg/kg body weight) for five consecutive days as described previously (Liadis et al. 2003). Blood glucose was measured weekly after the injection.

Activation of c-Myc

The c-Myc-ER (TAM) transgene was activated by daily i.p. injection of 1 mg tamoxifen (Sigma) dissolved in peanut oil (Sigma), at a final concentration of 10 mg/ml in adult mice. Blood glucose levels were measured at specific time points.

Metabolic studies

Mice were fasted overnight for 14–16 h prior to experiments. Blood glucose levels were determined from tail venous blood. Glucose tolerance tests were performed on fasted mice using i.p. injected glucose at a dose of 2 g/kg body weight. Insulin tolerance tests were performed using human regular insulin at a dose of 1 U/kg body weight i.p. injected. Blood glucose measurements were obtained at 0, 15, 30, 45, 60, and 120 min after glucose or insulin injection.

Immunohistochemistry, immunofluorescent staining, and islet morphometry

Pancreata were isolated from mice at days 0, 1, and 30 after tamoxifen treatment. Pancreatic tissue was fixed for 24 h in 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Samples were dehydrated and prepared as paraffin blocks. Sections (7 μm thick) were obtained at 150 μm intervals on three levels and stained for insulin, Ki67 (DAKO, Burlington, Ontario, Canada), and TUNEL (ID Labs, London, Ontario, Canada).

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which is about half of the expected Mendelian ratio, as previously reported (Hao et al. 2005). Surviving mice appeared generally healthy similar to previous observations (Hao et al. 2005). To confirm the absence of cytochrome c-mediated apoptotic activation in the Cytc\(^{KA/KA}\) mice, we examined caspase-9 cleavage by western blot analyses in the islets on apoptotic stimuli with c-Myc activation (to be discussed in detail in the next section). Activation of caspase-9 was absent in islets of Cytc\(^{KA/KA}\) mice, as evidenced by a lack of cleavage of pro-caspase-9, confirming the lack of cytochrome c-mediated apoptosis in these mice (Fig. 1).

The intrinsic pathway is known to be involved in β-cell apoptosis (Thomas & Biden 2009). However, it is not clear whether the cytochrome c-mediated apoptotic pathway is required for β-cell mass and glucose homeostasis. To assess the essential role of this apoptotic pathway in pancreatic β-cells, we performed metabolic studies and examined pancreatic morphology in Cytc\(^{KA/KA}\) mice. No differences were found in body weight (Fig. 2A) nor fed (Fig. 2B) or fasting blood glucose levels (Fig. 2C) between Cytc\(^{KA/KA}\) mice and their wild-type controls. Glucose tolerance and insulin tolerance tests also did not show any differences between the genotypes (Fig. 2D and E). β-Cell area was examined by insulin

**Results**

**Lack of apoptotic function of cytochrome c in pancreatic islets has no effect on glucose homeostasis or β-cell mass**

Mice with the cytochrome \(c\) knock-in mutation (Cytc\(^{KA/KA}\)) were born at a frequency of 12% from heterozygous breeding,

Statistical analysis

Data are presented as mean±S.E.M. and were analyzed by one-sample t-test, independent samples t-test, and one-way ANOVA with the post hoc Tukey least significant difference test, where appropriate. Data were analyzed using the statistical package SPSS for Windows version 17.0.

Figure 1  Cytochrome c inactivation in the Cytc\(^{KA/KA}\) mice. Western blot analyses on isolated islets show increased cleaved caspase-9 expression in the islets of Cytc\(^{-/-}\) mice after c-Myc activation, whereas decreased cleaved caspase-9 expression levels were observed in the islets of Cytc\(^{+/+}\) mice after c-Myc activation for 1 day, \(n=3\) per genotype. Results represent mean±S.E.M. *\(P<0.05\).

