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ORIGINAL ARTICLE

Adrenomedullin has a cytoprotective role against endoplasmic reticulum stress for pancreatic β-cells in autocrine and paracrine manners

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ABSTRACT

Aims/Introduction: Pancreatic β-cells are sensitive to endoplasmic reticulum (ER) stress, which has a major role in the context of β-cell death. Adrenomedullin (ADM) has been shown to exert a cytoprotective effect under various pathophysiological conditions. Several studies have suggested that thiazolidinediones have protective effects on β-cells. During the course to elucidate the molecular mechanisms by which pioglitazone prevents β-cell death, ADM emerged as a candidate. Here, we studied the regulation of ADM expression, including the effects of pioglitazone, and its role in pancreatic islets.

Materials and Methods: We analyzed ADM expression in islet cell lines treated with pioglitazone. The effects of ER stress on ADM and ADM receptor expressions were investigated by analyzing thapsigargin-treated MIN6 cells and islets isolated from *Wfs1*$^{-/-}$ and *db/db* mice. To study the anti-apoptotic effect of ADM, ER stress-exposed MIN6 cells were treated with ADM peptides or transfected with ADM expression plasmid.

Results: Pioglitazone increased the production and secretion of ADM in islets through peroxisome-proliferator activated receptor-γ-dependent mechanisms. Thapsigargin treatment increased expressions of both ADM and ADM receptor, composed of *Ramp2*, *Ramp3* and *Crlr*, in MIN6 cells. ADM and ADM receptor expressions were also increased in isolated islets from *Wfs1*$^{-/-}$ and *db/db* mice. ADM peptides and ADM overexpression protected MIN6 cells from thapsigargin-induced apoptosis.

Conclusions: ER stress stimulates ADM production and secretion in islets. ADM signaling might protect β-cells from ER stress-induced apoptosis, and might be one of the self-protective mechanisms. β-Cell protection by pioglitazone is partly through induction of ADM. ADM-based therapy could be a novel strategy for treating diabetes.

INTRODUCTION

Pancreatic β-cell failure and death are the critical pathogenesis of most forms of type 2 diabetes mellitus. Uncovering the molecular mechanisms leading to β-cell failure and death is therefore essential to develop type 2 diabetes mellitus treatments based on β-cell protection. Although monogenic forms of diabetes are uncommon, they sometimes provide meaningful insights into common molecular pathways underlying type 2 diabetes mellitus.

Wolfram syndrome (WFS) is a rare genetic disorder represented by young-onset, insulin-deficient diabetes, optic atrophy, deafness and neurological problems[1]. Most WFS patients harbor mutations in the *WFS1* gene[2]. WFS1 protein (wolframin) is a 100 kDa transmembrane glycoprotein localized in the endoplasmic reticulum (ER)[3]. WFS1 protein is involved in ER stress responses, and loss of WFS1 protein causes ER stress in the β-cells, resulting in the ER stress-induced dysfunction and

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apoptosis[4–6]. Importantly, common polymorphisms in the *WFS1* gene are associated with type 2 diabetes mellitus, showing that β-cell failure in WFS might be an extreme example of that of type 2 diabetes[5,7–11]. In fact, there is abundant evidence showing that β-cell ER stress is involved in the development of type 2 diabetes[11]. During the attempts to investigate the mechanism whereby loss of WFS1 protein causes β-cell failure, we found that pioglitazone (PIO) remarkably prevented diabetes development in the *Wfs1*$^{-/-}$*A*^{y}*/a* mouse, a mouse model of WFS[5]. By microarray gene expression analysis of the islets from PIO-treated *A*^{y}*/a* mice, upregulation of adrenomedullin (ADM) was shown.

Adrenomedullin is a vasodilator peptide composed of 52 amino acid residues, identified from human pheochromocytoma[12]. ADM belongs to the calcitonin/calcitonin gene-related peptide family, and the ADM signal is conveyed by the calcitonin receptor-like receptor (CRLR). CRLR communicates with receptor activity-modifying protein (RAMP). RAMP2 and RAMP3 transport CRLR to the cellular membrane to produce ADM receptors 1 and 2, respectively[13]. ADM is secreted from a variety of cell types, functioning in both an autocrine and paracrine fashion, and is considered to act as an anti-oxidant, anti-inflammatory, anti-fibrotic and anti-apoptotic mediator[14–16].

Numerous studies have shown ADM to have a broad distribution in many tissues and organs, which is reflected by its diverse physiological functions under both healthy and disease conditions. ADM exerts potent protective effects under various pathological conditions, playing its anti-inflammatory and anti-apoptotic roles. We hypothesized that ADM has protective effects on the stressed pancreatic β-cells and night mediate, at least in part, the protective effects of PIO. The present study aimed to investigate the regulation of ADM expression and its anti-apoptotic effects under ER stress conditions in pancreatic islets.

METHODS

Animal experiments

We carried out all mouse experiments under the approval of the Ethics of Animal Experimentation Committee at Yamaguchi University School of Medicine. The mouse facility was temperature-controlled under a 12-h light/12-h dark cycle. The starting time of the light period was Zeitgeber time 0, and that of the dark period was Zeitgeber time 12. PIO, supplied by Takeda Pharmaceutical (Osaka, Japan), was given within normal mouse chow with 0.01% PIO (wt/wt) from 4 weeks-of-age.

The yellow agouti (*A*^{y}*/a*) mice used for these experiments were a kind gift from Professor M Nishimura (Nagoya University Graduate School of Medicine, Nagoya, Japan). WFS1-deficient *A*^{y}*/a* mice were generated by breeding *Wfs1*$^{-/-}$ mice with *A*^{y}*/a* mice to make the compound heterozygote (*Wfs1*$^{+/-}$ *A*^{y}*/a*). Then, the heterozygote was mated with *Wfs1*$^{+/-}$ *a/a*. Male *db/db* mice and non-diabetic controls were obtained from CLEA Japan (Tokyo, Japan). All mice are on the C57BL/6J background, and experiments were carried out with male mice.

Microarray experiment

Total ribonucleic acid (RNA) was extracted from mouse islets using ISOGEN (Nippon Gene, Tokyo, Japan) and the RNeasy Kit (Qiagen, Hilden, Germany). Labeling, hybridization (Affymetrix Mouse Genome 430 2.0 Array; Santa Clara, CA, USA) and scanning were carried out at KURABO (Osaka, Japan), following standard Affymetrix procedures.

