Glucose metabolism-related protein 1 (GMRP1) regulates pancreatic beta cell proliferation and apoptosis via activation of Akt signalling pathway in rats and mice

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Abstract

Aims/hypothesis We attempted to elucidate the impacts on and possible mechanisms by which glucose metabolism-related protein 1 (GMRP1) affects beta cell survival.

Methods Adenovirus-mediated GMRP1 overproduction and siRNA-mediated knockdown were performed in INS-1E cells and rat islets, after which cell proliferation or apoptosis were determined, and phosphorylation of Akt and BCL2-associated agonist of cell death (BAD) investigated. INS-1E cells and rat islets were cultured at 5.6 (low) or 25 mmol/l (high) glucose for 24 or 48 h, and cell proliferation or apoptosis and GMRP1 levels were investigated. INS-1E cells were treated for 24 h with 0, 10, 50 and 100 nmol/l insulin, and GMRP1 levels were determined. After INS-1E cells were transfected with siRNA for 72 h, high glucose-induced cell proliferation and insulin-stimulated Akt phosphorylation were investigated. Glucose-infused rat models were established and beta cell proliferation and mass were evaluated. Levels of GMRP1, and phosphorylation of Akt and BAD were determined in

glucose-infused islets. The GMRP1-mediated Akt pathway was also investigated in *db/db* mice.

Results Overproduction of GMRP1 promoted beta cell proliferation via increased phosphorylation of Akt. Knockdown of *Gmrp1* (also known as *Btbd10*) reduced phosphorylation of Akt with enhanced beta cell apoptosis. High glucose increased GMRP1 levels and cell proliferation in INS-1E cells and islet cells. Knockdown of *Gmrp1* decreased high glucose-induced cell proliferation and insulin-stimulated Akt phosphorylation. Increased GMRP1 levels were involved in the enhancement of beta cell proliferation and mass in glucose-infused islets. Decreased GMRP1 levels may participate in beta cell apoptosis of *db/db* mice.

Conclusions/interpretation GMRP1 regulates pancreatic beta cell proliferation and apoptosis via activation of Akt signalling pathway.

Keywords Akt · BAD · Beta cell survival · *db/db* mice · Glucose-infused rat · GMRP1

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Abbreviations

Ad-GFP	Recombinant adenovirus expressing enhanced green fluorescent protein
Ad-GMRP1	Recombinant adenovirus containing full-length cDNA of rat <i>Gmrp1</i>
BAD	BCL2-associated agonist of cell death
GFP	Green fluorescent protein
GMRP1	Glucose metabolism-related protein 1
PKC- δ	3-Phosphoinositide-dependent protein kinase-1
siRNA	Small interfering RNA

Introduction

Diabetes mellitus, whether type 1 or type 2, results from an inadequate mass of functional pancreatic beta cells [1]. The control of beta cell mass is a dynamic process and depends on a subtle balance between cell proliferation and growth, and cell death [2, 3]. Recent data suggest that adult pancreatic beta cells retain a significant proliferative capacity in vivo and that replication is a major component involved in maintenance of beta cell mass in adult animals [4, 5]. Beta cell replication can be induced by many strategies and intrinsic factors [5–16]. The molecular mechanisms involved in regulation of beta cell proliferation are not fully understood. Several lines of evidence suggest that Akt and its mediated signalling pathway play critical roles in regulation of beta cell mass and function [12, 17–20]. In most cases Akt activation is dependent on phosphoinositide-3-kinase. Once activated, Akt in turn phosphorylates a number of downstream targets that in general promote cell growth and survival [21]. BCL2-associated agonist of cell death (BAD) is a well-established target of Akt [22, 23]. It has been shown that Akt-mediated phosphorylation of BAD played an important role in the regulation of beta cell proliferation or apoptosis [19, 24].

In previous studies of our group, cDNA libraries of human hypothalamus, pituitary, adrenal and insulinoma tissues were established and several novel full-length cDNAs cloned [25, 26]. A novel full-length cDNA cloned from human hypothalamus tissue was selected for further study and found to be related with glucose metabolism (X. Wang and R. Hu, unpublished data). We named this novel gene glucose metabolism-related protein 1 (*GMRP1*). The sequence data of human *GMRP1* gene have been submitted to GenBank database under accession number AF329102. *GMRP1* is the alias of *BTBD10*, the official name given by HUGO Gene Nomenclature Committee. *BTBD10* was first described by Chen et al. [27] and found to be downregulated in gliomas. In another study by Nawa et al. [28], BTB (POZ) domain containing 10, the protein encoded by *BTBD10*, was found to interact with Akt by yeast two-hybrid and appeared to behave as a suppressor of neuronal cell death and an enhancer of cell growth via its positive regulation of Akt phosphorylation.

