Inhibitor of differentiation proteins protect against oxidative stress by regulating the antioxidant–mitochondrial response in mouse beta cells

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Abstract

Aims/hypothesis Oxidative stress is implicated in beta cell glucotoxicity in type 2 diabetes. Inhibitor of differentiation (ID) proteins are transcriptional regulators induced by hyperglycaemia in islets, but the mechanisms involved and their role in beta cells are not clear. Here we investigated whether or not oxidative stress regulates ID levels in beta cells and the role of ID proteins in beta cells during oxidative stress. **Methods** MIN6 cells were cultured in H₂O₂ or ribose to induce oxidative stress. ID1, ID3 and small MAF proteins (MAFF, MAFG and MAFK) were inhibited using small interfering RNA. Isolated islets from *Id1*^{−/−}, *Id3*^{−/−} and diabetic *db/db* mice were used.

Results ID1–4 expression was upregulated in vivo in the islets of diabetic *db/db* mice and stimulated in vitro by ribose and H₂O₂. *Id1/3* inhibition reduced the expression of multiple antioxidant genes and potentiated oxidative stress-induced apoptosis. This finding was associated with increased levels of intracellular reactive oxygen species, altered mitochondrial morphology and reduced expression of *Tfam*, which encodes a mitochondrial transcription factor, and respiratory chain components. *Id1/3* inhibition also reduced the expression of small MAF transcription factors (*MafF*, *MafG* and *MafK*), interacting partners of nuclear factor, erythroid 2-like 2

(NFE2L2), master regulator of the antioxidant response. Inhibition of small MAFs reduced the expression of antioxidant genes and potentiated oxidative stress-induced apoptosis, thus recapitulating the effects of *Id1/3* inhibition.

Conclusions/interpretation Our study identifies IDs as a novel family of oxidative stress-responsive proteins in beta cells. IDs are crucial regulators of the adaptive antioxidant–mitochondrial response that promotes beta cell survival during oxidative stress through a novel link to the NFE2L2–small MAF pathway.

Keywords Apoptosis · Beta cell · Islets of Langerhans · Oxidative stress · Reactive oxygen species · Type 2 diabetes

Abbreviations

8OHdG	8-Hydroxyguanosine
BMP	Bone morphogenetic protein
ETC	Electron transport chain
GPX	Glutathione peroxidase
HMOX1	Heme oxygenase 1
ID	Inhibitor of differentiation
NFE2L2	Nuclear factor, erythroid 2-like 2
ROS	Reactive oxygen species
siRNA	Small interfering RNA
SOD2	Superoxide dismutase 2
TFAM	Transcription factor A, mitochondrial

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Introduction

Beta cell failure in type 2 diabetes is characterised by dysfunctional insulin secretion and reduced beta cell mass, which has been linked to an increased rate of apoptosis [1–3]. Several lines of evidence underscore a role for chronic

hyperglycaemia—termed ‘glucotoxicity’—in increased beta cell apoptosis. Thus, elevated glucose concentrations trigger apoptosis in cultured islets and beta cell lines [4–8] and in animal models of type 2 diabetes [9–11].

Oxidative stress has been proposed as a central mechanism of hyperglycaemia-induced beta cell demise [12, 13]. Elevated glucose or ribose levels have been shown to increase reactive oxygen species (ROS) production in beta cells [8, 14–16], and islets of diabetic mice exhibit higher ROS content, mitochondrial dysfunction and oxidative damage [15, 17–19].

The vulnerability of beta cells to oxidative stress may be owed to their low expression of several key antioxidant genes, namely *Gpx1*, *Sod1–2* and catalase [20]. However, other genes of the antioxidant arsenal either display strong expression in beta cells (e.g. other *Gpx* isoforms, *Hmox1*, *Srxn1*, *Prdxs*, *G6pdx*), or are markedly upregulated under oxidative stress conditions [6, 7, 21, 22]. The signalling pathways that regulate redox status and antioxidant gene expression in beta cells are only partially understood. Nuclear factor, erythroid 2-like 2 (NFE2L2, also known as NRF2) is a primary regulator of the antioxidant response and its activation has been shown