Cell and islet culture

The mouse pancreatic β-cell line (MIN6 cells[17]) was a kind gift from Dr J Miyazaki. The pancreatic α-cell line, alpha-TC1, was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). We cultured MIN6 cells and alpha-TC1 cells, at passages 23–30, in 25 mmol/L glucose Dulbecco's modified Eagle's medium containing 15% fetal calf serum, 71.5 μmol/L beta-mercaptoethanol, 50 mg/L streptomycin and 75 mg/L penicillin sulfate under 5% CO_2 at 37°C. Pancreatic islets were isolated from C57BL/6 mice by ductal perfusion with collagenase. The islets were hand-picked and kept in RPMI medium with 10% fetal calf serum.

RNA extraction and quantitative reverse transcription polymerase chain reaction

MIN6 RNA was extracted by using the RNeasy Mini Kit (Qiagen). Mouse islet RNA was extracted with Isogen (Nippon Gene) and the RNeasy Kit. Complementary deoxyribonucleic acid (DNA) synthesized with Superscript II Reverse Transcriptase (Life Technologies, Carlsbat, CA, USA) was subjected to real-time polymerase chain reaction (PCR) on the Step one Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR Master Mix (Life Technologies). Each complementary DNA was quantitated using the ΔCt method and normalized to the amount of *Gapdh*.

Primer sequences, designed for mouse genes, are listed below.

m*Gapdh* forward: 5′-AGTATGACTCCACTCACGGCAA-3′
m*Gapdh* reverse: 5′-TCTCGCTCCTGGAAGATGGT-3′
m*Ddit3* forward: 5′-GGAGGTCTTCCTCAGATG-3′
m*Ddit3* reverse: 5′-GGACGCAGGGTCAAGAGTAG-3′
m*Adm* forward: 5′-CCCAGACTCTTGATCCATTCC –3′
m*Adm* reverse: 5′-GTAGCGTTTGACACGAATGTG –3′
m*Ramp2* forward: 5′-CTCATCCCACTGAGGACAGC-3′
m*Ramp2* reverse: 5′-TGCACCAGTCCTTGACAGAG-3′
m*Ramp3* forward: 5′-CTGTCTGGAAGTGGTGCAAC-3′
m*Ramp3* reverse: 5′-CTCGGTGCAGTTAGTGAAGC-3′
m*Crlr* forward: 5′-CAGAAGGCCTTTACTGCAATAGG-3′
m*Crlr* reverse: 5′-TCCCTGCTGCAACGTCATT-3′

Reporter gene assay

Transfection of the *Adm* promoter luciferase reporter, constructed in the pGL plasmid (Promega, Madison, WI, USA), to MIN6 cells was carried out after a 15-h culture in six-well plates using Lipofectamine 2000 (Thermofisher, Waltham, CA, USA). Then, 24 h after the transfection, cells were treated with

or without 10 μmol/L PIO. At 48 h post-transfection, cells were analyzed for luciferase activity using a PicaGene LUC assay kit (Toyo Ink, Tokyo, Japan). Luciferase activities were normalized to protein content.

Chromatin immunoprecipitation

MIN6 cells were cultured with or without 10 μmol/L PIO for 48 h in a 10-cm dish. We carried out the chromatin immunoprecipitation (ChIP) assay using an EZ-ChIP chromatin immunoprecipitation kit (Merck Millipore, Billerica, MA, USA). After fixing with 1% formaldehyde, cells were lysed, briefly sonicated and immunoprecipitated at 4°C overnight. The following antibodies were used in the ChIP reactions: normal rabbit immunoglobulin G (Santa Cruz Biotechnology, Dallas, TX, USA), anti-acetyl histone H3 (Merck Millipore) and anti-peroxisome-proliferator activated receptor (PPAR; Santa Cruz Biotechnology). We washed the ChIP reactions, and eluted chromatin based on the manufacturer's protocol. Chromatin was purified with PCR clean up columns (Qiagen), and PCRs were carried out with AmpliTaq Gold PCR Master Mix (Thermofisher).

The following primers, designed for mouse genes, were used.
ADM-P1 forward: 5′- CAAACTTGGCAAGCACTCAG-3′
ADM-P1 reverse: 5′- AATGGGCTAGGACACACTCC-3′
ADM-P2 forward: 5′- CAAACTTGGCAAGCACTCAG-3′
ADM-P2 reverse: 5′- ACGGGTACTCCAAATGAAGG-3′
ADM-P3 forward: 5′- AAACCCCAATTTCCAATTCAG-3′
ADM-P3 reverse: 5′- GAAGGGGAACCAGAACAACTC-3′

Immunofluorescence

Adult mouse pancreatic tissues were harvested and processed, as previously described[18]. After antigen retrieval on tissue sections, overnight incubation was carried out with each of the primary antibodies: guinea pig anti-insulin (1:4,000; Sigma, St. Louis, MD, USA), mouse anti-glucagon (1:4,000; Abcam, Cambridge, MA, USA) and rabbit anti-ADM (1:200; Santa Cruz Biotechnology). Coverslips were placed in antifade solution containing 4′,6-diamidino-2-phenylindole (Vectashield; Vecta Laboratories, Burlingame, CA, USA). To visualize the antigens, fluorescein FITC and cyanine Cy3-conjugated appropriate secondary antibodies (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used. Digital images acquired were compiled using a BZ Analyzer (Keyence, Osaka, Japan).

Measurement of ADM peptide

The serum and secreted ADM levels were measured using an enzyme immunoassay (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), following the manufacturer's instructions.

Treatments with ADM peptide

Rat ADM (1–50) and human ADM (22–52) were purchased from Peptide Institute (Osaka, Japan). MIN6 cells were cultured for 24 h with ADM peptide at 0.1 or 100 nmol/L in the presence or absence of 1 μmol/L thapsigargin (TG).

Western blot analysis

MIN6 cells, alpha-TC1 cells and isolated islets were lysed in lysis buffer (Cell Signaling Technology, Danvers, MA, USA), and the lysates were separated by 4–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). Western blot analyses were carried out with anti-cleaved caspase 3 (1:1,000; Cell Signaling Technology) and α-tubulin (1:1,000; Sigma-Aldrich) antibodies.

