Mimitin, a novel mitochondrial protein, has been shown to act as a molecular chaperone for the mitochondrial complex I and to regulate ATP synthesis. During Type 1 diabetes development, pro-inflammatory cytokines induce mitochondrial damage in pancreatic β-cells, inhibit ATP synthesis and reduce glucose-induced insulin secretion. Mimitin was expressed in rat pancreatic islets including β-cells and decreased by cytokines. In the ob/ob mouse, a model of insulin resistance and obesity, mimitin expression was down-regulated in liver and brain, up-regulated in heart and kidney, but not affected in islets. To further analyse the impact of mimitin on β-cell function, two β-cell lines, one with a low (INS1E) and another with a higher (MIN6) mimitin expression were studied. Mimitin overexpression protected INS1E cells against cytokine-induced caspase 3 activation, mitochondrial membrane potential reduction and ATP production inhibition, independently from the NF-κB (nuclear factor κB)–iNOS (inducible NO synthase) pathway. Mimitin overexpression increased basal and glucose-induced insulin secretion and prevented cytokine-mediated suppression of insulin secretion. Mimitin knockdown in MIN6 cells had opposite effects to those observed after overexpression. Thus mimitin has the capacity to modulate pancreatic islet function and to reduce cytokine toxicity.

Key words: ATP, cytokine, diabetes, insulin-secreting cell, mimitin, mitochondrion.

INTRODUCTION

Mimitin is a small 20 kDa mitochondrial protein that is a direct target for c-Myc and is involved in cell proliferation [1]. Mimitin contains an ATP/GTP-binding motif and a domain called Complex1_17_2 kDa [1]. Mimitin is thought to play the role of a molecular chaperone for assembly of the mitochondrial respiratory chain complex I and to be involved in ATP generation in mitochondria [1]. Previous studies have shown that mimitin may modulate cell death [2]. In hepatoma cells, the expression of mimitin was found to be increased upon exposure to IL (interleukin)-1β and IL-6 [2]. It has also been shown that blockade of mimitin expression by siRNA (small interfering RNA) technology leads to a decrease in HepG2 cell proliferation and accelerates TNFα (tumour necrosis factor α) and cycloheximide-induced apoptosis [2].

Synthesis of ATP is crucial for glucose-induced insulin secretion [3], and since this process is disturbed by cytokines during Type 1 diabetes development [4], mimitin attracted our attention. So far there is no information about mimitin in pancreatic islets. It was therefore the aim of the present study to elucidate the role of mimitin in primary islet cells and in insulin-secreting cell lines with special reference to its effects on the action of pro-inflammatory cytokines, which are responsible for pancreatic β-cell damage during Type 1 diabetes development [5,6]. Moreover, the influence of mimitin on β-cell function with special emphasis on the regulation of glucose-induced insulin secretion was studied. We show that mimitin can act as a modulator of glucose-induced insulin secretion and prevent its inhibition by pro-inflammatory cytokines.

MATERIALS AND METHODS

Chemicals

Cytokines and the dNTP mixture were obtained from PromoCell. The jetPEITM transfection reagent was purchased from PEQLAB Biotechnologie, and Biotherm™ Taq polymerase was from GeneCraft. The SuperScript II RT™ reverse transcriptase and all tissue culture equipment were from Invitrogen. Hybond N nylon membranes and the GeneRuler ladder were from Invitrogen. Hybond N nylon membranes and the GeneRuler ladder were from Invitrogen. Primers were from MILLEPIRE. All other reagents were from Sigma–Aldrich.

Rat and mouse tissue preparation

Pancreatic islets and other tissues were from 250–300 g male Lewis rats or from ob/ob mice (50–70 g) or their lean littermates (25–30 g) bred in our institution according to German animal law. The ob/ob mice [7] used in the present study originate from a colony described previously [8] and are characterized by a moderate hyperglycaemia together with hyperinsulinaemia due to islet hypertrophy, without any defects in β-cells.

Rat islet isolation, culture and treatment

Pancreatic islets were isolated by collagenase digestion and handpicked under a stereomicroscope. Isolated islets were cultured on 35 mm ECM (extracellular matrix)-coated plates (Novamed), the ECM being derived from bovine corneal endothelial cells, in RPMI 1640 tissue culture medium containing 5 mM glucose, 10% FBS (fetal bovine serum), 200 units/ml penicillin and 0.2 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2. For immunofluorescence studies, islets were allowed to adhere and to spread for 10–12 days on the ECM plates. After transfer on to collagen-coated chamber slides, they were allowed to attach for 24 h. The islets were treated with IL-1β (600 units/ml) or a cytokine mixture [IL-1β, 60 units/ml; TNFα, 185 units/ml; and IFNγ (interferon γ), 14 units/ml] for 24 h [5,6].

Abbreviations used: AMPK, AMP-activated protein kinase; BrdU, bromodeoxyuridine; ECL, enhanced chemiluminescence; ECM, extracellular matrix; FBS, fetal bovine serum; FW, forward; IL, interleukin; IFNγ, interferon γ; iNOS, inducible NO synthase; Ins2, insulin 2; NF-κB, nuclear factor κB; REV, reverse; RT, reverse transcription; SEAP, secreted alkaline phosphatase; shRNA, short hairpin RNA; siRNA, small interfering RNA; TNFα, tumour necrosis factor α.

