DIABETES/METABOLISM RESEARCH AND REVIEWS
Diabetes Metab Res Rev 2007; **23**: 234–238.
Published online 4 September 2006 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/dmrr.680

RESEARCH ARTICLE

Gliclazide protects human islet beta-cells from apoptosis induced by intermittent high glucose

S Del Guerra
M Grupillo
M Masini
R Lupi
M Bugliani
S Torri
U Boggi
M Del Chiaro
F Vistoli
F Mosca
S Del Prato
P Marchetti*

Department of Endocrinology and Metabolism, Metabolic Unit, University of Pisa, Pisa – Italy

*Correspondence to: P Marchetti, Department of Endocrinology and Metabolism, Metabolic Unit, Ospedale Cisanello, via Paradisa 2, 56100 Pisa – Italy. E-mail: marchant@immr.med.unipi.it

Abstract

Background Decreased beta-cell mass, mainly due to apoptosis, is crucial for the development and progression of type 2 diabetes. Chronic exposure to high glucose levels is a probable underlying mechanism, whereas the role of oral anti-diabetic agents (sulphonylureas in particular) is still unsettled.

Methods To directly investigate more on such issues, we prepared isolated human islets, which were then cultured for 5 days in continuous normal glucose concentration (NG, 5.5 mmol/L) or normal and high (HG, 16.7 mmol/L) glucose levels (alternating every 24 h), with or without the addition of therapeutical concentration (10 μmolL) of gliclazide or glibenclamide.

Results Intermittent high glucose caused a significant decrease of glucose-stimulated insulin secretion, which was not further affected by either sulphonylurea. Apoptosis, as assessed by electron microscopy, was also significantly increased by alternating high glucose exposure, which was accompanied by altered mitochondria morphology and density volume, and increased concentrations of nitrotyrosine, a marker of oxidative stress. Gliclazide, but not glibenclamide, was able to significantly reduce high glucose induced apoptosis, mitochondrial alterations, and nitrotyrosine concentration increase.

Conclusion Therefore, gliclazide protected human beta-cells from apoptosis induced by intermittent high glucose, and this effect was likely to be due, at least in part, to the anti-oxidant properties of the molecule. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords pancreatic beta-cells; apoptosis; gliclazide; oxidative stress

Introduction

Diabetes mellitus is a chronic disease caused by insulin deficiency, combined with varying degrees of ineffectiveness of the insulin produced [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 including retinopathy, nephropathy, neuropathy, and cardiovascular disease [1–4]. There are two main forms of diabetes, type 1 and type 2: the former is characterized by an absolute insulin insufficiency due to the immunological destruction of pancreatic beta-cells, and accounts for approximately 10% of all cases; the latter is associated with insulin deficiency and reduced insulin

Received: 24 May 2006
Accepted: 21 July 2006

action, and it represents the most common form (around 90% of all cases) [1–4].

Intensive management of diabetes to optimize glycemic control is associated with reduced development and/or severity of degenerative complications in both type 1 and type 2 diabetes [4–6]. Diet, exercise and, when needed, weight loss are at the centre of any therapeutical programme for diabetes. Oral anti-diabetic agents are required in type 2 diabetes when such an approach is unable to attain targets of glycemic control [4,6,7]. In the UKPDS [6], insulin, sulphonylureas (chlorpropamide and glibenclamide) or metformin were used to demonstrate that improved diabetes control was effective in positively affecting the course of microvascular complications, with no difference between the compounds. However, a decline of pancreatic beta-cell function was observed with time, whichever agent was used, which clearly contributed to the progression of diabetes [6,8].

Gliclazide is a second-generation sulphonylurea, widely used in the treatment of type 2 diabetes [9–12]. Similar to the other compounds of the same pharmacological family, the drug reduces blood glucose level by inducing insulin release from pancreatic beta-cells through interaction with the sulphonylurea receptor (SUR1) of the K_{ATP}-channel [9,10,12,13]. The binding of gliclazide to SUR1 receptor is very rapidly reversible in contrast to glibenclamide, which shows prolonged binding to the pancreatic beta-cell. Besides its hypoglycemic effects, gliclazide has anti-oxidant properties, which are independent of any effects on glucose levels [14–16]. Since pancreatic beta-cell damage due to environmental factors such as high glucose (HG) or free fatty acid levels [17,18], as well as the diabetic milieu [19–21] is associated with increased oxidative stress, we decided to investigate whether gliclazide may have direct protective actions on isolated human pancreatic islets exposed to HG. Morphological, functional, and molecular studies were performed, and the results were compared with those obtained with glibenclamide, a sulphonylurea which is also largely employed in the therapy of type 2 diabetic patients [9].