Quantification of DNA fragmentation

MIN6 cells were incubated in 12-well plates overnight before transfection of pCDNA-ADM or pCDNA-LacZ using Lipofectamine 2000 (Thermofisher). Then, 24 h post-transfection, these cells were cultured in the presence or absence of 1 μmol/L TG. At 48 h post-transfection, floating and detached cells were lysed, and chromosomal DNA was arranged using the Quick Apoptotic DNA Ladder Detection Kit (BioVision Inc., Milpitas, CA, USA), according to the manufacturer's instructions. A total of 20 μL of each sample were then electrophoresed on a 1.2% agarose/EtBr gel.

Measurement of intracellular cyclic adenosine monophosphate level

MIN6 cells were incubated in 12-well plates overnight before transfection of pCDNA-ADM or pCDNA-LacZ using Lipofectamine 2000 (Thermofisher). Then, 24 h post-transfection, cells were cultured with or without 1 μmol/L TG. At 48 h post-transfection, cells were lysed with 0.1 mol/L HCl. Intracellular cyclic adenosine monophosphate (cAMP) levels were analyzed with a Cyclic AMP EIA kit (Cayman Chemical, Ann Arbor, MI, USA), referring to the manufacturer's instructions.

Statistical analysis

Student's *t*-test or Welch's *t*-test were used to compare the study groups with the control group. Data are shown as the mean ± standard error of the mean, and differences were considered to be significant at $P < 0.05$.

RESULTS

In vivo and *in vitro* assessments of direct effects of PIO on *Adm* messenger RNA expression in pancreatic islet cells

The original aim of the present study was to determine the molecular mechanisms by which PIO prevents cell apoptosis by exerting a direct action on pancreatic β-cells. We previously analyzed gene expression profiles of islets using microarray in A^y/a mice fed standard chow with or without PIO for 4 weeks (Table S1). We noticed *D7Wsu130e*, an expressed sequence tag, that was mapped very close to the *Adm* gene was among the genes upregulated by PIO feeding. Therefore, we took the *Adm* as a functional candidate, and analyzed further. Real-time PCR confirmed significant upregulation, of approximately 1.3-fold (Figure 1a). Basal messenger RNA (mRNA) expression of *Adm* was significantly higher in alpha-TC1 cells than in MIN6 cells

(Figure 1b). It was found that 48 h of 10 μmol/L PIO treatment significantly increased *Adm* mRNA expression in both alpha-TC1 (1.3-fold) and MIN6 (2.3-fold) cells (Figure 1b). The reporter construct was constituted from a ~1.5 kb fragment of the *Adm* promoter containing three PPAR responsive element-like regions. Luciferase activity was analyzed based on the value of LacZ normalized by protein content. It was found that 24 h of treatment with 10 μmol/L PIO increased *Adm* promoter activity by 1.2-fold (Figure 1c). By ChIP assay, we confirmed PPARγ-mediated *Adm* induction by PIO. MIN6 cells were cultured with or without PIO, and chromatin extracts from respective cells were immunoprecipitated. PCR was carried out with the primers designed to encompass PPAR responsive element-like regions (ADM-P1-3). PIO activated PPARγ binding to the specific region (P3) within the *Adm* promoter (Figure 1d).

Adm and ADM receptor expressions in Wfs1$^{-/-}$ islets

ER stress was assessed by measuring the relative expression of the ER stress-associated protein CHOP (*Ddit3*) mRNA in islets from *Wfs1*$^{+/+}$ *a/a*, *Wfs1*$^{+/+}$ *A*^{y}*/a*, *Wfs1*$^{-/-}$ *a/a* and *Wfs1*$^{-/-}$ *A*^{y}*/a* mice. *Ddit3* expression was significantly elevated in islets from *Wfs1*$^{-/-}$ *a/a* and *Wfs1*$^{-/-}$ *A*^{y}*/a* mice, as compared with *Wfs1*$^{+/+}$ *a/a* mice (Figure 2a). *Adm* mRNA expression was significantly elevated in islets from *Wfs1*$^{-/-}$ *a/a* and *Wfs1*$^{-/-}$ *A*^{y}*/a* mice, as compared with those from *Wfs1*$^{+/+}$ *a/a* mice, in association with the increase in *Ddit3* expression (Figure 2a,b), whereas serum ADM was not elevated in either *Wfs1*$^{-/-}$ *a/a* or *Wfs1*$^{-/-}$ *A*^{y}*/a* mice, as compared with *Wfs1*$^{+/+}$ *a/a* mice (Figure 2c). Immunofluorescence analysis for endogenous ADM in islets from *Wfs1*$^{-/-}$ *A*^{y}*/a* mice showed that increased ADM was mainly expressed in non- β-cells (Figure 2d). ADM fluorescence appeared to be higher in insulin-positive cells in *Wfs1*$^{-/-}$ *A*^{y}*/a* mice than that in wildtype (WT) mice (Figures 2d,S1). This suggests that ER stress enhances ADM expression in β-cells *in vivo*. ADM receptors are composed of heterodimerization of the CRLR and a RAMP2 or RAMP3. Alterations in ADM receptor expression in islets from *Wfs1*$^{-/-}$ *a/a* mice were evaluated at the mRNA level using real-time PCR. *Ramp2*, *Ramp3* and *Crlr* mRNA levels were significantly increased in

