Insulin secretion defects of human type 2 diabetic islets are corrected in vitro by a new reactive oxygen species scavenger


Abstract

Oxidative stress is a putative mechanism leading to beta-cell damage in type 2 diabetes. We studied isolated human pancreatic islets from type 2 diabetic and non-diabetic subjects, matched for age and body mass index. Evidence of increased oxidative stress in diabetic islets was demonstrated by measuring nitrotyrosine concentration and by electron paramagnetic resonance. This was accompanied by reduced glucose-stimulated insulin secretion, as compared to non-diabetic islets (Stimulation Index, SI: 0.9 ± 0.2 vs. 2.0 ± 0.4, P < 0.01), and by altered expression of insulin (approximately −60%), catalase (approximately +90%) and glutathione peroxidase (approximately +140%). When type 2 diabetic islets were pre-exposed for 24 h to the new antioxidant bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate di-hydrochloride, nitrotyrosine levels, glucose-stimulated insulin secretion (SI: 1.6 ± 0.5) and gene expressions improved/normalized. These results support the concept that oxidative stress may play a role in type 2 diabetes beta-cell dysfunction; furthermore, it is proposed that therapy with antioxidants could be an interesting adjunctive pharmacological approach to the treatment of type 2 diabetes.

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Résumé

Un nouveau scavenger des espèces réactives de l’oxygène corrige in vitro les défauts de la sécrétion d’insuline des îlots diabétiques humains de type 2

Le stress oxydant est considéré un mécanisme important à l’origine des lésions des cellules β dans le diabète type 2. Dans des îlots humains isolés à partir de sujets diabétiques type 2 et non diabétiques, comparables par âge et le BMI, l’augmentation du stress oxydatif a été mise en évidence par l’évaluation des concentrations de nitrotyrosine et par résonance paramagnétique électronique. Par ailleurs, chez les îlots diabétiques, a été mise en évidence une réduction de la sécrétion d’insuline induite par le glucose par comparaison aux îlots témoins, avec en outre, une expression anormale de l’insuline (−60 %), de la catalase (+90 %) et du glutathion peroxydase (−140 %). Après prémétabolisation durant 24 heures des îlots diabétiques en présence d’un nouvel antioxydant, le bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decandioate di-hydrochloride, les concentrations de nitrotyrosine et la sécrétion d’insuline induite par le glucose, ainsi que l’expression des gènes mentionnés, se sont améliorées ou normalisées. Ces résultats confirment que le stress oxydant est impliqué dans les altérations des cellules β au cours du diabète type 2 et suggèrent qu’une thérapie antioxydante pourrait constituer une approche complémentaire très intéressante pour le traitement du diabète de type 2.

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1. Introduction

Diabetes mellitus is a chronic syndrome caused by insulin deficiency, combined with varying degrees of ineffective insulin action [1,2]. The worldwide figure of people with diabetes is set to rise from 150 million in the year 2000 to 300 million in 2025 [3], and the disease is often associated with long-term microvascular, neurological, and macrovascular complications [1–4]. There are two main forms of diabetes: type 1 diabetes is characterized by an absolute or nearly so insulin insufficiency due to the immunological destruction of pancreatic β-cells, and accounts for approximately 10% of all cases; type 2 diabetes is characterized by insulin deficiency and reduced insulin action, and it represents the most common form (around 90% of all cases) [1–4]. Several lines of evidence show that defects of the insulin secreting beta-cell are central to the development of type 2 diabetes, and commonly found alterations of insulin secretion include reduced or absent glucose-stimulated first phase release, blunted responses to non-glucose stimuli and secretion include reduced or absent glucose-stimulated first phase release, blunted responses to non-glucose stimuli and loss of normal oscillatory insulin secretion pattern [3–6]. These defects appear to be due to both decreased beta-cell mass and intrinsic alterations of beta-cell function [7–9]. Although the mechanisms causing beta-cell damage in type 2 diabetes are still unclear, the role of oxidative stress is receiving much attention. Enhanced production of reactive oxygen species (ROS) can be produced in beta-cells by several path-ways, including increased metabolism due to excessive glucose digestion and density gradient purification and viability assessed as previously described [21,22,25,26]. The main clinical characteristics of non-diabetic and diabetic donors were age 49 ± 16 and 65 ± 9.5 years, respectively; gender, four males/four females and three males/two females; body mass index, 25.6 ± 2.5 and 27.5 ± 4.0 kg/m². Aliquots of approxi-mately 500 hand-picked islets were then cultured free-floating in 37 °C for 24 h in M199 culture medium (containing 10% bovine serum and antibiotics) either plain or containing 10 or 100 μmol/l IAC (kindly provided by Medestea, Torino, Italy). The final pH of all media was buffered at pH 7.4.