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**Immunofluorescence**

For immunofluorescence staining, rat islet cells or INS1E cells were seeded onto to collagen-coated glass slides and incubated as described above following an overnight fixation with 4% (w/v) paraformaldehyde in PBS. After fixation, cells were washed three times with PBS for 5 min. After a 20 min blocking in PBS with 0.1% Triton X-100 and 1% (v/v) BSA at room temperature (20 °C) and washed again as above. The slides were incubated with primary antibodies diluted in PBS with 0.1% Triton X-100 and 0.1% BSA at room temperature for 1 h {rabbit polyclonal anti-mimitin antibody, 1:100 dilution (Abcam); guinea pig polyclonal anti-insulin antibody, 1:100 dilution (Dianova)]. For nuclear counterstaining, 300 nM DAPI (4′,6-diamidino-2-phenylindole) was used for 5 min at room temperature. Slides were thereafter mounted with Mowiol (Sigma–Aldrich). Images were captured and analysed using a Cell®/Olympus IX81 inverted microscope system.

**Cell culture and cytokine incubation**

Insulin-secreting INS1E cells were cultured as described previously [9] in fully supplemented RPMI 1640 medium, with 10 mM glucose, 10% (v/v) FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere at 37 °C and 5% CO₂. Insulin-secreting MIN6 cells were grown in DMEM (Dulbecco’s modified Eagle’s medium) medium supplemented with 25 mM glucose, 10% (v/v) FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere at 37 °C and 5% CO₂. IL-1β was used at a concentration of 600 units/ml, and the cytokine mixture contained IL-1β (60 units/ml), TNFα (185 units/ml) and IFNγ (14 units/ml).

**Overexpression of mimitin in insulin-secreting INS1E cells**

Human mimitin cDNA was stably overexpressed in insulin-secreting INS1E cells using the jetPEI™ transfection reagent. INS1E cells transfected with an empty pcDNA3 vector were used as a control (two clones). Positive clones were selected against G418 (125 μg/ml) and the mimitin expression levels were confirmed by Western blotting.

**Knockdown of mimitin in insulin-secreting MIN6 cells**

MIN6 cells were infected with lentiviral particles (self-made [9a]) containing mouse mimitin or control shRNA (short hairpin RNA; both vectors from Santa Cruz Biotechnology) and the success of the knockdown was verified by Western blotting.

**Confirmation of mitochondrial localization of mimitin**

Insulin-secreting INS1E cells transfected with pmaxFP™-green mimitin were seeded on to Mat-Tek dishes (MatTek) 24 h before loading with MitoTracker Deep Red (Invitrogen) for 15 min at 37 °C. Images were captured and analysed using a Cell®/Olympus IX81 inverted microscope system.

**Proliferation assay**

The proliferation rate of INS1E cells was quantified by a colorimetric method using the Cell Proliferation BrdU (bromodeoxyuridine) ELISA (Roche). Cells were seeded at a concentration of $4 \times 10^4$ cells/well in 96-well microtitre plates and allowed to attach for 24 h. Thereafter cells were incubated with IL-1β (600 units/ml) or a cytokine mixture (IL-1β, 60 units/ml, TNFα, 185 units/ml and IFNγ, 14 units/ml) for 24 h. The assay was performed according to the manufacturer’s instructions. $A_{450}$ (reference wavelength of 650 nm) was measured. Results were expressed as the percentage of untreated cells.

**NF-κB (nuclear factor κB) reporter gene assay**

For the NF-κB enhancer element activity studies, $2 \times 10^4$ cells/well were seeded in 96-well plates 24 h before transient transfection was performed (jetPEI™ transfection reagent) and 48 h before addition of cytokines for 24 h. The pSEAP-NF-κB construct was used as described previously [10]. SEAP (secreted alkaline phosphatase) was measured using the Phospha-Light™ System kit (Applera).

**Nitrite measurement**

Nitrite accumulation after 24 h of cytokine treatment was determined spectrophotometrically at 562 nm by the Griess reaction [11].

**Activity assay of caspases 3 and 9**

INS1E cells were seeded at a density of $5 \times 10^4$ cells/well on to six-well plates 24 h before addition of cytokines. MIN6 cells were seeded at a density of $3 \times 10^4$ cells/well on to six-well plates 2 days before lentiviral particles were added. After a 4 h incubation with lentivirus, the medium was changed and the experiments were started on the following day. Activation of caspases 3 and 9 was quantified after a 24 h exposure to cytokines, using red caspase 3 and green caspase 9 staining kits (PromoCell) according to the manufacturer’s instructions. After staining and washing, cell suspensions were promptly read in the CyFlow ML cytomter (Partec). A total of $2 \times 10^4$ events were acquired. Non-labelled cells were used as a negative control and for the determination of the gates. Data were analysed by the FlowJo software (Tree Star). Results are expressed as the percentage of positive cells without exposure to cytokines.

**RNA isolation and cDNA preparation**

For RNA extraction, cells were plated at a density of $6 \times 10^5$ cells/well on to six-well plates and grown to confluence within 2 days. Total RNA from insulin-secreting INS1E cells or rat tissues was obtained using Nucleo-Spin RNA columns (Macherey-Nagel). The quality of the total RNA was verified by agarose gel electrophoresis. RNA was quantified spectrophotometrically at 260/280 nm. Thereafter, 2 μg of RNA was reverse-transcribed into cDNA using Random-Hexamer primers and a reverse transcriptase.