The current study attempted to investigate whether glucose metabolism-related protein 1 (*GMRP1*) regulates Akt signalling pathway in beta cells and whether *GMRP1* exerts impacts on beta cell proliferation or apoptosis.

Methods

Animals We used Sprague–Dawley male rats (age 2 months), male *db/db* mice (age 5 months) and age-matched

C57BLKS/J mice, all obtained from the Experimental Animal Center, Shanghai Medical College of Fudan University. All experimental procedures were approved by the Shanghai Animal Care and Use Committee on Animals and followed the policies of the International Association for the Study of Pain on the use of laboratory animals. All efforts were made to minimise animal suffering and reduce the numbers of the animals used.

Northern blot analysis Target gene fragments of human *GMRP1* were cloned into pDrive vectors (Qiagen, Chatsworth, CA, USA). Digoxigenin-labelled probes were generated by transcription with *SP6/T7* RNA polymerase using a digoxigenin RNA labelling kit (Roche Diagnostics, Indianapolis, IN, USA). Human tissues including human islets were obtained from Shanghai Body Donation Center. Use of those tissues was approved by the Medical Ethics Committee at Huashan Hospital, Fudan University. Northern blotting was performed using a kit (non-isotopic digoxigenin Northern Starter; Roche Diagnostics) as described previously [29].

Preparation of polyclonal antibody against human *GMRP1* The complete cDNA sequence of human *GMRP1* gene was subcloned into pET32 vector (Novagen, Madison, WI, USA). The recombinant pET32-*GMRP1* vector was transformed into *E. coli* strain BL21. Recombinant *GMRP1* protein was produced and purified, and polyclonal antibody against human *GMRP1* generated by immunising rabbits as described previously [29].

Immunostaining analysis Pancreatic tissues from (1) Sprague–Dawley rats infused with saline and high glucose, (2) *db/db* mice and (3) C57BLKS/J mice were fixed overnight in formalin at room temperature, along with human insulinoma tissues, and then dehydrated, embedded in paraffin and sectioned (5 μ m). For *GMRP1* and insulin double immunohistochemistry, rabbit anti-*GMRP1* polyclonal antibody and goat anti-insulin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to incubate the same section sequentially and the sections were incubated with anti-rabbit IgG antibody conjugated with alkaline phosphatase (Pierce, Rockford, IL, USA) to stain for *GMRP1*, followed by incubation with horseradish peroxidase-conjugated anti-goat IgG antibody (Pierce) to stain for insulin. The sections were then observed and imaged using a microscope (Leica Microsystems, Wetzlar, Germany). For Ki67 and insulin immunofluorescence, guinea pig anti-insulin polyclonal antibody (Abcam, Cambridge, MA, USA) and rabbit monoclonal antibody against Ki67 (Abcam) were used as primary antibodies, followed by staining for insulin with FITC-conjugated anti-guinea pig IgG antibody (Jackson Immuno-

noResearch Laboratories, West Grove, PA, USA) and for Ki67 with Rhodamine-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch). Images were taken under a fluorescence microscope equipped with a charge-coupled device camera (Leica Microsystems).

Western blot analysis Total proteins were extracted from isolated islet cells and INS-1E cells using RIPA buffer (Pierce). Western blotting was performed as described previously [29].

Cell culture INS-1E cells kindly supplied by P. Maechler (Department of Cell Physiology and Metabolism, Faculty of Medicine, University of Geneva, Geneva, Switzerland) [30], were cultured according to the protocol described [30]. For experiments on regulation of GMRP1 by glucose or insulin, INS-1E cells were cultured to 60% confluence and treated with 5.6 or 25 mmol/l glucose for 24 or 48 h, after which 0, 10, 50 and 100 nmol/l insulin was administered for 24 h. GMRP1 protein levels were investigated by western blotting. Apoptosis of INS-1E cells

Fig. 1 Expression patterns of *GMRP1* and the protein it encodes. **a** Gene expression profile of human *GMRP1*. mRNA levels of *GMRP1* were detected in human tissues by northern blotting. Total RNA (10 μg) isolated from human tissues as indicated was hybridised with a digoxigenin-labelled *GMRP1* RNA probe. 18S rRNA was used as control to confirm comparable RNA loading. The locations of 18S and 28S rRNA are indicated on the right. Af, abdominal fat; Ao, aorta; Br, brain; Co, colon; Du, duodenum; Ht, heart; Hy, hypothalamus; In, insulinoma; Kd, kidney; Li, liver; Lu, lung; Pa, pancreas; Pr, prostate; Pu, pituitary; Si, small intestine; Sm, skeletal muscle; Sp, spleen; St, stomach; Te, testis; Th, thymus. **b** Differential expression of *GMRP1*