Fig. 1 ID protein expression is upregulated in the islets of diabetic mice in parallel with antioxidant genes. Immunostaining of (a) ID1, (b) ID3 and (c) NFE2L2 in islets of control *db/+* and diabetic *db/db* mice. Images are representative of four animals per group. (d–r) Changes in the mRNA

levels of *Id1–4* and antioxidant genes in the islets of *db/+* and *db/db* mice. $n=6–10$ animals per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs *db/+*. Scale bar, 50 μ m

to protect beta cells against oxidative damage [23]. NFE2L2 activation of antioxidant gene expression requires its dimerisation with small MAF proteins (MAFF, MAFG and MAFK) [24–26].

The inhibitor of differentiation (ID) proteins are transcriptional regulators that play important roles in both physiology (e.g. development) and pathology (e.g. tumourigenesis). They have been proposed to act as repressors of basic helix-loop-helix transcription factors thereby modulating cell differentiation and proliferation [27, 28]. Previous reports have demonstrated that the expression of ID1 and ID3 is induced by glucose stimulation in human islets and beta cell lines [29, 30]. Further studies link the upregulation of *Id1* mRNA levels to hyperglycaemia in islets of diabetic mice [31] and suggest an influence of *Id1* expression in insulin secretion [32]. However,

the mechanisms underlying the induction of ID proteins by hyperglycaemia and their precise role in beta cell pathophysiology are not clear.

Here, we demonstrate that IDs are novel oxidative stress-responsive proteins in beta cells. We also identify an unexpected role of ID expression in the induction of the antioxidant response under oxidative stress conditions. Inhibition of *Id1/3* reduces the expression of multiple antioxidant genes and leads to increased ROS production and apoptosis, and altered mitochondrial function and morphology. Our studies also suggest a mechanism for these effects via a previously unrecognised interaction of IDs with the NFE2L2MAF antioxidant pathway. Our results, therefore, suggest that IDs are key regulators of the adaptive antioxidant-mitochondrial

Fig. 2 Oxidative stress upregulates Id genes in beta cells. MIN6 cells were cultured for 48 h in the absence or presence of (a–e) H₂O₂ (100–300 μmol/l) or (f–j) ribose (5–50 mmol/l). *n*=3 experiments. **p*<0.05,

p*<0.01, *p*<0.001 vs control. Immunostaining of (k) ID1 (green) and (l) ID3 (red). Images are representative of four experiments. Scale bar, 10 μm

response that promote beta cell survival under oxidative stress.

Methods

Reagents Ribose was obtained from Sigma (St Louis, MI, USA) and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) and Deep Red Mitotracker were obtained from Invitrogen (Carlsbad, CA, USA). Control non-targeting and ON-TARGETplus SMARTpool small interfering RNA (siRNA) and transfection reagent DharmaFECT3 were sourced from Thermo Fisher Scientific (Lafayette, CO, USA).

Mice C57BL/KsJ *db/db* and their age-matched lean control *db/+* mice were taken from the Garvan Institute (Sydney, NSW, Australia) breeding colonies at the age of 14–16 weeks. Wild-type (C57BL/6/129/Sv), *Id1*^{−/−} and *Id3*^{−/−} mice were bred in-house using animals provided by Robert Benezra (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) and used at the age of 8–10 weeks. All experiments were approved by the Garvan Institute/St Vincent's Hospital Animal Experimentation Ethics Committee.

Islet culture Wild-type, *Id1*^{−/−} and *Id3*^{−/−} islets were cultured in RPMI medium containing 11.1 mmol/l glucose (Invitrogen), 0.2 mmol/l glutamine, 10% heat-inactivated FBS, 100 units/ml penicillin and 100 mg/ml streptomycin in the presence or absence of 50 mmol/l ribose. To assess

mitochondrial morphology, islets were dispersed with trypsin and cells were seeded on poly-L-lysine-treated glass coverslips before treatment.

Cell culture and treatment MIN6 beta cells (P26-39) were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 25 mmol/l glucose, 10 mmol/l HEPES, 10% FCS, 50 units/ml penicillin and 50 mg/ml streptomycin. Cells were transfected with either control, *Id1* and/or *Id3* or *MaqF/G/K* siRNA. After 24 h, the transfection medium was changed and cells were cultured for 48 h in the absence or presence of H₂O₂ or ribose.