**Real-time RT (reverse transcription)—PCR**

The QuantiTect SYBR Green™ technology (Qiagen) based on a fluorescent dye that binds only double-stranded DNA was employed. The reactions were performed using the DNA Engine Opticon™ Sequence Detection System (Biozym Diagnostik). A total volume of 25 μl was used for the PCR reactions. Samples
were first denatured at 94°C for 3 min followed by up to 40 PCR cycles. Each PCR cycle comprised a melting at 94°C for 30 s, an annealing at 62°C [mimitin and Ins2 (insulin 2)] for 30 s and an extension at 72°C for 30 s. Each PCR amplification was performed in triplicate. The optimal parameters for the PCR reactions were defined empirically. The purity of the amplified products was verified by melting curves. Results for human mimitin, rat mimitin, mouse mimitin and rat Ins2 were normalized to β-actin. The sequences of the primers used in the present study were as follows: human mimitin, FW (forward), 5′-AGGAACCTGCTGCCTCAACA-3′; and REV (reverse), 5′-CTCGTGGCAGATGCTT-3′; rat mimitin, FW, 5′-GGAGATGGTGTCGCTGGTT-3′, and REV, 5′-CAGATGTCTGACCCTTCA-3′; rat Ins2, FW, 5′-AGGACCCCAAGTGCCACAA-3′; and REV, 5′-AGCAGCTGCTTGTCGCGTG-3′, and REV, 5′-CAGATGTCTGACCCTTCA-3′; mouse mimitin, FW, 5′-ATAGAAGCGTGTCGCCGCGTG-3′, and REV, 5′-CAGATGTCTGACCCTTCA-3′; rat β-actin, FW, 5′-GAAACGGGGCAGCTGATACT-3′, and REV, 5′-GGCCACACGGACAGCTCATTGA-3′; mouse β-actin, FW, 5′-AGAGGAAATCGTGCGTGAAC-3′, and REV, 5′-CAATAGTGTGACCTGGCCGT-3′.

Western blot analysis of mimitin

For protein analysis cells were plated at a density of 6 × 10⁴ cells/well on to six-well plates and grown to confluence within 2 days. Cells were exposed to the desired concentrations of cytokines for 24 h. Thereafter, cells were homogenized in ice-cold PBS using short bursts (10 s using a Braun-Sonic 125 Homogenizer). Protein content was determined by the BCA (bicinchoninic acid) assay (Pierce). For Western blotting, 10 μg of total protein was resolved by SDS/PAGE (10% gel) and then electroblotted on to membranes. Immunodetection was performed using specific primary antibodies against mimitin (polyclonal, self-made [2]) followed by exposure to secondary peroxidase-conjugated AffiniPure donkey anti-[rabbit IgG (H + L)] (Dianova). Hybridized antibodies were visualized through chemiluminescence using the ECL detection system and captured by the INTAS® chemiluminescence detection system (Intas Science Imaging Instruments). The blots were stripped (ReBlot Plus Strong Solution, Millipore), blocked and incubated with primary antibodies against actin (goat polyclonal, Santa Cruz Biotechnology), followed by exposure to secondary peroxidase-conjugated anti-goat antibodies (Dianova) and analysed as described above.

Mitochondrial membrane potential estimation

Cells were seeded at a density of 4 × 10⁴ cells/well on to black 96-well plates 24 h before addition of cytokines. Thereafter cells were incubated for 20 min with rhodamine 123 (50 μM) (Invitrogen). After washing, plates were analysed at 480/520 nm excitation/emission using a Victor2 1420 Multilabel Counter fluorescence reader (PerkinElmer). Each condition was measured at least in duplicate. Results were expressed as the percentage of untreated cells.

ATP measurements

ATP was determined using the ATPlite Detection Assay System (PerkinElmer)[12]. INS1E cells were seeded at a density of 4 × 10⁴ cells/well and MIN6 cells at a density of 2 × 10⁵ cells/well on to black 96-well plates 24 h before the addition of cytokines. After 24 h, cells were cultured in the absence of glucose for 1 h followed by a 2 h incubation with 10 mM glucose. Cells were then lysed and used for ATP concentration measurements according to the manufacturer’s instructions. Results were normalized to protein content.

Insulin secretion and content

Insulin secretion and content in control and mimitin-overexpressing insulin-secreting INS1E and MIN6 cell clones were measured by radioimmunoassay [13]. INS1E cells were seeded at a density of 3.5 × 10⁵ cells/well on to six-well plates 2 days before test components were added. MIN6 cells were seeded at a density of 3 × 10⁵ cells/well on to six-well plates 2 days before lentiviral particles were added. After a 4 h incubation with lentivirus, the medium was changed and the experiments were started on the following day. A 24 h incubation with cytokines was followed by a 1 h incubation without glucose and a 2 h stimulation with glucose (3, 10 or 30 mM). Thereafter supernatants were collected for radioimmunoassay. Insulin values were normalized to the DNA content of the incubated cells.

Data analysis

Analyses of the real-time RT–PCR data were performed using the Opticon Monitor v.1.07 (MJ Research). All results are means ± S.E.M. Statistical analyses were performed using the Prism analysis program (GraphPad); P < 0.05 was considered significant.