gene in human islets and insulinomas by northern blotting. **c** Levels of GMRP1 protein in rat islets and INS-1E cells by western blotting. **d** Statistical analysis of western blot (**c**). Level of GMRP1 in rat islets was set to 1; ** $p < 0.01$. **e** Cellular distribution of GMRP1 protein in mouse islets. The same pancreatic section from a C57BLKS/J mouse was immunostained for GMRP1 and insulin sequentially. **f** Immunohistochemistry analysis of GMRP1 in human insulinoma tissue and peritumoral islets. The same sections of insulinoma tissue and peritumoral islets were immunostained for GMRP1 and insulin sequentially. Scale bar, 100 μm

was evaluated by determination of histone-complexed DNA fragments using a kit (Cell Death Detection ELISA; Roche Molecular Biochemicals, Mannheim, Germany). The relative frequency of apoptosis was photometrically determined by measuring the peroxidase activity of the immunocomplexes at 405 nm. Cell proliferation was assessed by [3 H] thymidine incorporation according to the protocol described by El Ouamari [31].

Islet isolation and treatment Islets of Langerhans were isolated from adult Sprague–Dawley rats, saline/high glucose-infused rats, C57BLKS/J mice and *db/db* mice by *in situ* pancreas collagenase infusion and separated by density gradient centrifugation at 800 *g* according to the modified protocol described by Kinasiewicz [32]. Isolated islets were transferred to 24-well plates and cultured in RPMI containing different glucose concentrations (5.6 and 25 mmol/l) for different periods (24 and 48 h) respectively. GMRP1 protein levels were investigated by western blotting and apoptosis of islet cells was evaluated by DNA fragmentation. Cell proliferation was assessed by [3 H]thymidine incorporation.

Adenovirus generation and infection Recombinant adenovirus containing full-length cDNA of rat *Gmrp1* (Ad-GMRP1) was generated using the AdMax cloning system (Microbix Biosystems, Toronto, ON, Canada) according to the protocol provided by the manufacturer. Recombinant adenovirus expressing enhanced green fluorescent protein (GFP) (Ad-GFP) was used as a control adenovirus. INS-1E cells or rat islet cells were cultured in six-well plates to 60% confluence and then infected with Ad-GMRP1 or Ad-GFP for 90 min. Viruses were removed and fresh medium was added for an additional 72 h of incubation.

Small interfering RNA-mediated knockdown of *Gmrp1* For *Gmrp1* small interfering RNA (siRNA) experiments, 20- to 25-nucleotide stealth-prevalidated siRNA duplexes designed for rat *Gmrp1* (Invitrogen, Carlsbad, CA, USA) were used. INS-1E cells were seeded in six-well plates at a density of $\sim 5 \times 10^5$ cells per well in culture medium without antibiotics and transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were transfected for 6 h with the *Gmrp1* siRNA at a final concentration of 50 nmol/l or with control

Fig. 2 Adenovirus-mediated overproduction of GMRP1 increased beta cell proliferation via Akt–BAD pathway. INS-1E cells (**a–d**) or rat islets (**e–h**) were transfected with Ad-GMRP1 or Ad-GFP for 72 h, after which western blotting was used to detect levels of GMRP1, β -actin, total AKT, phosphorylated Akt (p-Akt) at Thr 308 or at Ser 473, total BAD and phosphorylated BAD (p-BAD) at Ser 136 in INS-1E cells (**a**) or rat islets (**e**). **b, f** Results of statistical analysis of blots (**a, e**). Phosphorylation of Akt at Thr 308 or at Ser 473 was measured using p-Akt at Thr 308 or at Ser 473 divided by total Akt. Phosphorylation of BAD was evaluated similarly. The relative levels

of GMRP1, p-AKT (Thr 308), p-AKT (Ser 473) and p-BAD (Ser 136) in Ad-GMRP1-transfected (black bars) INS-1E cells or rat islets are shown, vs corresponding levels in Ad-GFP-transfected (grey bars) cells or islets, which were set to 1; * $p < 0.05$ and *** $p < 0.001$. **c, d** INS-1E cells or (**g, h**) rat islets were infected with Ad-GMRP1 or Ad-GFP for 90 min. Viruses were removed and fresh medium was added for an additional 72 h of incubation. Cell proliferation of INS-1E cells (**c**) or rat islets (**g**) was assessed by measuring [3 H]thymidine incorporation; ** $p < 0.01$. Cell apoptosis of INS-1E cells (**d**) or rat islets (**h**) was determined by DNA fragmentation assay. OD, optical density

siRNA (non-targeting siRNA) at the same concentration before switching to fresh culture medium including antibiotics. At 72 h after transfection, cells were lysed to extract total protein or to detect apoptotic cells by DNA fragmentation or to investigate cell proliferation by [3 H]thymidine incorporation. After transfection for 72 h with *Gmrp1* siRNA or control siRNA, INS-1E cells were treated with 5.6 or 25 mmol/l glucose for an additional 24 h. Cell proliferation was then assessed by [3 H]thymidine incorporation or cells were stimulated for 10 min with 100 nmol/l insulin, followed by western blot to determine phosphorylation of Akt at Thr 308.