Real-time RT-PCR Real-time RT-PCR was performed as previously described [33]. Primer sequences are listed in Electronic Supplementary Material (ESM) Table 1. The value obtained for each specific gene product was normalised to the control gene cyclophilin A and expressed as a fold change of the value in control condition.

Western blots Western blotting was performed as previously described [32]. Antibodies are listed in ESM Table 2.

Functional assays Glutathione peroxidase (GPX) activity was determined indirectly as the decrease in NADPH absorption at 340 nm as previously described [34]. Cell death was determined with the use of a Cell Death Detection ELISA (Roche Diagnostics, Castle Hill, NSW, Australia) [6]. ATP generation was determined using ATPlite kit (PerkinElmer, Melbourne, VIC, Australia). Oxygen consumption was measured using a Clark-type oxygen

Fig. 3 Oxidative stress time-dependently increases mRNA levels of *Id1*, *Id3*, antioxidant and proapoptotic genes. MIN6 cells were cultured from 0–48 h in the absence (open circles) or presence (closed circles) of

50 mmol/l ribose. *n*=3 experiments. **p*<0.05, ***p*<0.01, ****p*<0.001 vs time 0; †*p*<0.05, ††*p*<0.01, †††*p*<0.001 vs same group in the absence of ribose

electrode system as previously described [34]. For ROS measurement, cells were incubated with 2 $\mu\text{mol/l}$ CM-H₂DCFDA or DMSO for 30 min at 37°C. Fluorescence intensity was measured (ex/em at 485/520 nm) using a plate reader (FluoStar OPTIMA, BMG Lab Technologies, Mornington, VIC, Australia).

Immunofluorescence Immunostaining was performed as previously described [35]. Images were acquired using a Leica SP8 confocal microscope.

Statistical analysis Results are given as mean $\pm$ SEM for the indicated number of experiments. Statistical significance was

Fig. 4 Inhibition of *Id1* and/or *Id3* attenuates the global antioxidant response. MIN6 cells transfected with either control siRNA (si-C), siRNA against *Id1* (si-Id1), *Id3* (si-Id3) or both (si-Id1/3) were cultured for 48 h in the absence (white bars) or presence of 50 mmol/l ribose (black bars). **(a–m)** Changes in mRNA levels. Graphs are representative of three experiments. * p <0.05, ** p <0.01, *** p <0.001 vs same group in the absence of ribose; † p <0.05, †† p <0.01, ††† p <0.001 vs control siRNA. **(n)** Changes in protein levels. n =3–5 experiments. C, control; H, H₂O₂ 100 $\mu\text{mol/l}$; R, ribose 50 mmol/l. **(o)** Changes in GPX enzymatic activity. n =5 experiments. * p <0.05, *** p <0.001 vs same group in the absence of ribose. U, untreated

assessed by unpaired two-tailed Student's *t* test, one-way ANOVA and a post-test of Newman–Keuls or two-way ANOVA and a post-test of Bonferroni.

Results

ID expression is increased in the islets of diabetic mice in parallel with the antioxidant response By immunofluorescence on histological pancreatic sections we observed a marked increase in the expression of ID1 and ID3 in the islets of diabetic *db/db* mice in comparison with normoglycaemic control *db/+* mice (Fig. 1a,b). These effects were paralleled by increased expression of NFE2L2, a master transcriptional regulator of antioxidant gene expression (Fig. 1c). In agreement with these observations, mRNA levels of *Id1–4* were upregulated in the islets of *db/db* mice (Fig. 1d–g). Furthermore, mRNA levels of the antioxidant genes *Hmox1*, *Gpx1–2*,

Txnrd1, *G6pdx*, *Srxn1* and *Prdx1–4* were increased (Fig. 1h–q). The mRNA levels of *Nfe2l2* were unchanged (Fig. 1r). These results demonstrate an in vivo association between the upregulation of IDs and the antioxidant response in islets of diabetic *db/db* mice.