RESULTS

Expression of mimitin in rat and mouse tissues

The mimitin gene expression level in rat liver was set as 100%. In tests and pancreas mimitin expression was higher than in liver (Table 1). In all other tissues analysed, including pancreatic islets, the expression was significantly lower than in liver (Table 1). Particularly in heart muscle, brain and kidney, mimitin expression was extremely low in the range of 1% of the liver (Table 1). Mimitin gene expression in the mouse insulin-producing MIN6 cell line was strong (Figure 1A). In contrast, the gene expression of mimitin in the rat insulin-secreting cell lines INS1E, INS1 and RINm5F was lower than in rat pancreatic islets (Figure 1A). To address whether native pancreatic β-cells express mimitin, a detailed immunofluorescence analysis of the mimitin distribution in rat islet cells was performed showing expression in all islet cell types (Figure 2B). The mimitin expression level was

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mimitin (%)</th>
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<tbody>
<tr>
<td>Testis</td>
<td>300 ± 21 (5)*</td>
</tr>
<tr>
<td>Pancreas</td>
<td>290 ± 19 (6)*</td>
</tr>
<tr>
<td>Liver</td>
<td>100 ± 9 (8)</td>
</tr>
<tr>
<td>Intestine</td>
<td>49 ± 5 (4)*</td>
</tr>
<tr>
<td>Lung</td>
<td>35 ± 4 (5)*</td>
</tr>
<tr>
<td>Pancreatic islets</td>
<td>23 ± 3 (5)*</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>18 ± 1 (5)*</td>
</tr>
<tr>
<td>Heart muscle</td>
<td>1 ± 0 (5)*</td>
</tr>
<tr>
<td>Brain</td>
<td>1 ± 0 (5)*</td>
</tr>
<tr>
<td>Kidney</td>
<td>1 ± 0 (5)*</td>
</tr>
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</table>

*P < 0.05 compared with liver, ANOVA followed by a Bonferroni test.
higher in α-cells than in β-cells, but nevertheless was clearly detectable, in contrast with the almost complete lack of expression in INS1E cells (Figures 2A and 2B). Cells negative for both insulin and glucagon expressed more mimitin than α- or β-cells (Figure 2B). A significant cytokine-induced reduction in mimitin gene expression was observed in rat islets (Figure 1B). The reduction in mimitin expression was particularly strong in rat α- and β-cells (Figure 2B), and somewhat weaker in other cell types (Figure 2B).

In isolated islets from spontaneously hyperglycaemic ob/ob mice, an animal model of insulin resistance and obesity, mimitin expression was comparable with that in islets from lean littermates (Figure 1C and Table 2). In contrast, a significant reduction of approximately 40% in mimitin expression in the liver as well as in the brain between ob/ob mice and their lean littermates was observed (Table 2). In the heart and the kidney of ob/ob mice, mimitin was expressed at a higher level when compared with the lean littermates (Table 2). The expression of mimitin in the skeletal muscle was 2-fold higher than in the liver in both lean and ob/ob mice (Table 2).

To analyse the role of mimitin in pancreatic β-cells, the effects of mimitin overexpression and mimitin knockdown were analysed with special reference to cell function and insulin secretory capacity.

**Stable overexpression of mimitin in insulin-secreting INS1E cells**

The endogenous level of mimitin expression in the INS1E rat β-cell line was low, particularly when compared with the MIN6 mouse β-cell line (Figure 1A). So the INS1E insulin-secreting cell line can serve as a good model for studies on the effects of mimitin overexpression. Therefore cDNA for mimitin was introduced and several positive clones were obtained. To exclude a possible influence of clonal variation on the results, for further analyses three clones were selected, INS1E-mimitin K1, K2 and K3 (Figure 3A). Mimitin expression was estimated at the protein level (Figure 3A). Insulin-secreting INS1E cells transfected with the empty pcDNA3 vector were used as control cells (two clones, INS1E-control 1 and INS1E-control 2). The mitochondrial localization of mimitin was confirmed using MitoTracker Deep Red staining (Figure 2C). Mimitin expression in untransfected and transfected INS1E-control as well as mimitin-overexpressing cells was only slightly affected by pro-inflammatory cytokines [for INS1E cells, IL-1β (600 units/ml) 96 ± 12%, cytokine mixture 80 ± 6%; and for INS1E-control cells, IL-1β (600 units/ml) 91 ± 13%, cytokine mixture 100 ± 12%; for INS1E-mimitin K3, IL-1β (600 units/ml) 87 ± 4%, cytokine mixture 89 ± 8%; compared with untreated 100%, n = 14].

**Effects of mimitin overexpression on cell viability and proliferation**

Caspace 3 was 1.5-fold increased by IL-1β and even more by a cytokine mixture in INS1E-control 1 and 2 cell clones (Table 3). Importantly, mimitin overexpression abolished the activation of caspase 3 in the presence of cytokines (Table 3). The protective effects of mimitin were specific for cytokine-mediated toxicity, because the activation of caspase 3 by camptothecin (0.5 μM) did not differ in INS1E-control 1 and 2 and INS1E-mimitin cells (results not shown).