Materials and methods

Pancreatic islet isolation and culture

Pancreata were obtained from 9 non-diabetic, non-obese multiorgan donors (age: 59 ± 17 years, gender: six males and three females, body mass index: 24.4 ± 3.3 kg/m^2). Isolated islets were prepared by collagenase digestion and density gradient purification as previously reported [18,19,21], with the approval of our local Ethics Committee. Cells were maintained free floating in M199 culture medium, at 5.5 mmol/L glucose concentration, for 2–3 days; then, aliquots of approximately 100 islets were incubated for 5 days under the following conditions: (1) control M199 medium containing normal (5.5 mmol/L) glucose (NG); (2) NG or 16.7 mmol/L HG, alternating every 24 h; (3) as (2) with the addition of 10 μmol/L gliclazide; (4) as (2), with the addition of 10 μmol/L glibenclamide. Insulin concentration in the incubation medium was measured every 24 h. The concentrations of sulphonylureas we used were in the therapeutical range [22,23]. Functional and morphological studies were performed at the end of the 5-day incubations.

Insulin secretion studies

Insulin secretion studies were performed as reported elsewhere [18,19,21]. Briefly, following a 45-min pre-incubation period at 3.3 mmol/L glucose, groups of 30 islets of comparable size were kept at 37 °C for 45 min in Krebs-Ringer bicarbonate solution, 0.5% albumin, pH 7.4, containing 3.3 mmol/L glucose. At the end of this period, the medium was completely removed and replaced with Ringer bicarbonate solution containing either 3.3 or 16.7 mmol/L glucose. After additional 45 min incubation, the medium was removed. The samples (500 μL) from the different media were stored at −20 °C until insulin concentrations were measured by IRMA (Pantec Forniture Biomediche, Turin, Italy).

Electron microscopy evaluation

Electron microscopy studies were performed as previously described [18,19,21,24]. Samples were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4 for 1 h at 4 °C. After rinsing in cacodylate buffer, the tissue was post-fixed in 1% cacodylate buffered osmium tetroxide for 2 h at room temperature, then dehydrated in a graded series of ethanol, briefly transferred to propylene oxide and embedded in Epon–Araldite. Ultrathin sections (60–80 nm thick) were cut with a diamond knife, placed on formvar-carbon coated copper grids (200 mesh), and stained with uranyl acetate and lead citrate. The amount of apoptotic cells, mitochondria morphology and density volume were then examined on these preparations.

Determination of nitrotyrosine concentration

Nitrotyrosine concentration was determined by an ELISA method as previously described [19]. White 96-well plates (Iwaki, Japan) were coated with 200 μL of standard curve samples (15–0.166 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-coniugated goat anti-mouse IgG secondary antibody for 45 min at 37 °C. The peroxidase reaction product

DOI: 10.1002/dmrr

was generated using tetramethyl-benzidine microwell peroxidase substrate (Sigma–Aldrich) (150 μL/well). The plates were incubated 5–10 min at room temperature and optical density was read at 492 nm in a microplate reader.

Statistical analysis

The results are expressed as mean ± SD. Data were compared by analysis of variance (ANOVA), followed by the Bonferroni correction.

Results

Insulin release in the incubation medium (average values during the 5-day incubation) differed significantly ($p < 0.01$ by ANOVA) between the experimental groups. The secretion of the hormone (μU/mL) was 155 ± 81 at 5.5 mmol/L glucose (NG) and 289 ± 62 at intermittent high (16.7 mmol/L, HG) glucose ($p < 0.05$ by the Bonferroni test). The presence of gliclazide (338 ± 39, $p < 0.05$ *vs* NG) or glibenclamide (346 ± 54, $p < 0.05$ *vs* NG) caused a further, although non-significant, increase of insulin secretion, with no difference between the two molecules.

At the end of the 5-day incubation with 5.5 mmol/L glucose the islets were acutely challenged. Insulin release at 3.3 mmol/L glucose was 19.7 ± 6.1 μU/mL and increased to 45.3 ± 13.1 μU/mL at 16.7 mmol/L glucose ($p < 0.01$), corresponding to a stimulation index (i.e. the ratio of insulin release at 16.7 mmol/L glucose over insulin release at 3.3 mmol/L glucose) of 2.3 ± 0.8. As shown in Figure 1, culturing the islets at alternating normal and HG determined a significant decrease of stimulation index, which was not further affected by either gliclazide or glibenclamide.