***Id* genes are induced by oxidative stress in beta cells** MIN6 cells [32] were treated with the pro-oxidant agents H_2O_2 or ribose. Ribose is a sugar that produces ROS more potently and in a shorter time than glucose and is an established model in which to study beta cell glucotoxicity in vitro [14, 36]. Treatment with H_2O_2 (Fig. 2a–e) and ribose (Fig. 2f–j) increased *Id1–4* and *Hmox1* mRNA levels in a concentration-dependent manner. Accordingly, ribose treatment increased the (peri)nuclear expression of ID1 (Fig. 2k) and ID3 proteins (Fig. 2l).

Id1 and *Id3* mRNA levels were induced by ribose in a time-dependent manner. The effect of ribose was significant at 6 h and reached a maximum at 48 h (Fig. 3a,b). The mRNA levels

Fig. 5 Inhibition of *Id1* and *Id3* increases ROS levels, oxidative damage and apoptosis. MIN6 cells transfected with either control siRNA (si-C) or siRNA against *Id1* and *Id3* (si-Id1/3) were cultured for 48 h in the absence (white bars) or presence of 50 mmol/l ribose (black bars) or 100 μ mol/l H_2O_2 (grey bars). Changes in (a) intracellular ROS and (b) apoptosis levels. (c) Changes in apoptosis levels in isolated wild-type (WT), *Id1*^{−/−} and *Id3*^{−/−} mouse islets cultured for 48 h in the absence (white bars) or presence of 50 mmol/l ribose (black bars). (d–g) Changes in mRNA levels in MIN6 cells. *n*=3–4 experiments. **p*<0.05, ***p*<0.01, ****p*<0.001 vs same group in the absence of ribose or H_2O_2 ; †*p*<0.05, ††*p*<0.01, †††*p*<0.001 vs control siRNA or wild-type. (h–k) Immunostaining of 8OHdG (red). Images are representative of three experiments. Scale bar, 10 μ m

of antioxidant genes were also induced by ribose in a time-dependent manner. *Hmox1*, *Srxn1* and, to a lesser extent, *Gpx2* were induced at 6 h and reached the maximum at 24 h (Fig. 3c–e) while *Prdx1* was significantly induced between 24–48 h and reached the maximum at 48 h (Fig. 3f). In parallel, *Nfe2l2* tended to be induced at 6 h and reached the maximum at 48 h (Fig. 3g). The mRNA levels of the proapoptotic gene *Bax* were upregulated by ribose treatment slightly later than *Id* and antioxidant genes (Fig. 3h). These findings suggest a close association between IDs and antioxidant gene expression under oxidative stress conditions in MIN6 beta cells.

Furthermore, the ribose-induced upregulation of mRNA levels for *Id* genes was prevented by treatment with the antioxidant MnTBAP (ESM Fig. 1a–j). Together these results demonstrate that IDs are novel oxidative stress-responsive proteins in MIN6 beta cells.

Fig. 6 Inhibition of *Id1* and *Id3* impairs mitochondrial homeostasis and alters mitochondrial morphology. MIN6 cells transfected with either control siRNA (si-C) or siRNA against *Id1* and *Id3* (si-Id1/3) were cultured for 48 h in the absence (white bars) or presence of 50 mmol/l ribose (black bars) or 100 μ mol/l H_2O_2 (grey bars). (a) Changes in oxygen consumption, (b) ATP levels, (c–h) protein levels of ETC complex components and (i) mRNA levels of the mitochondrial transcription factor *Tfam*. $n=3$ –5 experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs same group in the absence of ribose; † $p<0.05$, †† $p<0.01$, ††† $p<0.001$ vs control siRNA. C, control; H, H_2O_2 100 μ mol/l; R, ribose 50 mmol/l. (j–m) Mitochondrial (Mito) morphology (red) in MIN6 cells cultured for 48 h in the absence or presence of 50 mmol/l ribose. Images are representative of five experiments. Scale bar, 10 μ m