Infusions Male Sprague–Dawley rats (~250 g) were randomly divided into six groups as follows: (1) rats infused with saline for 24 h (0.45% [wt/vol.] NaCl infusion); (2) rats infused with saline for 48 h; (3) rats infused with saline for 72 h; (4) rats infused with glucose for 24 h (50% [wt/vol.] dextrose infusion, at 2 ml/h); (5), rats infused with glucose for 48 h; and (6) rats infused with glucose for 72 h. The infusion technique was similar to that described by Bonner-Weir et al. [33]. At the end of the infusion period, animals were removed from the restraint system, returned to their cages and eventually killed for morphological assessment of beta cell mass and proliferation, or for islet isolation.

Beta cell replication Beta cell replication was evaluated using measurement of Ki67-positive beta cells in islets of rats infused with saline or glucose. Pancreatic sections were double-stained with anti-Ki67 antibody and anti-insulin antibody as described above. On these sections, beta cells showed green cytosol and Ki67-positive cells appeared with red nuclei. Ki67-positive beta cells were counted by systematically sampling all of the beta cells identified by insulin staining in each section using a light microscope under high magnification ($\times 1,000$). The proportion of Ki67-positive beta cell nuclei to total beta cell nuclei was calculated.

Measurement of beta cell mass To measure beta cell mass, pancreatic sections (5 μ m) were incubated overnight at 4°C with guinea pig anti-insulin polyclonal antibody (1:200 dilution; Abcam) and then incubated with biotinylated anti-guinea pig IgG antibody and with avidin–biotin–peroxidase complex (Vector, Burlingame, CA, USA). Point-counting morphometry of insulin immunostained pancreatic sections as applied by Montaña [34] was used to calculate the relative beta cell volume as a measure of beta cell mass.

TUNEL assay Pancreas sections of *db/db* or control mice were double immunostained for insulin and for fragmented DNA by TUNEL assay. TUNEL staining was carried out using a DNA fragmentation detection kit (FragEL; Merck, Darmstadt, Germany) according to the manufacturer's

instructions. Cells were counted (1,000 cells per sample) and the number of TUNEL-positive cells quantified and expressed as a percentage of the total.

Statistical analysis Data are presented as means $\pm$ SEM. Statistical significance was determined by ANOVA, with $p < 0.05$ considered significant.

Results

Expression pattern of *GMRP1* gene and its protein We established gene expression profile of human *GMRP1*. The results of northern blotting demonstrated that *GMRP1* mRNA was ubiquitously expressed in human tissues, with high expression especially in brain, aorta, testis and insulinoma tissues (Fig. 1a). We also compared *GMRP1* mRNA levels between human islets and insulinomas, and found much greater expression of *GMRP1* in insulinoma tissues than in human islets (Fig. 1b). The results of

Fig. 3 siRNA-mediated knockdown of *GMRP1* inhibited proliferation and induced apoptosis of INS-1E cells. INS-1E cells were transfected with *Gmrp1* siRNA or control siRNA (Con) for 72 h, after which levels of *GMRP1*, β -actin, total AKT, phosphorylated Akt (p-Akt) at Thr 308 or at Ser 473, as well as total BAD and phosphorylated BAD (p-BAD) at Ser 136 were determined by western blotting (a). **b** Statistical analysis of blot (a). Phosphorylation of Akt at Thr 308 or at Ser 473 was measured using p-Akt at Thr 308 or at Ser 473 divided by total Akt. Phosphorylation of BAD was evaluated similarly. The relative levels of *GMRP1*, p-AKT (Thr 308), p-AKT (Ser 473) and p-BAD (Ser 136) in *Gmrp1* siRNA-transfected (black bars) INS-1E cells are shown, with control siRNA-transfected (grey bars) cells set to 1; *** $p < 0.001$ and ** $p < 0.01$. After INS-1E cells were transfected with *Gmrp1* siRNA or control siRNA for 72 h, cell proliferation (c) was assessed by measuring [3 H]thymidine incorporation; ** $p < 0.01$. **d** Cell apoptosis was determined by DNA fragmentation assay; * $p < 0.05$. OD, optical density

immunohistochemistry also indicated higher levels of GMRP1 protein in insulinoma tissue than in peritumoral normal islets (Fig. 1f). Western blot analysis demonstrated that levels of GMRP1 in INS-1E cells increased ~3.2-fold in comparison with those in rat islets (Fig. 1c, d). Immunohistochemistry analysis also showed that GMRP1 was predominantly produced in islets of mouse pancreas tissue and was hardly detected in exocrine tissue. In detail, according to the architecture of islets and insulin staining, GMRP1 appeared to be more abundant in non-beta cells than in beta cells (Fig. 1e).