The amount of apoptotic beta-cells was assessed by electron microscopy, based on typical ultrastructure changes, and in particular on nuclear alterations

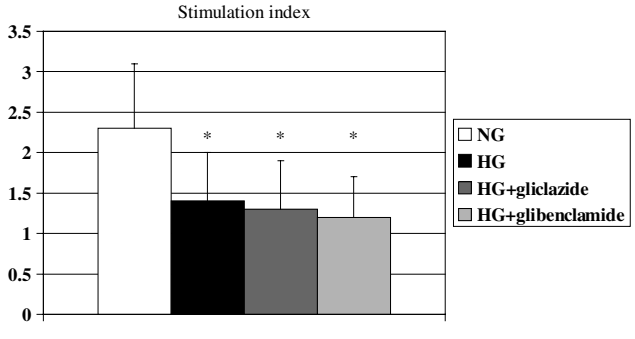

Figure 1. Insulin release (expressed as stimulation index, i.e. the ratio of insulin release at 16.7 mmol/L glucose over insulin release at 3.3 mmol/L glucose) from islets pre-exposed for 5 days to 5.5 mmol/L glucose (NG), alternating 5.5 and 16.7 mmol/L glucose (HG), HG with gliclazide or HG with glibenclamide. Groups differed significantly ($p < 0.01$) by ANOVA; *$p < 0.05$ *vs* NG after the Bonferroni correction

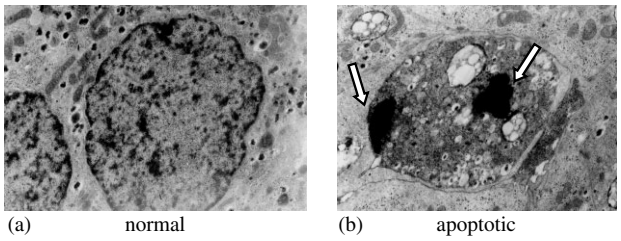

Figure 2. Normal beta-cell (a) and apoptotic beta-cell (b); the latter shows marked chromatin condensation (arrows) in the nucleus

(Figure 2). Count showed that apoptotic beta-cells (Figure 3) were $0.8 \pm 0.2\%$ in NG maintained islets and $10.6 \pm 4.8\%$ following HG incubation ($p < 0.05$ by the Bonferroni test); the combination of HG and glibenclamide ($18.3 \pm 8.2\%$) tended to increase the amount of apoptosis, as compared with HG alone, and the presence of gliclazide determined a significant reduction of apoptosis ($5.3 \pm 3.4\%$, NS *vs* NG, $p < 0.05$ *vs* HG and HG + glibenclamide).

Beta-cell mitochondria morphology and density volume were also assessed by electron microscopy. Mitochondria of beta-cells cultured in normal glucose (NG) had an oval shape, with well defined membranes and cristae (Figure 4); however, after culture in alternating normal and high glucose, these organelles appeared rounded

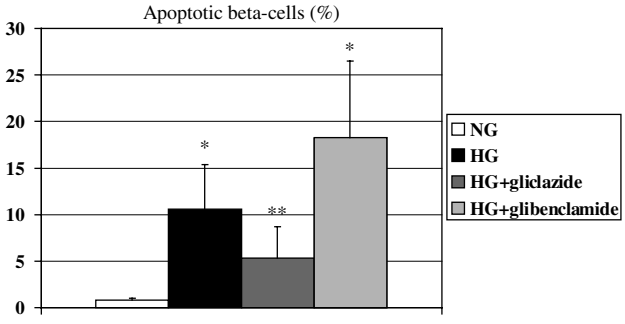

Figure 3. Beta-cell apoptosis (as assessed by electron microscopy) in islets pre-exposed for 5 days to 5.5 mmol/L glucose (NG), alternating 5.5 and 16.7 mmol/L glucose (HG), HG with gliclazide or HG with glibenclamide. Groups differed significantly ($p < 0.01$) by ANOVA; *$p < 0.05$ *vs* NG; and **$p < 0.05$ *vs* HG and HG + glibenclamide after the Bonferroni correction