Inhibition of *Id1* and/or *Id3* leads to global attenuation of the antioxidant response To explore the role of ID induction under oxidative stress in beta cells, we used siRNA to inhibit the expression of *Id1* and/or *Id3* (Fig. 4a,b). Interestingly, in ribose-treated cells, inhibition of *Id1* significantly reduced the mRNA levels of *Hmox1*. When both *Id1* and *Id3* were inhibited a stronger effect was observed (Fig. 4c). This additive inhibitory effect was observed for all the other antioxidant genes studied, including *Gpx1*–2, *Srxn1*, *Prdx1*–4, *Txnrd1* and *G6pdx* (Fig. 4d–l), while *Nfe2l2* was not significantly affected (Fig. 4m). In agreement with this finding, *Id1* inhibition decreased H_2O_2 - and ribose-induced upregulation of heme oxygenase 1 (HMOX1) protein levels and this reduction was stronger after inhibition of both isoforms (Fig. 4n). We also found that inhibition of *Id1/3* reduced the ribose-mediated upregulation of superoxide dismutase 2 (SOD2) protein levels. Moreover, ribose-induced upregulation of GPX

activity was partially prevented by approximately 21% after *Id1/3* inhibition (Fig. 4o). These results indicate a striking association between *Id1/3* inhibition and a global attenuation of the antioxidant response.

Inhibition of *Id1* and/or *Id3* increases ROS levels, apoptosis and oxidative damage We assessed the influence of *Id* inhibition on ROS generation and cell viability. The attenuated antioxidant response may occur as a consequence of reduced ROS levels or it may lead to increased ROS generation. Interestingly, *Id1/3* inhibition strongly increased basal and ribose-stimulated H_2O_2 levels by approximately 2.4- and 1.8-fold respectively (Fig. 5a). In line with these results, *Id1/3* inhibition doubled basal and H_2O_2 - and ribose-induced apoptosis (Fig. 5b). Moreover, in cultured islets basal and ribose-induced apoptosis tended to be potentiated in *Id1*^{-/-} islets and ribose-induced apoptosis was significantly increased in *Id3*^{-/-} islets (Fig. 5c). The inhibition of *Id1* or *Id3* alone in MIN6 beta cells also potentiated ribose-induced apoptosis but the strongest effect was observed when both isoforms were inhibited (ESM Fig. 2). The absence of *Id1/3* was also associated with increased mRNA levels of *Bax* and DNA-damage inducible transcript 2 (Fig. 5d,e), and reduced mRNA levels of the anti-apoptotic genes *Bcl2* and *Bcl-xl* (Fig. 5f,g). Accordingly, DNA-damage was increased after inhibition of *Id1/3*. Thus, 8-hydroxyguanosine (8OHdG) immunostaining was barely detected in untreated cells and markedly increased by ribose treatment (Fig. 5h,i). This staining was further increased upon *Id1/3* inhibition in control and ribose-treated cells (Fig. 5j,k). Altogether, these results demonstrate that the inhibition of *Id1/3* leads to increased ROS levels, oxidative damage and apoptosis.

Inhibition of *Id1* and/or *Id3* impairs mitochondrial homeostasis An association between increased ROS generation and altered mitochondrial function has been demonstrated in islets of diabetic animals [15, 19]. We assessed mitochondrial function by measuring oxygen consumption and ATP generation. Ribose treatment tended to reduce oxygen consumption. After inhibition of *Id1/3*, oxygen consumption tended to be lower in untreated cells and was significantly reduced by approximately 20% after ribose treatment (Fig. 6a). Ribose treatment also markedly reduced ATP levels but this effect was stronger after inhibition of *Id1/3* (Fig. 6b).

This alteration in mitochondrial function was associated with changes in the expression of electron transport chain (ETC) components. Thus, inhibition of *Id1/3* tended to decrease the expression of CI (subunit NDUF8), CII (subunit SDHB) and CIII (subunit UQCRC2), significantly decreased the expression of CV (subunit ATP5A), and strongly decreased the expression of CIV (subunit MTCO1) (Fig. 6c–h). On the other hand, the expression of *Ucp2* was not affected by *Id1/3* inhibition (ESM Fig. 3a). MTCO1, a catalytic

