CLINICAL STUDY

Insulinotropic actions of nateglinide in type 2 diabetic patients and effects on dipeptidyl peptidase-IV activity and glucose-dependent insulinotropic polypeptide degradation

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Abstract

Background: Nateglinide restores early-phase insulin secretion to feeding and reduces postprandial hyperglycaemia in type 2 diabetes. This study evaluated the effects of nateglinide on dipeptidyl peptidase-IV (DPP-IV) activity and glucose-dependent insulinotropic polypeptide (GIP) degradation.

Research design and methods: Blood samples were collected from type 2 diabetic subjects (n=10, fasting glucose 9.36±1.2 mmol/l) following administration of oral nateglinide (120 mg) 10 min prior to a 75 g oral glucose load in a randomised crossover design.

Results: Plasma glucose reached 18.2±1.7 and 16.7±1.7 mmol/l at 90 min in control and placebo groups (P<0.001). These effects were accompanied by prompt 32% inhibition of DPP-IV activity after 10 min (19.9±1.6 nmol/ml per min, P<0.05), reaching a minimum of 1.9±0.1 nmol/ml per min at 120 min (P<0.001) after nateglinide. Insulin and C-peptide levels increased significantly compared with placebo, to peak after 90 min at 637.6±163.9 pmol/l (P<0.05) and 11.8±1.4 mg/l (P<0.01) respectively. DPP-IV-mediated degradation of GIP was significantly less in patients receiving nateglinide compared with placebo. Inhibition of DPP-IV activity corresponded with a time- and concentration-dependent inhibitory effect of nateglinide on DPP-IV-mediated truncation of GIP(1–42) in vitro. Comparison of in vitro inhibition of DPP-IV by nateglinide and vildagliptin revealed IC₅₀ values of 17.1 and 2.1 μM respectively.

Conclusions: Although considerably less potent than specified DPP-IV inhibitors, the possibility that some of the beneficial actions of nateglinide are indirectly mediated through DPP-IV inhibition and increased bioavailability of GIP and other incretins merits consideration.

European Journal of Endocrinology 161 877–885

Introduction

Development of type 2 diabetes and the transition from normal glucose tolerance to impaired glucose tolerance are characterised by a gradual impairment of the acute insulin response. Treatment of diabetes with nateglinide, a D-phenylalanine derivative, results in a rapid, transient secretion of insulin from the pancreatic β-cells, restoring postprandial early-phase insulin secretion in a glucose sensitive manner, without affecting basal insulin levels (1–4).

To date, many studies have confirmed that nateglinide is a highly effective insulin secretagogue with anti-hyperglycaemic properties when given immediately prior to a meal or an oral glucose load. In brief, nateglinide monotherapy has been shown to significantly reduce HbA1c and prevent mealtime glucose spikes (2, 5).

Combination therapy with insulin-sensitising agents, such as metformin or thiazolidinediones, ameliorates insulin secretion and insulin resistance, and reduces postprandial hyperglycaemia in pre-diabetic subjects with impaired glucose tolerance (1, 6). Pharmacologically, nateglinide acts on pancreatic β-cells via the closure of the Kₐ₅₆ channels by binding to sulphonylurea receptor subunits resulting in inhibition of ATP sensitive K⁺ channels causing cell depolarisation, Ca²⁺ influx and insulin release (7, 8). Notably, nateglinide inhibits Kₐ₅₆ channels more rapidly and with a shorter duration of action than sulphonylurea or other meglitinide oral insulin-releasing drugs (7, 8). It has been suggested that this accounts for the rapid action of nateglinide in restoring early-phase insulin secretion in response to a meal, important for postprandial nutrient regulation. The augmentation
of insulin release is rapid and short-lived as nateglinide is quickly eliminated from plasma (half-life ~ 1.5 h) by cytochrome P450 enzymes and hepatic metabolism (9).

There is continuous pressure to improve existing treatment methods and develop new therapies for diabetes. One novel diabetic therapeutic target is the inhibition of the ubiquitous enzyme, dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5). DPP-IV rapidly metabolises the incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotrophic polypeptide (GIP) resulting in their reduced bioavailability and rapid inactivation in the circulation (10). GLP-1 and GIP are incretin hormones, released in response to nutrient stimulation and act to augment nutrient-induced insulin release in a glucose-dependent manner (11). This combined with other beneficial effects contribute to the antidiabetic actions of the incretins (12–14). DPP-IV, a highly specialised endopeptidase, widely distributed in mammalian tissues (15), inactivates GIP and GLP-1 by removing the dipeptides, Tyr1–Ala2 and His7–Ala8 from the N-terminus of GIP and GLP-1 respectively (10). Owing to the rapid enzymatic degradation of these two key incretin hormones, much attention has been focused on the development of DPP-IV inhibitors to ameliorate insulin release and metabolic control (16–20). Recent studies in type 2 diabetes have revealed that DPP-IV activity is reduced and GLP-1(7-36) amide levels elevated in metformin-treated type 2 diabetic subjects (21–26). Other studies have suggested that DPP-IV activity is unchanged (27) or increased in diabetes (28). Few studies have assessed clinically the potential effects of other antidiabetic drugs on DPP-IV and incretin action, particularly GIP. In recent animal studies, we have shown that nateglinide administration enhanced active GLP-1 concentrations and lowered levels of DPP-IV activity, thereby improving glucose-lowering and the insulin-releasing activity of GLP-1 (29). In this study, we have explored the clinical effects of nateglinide, a rapid acting oral prandial insulin secretagogue, on DPP-IV activity and GIP degradation in type 2 diabetic patients.