The basal proliferation rate in untreated INS1E-mimitin cells was significantly higher in comparison with INS1E-control cells. The absolute absorbance values were in INS1E-control 1 cells 1.34 ± 0.14 and in INS1E-control 2 cells 1.35 ± 0.08. They increased significantly (P < 0.05) in the INS1E-mimitin K1 (1.37 ± 0.09), INS1E-mimitin K2 (2.58 ± 0.14) and INS1E-mimitin K3 (2.28 ± 0.17) clones (expressed as ΔA450/650, n = 4–13). Cytokines significantly decreased the proliferation of control INS1E cells, leading to a 55–60% and 70% loss of proliferative capacity, in the case of IL-1β and the cytokine mixture, respectively (Table 3). INS1E cells overexpressing mimitin were protected against the cytokine-mediated reduction of proliferation capacity (Table 3). Thus mimitin overexpression protects insulin-secreting cells against cytokine-mediated viability and proliferation loss. Since the largest protective effects on cell viability as well as proliferation rate were seen in the case of the INS1E-mimitin K3 clone, the subsequent experiments were performed with this K3 clone.

**Effects of mimitin overexpression on cytokine-stimulated mitochondrial dysfunction**

Cytokines significantly activated mitochondrial caspase 9 in INS1E-control cells (Figure 3B). Mimitin overexpression...
Mimitin in insulin-secreting cells

Figure 2  Immunofluorescence analysis of mimitin expression in insulin-secreting cells and primary rat islet cells

Insulin-secreting INS1E cells or rat islet cells were seeded on to chamber slides, fixed and immunostained. (A) INS1E cells, mimitin (green) and insulin (red). (B) Rat islet cells, mimitin (green), insulin (red) and glucagon (red); double-positive cells appear yellow. (C) Mitochondrial localization of mimitin in insulin-secreting INS1E transfected with pmaxFP-green mimitin vector: mimitin (green) and mitochondria (red); in the overlay, mitochondrially localized mimitin appears yellow. White arrows show β-cells, white stars show cells negative for insulin or negative for glucagon, and white V indicates α-cells.

...attenuated cytokine-mediated caspase 9 activation (Figure 3B). The protective effect of mimitin was specific for cytokine-mediated toxicity, because the activation of caspase 9 by camptothecin (0.5 μM) did not differ in INS1E-control and INS1E-mimitin cells (Figure 3B).

Mitochondrial membrane potential in INS1E control and mimitin-overexpressing cells was identical under control conditions (results not shown). Exposure of INS1E-control cells to cytokines for 24 h reduced mitochondrial membrane potential (a 25 % decrease after exposure to IL-1β and a nearly 40 % decrease in the case of the cytokine mixture; Figure 3C). The cytokine-mediated decrease in mitochondrial membrane potential was not present after IL-1β exposure and significantly smaller in the case of the cytokine mixture in INS1E-mimitin K3 cells when compared with control cells (Figure 3C).

The ATP content in INS1E-control cells was 3.4 ± 0.2 (clone 1) and 3.2 ± 0.3 (clone 2; not depicted in Figure 3D) nmol/mg of protein and was significantly decreased after a 24 h incubation with cytokines (Figure 3D). Interestingly, mimitin-overexpressing cells contained significantly more ATP than control cells (Figure 3D) and this ATP content was only very slightly reduced by cytokines (Figure 3D).

Table 2  Mimitin gene expression in different mouse tissues

Mimitin expression in different mouse tissues (from lean and ob/ob mice) was determined by quantitative real-time RT-PCR and normalized to β-actin. The value for mouse liver from lean littermates was 0.044 ± 0.004 (arbitrary units) and was set as 100 %. Results are means ± S.E.M. with the numbers of experiments given in parentheses (arbitrary units). *P < 0.05 compared with liver in lean littermates; #P < 0.05 compared with the same tissue in lean littermates; Student’s t test.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Lean littermates (% of liver)</th>
<th>ob/ob mice (% of liver in lean mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>100 ± 9 (23)</td>
<td>63 ± 5 (22)*</td>
</tr>
<tr>
<td>Pancreatic islets</td>
<td>53 ± 13 (16)*</td>
<td>54 ± 7 (20)*</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>245 ± 71 (4)*</td>
<td>206 ± 71 (6)*</td>
</tr>
<tr>
<td>Heart muscle</td>
<td>150 ± 18 (11)</td>
<td>214 ± 14 (11)*#</td>
</tr>
<tr>
<td>Brain</td>
<td>86 ± 24 (10)</td>
<td>54 ± 7 (12)*#</td>
</tr>
<tr>
<td>Kidney</td>
<td>72 ± 4 (4)*</td>
<td>100 ± 11 (6)*#</td>
</tr>
</tbody>
</table>

Effects of mimitin overexpression on cytokine-induced NF-κB activation and nitrite production

Incubation of insulin-secreting INS1E-control 1 cells with cytokines induced NF-κB activation (Table 4). A similar induction...
Table 3 Effects of mimitin overexpression in insulin-secreting INS1E cells on caspase 3 activation and cell proliferation after exposure to cytokines

Insulin-secreting INS1E cells overexpressing mimitin as well as control cells, were incubated for 24 h with either IL-1β (600 units/ml) alone or with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNFα and 14 units/ml IFNγ). Caspase 3 activity was measured by flow cytometry and cell proliferation by a BrdU incorporation assay. Results are expressed as the percentage of untreated cells and are means ± S.E.M. for the indicated numbers (in parentheses) of independent experiments, each measured for at least three repetitions. *P < 0.05 compared with untreated; #P < 0.05 compared with control clone 1 treated in the same way; ANOVA followed by a Bonferroni test.