Figure 2 Lack of functional cytochrome c did not affect glucose homeostasis. No differences were found in weight (A), fed blood glucose (B), or fasting blood glucose level (C) between Myc\(^{-/-}\) Cytc\(^{+/+}\) and Myc\(^{-/-}\) Cytc\(^{KA/KA}\) mice, \(n=8\) per genotype. In addition, glucose tolerance tests (GTTs) at a dose of 2 g/kg BW, (D) and insulin tolerance tests (ITTs) at a dose of 1 U/kg BW, (E) revealed no significant differences between the genotypes as well, \(n=5\) per genotype. Mice used for the GTTs and ITTs were between 8 and 12 weeks of age. Abbreviation: +/+; Cytc\(^{+/+}\); KA/KA, Cytc\(^{KA/KA}\). Results represent mean±S.E.M.
Cytochrome c did not play an essential role in regulating β-cell mass. (A) β-Cell area per pancreatic area analyses on insulin-stained sections showed no difference between the genotypes, n = 3 per genotype. Original magnification ×10. Scale bars = 200 μm. Results represent mean ± s.e.m. (B) Insulin and glucose double staining by immunofluorescence show intact islet architecture in the Cytc-KA/KA mice, similar to controls, n = 3 per genotype. All mice used in experiments were between 8 and 12 weeks of age. Original magnification ×20. Scale bars = 40 μm.

Cytochrome c-mediated apoptotic pathway is not required for STZ-induced diabetes and pancreatic β-cell destruction

STZ is a glucose analog that selectively destroys pancreatic β-cells. Apoptosis is the major mode of cell death that is responsible for diabetes development in this model (Saini et al. 1996). To determine the essential role of the intrinsic pathway on STZ-induced β-cell apoptosis and diabetes in vivo, we subjected the mice to MLDS-induced diabetes. Cytc-KA/KA mice developed diabetes as evidenced by increased blood glucose levels that were similar to their control counterparts (Fig. 4A). Insulin-immunostained pancreatic sections also showed similar decreases in β-cell area/pancreatic area between the genotypes (Fig. 4B). These results demonstrate that apoptosis in β-cells can proceed without the cytochrome c-mediated intrinsic apoptotic pathway at least in response to STZ.

Loss of the apoptotic function of cytochrome c does not confer protection against c-Myc-induced β-cell apoptosis and diabetes

Overexpression of c-Myc in the β-cells in mice results in rapid proliferation, followed by massive apoptosis, leading to fulminant diabetes and death shortly after birth (Laybutt et al. 2002). To circumvent this early lethality and to enable investigating the role of c-Myc in β-cells in adult mice, an inducible transgenic mouse expressing modified estrogen receptor ligand-binding domain fused to the human c-myc gene was generated. This mouse allows for the c-Myc transgene expression to be ‘switched on’ in β-cells in the presence of tamoxifen (Pelengaris et al. 2002). We crossed this transgenic mouse to Cytc-KA/KA mice to generate Myc+Cytc-KA/KA mice to test whether the cytochrome c-mediated apoptotic pathway plays a role in c-Myc-induced β-cell apoptosis and diabetes.

Myc+Cytc-KA/KA mice and their control Myc+Cytc+/- mice littermates were injected with tamoxifen for 30 consecutive days to sustain the activation of c-Myc in the pancreatic β-cells. Myc+Cytc+/-/+ mice developed hyperglycemia on c-Myc activation as early as day 3 of injection, similar to Myc+Cytc-KA/KA mice, and they reached similar degree of hyperglycemia (Fig. 5A), suggesting that cytochrome c-mediated apoptosis was not essential in c-Myc-induced diabetes. Pancreatic sections immunostained for insulin showed reduced β-cell area and near ablation of islets in both genotypes (Fig. 5B).

We assessed pancreatic sections for Ki67- and TUNEL-positive islet cells after 2 days of tamoxifen injection to determine the role of cytochrome c in c-Myc-induced islet proliferation and apoptosis respectively. We observed similar
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Given that the cytochrome c-mediated intrinsic apoptotic pathway was not required for c-Myc-induced apoptosis, we were interested in examining whether the extrinsic pathway was essential in c-Myc-induced β-cell apoptosis in vivo. Thus, Myc+RIPcre+Casp8fl/fl mice were generated using a similar strategy as Myc+CytcKA/KA mice, through breeding the inducible β-cell-specific c-Myc transgenic mice with RIPcre+Casp8fl/fl mice.