2. Materials and methods

2.1. Islet isolation and culture

Isolated islets were prepared from the pancreata of eight non-diabetic and five type 2 diabetic multiorgan donors (with the approval of the local Ethics Committee) by collagenase digestion and density gradient purification and viability assessed as previously described [21,22,25,26]. The redox balance of isolated islets was determined by measuring nitrotyrosine concentrations and determining the amount of nitrooxide originated from IAC [23,24] by electron paramagnetic resonance (EPR). Nitrotyrosine concentrations were assessed by an ELISA method as previously described [25]. White 96-well plates (Iwaki, Japan) were coated with 200 μl of standard curve samples (15–0.66 nmol/l) or 1 μg/μl of islet cell lysates (65 μl/well) in 0.1 mol/l carbonate–bicarbonate buffer (135 μl), pH 9.6, kept overnight at 4 °C. Non-specific binding was blocked by 1% BSA in PBS-T (PBS plus 0.05% Tween 20), for 1 h at 37 °C and the wells were incubated with purified monoclonal anti-nitrotyrosine mouse IgG (Upstate, NY) for 1 h at 37 °C. Then, the plates were washed and incubated with a peroxidase-conjugated goat anti-mouse IgG secondary antibody for 45 min at 37 °C. The peroxidase membrane permeant [23]. The molecule acts as a broad, effective and self-regenerating intracellular scavenger of reactive oxygen and nitrogen species at an early stage, i.e. prior to the generation of ROS-derived toxic products [23]. Following reaction with the majority of radical species of biological relevance, including alkyl, alcoxyl, peroxyl, hydroperoxyl and hydroxyl radicals, as well as superoxide, this peculiar antioxidant transforms completely and instantaneously to the corre-sponding nitroxide [24]. Our results confirmed the presence of increased oxidative stress in the diabetic islets and showed that IAC exposure reduced oxidative stress and improved glucose-stimulated insulin secretion; finally we investigated on some of the possible molecular mechanisms involved in these effects.

2.2. Determination of oxidative stress

The redox balance of isolated islets was determined by measuring nitrotyrosine concentrations and determining the amount of nitrooxide originated from IAC [23,24] by electron paramagnetic resonance (EPR). Nitrotyrosine concentrations were assessed by an ELISA method as previously described [25]. White 96-well plates (Iwaki, Japan) were coated with 200 μl of standard curve samples (15–0.66 nmol/l) or 1 μg/μl of islet cell lysates (65 μl/well) in 0.1 mol/l carbonate–bicarbonate buffer (135 μl), pH 9.6, kept overnight at 4 °C. Non-specific binding was blocked by 1% BSA in PBS-T (PBS plus 0.05% Tween 20), for 1 h at 37 °C and the wells were incubated with purified monoclonal anti-nitrotyrosine mouse IgG (Upstate, NY) for 1 h at 37 °C. Then, the plates were washed and incubated with a peroxidase-conjugated goat anti-mouse IgG secondary antibody for 45 min at 37 °C. The peroxidase...
reaction product was generated using TMB (Tetramethylbenzidine) Microwell Peroxidase Substrate (Sigma-Aldrich) (150 μl/well). Plates were incubated 5–10 minutes at room temperature and optical density was read at 492 nm in a microplate reader.