Fig. 7 Altered mitochondrial morphology in beta cells from *Id3*^{-/-} mice. Mitochondrial (Mito) morphology (red) in dispersed wild-type (WT) and *Id3*^{-/-} (KO) mouse islets cultured for 48 h in the absence or presence of 50 mmol/l ribose. Images are representative of five experiments. Scale bar, 10 μ m

subunit of cytochrome c oxidase, is regulated by transcription factor A, mitochondrial (TFAM). The mRNA levels of *Tfam* were upregulated by ribose treatment (Fig. 6i) as well as in the islets of diabetic *db/db* mice (ESM Fig. 3b). Interestingly, inhibition of *Id1/3* reduced the mRNA levels of *Tfam* (Fig. 6i).

Increased ROS production has been associated with altered mitochondrial morphology [15, 19]. Exposure to ribose modified the mitochondrial morphology into a more condensed-globular pattern (Fig. 6j,k). Interestingly, *Id1/3* inhibition

Fig. 8 Inhibition of *Id1* and *Id3* increases the nuclear accumulation of NFE2L2 while reducing the expression of its interacting partners, the MAFs. MIN6 cells transfected with either control siRNA (si-C) or siRNA against *Id1* and *Id3* (si-*Id1/3*) were cultured for 48 h in the absence (white bars) or presence (black bars) of 50 mmol/l ribose. (a–d) Immunostaining of NFE2L2. Images are representative of three experiments. Scale bar, 10 μ m. (e) Analysis of NFE2L2-interacting partners using Cytoscape (2.8.3) and MiMI plug-in (www.cytoscape.org). (f–h) Changes in *MafF*, *MafK* and *MafG* mRNA levels. $n=4$ experiments. * $p<0.05$, *** $p<0.001$ vs same group in the absence of ribose; † $p<0.01$, †† $p<0.001$ vs control siRNA. (i) Changes in MAFK protein levels. $n=4$ experiments. C, control; R, ribose 50 mmol/l. (j–l) Changes in mRNA levels in islets of *db/+* and *db/db* mice. $n=6$ animals/group. * $p<0.05$, *** $p<0.001$ vs *db/+*

altered mitochondrial morphology both in untreated cells (Fig. 6l) and after ribose treatment (Fig. 6m). Similarly, in primary beta cells from wild-type mice, mitochondrial morphology was altered by ribose treatment (Fig. 7a). In beta cells

from *Id3*^{-/-} mice, altered morphology was observed under control and ribose conditions (Fig. 7b).

Together, these results show that the inhibition of *Id1/3* is associated with altered mitochondrial function and

morphology. These alterations were associated with reduced expression of components of the ETC, particularly MTCO1, as well as the upstream transcription factor *Tfam*.

Inhibition of *Id1* and *Id3* reduces the expression of MAFs, interacting partners of NFE2L2 Under control conditions, NFE2L2 was barely detected in the cytosol while ribose stimulation increased its nuclear localisation (Fig. 8a,b). Surprisingly, inhibition of *Id1/3* increased NFE2L2 nuclear expression under control conditions and after ribose treatment (Fig. 8c,d) without affecting the expression of the cytoplasmic adaptor protein kelch-like ECH-associated protein (ESM Fig. 4a,b). This observation is in agreement with the finding of increased oxidative stress in the absence of *Id1/3*, but does not explain the inhibitory effect on antioxidant gene expression. We therefore hypothesised that IDs may affect an interacting partner of NFE2L2 (Fig. 8e). *Atf4* [37] and *c-Jun* [38] were potential candidates. However, *Atf4* mRNA levels (ESM Fig. 5a) and phospho-c-JUN protein levels (ESM Fig. 5b) were unaffected by *Id1/3* inhibition. The other potential candidates were the small MAF proteins [24–26, 38]. We found that ribose treatment markedly increased *MafF* and *MafK* mRNA levels. Interestingly, *Id1/3* inhibition strongly prevented this increase (Fig. 8f,g). *MafG* mRNA levels were slightly increased by ribose treatment and slightly reduced by *Id1/3* inhibition (Fig. 8h). Furthermore, MAFK protein levels were reduced by *Id1/3* inhibition (Fig. 8i). These findings demonstrate that the inhibition of *Id1/3* leads to reduced expression of small MAF proteins. Confirming the potential physiological relevance of these findings, *MafF* and *MafK* mRNA levels were markedly increased in the islets of diabetic *db/db* mice while the mRNA levels of *MafG* were not significantly affected (Fig. 8j–l). These findings suggest a novel mechanism whereby IDs regulate antioxidant gene expression by modifying the expression of small MAF proteins, NFE2L2-interacting partners.