Mice were injected with tamoxifen daily for 30 days to activate c-Myc in the islets as previously shown (Radziszewska et al. 2009). Similar to Myc+CytcKA/KA mice, Myc+RIPcre+Casp8fl/fl mice and their wild-type littermates developed diabetes within 3 days of c-Myc activation. Hyperglycemia continued throughout the period of tamoxifen injection without any differences between the two genotypes (Fig. 7A). The pancreatic sections immunostained for insulin at the end of the 30-day injections revealed near-complete islet destruction in the mice of both genotypes (Fig. 7B). The similar progression of diabetes in both Myc+RIPcre+Casp8fl/fl mice and Myc+RIPcre+Casp8fl/+ littermates indicates that the caspase-8-mediated pathway is also not essential for c-Myc-induced β-cell death.

**Discussion**

Cytochrome c released from the mitochondria is an essential component of the intrinsic apoptotic pathway in response to DNA damage and other forms of cellular stress (Parone et al. 2002). However, due to the essential requirement of cytochrome c in mitochondrial respiration, in vivo analysis of cytochrome c in apoptotic signaling was not possible. To circumvent this limitation, a cytochrome c ‘knock-in’ mouse (CytcKA/KA mouse) in which a K72 allele mutation was generated (Hao et al. 2005). In this model, cytochrome c is released normally from the mitochondria on apoptotic stimuli but is unable to engage with APAF-1 to activate downstream caspase-9 (Hao et al. 2005). This study examined the role of cytochrome c-mediated apoptosis in the pancreatic β-cells and in glucose homeostasis. Surprisingly, no significant defects in glucose metabolism or β-cell mass were found in CytcKA/KA mice, demonstrating that cytochrome c-mediated apoptosis is not essential in modulating pancreatic β-cell mass.

Mouse embryonic fibroblasts harboring the Cytcfl/fl mouse show reduced caspase-3 activation and were resistant to a variety of apoptotic stimuli including u.v. irradiation, serum withdrawal, or staurosporine (Li et al. 2000, Hao et al. 2005). Moreover, a positive feed forward is proposed to exist between caspase-3 activation and cytochrome c release. This is based on the finding that caspase-3 cleaves the anti-apoptotic BCL-2 family member BCL-XL, which promotes mitochondrial outer membrane permeabilization (Woo et al. 1999, Basanez et al. 2001). In pancreatic β-cells, the absence of caspase-3 has been shown to confer protection against STZ- and c-Myc-induced apoptosis (Liadis et al. 2003, Radziszewska et al. 2009). These findings prompted further study on the role of the cytochrome c-mediated apoptotic pathway in β-cell apoptosis.
Our results show that loss of the cytochrome c-mediated apoptotic pathway did not protect mice from STZ-induced diabetes and β-cell destruction, which is in contrast to the phenotype observed in mice with β-cell-specific caspase-8 deletion, where the mice are protected from STZ-induced β-cell apoptosis and death (Liadis et al. 2007). Caspase-3 is known to be the central executioner of apoptosis downstream of both the intrinsic and the extrinsic apoptotic pathways (Lakhani et al. 2006). Mice lacking either caspase-3 or caspase-8 specifically in the β-cells were protected against STZ-induced diabetes. Therefore, the lack of protection from mice with defects in the cytochrome c-mediated intrinsic apoptotic pathway would suggest that a redundant or compensatory mechanism is in place for intrinsic but not extrinsic apoptotic pathway in β-cells, at least in response to STZ. Other mitochondrial proteins that are released during apoptosis, including apoptosis-inducing factor, Smac/DIABLO, endonuclease G, and Omi/HtrA2, have been implicated in various aspects of the cell death process (van Loo et al. 2002). These mitochondrial proteins therefore potentially play redundant roles and compensate for the loss of cytochrome c.

Figure 6 Tamoxifen treatment after 2 days increases islet cell proliferation and apoptosis in both Myc+Cytc+/+ and Myc+CytcK/A/A mice. (A) Ki67 immunostaining and (B) TUNEL assay of pancreatic sections showed no differences in islet cell proliferation or apoptosis, respectively, between Myc+Cytc+/+ and Myc+CytcK/A/A mice, n=4 per genotype. Original magnification ×20. Scale bars=40 μm. Results represent mean±S.E.M. *P<0.05 compared to untreated controls.