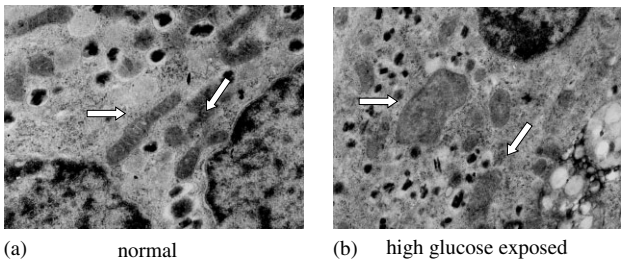

Figure 4. Normal beta-cell (a) and intermittent high glucose exposed beta-cell (b), showing mitochondria (arrows) with rounded shape and ill-defined cristae

DOI: 10.1002/dmrr

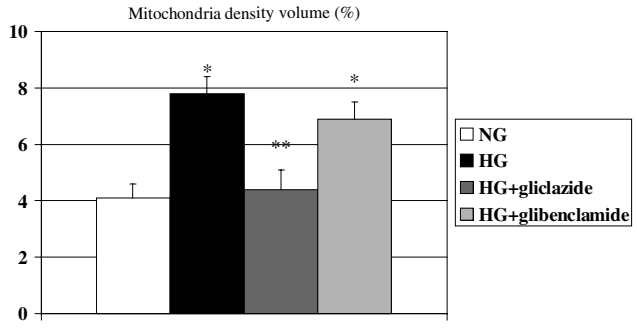

Figure 5. Mitochondria density volume in islets pre-exposed for 5 days to 5.5 mmol/L glucose (NG), alternating 5.5 and 16.7 mmol/L glucose (HG), HG with gliclazide or HG with glibenclamide. Groups differed significantly ($p < 0.01$) by ANOVA; *$p < 0.05$ *vs* NG; and **$p < 0.05$ *vs* HG and HG + glibenclamide after the Bonferroni correction

(leading to increased density volume) and with ill-defined cristae (Figure 4). The presence of gliclazide, but not of glibenclamide, prevented these changes (Figure 5).

Finally, the concentrations of nitrotyrosine, a marker of oxidative stress, were measured, showing a beneficial effect of gliclazide. In fact, nitrotyrosine levels (nmol/L) were 5.6 ± 2.3 at NG, 14.5 ± 7.7 at HG ($p < 0.05$ *vs* NG), 6.1 ± 3.4 at HG plus gliclazide ($p < 0.05$ *vs* HG) and 8.8 ± 4.8 at HG plus glibenclamide.

Discussion

The results of the present study confirm that prolonged exposure of isolated human islets to increased glucose concentrations causes functional and survival defects of beta-cells, which are probably due, at least in part, to increased oxidative stress [17,25–28]. In fact, we found increased production of nitrotyrosine, which derives from the reaction of superoxide and nitric oxide, and is considered a reliable marker of oxidative stress [29]. In addition, following high glucose incubation mitochondrial alterations occurred, which again are associated with unbalanced redox reactions [17,25,30]. Notably these changes were demonstrated by experiments in which high glucose was present intermittently, to better mimic the situation of clinical diabetes [29], and by using electron microscopy, which remains the gold standard to assess cell survival. Altogether, these findings further support the concept that in patients with type 2 diabetes, elevated blood glucose levels can contribute to the progressive loss of beta-cell functional mass, leading to relentless deterioration of diabetes control with years [6,8]. It was recently proposed that sulphonylureas can rapidly induce beta-cell apoptosis in human islets *in vitro*, an effect that with glibenclamide was observed following exposure to both 5.5 and 11.1 mmol/L glucose [31]. Although other studies showed no major pro-apoptotic effect of sulphonylureas on human islet cells kept in 5.5 mmol/L glucose for 72 h [32], the fact that sulphonylureas could precipitate the decrease in beta-cell mass in type 2 diabetes patients deserves careful examination. In this regard, the most important finding of the present study is that gliclazide, but not glibenclamide, is able to partly protect beta-cells from high glucose induced apoptosis. This result was obtained despite the fact that insulin release in the incubation medium was similar with the two sulphonylurea compound. Therefore, gliclazide might have a direct protective effect on islet cells. The molecule possesses anti-oxidant properties, as shown by inhibition of LDL oxidation, reduction of platelet activity, and decrease of free radical production [14–16]. It was also recently shown that beta-cell death induced by hydrogen peroxide was partially suppressed by the addition of gliclazide, but not of glibenclamide, using MIN6 cells [33]. Accordingly, in our study, gliclazide incubation was associated with reduced nitrotyrosine formation and improved mitochondria morphology. These findings are in line with data from several groups, showing that conditions responsible to improve the redox balance, such as glutathione, metformin, ACE-inhibitors and others exert protective action on beta-cells exposed to high glucose or high free fatty acids, and can even reverse, at least in part, the functional and molecular alterations of islet cells in human type 2 diabetes [17,19,21,34,35]. Notably, however, the present results are the first to show that a sulphonylurea compound can have such effects on human beta-cells.