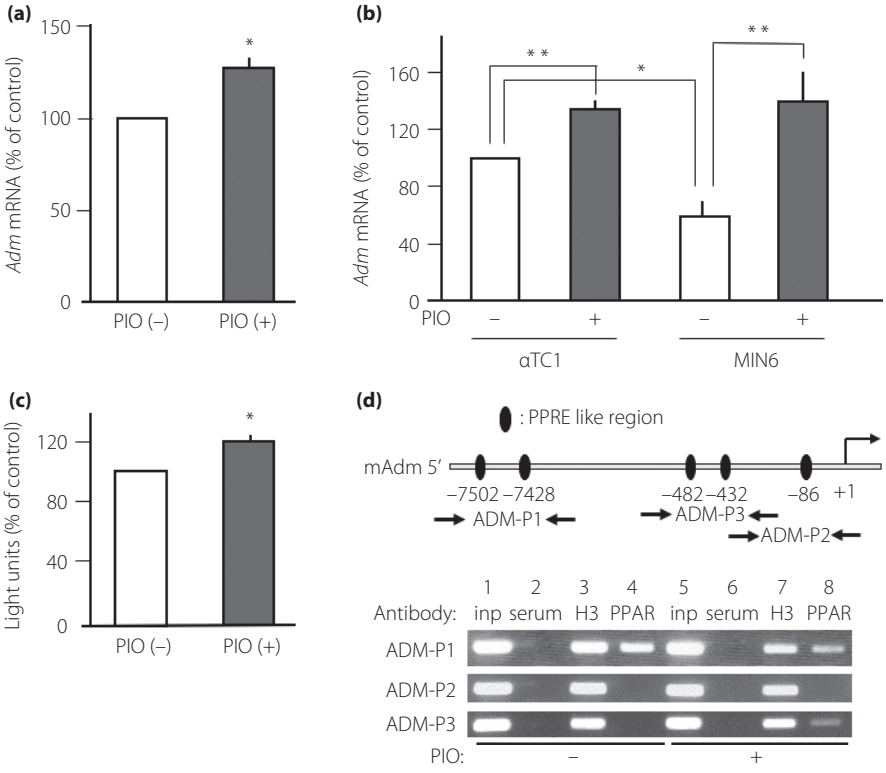

Figure 1 | Effects of pioglitazone (PIO) on adrenomedullin (*Adm*) messenger ribonucleic acid (mRNA) expression in pancreatic islet cells. (a) *Adm* mRNA expression in isolated islets from 8-week-old *A*^{y}*/a* mice maintained on a normal chow diet or a chow diet with 0.1% PIO. Data are the mean ± standard error of the mean (n = 3). *P < 0.05. (b) *Adm* mRNA expression in αTC1 or MIN6 cells cultured for 48 h in the presence or absence of 10 mmol/L PIO. Data are the means ± standard error of the mean (n = 4). **P < 0.01, *P < 0.05. (c) Activities of the mouse *Adm* promoter reporter construct in MIN6 cells treated with or without 10 μmol/L PIO. Normalized luciferase activities are shown as the means ± standard error of the mean (n = 3). *P < 0.05. (d) PIO-dependent peroxisome-proliferator activated receptor-γ (PPARγ) binding to the *Adm* promoter. Chromatin immunoprecipitation analysis of the *Adm* promoter in MIN6 cells treated with or without 10 μmol/L PIO for 48 h was carried out using the indicated antibodies. Lanes 1 and 5, input deoxyribonucleic acid (inp); lanes 2 and 6; normal rabbit immunoglobulin G (serum); lanes 3 and 7, anti-acetyl-histone H3 (H3); lanes 4 and 8, anti-PPAR antibody (PPAR). PPRE, PPAR responsive element.

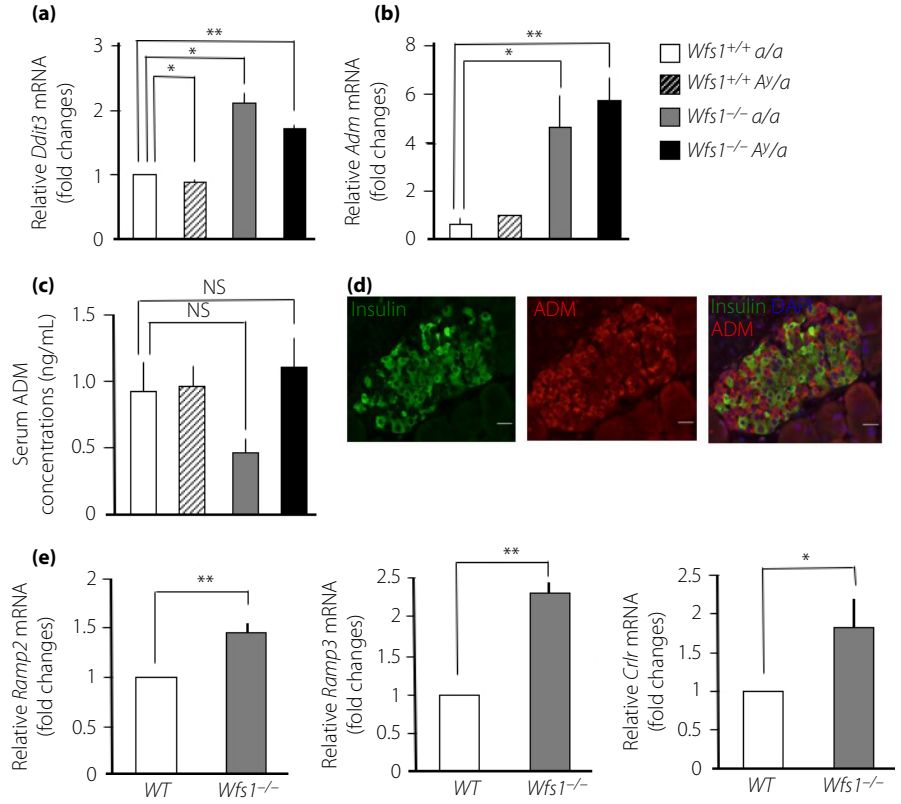

Figure 2 | Adrenomedullin (*Adm*) and ADM receptor expressions are upregulated in $Wfs1^{-/-}$ islets. (a) *Ddit3* and (b) *Adm* messenger ribonucleic acid (mRNA) expressions were analyzed by real-time polymerase chain reaction in $Wfs1^{+/+}$ *a/a*, $Wfs1^{+/+}$ A^y/a, $Wfs1^{-/-}$ *a/a* and $Wfs1^{-/-}$ A^y/a islets. Individual RNA samples were purified from 100–400 pancreatic islets from mice at 8–10 weeks-of-age. Data are the mean ± standard error of the mean (*Ddit3*; $n = 3$ mice/genotype, *Adm*; $n = 6$ mice/genotype). $^{**}P < 0.01$, $^{*}P < 0.05$. (c) Plasma ADM concentrations in $Wfs1^{+/+}$ *a/a*, $Wfs1^{-/-}$ *a/a* and $Wfs1^{-/-}$ A^y/a at 10 weeks-of-age were measured by enzyme-linked immunosorbent assay. Data are the mean ± standard error of the mean ($n = 5$–12 mice/genotype). (d) Immunofluorescence analysis for endogenous ADM in $Wfs1^{-/-}$ A^y/a islets at 10 weeks-of-age. Scale bars, 20 μm. (e) *Ramp2, Ramp3 and Crlr* mRNA expressions were analyzed by real-time polymerase chain reaction in $Wfs1^{+/+}$ *a/a* and $Wfs1^{-/-}$ *a/a* islets. RNA was purified from 200–400 pancreatic islets from three mice at 8–10 weeks of age. Data are the mean ± standard error of the mean ($n = 3$ or five mice/genotype). $^{**}P < 0.01$, $^{*}P < 0.05$. A^y/a, yellow agouti; Crlr, calcitonin receptor like receptor; Ddit3, deoxyribonucleic acid damage inducible transcript 3; NS, no significant difference; Ramp, receptor activity-modifying protein.

islets from $Wfs1^{-/-}$ *a/a* mice, as compared with $Wfs1^{+/+}$ *a/a* mice (Figure 2e). These findings raise the possibility of the paracrine and autocrine actions of ADM being enhanced in pancreatic islets subjected to ER stress.