(a) Caspase 3 activity (% of untreated)

Table 4 Effects of mimitin overexpression on cytokine-induced NF-κB activation and nitrite production in insulin-secreting INS1E cells

For estimation of transcription factor activation, insulin-secreting INS1E cells were transfected 24 h prior to cytokine treatment and then incubated with either IL-1β (600 units/ml) alone or with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNFα and 14 units/ml IFNγ) for 24 h. Thereafter the medium was collected and a SEAP-reporter gene assay was performed. Nitrite concentrations were determined by a Griess assay. Results are means for the indicated numbers (in parentheses) of independent experiments, each measured for at least three repetitions. *P < 0.05 compared with untreated; ANOVA followed by a Bonferroni test. n.d., not detectable.

(a) NF-κB (% of untreated)

was observed in cells overexpressing mimitin (Table 4). The iNOS (inducible NO synthase) protein expression analysis revealed a significant induction after exposure to cytokines in INS1E-control 1 as well as INS1E-mimitin K3 cells (results not shown). Measurements of nitrite production in INS1E-control 1 as well as INS1E-mimitin K3 cells revealed no significant nitrite accumulation under control conditions (Table 4). Incubation of INS1E-control 1 as well as INS1E-mimitin K3 cells with cytokines induced NO production as documented by a significant rise in accumulated nitrite (Table 4). Thus mimitin overexpression did not block the NF-κB–iNOS pathway and therefore did not rescue insulin-producing cells from cytokine toxicity by reduction of NO production.
Figure 4  Effects of mimitin overexpression in insulin-secreting INS1E cells on insulin secretion and content

Insulin-secreting INS1E cells were incubated with either IL-1β alone (600 units/ml) or with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNFα and 14 units/ml IFNγ) for 24 h. Thereafter cells were cultured in the absence of glucose followed by a 1 h incubation with glucose (3, 10 or 30 mM). Insulin was measured by radioimmunoassay:

(A) glucose-induced insulin secretion;  
(B) insulin content;  
(C) glucose-induced insulin secretion at 10 mM glucose (Glc) in the absence or presence of cytokines;  
(D) insulin content at 10 mM glucose in the absence or presence of cytokines. White bars, INS1E-control 1 cells; striped bars, INS1E-control 2 cells, black bars, INS1E-mimitin K3 cells. Results are means ± S.E.M. for six independent experiments. * P < 0.05 compared with untreated; #P < 0.05 compared with INS1E-control 1 cells treated in the same way, §P < 0.05 compared with INS1E-control 2 cells treated in the same way; ANOVA followed by a Bonferroni test.

Effects of mimitin overexpression on Ins2 gene expression, insulin secretion and content

The expression of the Ins2 gene in insulin-secreting INS1E-mimitin K3 cells did not significantly differ from control cells (INS1E-control 1 cells, untreated 6.0 ± 0.6, IL-1β 2.4 ± 0.2, cytokine mixture 1.3 ± 0.3; compared with INS1E-mimitin K3 cells, untreated 7.2 ± 1.0, IL-1β 2.7 ± 0.3, cytokine mixture 1.8 ± 0.3; arbitrary units, n = 6).

INS1E-control 1 cells secreted 0.09 ± 0.01 and INS1E-control 2 cells 0.07 ± 0.01 ng/μg of DNA/h insulin at 3 mM glucose (Figure 4A). Incubation in the medium containing 30 mM glucose stimulated insulin secretion up to 0.17 ± 0.01 (INS1E-control 1) and 0.18 ± 0.02 (INS1E-control 2) ng/μgDNA/h (Figure 4A). The basal insulin secretion at 3 mM glucose in INS1E-mimitin K3 cells was 0.23 ± 0.02 ng/μgDNA/h, approximately 2.5-fold higher than in control INS1E cells (Figure 4A). Insulin secretion induced by 30 mM glucose in INS1E-mimitin K3 cells reached 0.98 ± 0.08 ng/μg of DNA/h and was significantly higher than glucose-induced insulin secretion in control cells.

Incubation of INS1E-control cells with IL-1β or a cytokine mixture significantly reduced insulin secretion (10 mM glucose; control 1, IL-1β 0.09 ± 0.01, cytokine mixture 0.10 ± 0.01, compared with untreated 0.18 ± 0.01 ng/μg of DNA/h; control 2, IL-1β 0.09 ± 0.01, cytokine mixture 0.10 ± 0.02, compared with untreated 0.17 ± 0.02 ng/μg of DNA/h; Figure 4C). In INS1E-mimitin K3 cells, IL-1β did not reduce insulin secretion and incubation with the cytokine mixture caused only a 28% decrease (Figure 4C). Although glucose-induced insulin secretion was decreased by cytokines in INS1E-mimitin K3 cells, the amount of secreted insulin upon glucose stimulation was significantly higher than in control cells (IL-1β 3.3-fold higher, and cytokine mixture 2.4-fold higher, compared with INS1E-control cells treated in the same way, P < 0.05; Figure 4C).