Overproduction of GMRP1 promoted beta cell proliferation via Akt–BAD pathway INS-1E cells or rat islets were transfected with Ad-GMRP1 or Ad-GFP for 72 h, and phosphorylation of Akt or BAD, and cell proliferation or apoptosis were determined. When compared with Ad-GFP, Ad-GMRP1 transfection significantly increased phosphorylation of Akt at Thr 308, namely 4.5-fold for INS-1E cells ($p<0.001$; Fig. 2a, b) and 3.6-fold for rat islets ($p<0.001$; Fig. 2e, f). It also significantly increased phosphorylation of Akt at Ser 473, namely 2.1-fold for INS-1E cells ($p<0.05$; Fig. 2a, b) and 1.8-fold for rat islets ($p<0.05$; Fig. 2e, f), as

Fig. 4 Impacts of glucose on GMRP1 levels and cell survival. **a–d** INS-1E cells or **(e–h)** rat islets were treated with 5.6 or 25 mmol/l glucose for 24 and 48 h respectively. Production of GMRP1 protein in INS-1E cells (**a**) or rat islets (**e**) was investigated using western blotting and quantified by statistical analysis with GMRP1 protein levels at 5.6 mmol/l glucose (grey bars) set to 1 for (**b**) INS-1E cells ($***p<0.001$) and (**f**) for rat islets ($*p<0.05$, $**p<0.01$ and $***p<0.001$). Black bars, 25 mmol/l. **c** [3 H]Thymidine incorporation was

used to assess cell proliferation in INS-1E cells ($**p<0.01$) and (**g**) rat islets ($*p<0.05$ and $**p<0.01$). **d** DNA fragmentation assay was performed to evaluate apoptosis in INS-1E cells ($**p<0.01$) and (**h**) rat islets ($*p<0.05$). **i** To investigate whether GMRP1 is involved in glucose-regulated cell survival, INS-1E cells were transfected with *Gmrp1* siRNA or control siRNA for 72 h and then treated with 5.6 or 25 mmol/l glucose for an additional 24 h, after which cell proliferation was determined; $*p<0.05$

well as phosphorylation of BAD at Ser 136 (4.2-fold for INS-1E cells, $p<0.001$; 3.7-fold for rat islets, $p<0.001$; Fig. 2a, b, e, f). [3 H]Thymidine incorporation analysis demonstrated that overproduction of GMRP1 led to a two-fold increase of proliferation in INS-1E cells ($p<0.01$; Fig. 2c) and a 2.3-fold increase in rat islets ($p<0.01$; Fig. 2g) compared with GFP control. However, DNA fragmentation showed that there were no significant differences in cell apoptosis between Ad-GMRP1 and Ad-GFP transfected INS-1E cells (Fig. 2d) or rat islets (Fig. 2h).

siRNA-mediated knockdown of *Gmrp1* inhibited proliferation and induced apoptosis of INS-1E cells After INS-1E cells were transfected with *Gmrp1* siRNA or control siRNA for 72 h, phosphorylation of Akt or BAD, and cell proliferation or apoptosis were determined. Western blot analysis indicated that transfection with *Gmrp1* siRNA resulted in ~75% ($p<0.001$) reduction of GMRP1 protein, and in ~70% ($p<0.001$), ~55% ($p<0.01$) and ~80% ($p<0.001$) decreases in phosphorylation of Akt at Thr 308 and Ser 473, and of BAD at Ser 136 respectively compared with control siRNA (Fig. 3a, b). Knockdown of *Gmrp1* reduced cell proliferation by ~60% ($p<0.01$; Fig. 3c) and increased cell apoptosis by 80% in comparison with controls ($p<0.05$; Fig. 3d).

Impact of glucose on GMRP1 levels and cell survival To investigate whether GMRP1 production was regulated by glucose, INS-1E cells or rat islets were cultured at 5.6 or 25 mmol/l glucose for 24 and 48 h respectively. GMRP1 protein in INS-1E cells was augmented 3.1-fold ($p<0.001$) and 3.6-fold ($p<0.001$) by exposure to 25 mmol/l glucose for 24 and 48 h respectively, when compared with 5.6 mmol/l glucose (Fig. 4a, b). In rat islets, high glucose also significantly increased GMRP1 levels during 24 h (2.6-fold, vs low glucose, $p<0.01$) and 48 h (1.8-fold, vs low glucose, $p<0.05$) of culture (Fig. 4e, f).