The possible clinical relevance of our findings remains to be fully assessed, particularly concerning the evaluation of the drug impact on possible prevention of progressive beta-cell damage. Gliclazide shows specific insulin secretion features, which might be explained by a rapidly reversible binding to pancreatic K_{ATP}-channels in contrast to glibenclamide [13]. Furthermore, the comparison of their insulin secretory effects reveals that gliclazide restores a physiological biphasic profile of insulin secretion, which is lost during development of type 2 diabetes, as opposed to the late monophasic response to glibenclamide [36].

All this might contribute to the long-term maintenance of glycemic control with gliclazide, as demonstrated during a prospective 5-year study comparing the rates of secondary failure with various sulphonylureas [37]. In addition, a retrospective study evaluating difference in secondary failure showed that the period until the start of insulin treatment is significantly longer in patients receiving gliclazide than in those receiving glibenclamide [38]. Finally, in a recent 52-week study, gliclazide caused a more marked and sustained beta-cell activity, as assessed by the surrogate marker HOMA-%B, compared to other oral anti-diabetic agents [39], but this does not necessarily imply improved beta-cell health or pathology.

Together, the available evidence suggests that gliclazide might play a role in slowing beta-cell functional mass decline in type 2 diabetic patients. Prospective studies are highly recommended to investigate this issue.