Adm and ADM receptor expressions in *db/db* mouse islets

Blood glucose levels were markedly higher in *db/db* mice than those in *WT* mice (Figure 3a). Serum ADM levels in *db/db* (3.5 ± 0.7 ng/mL [mean ± SEM]) mice were also obviously higher than those in *WT* mice (1.9 ± 0.2 ng/mL [mean ± SEM]; Figure 3b). *Ddit3* expression was significantly increased in islets from *db/db* mice, as compared with *WT* mice (Figure 3c). *Adm*, *Ramp2*, *Ramp3* and *Crlr* mRNA levels were also increased significantly in islets from *db/db* mice, as compared with the *WT* control mice (Figure 3d–g). Serum ADM elevations in *db/db* mice appeared to be associated with hyperglycemia, which in turn could induce systemic *Adm* mRNA expression and ADM secretion mainly in the vasculature. In contrast, blood glucose levels in $Wfs1^{-/-}$ *a/a* mice were normal (11.3 ± 0.51 mmol/L in $Wfs1^{+/+}$ *a/a* and 10.1 ± 0.38 mmol/L in $Wfs1^{-/-}$ *a/a* [mean ± SEM]), whereas *Adm* mRNA expression was significantly elevated in $Wfs1^{-/-}$ *a/a* islets (Figure 2b). These findings suggest that ER stress, without hyperglycemia, might enhance *Adm* expression, particularly in pancreatic islets.

Adm and ADM receptor expressions in MIN6 cells treated with TG

Adm and ADM receptor expressions were increased in $Wfs1^{-/-}$ and *db/db* islets in association with ER stress. These data show an interesting association between upregulations of *Adm* and the ADM receptor and increased ER stress *in vivo*. To confirm this association *in vitro*, we next examined the expression levels of *Adm* and the ADM receptor in the pancreatic β-cell line,

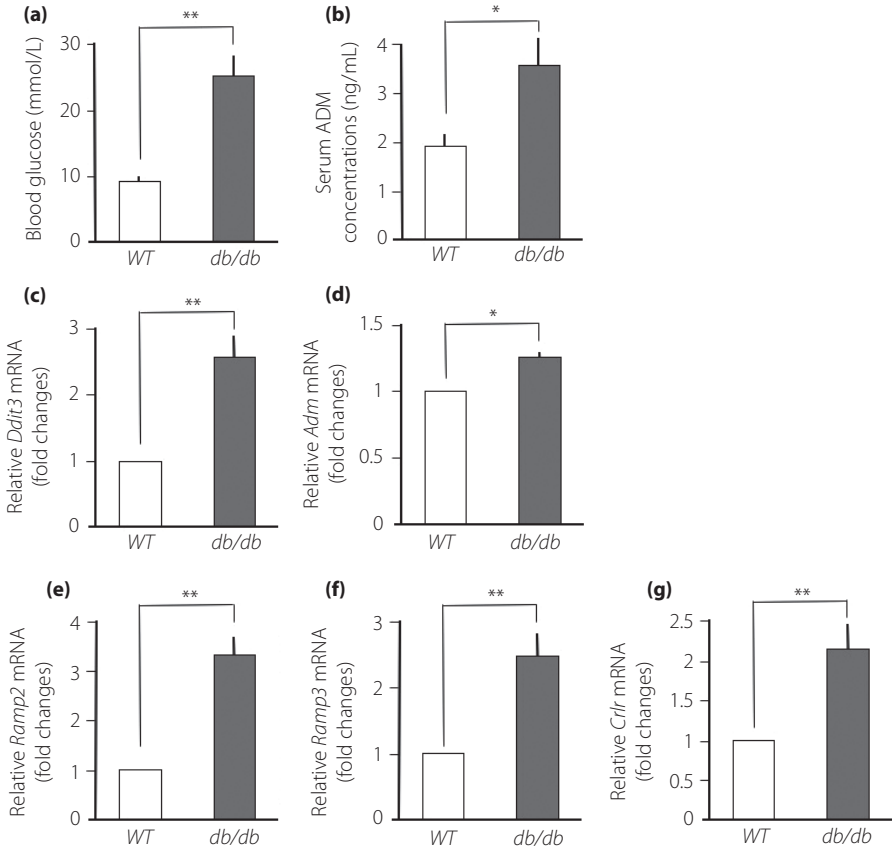

Figure 3 | Adrenomedullin (*Adm*) and ADM receptor expressions are upregulated in *db/db* islets. (a) Non-fasting blood glucose levels of wild-type (*WT*) and *db/db* mice. Data are the mean ± standard error of the mean ($n = 8$ in each group). $^{**}P < 0.01$. (b) Plasma ADM concentrations in *WT* and *db/db* mice were measured by enzyme-linked immunosorbent assay at 10 weeks-of-age. Data are the mean ± standard error of the mean (*WT*, $n = 5$; *db/db*, $n = 5$). $^{*}P < 0.05$. (c) deoxyribonucleic acid damage inducible transcript 3 (*Ddit3*), (d) *Adm*, (e) receptor activity-modifying protein (*Ramp*)*2,* (f) *Ramp3 and* (g) calcitonin receptor like receptor (*Crlr*) messenger ribonucleic acid (mRNA) expressions were analyzed by real-time polymerase chain reaction in *WT* and *db/db* islets. Individual RNA samples were purified from 100–400 pancreatic islets from mice at 8–10 weeks of age. Data are the mean ± standard error of the mean (*WT*, $n = 4$ mice; *db/db*, $n = 6$ mice). $^{**}P < 0.01$, $^{*}P < 0.05$.

MIN6, treated with TG. *Ddit3* mRNA was increased 20-fold (Figure 4a). Treatment with 1 μmol/L TG for 24 h significantly increased MIN6 *Adm* (9.3-fold), *Ramp2* (1.47-fold), *Ramp3* (3.55-fold) and *Crlr* (2.97-fold) mRNA (Figure 4b–e). Much more ADM was secreted from TG-treated MIN6 cells than from control MIN6 cells in accordance with the transcriptional levels (Figure 4f).