Proliferation of INS-1E cells was increased 2.2-fold ($p<0.01$) and 2.4-fold ($p<0.01$) by exposure to 25 mmol/l glucose for 24 and 48 h respectively when compared with 5.6 mmol/l glucose (Fig. 4c). Cell proliferation of rat islets was also significantly increased by high glucose for 24 h (1.5-fold vs low glucose, $p<0.05$) and 48 h (2.1-fold vs low glucose, $p<0.01$; Fig. 4g). DNA fragmentation analysis demonstrated that high glucose reduced apoptotic INS-1E cells about 60% ($p<0.01$) and 55% ($p<0.01$) for 24 and 48 h of culture respectively, when compared with low glucose (Fig. 4d). For rat islet cells, high glucose culture for 24 and 48 h led to 53% ($p<0.05$) and 41% ($p<0.05$) reductions of the apoptotic cells respectively (Fig. 4h).

To confirm whether GMRP1 is involved in beta cell survival regulated by glucose, INS-1E cells were transfected with *Gmrp1* siRNA or control siRNA for 72 h and

then treated with 5.6 or 25 mmol/l glucose for an additional 24 h, after which cell proliferation was determined. Knockdown of *Gmrp1* lowered high glucose-induced cell proliferation by ~30% ($p<0.05$; Fig. 4i).

Involvement of GMRP1 in insulin signalling pathway To determine the mechanism by which glucose regulates GMRP1 production, we investigated whether insulin administration without change of glucose concentration affected GMRP1 levels. INS-1E cells were cultured at 0, 10, 50 and 100 nmol/l insulin for 24 h, and GMRP1 levels determined. Insulin at 50 and 100 nmol/l significantly increased GMRP1 production compared with no insulin administration ($p<0.01$; Fig. 5a, b). After transfection for 72 h with *Gmrp1* siRNA or control siRNA, INS-1E cells were exposed to 100 nmol/l insulin for 10 min and Akt phosphorylation determined. Insulin-stimulated Akt phosphorylation significantly decreased in cells transfected with *Gmrp1* siRNA compared with control siRNA-transfected cells ($p<0.01$; Fig. 5c, d).

GMRP1-mediated Akt–BAD pathway contributed to beta cell proliferation in glucose-infused rats Pancreas sections stained for Ki67 and insulin were used to identify replicating beta cells in saline- or glucose-infused rats. Glucose infusion for 48 and 72 h significantly increased beta cell replication. Compared with saline infusion, glucose infusion resulted in approximately sevenfold ($p<0.001$) and tenfold ($p<0.001$) increases of beta cell replication for 48

Fig. 5 Involvement of GMRP1 in insulin signalling pathway. **a** INS-1E cells were cultured with insulin as indicated for 24 h and GMRP1 levels determined by western blotting, with **(b)** quantification of GMRP1. GMRP1 protein levels at 0 nmol/l insulin were set to 1; ** $p<0.01$. **c** After transfection with *Gmrp1* siRNA or control siRNA for 72 h, INS-1E cells were exposed to 100 nmol/l insulin for 10 min, after which levels of phosphorylated Akt (p-Akt) at Thr 308 and total Akt were determined by western blotting. Akt phosphorylation (p-Akt:total Akt) was quantified **(d)** using p-Akt at Thr 308 divided by total Akt while Akt phosphorylation in control siRNA-transfected INS-1E cells treated with 0 nmol/l insulin was set to 1; ** $p<0.01$

and 72 h respectively (Fig. 6a, b). Beta cell mass also showed 1.3-fold ($p<0.05$) and 1.6-fold ($p<0.05$) enhancements in rats infused with glucose for 48 and 72 h respectively when compared with saline-infused animals (Fig. 6c).

Islets were isolated from glucose- and saline-infused rats, and levels of GMRP1, total Akt, phosphorylated Akt, total BAD and phosphorylated BAD were investigated (Fig. 6d, e). Western blot analysis demonstrated that glucose infusion significantly enhanced GMRP1 production for 24 h (2.5-fold vs saline infusion, $p<0.01$), 48 h (3.2-fold, $p<0.01$) and 72 h (3.6-fold, $p<0.01$). Compared with saline infusion, glucose infusion for 24, 48 and 72 h respectively increased phosphorylation of Akt by 1.7- ($p<0.05$), 2.6- ($p<0.01$) and 3.1-fold ($p<0.01$). Glucose infusion as above also enhanced phosphorylation of BAD by 1.5- ($p<0.05$), 2.7- ($p<0.01$) and 2.8-fold ($p<0.01$) respectively.

Immunostaining data demonstrated that GMRP1 production in saline-infused rats was much weaker in beta cells than in non-beta cells. However, glucose infusion markedly increased GMRP1 production in beta cells (Fig. 6f).