Insulin content in INS1E-control cells cultured at 3 mM glucose was in the control 1 clone 45 ± 5 ng/μg of DNA, in the control 2 clone 42 ± 2 ng/μg of DNA, and was decreased in both clones after incubation at 30 mM glucose (Figure 4B). INS1E-mimitin K3 cells had a similar insulin content to control cells (47 ± 4 ng/μg of DNA), which was slightly reduced after
Effects of mimitin knockdown in insulin-secreting MIN6 cells

In the mouse insulin-secreting MIN6 cells, mimitin was expressed, in contrast with the very weak expression in the rat insulin-secreting INS1E cell line (Figure 1A, Figure 3A and Figure 5A). Mimitin was successfully knocked down in MIN6 cells (Figure 5A). Mimitin expression in untransfected as well as transfected MIN6 cells was unaffected by pro-inflammatory cytokines (results not shown). The suppression of mimitin in MIN6 cells was confirmed by Western blotting (Figure 5A). Mimitin expression in untransfected as well as transfected MIN6 cells was unaffected by pro-inflammatory cytokines (results not shown). The suppression of mimitin in MIN6 cells was confirmed by Western blotting (Figure 5A). Mimitin expression in untransfected as well as transfected MIN6 cells was unaffected by pro-inflammatory cytokines (results not shown). The suppression of mimitin in MIN6 cells was confirmed by Western blotting (Figure 5A). Mimitin expression in untransfected as well as transfected MIN6 cells was unaffected by pro-inflammatory cytokines (results not shown). The suppression of mimitin in MIN6 cells was confirmed by Western blotting (Figure 5A). Mimitin expression in untransfected as well as transfected MIN6 cells was unaffected by pro-inflammatory cytokines (results not shown).
of mimitin in MIN6 cells had opposite effects when compared with the effects observed after mimitin overexpression in INS1E cells.

The ATP content in the MIN6-shRNA-control cells was 4.4 ± 0.4 nmol/mg of protein and was significantly reduced by a knockdown of mimitin to 1.8 ± 0.2 nmol/mg of protein (Figure 5B). Pro-inflammatory cytokines slightly decreased ATP content in MIN6-shRNA-control cells, reaching a significant effect in the case of the cytokine mixture (Figure 5B). In the case of MIN6-shRNA-mimitin cells, a further reduction in ATP content was also observed with cytokines, however, to a lesser extent than in the control cells (Figure 5B).

A 24 h incubation with IL-1β (600 units/ml) or a mixture of cytokines (IL-1β, TNFα and IFNγ) did not induce caspase 3 activation in MIN6-shRNA-control cells (Figure 5C). Mimitin knockdown resulted in increased cytokine-induced caspase 3 activation (Figure 5C).

The insulin content in MIN6-shRNA-control and in MIN6-shRNA-mimitin cells was comparable (3.0 ± 0.3 compared with 3.7 ± 0.5 ng/μg of DNA respectively). The MIN6-shRNA-control cells secreted significantly more insulin at the basal condition as well as upon stimulation with glucose when compared with insulin-secreting INS1E-control cells (Figure 5D and Figure 4A). Mimitin knockdown in MIN6 cells decreased glucose-induced insulin secretion (Figure 5D). The MIN6-shRNA-mimitin cells, however, did not lose their insulin secretory responsiveness to the glucose stimulus, indicating that mimitin acts as a potentiator of glucose-stimulated insulin secretion. Incubation of MIN6-shRNA-control cells with pro-inflammatory cytokines dampened glucose-induced insulin secretion very weakly (Figure 5E). Vice versa, mimitin knockdown resulted in an enhancement of the cytokine-mediated inhibition of glucose-induced insulin secretion (Figure 5E).

**DISCUSSION**

Mimitin attracted our attention since it had been shown to act as a regulator of ATP synthesis [1] and the formation of ATP plays a crucial role in glucose-induced insulin secretion [3] and pancreatic β-cell survival upon exposure to pro-inflammatory cytokines [4].

Mimitin was expressed in different cell types of primary rat and mouse pancreatic islets. Pro-inflammatory cytokines significantly reduced mimitin expression in primary islet cells, but, however, had a weaker effect in the insulin-secreting INS1E cell line, which is characterized by a very weak expression of mimitin. These data indicate also that in a complex system composed of different cell types (islets) the regulation of mimitin expression can be influenced by additional yet-unknown modulatory factors.

Although the mimitin expression level of isolated islets from ob/ob mice and their lean littermates did not differ, a significant difference was observed in the case of other tissues, suggesting a possible regulation of mimitin in this animal model of insulin resistance and obesity and the involvement of mimitin dysregulation in the development of metabolic disturbances. Because mimitin regulates the ATP production, one of the possible targets influenced by the changes in mimitin expression could be AMPK (AMP-activated protein kinase). AMPK is a central cellular energy sensor, activated by a decrease in ATP, which controls whole-body energy homeostasis [14] and which is an important target for diabetes therapy [15]. It has been shown that leptin reduces the AMPK activity in the brain [16] and that the inhibition of the hypothalamic AMPK leads to the reduction of food intake and the increase in energy expenditure [17].