Inhibition of MAFs reduces antioxidant gene expression and potentiates apoptosis, recapitulating the effects of *Id1/3* inhibition To verify whether the inhibition of small MAF proteins recapitulate the effects of *Id1/3* inhibition on antioxidant gene expression, we used siRNA against *MafF*, *MafK* and *MafG*. By this means, the ribose-mediated upregulation of *MafF*, *MafK* and *MafG* was inhibited to a similar extent to that observed after inhibition of *Id1/3* (Fig. 9a–c). Strikingly, the inhibition of MAFs resulted in reduced mRNA levels of several antioxidant genes, including *Hmox1*, *Gpx1*, *Txnrd1*, *Prdx2–3* and, to a lesser degree, *G6pdx* (Fig. 9d–i). Furthermore, inhibition of MAFs potentiated ribose-induced apoptosis by approximately 1.8-fold (Fig. 9j).

These results demonstrate for the first time the importance of small MAF proteins (F/K/G) in the regulation of antioxidant gene expression and beta cell survival under conditions

of oxidative stress. Our results strongly suggest that *Id1/3* regulate antioxidant gene expression via the regulation of *Maf* expression, thereby providing a novel mechanism of the cellular antioxidant response.

Discussion

We have unveiled a novel cellular role for IDs in the regulation of redox status, mitochondrial integrity and beta cell survival under oxidative stress conditions. We have shown for the first time that IDs are oxidative stress-responsive genes in beta cells. All four members of the ID family were upregulated in the islets of diabetic *db/db* mice. This induction was paralleled by a global upregulation of the antioxidant response. Inhibition of *Id1/3* in beta cells led to mitochondrial impairment and global attenuation of the antioxidant response. Since oxidative stress is a major downstream mechanism of beta cell glucotoxicity [13], our results provide important insight into the molecular mechanisms regulating beta cell survival in type 2 diabetes.

IDs and the antioxidant response NFE2L2 is a key regulator of antioxidant gene expression and cellular homeostasis [39]. Unexpectedly, we found that the inhibition of *Id1/3* markedly increased NFE2L2 expression and nuclear localisation. This interesting observation, which is in line with the increased ROS accumulation under these conditions, suggested that *Id1/3* may affect an interacting partner rather than NFE2L2 itself. Indeed, we found that inhibition of *Id1/3* inhibited the expression of small MAF proteins F, K and G, known interacting partners of NFE2L2 that play an important role in the regulation of antioxidant gene expression [24–26, 40]. Interestingly, inhibition of *MafF/G/K* reduced the expression of several antioxidant genes and potentiated ribose-induced apoptosis, thereby mimicking the effects of *Id1/3* inhibition.

These striking findings provide a novel regulatory mechanism of the antioxidant response. Our results strongly suggest that *Id1/3* regulate antioxidant gene expression through the regulation of small MAF protein expression. Our results highlight the important role of small MAF proteins in beta cells. While recent reports have focused on the role of NFE2L2 in diabetes and beta cell pathophysiology [23, 41], less attention has been given to the role of small MAF proteins. Yet, our results show that increased expression and nuclear accumulation of NFE2L2 alone, in the absence of MAFs, was insufficient to maintain adequate expression of antioxidant genes under oxidative stress.