GMRP1 might be involved in suppression of beta cell apoptosis in db/db mice TUNEL analysis showed that apoptosis occurred in approximately 5.5% of beta cells in *db/db* mice, but only in around 0.2% of beta cells in control mice, indicating significant apoptosis of beta cells in *db/db* mice compared with control mice ($p<0.001$; Fig. 7a). Western blot analysis demonstrated that, compared with control mice, GMRP1 production was lowered by 60% ($p<0.01$) and phosphorylation of Akt and BAD reduced by 65% ($p<0.01$) and 70% ($p<0.01$) in islets of *db/db* mice (Fig. 7b, c). Immunohistochemistry data indicated that

Fig. 6 The roles of GMRP1-mediated Akt–BAD pathway in beta cell proliferation in rats infused as follows: 72 h with saline, 24 h with glucose (Glc), 48 h with glucose (Glc) and 72 h with glucose (Glc). **a** Male Sprague–Dawley rats were infused with 50% (wt/vol) dextrose at a dose of 2 ml/h for 24, 48 and 72 h respectively. Rats infused with saline for 72 h were used as controls. Pancreatic sections from these rats were immunostained with anti-insulin (green) and anti-Ki67 (red) antibodies. Scale bar, 50 μ m. **b** Beta cell proliferation in saline- or glucose-infused rats was assessed as percentage of Ki67-positive beta cells; *** $p<0.001$. **c** Beta cell mass of rats infused with saline (grey bars) or glucose (black bars) for indicated times; * $p<0.05$. **d** Levels of GMRP1, β -actin, total Akt, Akt phosphorylated (p-Akt) at Thr 308,

total BAD and BAD phosphorylated (p-BAD) at Ser 136 were investigated in islets isolated from saline- or glucose-infused rats. **e** Statistical analysis of above (**d**) results. Phosphorylation of Akt (p-Akt:total Akt) or BAD (p-BAD:total BAD) was measured using p-Akt or p-BAD divided by total Akt or BAD. Levels of GMRP1, p-Akt:total Akt and p-BAD:total BAD in glucose-infused rats are shown relative to levels in saline-infused rats set to 1; * $p<0.05$ and ** $p<0.01$. Grey bars, saline; hatched bars, glucose 24 h; striped bars, glucose 48 h; black bars, glucose 72 h. **f** Immunostaining for GMRP1 and insulin in pancreatic sections of 72 h saline- or glucose-infused rats. Scale bar, 100 μ m

decreased production in beta cells accounted for total reduction of GMRP1 in islets of *db/db* mice (Fig. 7d).

Discussion

In this study, we showed that GMRP1 acts as a novel regulator of Akt activity and exerts effects on beta cell proliferation or apoptosis. Mutagenesis studies have revealed that phosphorylation of Thr 308 and Ser 473 is required for Akt activity [23]. 3-Phosphoinositide-dependent protein kinase-1 (PDK-1), a well established upstream regulator of Akt, efficiently phosphorylates Akt at Thr 308 in a manner dependent on phosphoinositide-3-kinase [35]. In another way, PDK-1 interacts with protein kinase C-related kinase 2 and the PDK-1–protein kinase C-related kinase 2 complex is capable of phosphorylating Akt at Thr 308 and Ser 473 [36]. Integrin-linked kinase has also been suggested to regulate Akt by phosphorylation of Akt at Ser 473 [37]. The calcium/calmodulin-dependent kinase kinase has also been shown to activate Akt by directly phosphorylating it at Thr 308 [38]. Using a yeast two-hybrid screening, GMRP1 was identified as a protein that interacts with Akt3. Further studies have shown that GMRP1 bound to any Akt isoform including Akt1, Akt2 and Akt3 [28]. In vitro experiments on COS7 and NSC34

cells indicated that GMRP1 positively regulated Akt phosphorylation at Ser 473 and Thr 308 [28]. In INS-1E cells and rat islets, we have here presented evidence that overproduction of GMRP1 significantly increased phosphorylation of Akt at Thr 308 and at Ser 473, and that knockdown of *Gmrp1* decreased Akt phosphorylation. Taken together, these findings suggest that GMRP1 serves as a novel upstream regulator of Akt in islet beta cells.

It has been shown that overabundance of GMRP1 promoted NSC34 cell proliferation and suppressed neuronal death caused by mutant superoxide dismutase 1, soluble, and that the proliferative impact of GMRP1 was mediated by enhanced phosphorylation of Akt [28]. In our study, we found that overabundance of GMRP1 significantly increased proliferation of INS-1E cells and rat islets, and that knockdown of *Gmrp1* led to significantly reduced proliferation and enhancement of apoptosis in beta cells. We showed that expression of *GMRP1* gene and protein was much greater in insulinoma tissues than in normal islets. It is well known that insulinoma is derived from monoclonal proliferation of beta cells. We also observed that levels of GMRP1 protein in rat insulinoma cells (INS-1E cells) increased about 3.2-fold in comparison with those in rat islets. These data suggest that GMRP1 is involved in the regulation of beta cell proliferation. In vivo studies have indicated that transgenic mice overexpressing a constitu-