For the EPR experiments, the probe bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decandioate, obtained as previously described [23], was used to assess the amount of reactive oxygen species [24]. For the purpose of the present study, pancreatic islets were incubated at 37 °C in standard glass tubes in 0.01 mol/l Na’/K+ phosphate buffer (pH 7.4) in the presence of 1 mmol/l NADPH and 0.5 mol/l bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate. The nitroxide radical amount generated by oxidation of the probe was finally measured by EPR as described [24]. About 50 μl of the solution was transferred and sealed in a calibrated capillary glass tube, which was placed inside a standard EPR tube in the cavity of a Bruker ESP 300 EPR spectrometer. The actual amount of solution analyzed was chosen so as to cover the entire sensitive area of the instrument cavity. The spectra of the nitroxide radical generated by reaction of the hydroxylamine with the radicals produced in the biological sample were then recorded using the following instrumental settings: modulation amplitude = 1.0 G; conversion time = 163.84 ms; time constant = 163.84 ms; receiver gain = 1.0e5; microwave power = 6.3 mW. The intensity of the first time = 163.84 ms; time constant = 163.84 ms; receiver gain = 1.0e5; microwave power = 6.3 mW. The intensity of the first derivative of the EPR spectrum was used to quantify the amount of nitroxide per mL of sample, after calibration of the instrumental settings: modulation amplitude = 1.0 G; conversion time = 163.84 ms; time constant = 163.84 ms; receiver gain = 1.0e5; microwave power = 6.3 mW. The intensity of the first derivative of the EPR spectrum was used to quantify the amount of nitroxide per mL of sample, after calibration of the spectrometer response with known solutions of TEMPO-coline in water, using an artificial ruby crystal as internal standard. For simplicity, results were expressed as micromolar concentration (μM) of ROS in the sample.

2.3. Insulin secretion studies

Insulin secretion studies were performed by the batch incubation method, as detailed elsewhere [21,22,25,26]. Following a 45 min pre-incubation period at 3.3 mmol/l glucose, islets were kept at 37 °C for 45 min in Krebs–Ringer bicarbonate solution (KRB), 0.5% albumin, pH 7.4, containing 3.3 mmol/l glucose. At the end of this period, medium was completely removed and replaced with KRB containing either 3.3 or 16.7 mmol/l glucose. After additional 45 min incubation, medium was removed. Samples (500 μl) from the different media were stored at 20 °C until insulin concentrations were measured by IRMA.

2.4. Gene expression studies

Messenger RNA expression of catalase, glutathione peroxidase and insulin was measured by real-time quantitative reverse transcription reaction (Real-Time quantitative RT-PCR) [25,26]. Total RNA was extracted from the islets by using the RNaseasy Protect Mini Kit (QIAGEN) and quantified by absorbance at A260/A280 (ratio > 1.65) nm in a Perkin-Elmer spectrophotometer. Its integrity was assessed after electrophoresis in 1.0% agarose gels by ethidium bromide staining. The primers and probes sequences were obtained from PE Applied Biosystems (Pre-Developed TaqMan Assay Reagents Control Kits). PCR amplifications were performed in a total volume of 20 μl containing 20 ng of cDNA sample, 200 nmol/l of each primer, 100 nmol/l of the corresponding probe, 12.5 μl of TaqMan Universal PCR Master Mix. For each reaction the polymerase was activated by preincubation at 95 °C for 10 min. Amplification was then performed by 40 cycles of switching between 95 °C for 15 s and 60 °C for 60 s. The quantity of each cDNA sample was normalized to the housekeeping gene for b-actin.

2.5. Statistical analysis

Results are expressed as means ± SD. Comparison between two groups were performed by the two-tailed Student’s t-test, whereas comparisons between multiple groups were done by ANOVA, followed by the Bonferroni correction to test differences between specific conditions.

3. Results

3.1. Islet isolation

As shown in Fig. 1, at the end of the isolation process the islets appeared morphologically well preserved and viable, as assessed by different vital dyes.

3.2. Oxidative stress determination

Nitrotyrosine concentrations were significantly (P < 0.01) higher in type 2 diabetes (12.4±0.9 nmol/l) than in control (6.6 ± 0.3 nmol/l) islets (Fig. 2), confirming previous findings from this laboratory [21,22]. In addition, as shown in Fig. 2, increased oxidative stress in diabetic islets was demonstrated by EPR, which showed a 2-fold increase of nitroxide levels.

3.3. Insulin secretion

As expected, glucose-stimulated insulin release from type 2 diabetic islets was significantly lower than from non-diabetic islets. At 3.3 mmol/l glucose, insulin secretion (μU/islet/min) was 0.032 ± 0.005 from control cells and 0.028 ± 0.004 from diabetic cells (NS); at 16.7 mmol/l glucose, the release of the hormone was 0.064 ± 0.008 from non-diabetic islets and 0.027±0.004 from type 2 diabetic cells (P < 0.01). As a consequence, stimulation index (SI, the ratio of insulin release at 16.7 mmol/l glucose over insulin release at 3.3 mmol/l glucose) was 2.0 ± 0.4 and 0.9 ± 0.2, respectively (P < 0.01).