Figure 7 Caspase-8 deletion in the β-cells does not protect mice against c-Myc-induced diabetes and β-cell death. (A) Random blood glucose monitor during 30 days of tamoxifen treatment showed that both Myc+RIPcre+Casp8+/+ and Myc+RIPcre+Casp8fl/fl mice developed diabetes, n=5 per genotype. (B) β-Cell per pancreatic area calculated from insulin-stained pancreatic sections showed similar degrees of islet ablation in both Myc+ genotypes compared with Myc− controls, n=5 per genotype. Original magnification ×10. Scale bars=200 μm. All mice used in experiments were between 8 and 12 weeks of age. Results represent mean±S.E.M. *P<0.05.
c-Myc, a proto-oncogene, is a potent inducer of both proliferation and apoptosis but appears to have a predominant pro-apoptotic role in pancreatic β-cells (Jonas et al. 2001, Laybutt et al. 2002, Pelengaris et al. 2002, Van de Casteele et al. 2003). Activation of c-Myc sensitizes the cell to apoptosis by promoting cytochrome c release from the mitochondria to the cytosol. The molecular mechanisms through which c-Myc promotes cytochrome c release from the mitochondria are as yet unclear but are likely through its regulation on the expression, localization, or activity of BH3 proteins composed of anti-apoptotic (e.g. BCL-2 and BCL-XL) and pro-apoptotic (e.g. BAX and BAK) members. These proteins modulate each others’ activities by forming heterodimers that regulate the release of cytochrome c from the mitochondria (Li et al. 2000, Green 2003). In cultured fibroblasts, c-Myc-induced apoptosis was shown to be blocked by microinjection of anti-cytochrome c antibodies by inhibiting the activation of caspase-9 in a cell-free system, suggesting a necessary requirement of cytochrome c for c-Myc-induced cell death (Kluck et al. 1997b). These reports support a critical role of the cytochrome c-mediated apoptotic pathway in c-Myc-induced cell death.

Our previous study showed that caspase-3 deletion confers protection from c-Myc-induced apoptosis in pancreatic β-cells, suggesting that caspase-3 is required for c-Myc-induced apoptosis (Radziszewska et al. 2009). Given the central role of caspase-3 in both intrinsic and extrinsic apoptotic cascades, however, it was not known whether the activation of caspase-3 in this model was through the intrinsic or extrinsic pathway. In order to clarify the signaling pathways that mediate c-Myc-induced apoptosis in the pancreatic β-cell, we generated mice that have inducible c-Myc expression, localization, or activity of BH3 proteins modulate each others’ activities by forming heterodimers that regulate the release of cytochrome c from the mitochondria (Li et al. 2000, Green 2003). In cultured fibroblasts, c-Myc-induced apoptosis was shown to be blocked by microinjection of anti-cytochrome c antibodies by inhibiting the activation of caspase-9 in a cell-free system, suggesting a necessary requirement of cytochrome c for c-Myc-induced cell death (Kluck et al. 1997b). These reports support a critical role of the cytochrome c-mediated apoptotic pathway in c-Myc-induced cell death.

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We then examined the role of extrinsic pathway in c-Myc-induced apoptosis through deletion of its critical mediator, caspase-8, in the pancreatic β-cells. Unexpectedly, blockage of extrinsic pathway also did not protect mice from c-Myc-induced apoptosis and diabetes. Collectively, this study shows that both the intrinsic and the extrinsic pathways may be involved in β-cell death on c-Myc induction; however, the mice lacking either pathway alone are susceptible to c-Myc-induced β-cell death, suggesting a potential compensatory mechanism to take place in the event of a defect in one of the pathways.

In summary, this study reveals that cytochrome c-mediated apoptosis is not required in β-cell homeostasis, or for STZ- and c-Myc-induced apoptosis. It appears that for β-cell apoptosis induced by c-Myc activation, neither cytochrome c nor caspase-8 alone is essential. This likely reflects nature’s safety net of redundant mechanisms to avoid catastrophic loss of the potentially limited insulin-producing pancreatic β-cells.

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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