DOI: 10.1002/dmrr

References

1. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2006; **29**: S43–S48.
2. Ferranini E, Mari A. Beta cell function and its relation to insulin action in humans: a critical appraisal. *Diabetologia* 2004; **47**: 943–956.
3. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. *Nature* 2001; **414**: 782–787.
4. ADA. Standards of medical care in diabetes-2006. *Diabetes Care* 2006; **20**: S4–S42.
5. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993; **329**: 977–986.
6. UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 1998; **352**: 837–853.
7. Silvio E, Inzucchi MD. Oral antihyperglycemic therapy for type 2 diabetes. *JAMA* 2002; **287**: 360–372.
8. UK Prospective Diabetes Study Group. UK prospective diabetes study 16. Overview of 6 years' therapy of type 2 diabetes: a progressive disease. *Diabetes* 1995; **44**: 1249–1258.
9. Krentz AJ, Bailey CJ. Oral antidiabetic agents: current role in type 2 diabetes mellitus. *Drugs* 2005; **65**: 385–411.
10. Kecskemeti V, Bagi Z, Pacher P, Posa I, Kocsis E, Koltai M. New trends in the development of oral antidiabetic drugs. *Curr Med Chem* 2002; **9**: 53–71.
11. Frey N, Laveille C, Paraire M, Francillard M, Holford NH, Jochemsen R. Population PKPD modelling of the long-term hypoglycaemic effect of gliclazide given as a once-a-day modified release (MR) formulation. *Br J Clin Pharmacol* 2003; **55**: 147–157.
12. Mac Gavin JK, Perry CM, Goa KL. Gliclazide modified release. *Drugs* 2002; **62**: 1357–1364.
13. Gribble FM, Reimann F. Sulphonylurea action revisited: the post-cloning era. *Diabetologia* 2003; **46**: 875–891.
14. O'Brien RC, Luo M, Balazs N, Mercuri J. In vitro and in vivo antioxidant properties of gliclazide. *J Diabetes Complications* 2000; **14**: 201–206.
15. Jennings PE, Belch JJ. Free radical scavenging activity of sulfonylureas: a clinical assessment of the effect of gliclazide. *Metabolism* 2000; **49**: 23–26.
16. Fava D, Cassone-Faldetta M, Laurenti O, De Luca O, Ghiselli A, De Mattia G. Gliclazide improves anti-oxidant status and nitric oxide-mediated vasodilation in type 2 diabetes. *Diabet Med* 2002; **19**: 752–7570.
17. Robertson RP. Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cell in diabetes. *J Biol Chem* 2004; **279**: 42351–42354.
18. Lupi R, Dotta F, Marselli L, *et al*. Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* 2002; **51**: 1437–1442.
19. Del Guerra S, Lupi R, Marselli L, *et al*. Functional and molecular defects of pancreatic islets in human type 2 diabetes. *Diabetes* 2005; **54**: 727–735.
20. Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S. Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islets of Japanese Type II diabetic patients. *Diabetologia* 2002; **45**: 85–96.
21. Marchetti P, Del Guerra S, Marselli L, *et al*. Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. *J Clin Endocrinol Metab* 2004; **89**: 5535–5541.
22. Marchetti P, Navalesi R. Pharmacokinetic-pharmacodynamic relationship of oral hypoglycemic agents. An update. *Clin Pharmacokinet* 1998; **16**: 100–128.
23. Park JY, Kim KA, Park PW, Park CW, Shin JG. Effect of rifampin on the pharmacokinetics and pharmacodynamics of gliclazide. *Clin Pharmacol Ther* 2003; **74**: 334–340.
24. Marselli L, Dotta F, Piro S, *et al*. Th2 cytokines have a partial, direct protective effect on the function and survival of isolated human islets exposed to combined proinflammatory and Th1cytokines. *J Clin Endocrinol Metab* 2001; **86**: 4974–4978.
25. Brownlee M. A radical explanation for glucose-induced beta-cell dysfunction. *J Clin Invest* 2003; **112**: 1831–1842.
26. Davalli AM, Maffi P, Socci C, *et al*. Insights from a successful case of intrahepatic islet transplantation into a type 1 diabetic patient. *J Clin Endocrinol Metab* 2000; **85**: 3847–3852.
27. Lupi R, Del Guerra S, Tellini C, *et al*. The biguanide compound metformin prevents desensitization of human pancreatic islets induced by high glucose. *Eur J Pharmacol* 1999; **364**: 205–209.
28. Sesti G, Cardellini M, Marini MA, *et al*. A common polymorphism in the promoter of UCP2 contributes to the variation in insulin secretion in glucose-tolerant subjects. *Diabetes* 2003; **52**: 1280–1283.
29. Quagliaro L, Piconi L, Assaloni R, Martinelli L, Motz E, Ceriello A. Intermittent high glucose enhances apoptosis related to oxidative stress inhuman umbilical vein endothelial cells: the role of protein kinase C andNAD(P)H-oxidase activation. *Diabetes* 2003; **52**: 2795–2804.
30. Anello M, Lupi R, Spampinato D, *et al*. Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. *Diabetologia* 2005; **48**: 282–289.
31. Maedler K, Carr R, Bosco D, Zuellig R, Berney T, Donath M. Sulfonylureas induced β-cell apoptosis in cultured human Islets. *He J Clin Metabolism* 2005; **90**: 501–506.
32. Del Guerra S, Marselli L, Lupi R, *et al*. Effects of prolonged in vitro exposure to sulphonylureas on the function and survival of human islets. *J Diabetes Complications* 2005; **19**: 60–64.
33. Kimoto K, Kizaki T, Suzuki K, *et al*. Gliclazide protects pancreatic beta-cells from damage by hydrogen peroxide. *Biochem Biophys Res Commun* 2003; **303**: 112–119.
34. Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H. Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. *Diabetes* 2003; **52**: 581–587.
35. Lortz S, Tiedge M, Nachtwey T, Karlsen AE, Nerup J, Lenzen S. Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes. *Diabetes* 2000; **49**: 1123–1130.
36. Gregorio F, Ambrosi F, Cristallini S, Pedetti M, Filipponi P, Santeusanio F. Therapeutical concentrations of tolbutamide, glibenclamide, gliclazide and gliquidone at different glucose levels: in vitro effects on pancreatic A and B cell function. *Diabetes Res Clin Pract* 1992; **18**: 197–206.
37. Harrower ADB, Wong C. Comparison of secondary failure rate between three second-generation sulfonylureas. *Diabetes Res* 1990; **13**: 19–21.
38. Satoh J, Takahashi K, Takizawa Y. Secondary sulfonylurea failure: Comparison of period until insulin treatment between diabetic patients treated with gliclazide and glibenclamide. *Diabetes Res Clin Pract* 2005; **70**: 291–297.
39. Charbonnel B, Roden M, Urquhart R, *et al*. Pioglitazone elicits long-term improvements in insulin sensitivity in patients with type 2 diabetes: comparisons with gliclazide-based regiments. *Diabetologia* 2005; **48**: 553–560.

DOI: 10.1002/dmrr