3.4. The effects of the anti-oxidant IAC

Pre-culture of type 2 diabetic islets with 10 or 100 μmol/l IAC caused a significant reduction (P < 0.01 by ANOVA) of nitrotyrosine levels (nmol/l), which decreased from 12.4 ± 0.9 (non-treated cells) to 9.3 ± 0.5 at 10 μmol/l IAC (P < 0.05 vs. non-treated by the Bonferroni test) and 10.2 ± 1.4 at 100 μmol/l...
IAC (<0.05 vs. non-treated by the Bonferroni test). These changes were accompanied by improved glucose-stimulated insulin secretion, with SI values significantly increasing to 1.5 ± 0.4 and 1.6 ± 0.5 with 10 and 100 μmol/l IAC, respectively (Fig. 3). In non-diabetic islets, IAC exposure did not cause any significant change (not shown).

3.5. Molecular studies

As shown in Table 1, mRNA expression of catalase and glutathione peroxidase were significantly higher in type 2 diabetic than control islets, and 24 h exposure to IAC normalized the expression of the two ROS scavenging enzymes. This was accompanied by a significant increase of the expression of insulin, which was more than 50% lower in diabetic islets as compared to control cells prior to IAC pre-culture, and increased by approximately 30% afterwards (Table 1).

4. Discussion

This study confirms that human type 2 diabetes islets have increased oxidative stress, as assessed by measuring nitrotyrosine levels and nitroxide (this latter by EPR). Nitrotyrosine derives from the reaction of superoxide and nitric oxide, and is considered a reliable marker of oxidative stress [22,27]. Increased levels of nitrotyrosine in type 2 diabetic islet cells have been previously reported [22,28]. On the other hand, the probe that was used in the EPR experiments, due to its lipophilic properties, can readily cross the cell membrane to distribute...
in any compartment where the production of free radicals can take place [24,27], which makes the method a useful tool to measure the overall oxidative status of the system under investigation [24]. Proof of this principle has already been provided in biological systems of growing complexity from sub-cellular fractions [29], to the animal model [30], up to human tissues [23,24,31–33] over one decade, and virtues and limits of this EPR radical-probe approach have been thoroughly discussed in the literature [24].

For the first time this report shows that the use of the antioxidant molecule bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate di-hydrochloride (IAC), can significantly reduce the oxidative stress of type 2 diabetes islets. Besides its usefulness as EPR probe, this molecule is an antioxidant and radical scavenger, being able to react with the majority of radical and reactive oxygen and nitrogen species of relevance in a biological environment [23,24]. The effectiveness of IAC is demonstrated not only by the reduction of nitrotyrosine, but also by the normalization of catalase and glutathione peroxidase expression. These enzymes are involved in the removal of reactive oxygen species [34]. The decrease of their expression suggests that after IAC treatment an improved redox balance makes these enzymes less necessary.

Increased oxidative stress causes defective insulin gene expression (mainly due to the loss of critical proteins activating the insulin promoter), reduced insulin secretion and increased apoptosis [35,36]. Unsurprisingly, therefore, improving beta-cell redox balance can correct, at least in part, these defects, and, as a matter of fact, IAC was able to partially restore insulin expression in the present study.

It is to be underlined that reducing islet cell oxidative stress seems indeed to be an effective method to improve beta-cell function and survival [21,22]. In particular, metformin has been previously shown to reduce nitrotyrosine levels, improve insulin release, and decrease apoptosis in diabetic islets [22]. Altogether, the present study supports the concept that therapy with antioxidants could be an interesting adjunctive pharmacological approach to the treatment of type 2 diabetes. In this regard, the protection determined by IAC clearly suggests the potential usefulness of this compound. However, current evidence on the beneficial role of antioxidant therapy on type 2 diabetes in vivo is elusive [37,38]. This might mean that for in vivo studies the exposure of the beta-cell to the tested compounds (in terms of concentration and duration of exposure) might have been insufficient and/or that the unfavorable microenvironment of the beta-cell in vivo counteracts the possible beneficial effects of the therapy.

References