It is possible therefore that a reduced mimitin level in the brain of ob/ob mice when compared with their lean littermates may, additionally to the lack of leptin, enable higher neuronal AMPK activity, which as a consequence fosters food intake and obesity in this animal model. On the other hand, the reduced mimitin level in the liver of ob/ob mice may serve as an adaptive response, since increased AMPK in hepatocytes (e.g. by the action of metformin) has been shown to improve insulin sensitivity and inhibit glucose production [18]. In the heart, AMPK has been shown to activate phosphofructokinase 2 and to stimulate the glycolytic flux [14]. The expression of mimitin in the heart of ob/ob mice was significantly higher than in lean littermates. Such an increased expression of mimitin may result in a higher ATP content and may disturb the AMP/ATP ratio in the cells, leading to a decreased AMPK activity. In pancreatic β-cells, AMPK overexpression has been shown to impair cell function [19]. The pancreatic β-cells of ob/ob mice are not defective in their function [7,8] and in line with this we did not observe any differences between mimitin expression in islets isolated from lean and obese mice. Thus the mimitin data obtained from ob/ob mice and their lean littermates add a new aspect to the complex regulatory mechanisms in metabolic disorders.

To analyse the impact of mimitin on β-cell function and susceptibility to pro-inflammatory cytokines we chose for the present study two β-cell lines, the INS1E with a low expression level of mimitin and the MIN6 with a higher expression level of this protein.

Insulin-secreting INS1E cells are known for their high sensitivity to pro-inflammatory cytokines [20], which we confirmed in the present study. Overexpression of mimitin in INS1E cells prevented cytokine-induced caspase 3 activation. In contrast, MIN6 cells, which are resistant to cytokine toxicity, achieved sensitivity to pro-inflammatory cytokines after a knockdown of mimitin. These findings are in line with a previous report in which reduction of mimitin expression in hepatoma cells by the siRNA approach resulted in a potentiation of cytokine-induced apoptosis and inhibition of cell proliferation [2]. Overexpression of mimitin had opposite effects [2]. Moreover, the observed protective effect of mimitin overexpression is in line with reports showing that defects in the mitochondrial respiratory chain complex I are characteristic for many energy generation disorders and are possibly implicated in disturbed apoptotic signalling [21,22].

Pro-inflammatory cytokines cause pancreatic β-cell death via induction of mitochondrial stress and other responses [5,6,23]. The results of the present study clearly show that mimitin overexpression protects insulin-secreting cells against cytokine toxicity via suppression of mitochondrial stress, however, without concomitant inhibition of the NF-κB–iNOS pathway. Similar protection against cytokine-induced mitochondrial stress was recently reported by overexpression of prostacyclin synthase in insulin-producing cells, but this protective effect strongly depended on inhibition of the NF-κB signalling pathway [24]. Thus protection against cytokine toxicity via mimitin overexpression must involve other mechanisms.

Interestingly, mimitin-overexpressing cells exhibited a higher basal proliferation rate than control cells. This increased proliferative activity may result from an increased mitochondrial metabolism, as shown by a significantly higher ATP content in mimitin-overexpressing cells. This is in line with a previous report showing a correlation between a decreased rate of ATP production and a decreased β-cell proliferation [25]. It also confirms the results obtained in oesophageal carcinoma [1] and hepatoma cells [2], pointing to an important role of mimitin in cell proliferation.
The elevated ATP content in mimitin-overexpressing cells is indicative of an increased ATP production. Indeed mimitin has been shown to act as a molecular chaperone for the assembly of the mitochondrial respiratory chain complex I [1]. Therefore an increased level of mimitin may stimulate oxidative phosphorylation and raise ATP production. Our finding that mimitin knockdown in insulin-secreting MIN6 cells leads to a decrease in ATP content confirms the role of mimitin in ATP production.

In pancreatic β-cells, an increased ATP production may support proper insulin secretory responsiveness [3,25–27]. In the present study, insulin-secreting INS1E cells overexpressing mimitin indeed exhibited greater insulin secretory responsiveness to the physiological stimulus glucose. The expression of the Ins2 gene as well as the insulin content were similar in control and mimitin-overexpressing cells, suggesting that the prominent effect of mimitin overexpression is a potentiation of glucose-induced insulin secretion. The results obtained in the MIN6-shRNA-mimitin cells confirmed this observation, showing a decrease in overall insulin secretion, but no loss of glucose responsiveness. Thus mimitin seems to play a potentiating role in glucose-induced insulin secretion, but is not an obligatory component of it.

Importantly, mimitin overexpression counteracted cytokine-mediated inhibition of glucose-induced insulin secretion, and this went along with a preservation of the ATP content. In line with this, mimitin knockdown led to stronger cytokine-mediated inhibition of glucose-induced insulin secretion. Therefore mimitin protects against adverse cytokine effects, most probably via its chaperone-mediated preservation of ATP production.

On the other hand, an increased basal insulin secretion at a low glucose concentration as observed in INS1E-mimitin cells with a high mimitin expression level is an indicator for a potential hypoglycemia risk. Thus the rather moderate constitutive expression level of mimitin observed in primary β-cells might be necessary to avoid hypoglycemic episodes, though on the other hand it may contribute to the extraordinary vulnerability of the pancreatic β-cells [28].

The present study identified a novel mitochondrial protein, mimitin, as a mild modulator of the glucose-induced insulin secretion pathway. Mimitin prevented mitochondrial stress upon exposure to cytokines, and this protective effect was delivered independent of a suppression of the NF-κB pathway. Moreover, the data revealed that mimitin overexpression could prevent cytokine-induced inhibition of glucose-induced insulin secretion through maintenance of mitochondrial integrity.

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