ADM-mediated protection from apoptosis induced by TG

We studied the effect of ADM peptide on MIN6 cells exposed to TG. MIN6 cell death was assayed by measuring the level of cleaved caspase 3 expression. Both rat ADM 1–50 (full length) peptide and human ADM 22–52 (partial) peptide at 100 nmol/L significantly reduced the cleaved caspase 3 by 24 and 36%, respectively, but neither peptide exerted this effect at 0.1 nmol/L (Figure 5a). We also studied the anti-apoptotic effect of ADM overexpression on MIN6 cells treated with TG. The transcriptional levels of *Adm* increased markedly in MIN6 cells transfected with the *Adm* construct (pCDNA3-ADM), as compared with those transfected with the *LacZ* construct (pCDNA3-LacZ; Figure 5b). Much more ADM was secreted from pCDNA3-ADM transfected MIN6 cells than from MIN6 cells transfected with pCDNA3-LacZ, as reflected by the transcriptional levels, suggesting that MIN6 cells secrete ADM (Figure 5c). ADM overexpression significantly reduced the TG-evoked caspase 3 cleavage, by 49% (Figure 5d), and also significantly suppressed DNA fragmentation, by 56% (Figure 5e).

Effects of ADM on cAMP levels in MIN6 cells treated with TG

Previous studies have shown ADM to increase cAMP levels in both myocytes and oligodendroglial cells[35,48]. We investigated the possibility that ADM protects MIN6 cells from TG-induced apoptosis through elevation of intracellular cAMP levels. MIN6 cells transfected with the *adm* construct (pCDNA3-ADM) or the LacZ construct (pCDNA3-LacZ) were cultured for 24 h in the presence or absence of 1 μmol/L TG. After incubation, we

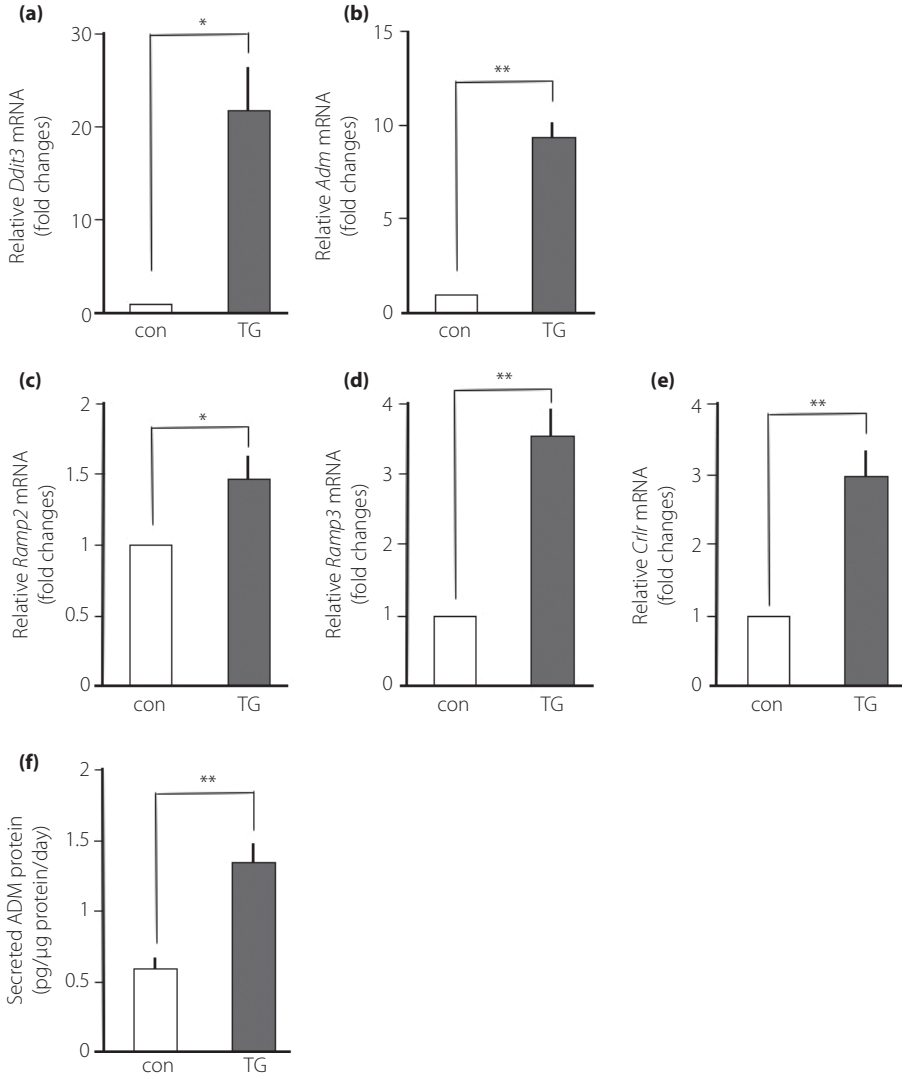

Figure 4 | Adrenomedullin (*Adm*) and ADM receptor expressions are upregulated in thapsigargin (TG)-treated MIN6 cells. (a) deoxyribonucleic acid damage inducible transcript 3 (*Ddit3*), (b) *Adm*, (c) receptor activity-modifying protein (*Ramp)2,* (d) *Ramp3 and* (e) calcitonin receptor like receptor (*Crlr*) messenger ribonucleic acid (mRNA) expressions were analyzed by real-time polymerase chain reaction. RNA was purified from MIN6 cells treated with dimethyl sulfoxide (DMSO) (con) or 1 μmol/L thapsigargin (TG) for 24 h. (f) Secreted ADM was quantified in the media from cells treated with DMSO (con) or 1 μmol/L TG by enzyme-linked immunosorbent assay. The values were normalized by the amounts of cellular α-tubulin quantified by western blotting. All data are presented as the mean ± standard error of the mean of three to five independent experiments. Welch's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$.

measured intracellular cAMP levels in these MIN6 cells. TG treatment decreased intracellular cAMP levels in MIN6 cells transfected pCDNA3-LacZ, from 37.2 ± 4.8 pmol/mg protein to 19.6 ± 2.8 pmol/mg protein, and ADM partially blunted the decrease in intracellular cAMP levels, to 30.5 ± 2.5 pmol/mg protein (Figure 6). These results suggest that the anti-apoptotic effects of ADM involve intercellular cAMP elevation in pancreatic β-cells exposed to ER stress.