Fig. 7 Impact of GMRP1 on apoptosis of beta cells in *db/db* mice. **a** Assessment of beta cell apoptosis in *db/db* and C57BLKS/J mice ($n=6$ in both groups). Pancreas sections from both groups were immunostained for insulin and by TUNEL assay. Beta cell apoptosis was assessed by percentage of TUNEL-positive (+) beta cells; *** $p<0.001$. **b** Levels of GMRP1, β -actin, total Akt, phosphorylated Akt (p-Akt) at Thr 308, total BAD and BAD phosphorylated (p-BAD) at Ser 136 were investigated in islets isolated from *db/db*

and C57BLKS/J (C57) mice. **c** Statistical analysis of the above (**b**) results, where phosphorylation of Akt (p-Akt:total [T] Akt) or BAD (p-BAD:total [T] BAD) was measured using p-Akt or p-BAD divided by T-Akt or T-BAD. Levels of GMRP1, p-Akt:total Akt and p-BAD:total BAD in *db/db* mice (black bars) are shown relative to those in C57BLKS/J (grey bars) mice set to 1; $n=4$ per group; ** $p<0.01$. **d** Immunostaining for GMRP1 and insulin in pancreatic sections of *db/db* and C57BLKS/J mice (**d**). Scale bar, 100 μ m

tively active form of Akt in islet beta cells exhibited striking increases in beta cell mass, proliferation, neogenesis and cell size [17, 18]. In vitro studies have also shown that enhanced Akt phosphorylation was necessary for and sufficient for glucose-promoted beta cell proliferation [39, 40]. Several targets of Akt have been identified that may contribute to Akt-mediated beta cell survival. It has been established that Akt phosphorylated BAD in vitro and in vivo, and that BAD phosphorylation inhibited cell apoptosis [22, 41]. Evidence also suggests that Akt-mediated phosphorylation of BAD inhibited beta cell apoptosis and promoted cell survival [19]. Our data demonstrate that overabundance of GMRP1 increased phosphorylation of Akt and BAD in beta cells, whereas knockdown of *Gmrp1*, by contrast, decreased phosphorylation of those proteins. However, it has not been ruled out that other downstream targets of Akt participate in the regulation of beta cell survival by GMRP1, e.g. ribosomal protein S6 kinase, p21, cyclin D1, cyclin D2, cyclin-dependent kinase 4 and glycogen synthase kinase 3 [12, 42–46]. These data imply that the impact of GMRP1 on beta cell survival is accomplished through the Akt–BAD pathway.

Glucose is one of the best stimuli for beta cell replication in vitro and in vivo [1]. We provided evidence that short-time (48, 72 h) glucose infusion markedly increased beta cell proliferation and mass, a finding that was similar to those of other studies [32, 47–49]. Western blot and immunohistochemistry analysis demonstrated that glucose infusion significantly increased GMRP1 production in beta cells of rat islets. It was also found that Akt and BAD phosphorylation were significantly enhanced by glucose infusion. Furthermore, our in vitro studies on INS-1E cells and isolated islets demonstrated that high glucose culture for short periods (24, 48 h) significantly increased GMRP1 production with enhancement of proliferation and reduction of cell apoptosis. It was also found that knockdown of *Gmrp1* significantly lowered cell proliferation induced by high glucose. This suggests that GMRP1 was involved in glucose-regulated survival of beta cells and that the impact of GMRP1 was effected through Akt signalling pathway. To investigate the mechanism by which glucose regulated GMRP1 production, the impact of insulin on GMRP1 production was determined. High insulin significantly increased GMRP1 levels, while knockdown of *Gmrp1* inhibited the activation of Akt signalling in response to insulin stimulation. We have shown that high glucose significantly increased insulin secretion in INS-1E cells and rat islets (X. Wang and R. Hu, unpublished data), implying that insulin acts as a mediator in glucose regulation of GMRP1, and also suggesting that GMRP1 is involved in insulin-stimulated activation of Akt signalling pathway. In another way, we attempted to investigate whether GMRP1

was involved in beta cell apoptosis in vivo. TUNEL analysis demonstrated that 5-month-old *db/db* mice had many more apoptotic beta cells than control mice. We also found that GMRP1 production, phosphorylation of Akt and phosphorylation of BAD were all significantly decreased in islets of *db/db* mice. Immunostaining analysis indicated that decreased GMRP1 levels in beta cells contributed to the reduction of GMRP1 production in *db/db* mice islets. Taken together, these data suggest that GMRP1 plays a role in beta cell apoptosis of *db/db* mice.

In summary, this study demonstrated that GMRP1 positively regulated phosphorylation of Akt and participated in regulation of beta cell proliferation or apoptosis. We present evidence that GMRP1 is involved in glucose-regulated cell proliferation and insulin-stimulated Akt signalling pathway in beta cells. We also found that GMRP1 might inhibit beta cell apoptosis. Thus, our data support the notion that the GMRP1-mediated Akt signalling pathway could be a novel component in the regulation of beta cell proliferation and apoptosis.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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