IDs and mitochondria Mitochondria have been shown to be a primary target for oxidative damage. Thus, acute exposure of rat islets and INS1 cells to H_2O_2 has been shown to impair

Fig. 9 Inhibition of MAFs reproduces the effects of *Idl/3* inhibition on antioxidant gene expression and apoptosis. MIN6 cells transfected with either control siRNA (si-C) or siRNA against *MafF*, *MafK* and *MafG* (si-FGK) were cultured for 48 h in the absence (white bars) or presence of 50 mmol/l ribose (black bars). Changes in (a–i) mRNA levels and (j) apoptosis. $n=3-4$ experiments. ** $p<0.01$, *** $p<0.001$ vs same group in the absence of ribose; † $p<0.05$, †† $p<0.01$, ††† $p<0.001$ vs control

siRNA. (k) The proposed model: IDs are oxidative stress-responsive proteins that regulate redox status, mitochondrial integrity and beta cell survival. IDs maintain the adaptive mitochondrial–antioxidant response during oxidative stress via (1) upregulation of TFAM and components of the ETC; and (2) upregulation of NFE2L2-interacting partners small MAF proteins and subsequent induction of antioxidant genes

mitochondrial function, reduce *Tfam* and ETC component expression, especially MTCO1, and increase mitochondrial ROS production and apoptosis [42]. These observations mirror the effects of *Idl/3* inhibition. The global attenuation of antioxidant gene expression induced by *Idl/3* inhibition may lead to ROS accumulation and mitochondrial alterations that enhance ROS generation, thus further exacerbating oxidative stress/damage and apoptosis. Alternatively, the downregulation of *Tfam* and ETC components by *Idl/3* inhibition may slow electron flow leading to enhanced ROS generation. Indeed, it is well established that beta cells generate high ROS levels when cultured in the presence of a low non-stimulatory glucose concentration in association with increased expression of antioxidant genes and apoptosis [6, 43, 44]. The resulting oxidative stress is further aggravated by the global attenuation of the antioxidant response. This view is supported by the observation that *Idl/3* inhibition leads to enhanced ROS formation, increased oxidative damage and potentiation of apoptosis under basal conditions in the absence of any

ribose or H_2O_2 stimulation. Moreover, in our model, chronic stimulation with H_2O_2 or ribose increases TFAM and ETC component expression. In line with this observation, mRNA levels of *Tfam* were upregulated in the islets of diabetic mice despite the presence of oxidative stress. Either way, these alterations were associated with altered mitochondrial morphology. The latter has been observed in beta cells of type 2 diabetes both in animal models and in humans, as well as in in vitro models of nutrient oversupply and oxidative stress [15, 19, 42, 45–47].

Potential exploitation of these new findings to protect beta cells Although antioxidant supplementation has been shown to be promising in type 2 diabetes animal models, it was overall ineffective in humans. Consequently, the activation of endogenous antioxidant genes could be a more promising strategy to reduce ROS-mediated apoptosis in beta cells. The identification of IDs as key upstream regulators of the antioxidant response, besides their parallel role in mitochondria, may serve for the development of a novel therapeutic strategy to

protect beta cells against oxidative stress. IDs are known downstream targets of the bone morphogenetic protein (BMP) signalling pathway. Emerging evidence indicates an important role of this pathway in beta cells [48–50]. However, one should keep in mind that the activation of IDs may also interfere with beta cell differentiation. We have previously shown that *Id1* was induced by elevated lipids in beta cells and plays a role in lipid-induced impairment of insulin secretion and differentiation [32]. Moderate physiological stimulation of this pathway may exert beneficial effects under oxidative stress while avoiding the alteration of the beta cell phenotype. Interestingly, our other studies show that low concentrations of recombinant BMP stimulated the expression of *Id1/3* in parallel with a significant protection against ribose-induced apoptosis in MIN6 cells (M. Bensellam, D. R. Laybutt, unpublished results).

In conclusion, we have identified IDs as a novel family of oxidative stress-responsive proteins in beta cells. We have demonstrated that *Id1/3* are crucial for the maintenance of an adaptive mitochondrial-antioxidant response that promotes beta cell survival under oxidative stress via a novel link to the NFE2L2–MAF signalling pathway. These novel findings may help the development of therapeutic strategies to protect beta cells against oxidative stress.

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Contribution statement MB and DRL conceived and designed experiments, acquired and analysed data and wrote the manuscript. MKM, JL and JYC designed experiments, acquired and analysed data and critically reviewed the manuscript. All authors approved the final version of the manuscript. DRL is responsible for the integrity of the work as a whole.

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