DISCUSSION

Herein, we showed that the ADM and ADM receptor expression levels are upregulated under ER stress conditions in pancreatic islets and in β-cell-derived cell lines. ADM, resulting from both autocrine and paracrine secretion, shows anti-apoptotic effects in pancreatic β-cells through upregulated ADM signaling and prevention of ER stress-induced reduction of intracellular cAMP.

We previously showed that PIO treatment ameliorates hyperglycemia and β-cell loss in *Wfs1*$^{-/-}$ *A*^{y}*/a* mice[5]. WFS1 deficiency and mild obesity as a result of hyperphagia in these mice led to ER stress-induced β-cell dysfunction and apoptosis, and PIO appeared to reduce ER stress in *Wfs1*$^{-/-}$ *A*^{y}*/a* islets and prevent diabetes[5]. In general, PIO reduces ER stress by improving peripheral insulin sensitivity. As an investigation of

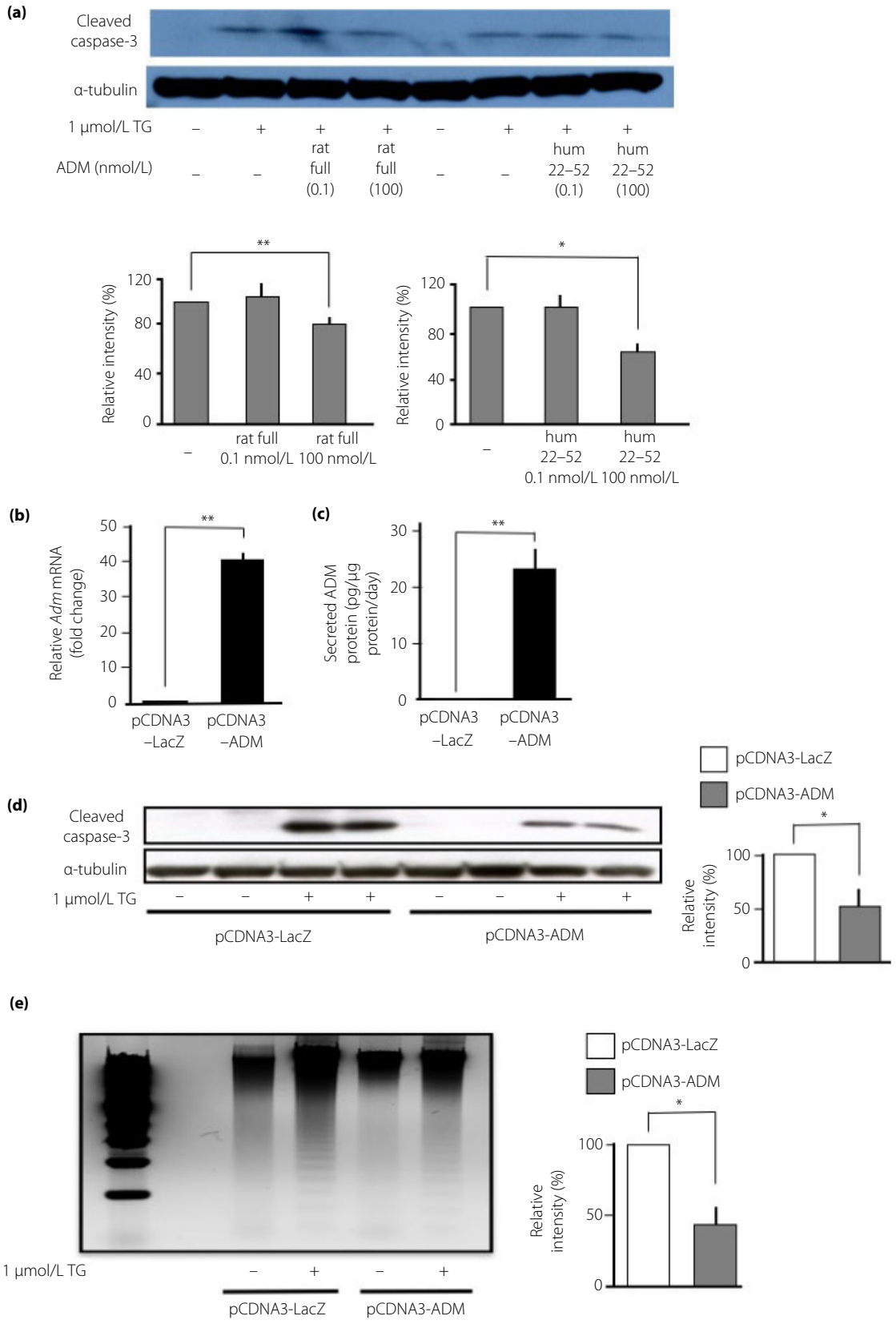

Figure 5 | Adrenomedullin (ADM) protects MIN6 cells from thapsigargin (TG)-induced apoptosis. (a) MIN6 cells were cultured for 24 h with rat full-length ADM peptide or human partial ADM peptide (hum 22–52) at 0.1 or 100 nmol/L in the presence or absence of 1 μmol/L TG. After the culturing, cleaved caspase 3 levels were determined. α-Tubulin was used as the protein loading control. All data are presented as the mean ± standard error of the mean of four or five independent experiments. Welch's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$. (b,c) pCDNA3-LacZ or pCDNA3-ADM was transfected into MIN6 cells. (b) Total messenger ribonucleic acid (mRNA) was extracted from the cells and subjected to real-time polymerase chain reaction analysis of *Adm*. ADM secreted in 24 h, under the relevant conditions, into the cell culture media was quantified by enzyme-linked immunosorbent assay. (c) ADM concentrations were normalized to the amount of protein in the cell lysate. Data are the mean ± standard error of the mean (n = 4–8). Welch's *t*-test: $^{**}P < 0.01$. (d) TG-induced (1 μmol/L) cleaved caspase 3 levels were determined in pCDNA3-LacZ (control condition) or pCDNA3-ADM transfected MIN6 cells. Data are means ± standard error of the mean (n = 4). Welch's *t*-test: $^{*}P < 0.05$. (e) Deoxyribonucleic acid fragmentation was assessed in pCDNA3-LacZ (control condition) and pCDNA3-ADM transfected MIN6 cells cultured for 24 h in the presence of 1 μmol/L TG. Welch's *t*-test: $^{*}P < 0.05$.

possible direct effects of PIO on the amelioration of ER stress-induced apoptosis in pancreatic islets, we hypothesized that ADM could be a candidate. We studied the effects of PIO on ADM mRNA expression both *in vivo* and *in vitro*. *In vivo*, PIO moderately, though significantly, increased ADM expression. In pancreatic α- and β-cell lines PIO strikingly increased ADM. Furthermore, we clearly showed PIO to increase ADM expression through PPARγ-dependent mechanisms in MIN6 cells. These results show that PPARγ agonists might protect β-cells against ER stress-induced apoptosis, at least in part through induction of ADM in islet cells.

Upregulations of ADM expressions have been detected in various tissues under diverse circumstances in both health and disease states[19,20]. The present study showed that ADM gene expression and production of the ADM protein in pancreatic islet cells were markedly augmented by ER stress. In fact, we found three ER stress response elements (−605, −264 and −649 bp to *Adm* transcription start site) in the *Adm* gene. *Adm* mRNA expression in islets, but not serum ADM concentrations, was significantly elevated in *Wfs1*$^{-/-}$ *a/a* and *Wfs1*$^{-/-}$ *A*^{y}*/a* mice in association with increased *Ddit3* expression (Figure 2a–d). ADM receptor expression was also significantly increased in islets from *Wfs1*$^{-/-}$ *a/a* mice (Figure 2e). These results suggest that under ER stress conditions, ADM paracrine and/or autocrine actions are exerted locally in pancreatic islets.

In contrast, serum ADM levels in *db*/*db* mice were markedly elevated in association with the increased blood glucose levels (Figure 3a,b), an observation consistent with the results of human studies[21,22]. Hyperglycemia reportedly induced ADM expression through protein kinase C in vascular smooth muscle cells[21]. The elevated ADM expression in aortic tissues implies that plasma ADM is derived from the vasculature in diabetic animals, including human with diabetes[21]. Therefore, ADM expression in the vasculature is a potential source of the serum ADM in *db*/*db* mice. In contrast, *Adm* and ADM receptor mRNA expressions were elevated in islets from *db*/*db* mice in proportion to the increase in *Ddit3* expression, suggesting the ADM signal to possibly be locally enhanced in states of ER stress, independently of the elevated serum ADM concentrations in *db*/*db* mouse islets.

We further examined the role of endogenous ADM on the regulation of β-cell function. The present results showed an anti-apoptotic action of ADM in pancreatic islets, which is beneficial in diabetes (Figure 5). In β-cells, an increased need for secreted insulin places a high demand on the ER for insulin synthesis, which leads to cellular stress[23]. In states of severe and chronic ER stress, β-cell apoptosis might be triggered. Long-term hyperglycemia enhances the metabolic flux into mitochondria and induces excessive production of reactive oxygen species, thereby leading to sustained oxidative stress[24]. Many kinds of inflammatory cytokines, elevated in diabetic pancreatic islets, also increase oxidative and ER stress[25,26]. In general, it is accepted that plasma ADM levels are positively associated with oxidative stress[27]. The present study clearly showed that, in pancreatic islets where oxidative stress and ER stress possibly coexisted, ER stress enhances the ADM signal, playing a critical role in β-cell homeostasis aimed at alleviating ER stress-induced apoptosis.

ADM can act as a potent vasodilator, and the vasodilatory effects are mediated by cAMP in vascular smooth muscle cells and the generation of nitric oxide in the epithelium[28,29]. TG

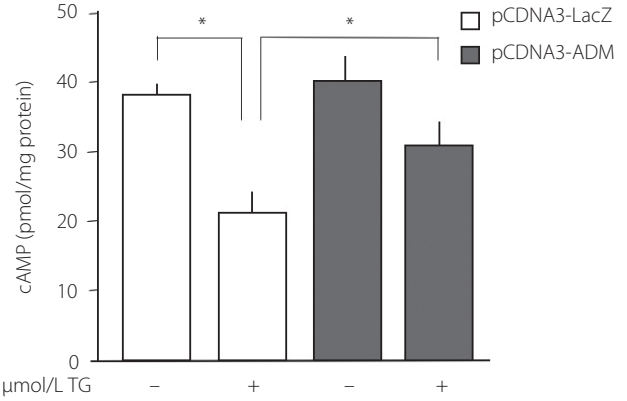

Figure 6 | Adrenomedullin (ADM) partially prevents the decrease in the intracellular cyclic adenosine monophosphate (cAMP) level in response to TG treatment. MIN6 cells transfected with pCDNA3-LacZ or pCDNA3-ADM were incubated for 24 h in the presence or absence of 1 μmol/L thapsigargin (TG). cAMP was extracted from cells with 0.1 mol/L HCl and measured by enzyme-linked immunosorbent assay. Values were normalized by protein concentration. Data are the mean ± standard error of the mean (n = 5). Welch's *t*-test: $^{*}P < 0.05$.

treatment decreased intracellular cAMP levels in MIN6 cells, and ADM partially restored intracellular cAMP (Figure 6), suggesting that the anti-apoptotic effects of ADM are brought about by intracellular cAMP elevation. The anti-apoptotic function of glucagon-like peptide-1 has been shown in animal models[30,31], wherein cAMP elevation activated EPAC2 (exchange protein directly activated by cAMP), thereby inhibiting caspase 3 activation and subsequent apoptosis[32]. It is thus possible that ADM and glucagon-like peptide-1 share a common anti-apoptotic signal pathway in pancreatic β-cells.

ADM plays a major role in blood flow regulation, and ADM functions as a potent vasodilator in the systemic circulation as well as in the vasculature of organs[33]. In addition, ADM was reported to be a cardioprotective peptide because of its anti-apoptotic effects on the vasculature and regulatory roles in cardiac endothelium cell proliferation [34]. ADM administration decreases vascular resistance and increases blood flow through coronary vasodilation, contributing to an elevation in cardiac output[35]. A clinical trial showed that intravenous ADM administration to patients suffering from acute myocardial infarction improved wall motion and infarct size[36]. ADM-based therapy might represent a novel therapeutic strategy for diabetes, in particular because of its cytoprotective effects against ER stress in pancreatic β-cells, which are independent of its cardioprotective benefits.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | The microarray list of significantly upregulated genes in islets from A^y/a mice with pioglitazone compared with islets from A^y/a mice without pioglitazone.

Table S1 | Immunofluorescence analysis for endogenous adrenomedullin in islets from wild-type mice.