Subjects and methods

Reagents

GIP was purchased from the American Peptide Company Inc (Sunnyvale, CA, USA). RPMI-1640, penicillin, streptomycin, foetal bovine serum, Hanks’ balanced saline solution, trypsin/EDTA were purchased from Gibco Life Technologies Ltd. Gly-Pro-MCA, 7-amino-4-methylcoumarin-(AMC), N,N-dimethyl-formamide and purified porcine kidney (DPP-IV) (EC 3.4.14.5) were supplied by Sigma. All water used was purified using a Milli-Q Water Purification System (Millipore Corporation, Milford, MA, USA). All other chemicals were of analytical grade.

Effects of nateglinide on DPP-IV-mediated degradation of GIP

GIP (3 μmol/l) was incubated with pooled plasma samples (150 min) from the placebo and nateglinide groups. Samples were incubated at 37 °C for 1, 2, 4 and 8 h. The reaction mixture was made up to a total volume of 500 μl using 50 mmol/l triethanolamine–HCl buffer, pH 7.8. TEA–H2O (10% v/v) was added to terminate the reaction after which samples were frozen (−20 °C) until reversed phase (RP)-HPLC analysis. In a separate series of experiments, GIP was similarly incubated for 0, 2, 4 and 8 h in normal pooled human plasma in the absence and presence of a range of nateglinide concentrations (6.25 μmol/l–1 mmol/l). Concentration-dependent effects of nateglinide on GIP degradation were studied using 5 μl purified porcine

Table 1 Characteristics of type 2 diabetic subjects (data are expressed as mean ± S.E.M.)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo group (n=10)</th>
<th>Nateglinide group (n=10)</th>
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<tbody>
<tr>
<td>F/M</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>9.6 ± 1.4</td>
<td>9.1 ± 1.0</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.3 ± 0.6</td>
<td>7.9 ± 0.9</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>99.8 ± 17.7</td>
<td>108.9 ± 19.8</td>
</tr>
<tr>
<td>C-peptide (μg/l)</td>
<td>3.3 ± 0.4</td>
<td>3.5 ± 0.5</td>
</tr>
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kidney DPP-IV. Peptide samples were separated using a Vydac C-18 (4.6×250 mm) column (the Separations Group, Hesperia, CA, USA) equilibrated with 0.12% (v/v) TFA–H₂O at a flow rate of 1.0 ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile–H₂O, the concentration of acetonitrile was raised from 0 to 45% over 15 min, 45 to 55% over 30 min and 55 to 100% over 5 min, using linear gradients. The absorbance was monitored at 206 nm. Percentage intact GIP(1–42) was calculated using peak areas. Peptide degradation products were confirmed using electrospray ionisation mass spectrometry (ESI–MS) (Finnigan MAT, Hemel Hempstead, UK). HPLC fractions were applied at a flow rate of 10 µl/min by syringe injection to the ESI source. Spectra were obtained at m/z 300–2000. Molecular masses were determined from ESI–MS profiles using prominent multiple-charged ions and determined using the equation \( M_r = iM_i - \alpha M_h \) (where \( M_r \) = molecular mass; \( M_i = m/z \) ratio; \( i = \) number of charges; \( M_h = \) mass of a proton).

**Biochemical analyses**

DPP-IV activity was determined fluorometrically by modification of the method by Fujiwara & Tsuru (1978) (30), by measuring free AMC liberated from the DPP-IV substrate, Gly-Pro-AMC. Plasma (20 µl), 50 mmol/l HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulphonic acid] pH 7.4) and 200 µmol/l Gly-Pro-AMC (Gly-Pro-amino methylcoumarin) were incubated at 37 °C for 60 min, followed by the addition of 1 ml of 3.0 mol/l acetic acid (pH 2.6) to terminate DPP-IV enzyme activity. Free AMC was measured at excitation wavelength of 370 nm and emission wavelength of 440 nm. Standards of 1.5–15 nmol/l AMC were used to construct a fluorescence-concentration curve. One unit of DPP-IV activity was defined as the enzyme activity that produced 1 nmol AMC of plasma in 1 min. Intra- and interassay coefficient of variations (CV) were 2.1 and 6.9% respectively.

HbA1c was measured in whole blood by ion exchange HPLC using the Menari HA-8140 kit (BIOMEN Ltd, Berkshire, UK). Glucose concentrations were measured in plasma using the glucose oxidase method (31). Serum creatinine was determined using the Johnston and Johnston Vitros 950 analyzer (Orthoclinical Diagnostics, Buckinghamshire, UK) using a multilayered dry slide aminohydrolase technique (32). Insulin was determined using the Abbott IMX insulin microparticulate enzyme immunoassay (Abbott Laboratories Ltd), which has a sensitivity of 6 pmol/l and an intra-assay CV of 4%. Cross-reactivity with proinsulin was <0.005% with no detectable reaction with C-peptide. C-peptide was measured using a commercial kit (Dako Diagnostics Ltd, Ely, Cambridge-shire, UK).

**Figure 1** Effects of nateglinide on circulating DPP-IV activity (A) and circulating concentrations of glucose (B), insulin (C) and C-peptide (D) in type 2 diabetic subjects. Nateglinide (120 mg) or placebo tablet was taken 10 min before an oral (75 g) glucose load. Values are mean ± S.E.M. of five observations. *P<0.05, **P<0.01 and ***P<0.001, compared with placebo at the same time point.
Inhibitory potency of DPP-IV catalysed degradation of GIP using nateglinide and vildagliptin and IC_{50} determination

A comparative experiment using vildagliptin was carried out in vitro to compare its relative inhibitory potency with nateglinide. DPP-IV activity was determined following in vitro incubation with the antidiabetic drugs, vildagliptin (0–10 μM) and nateglinide (0–500 μM). As above, GIP was incubated for 60 min at 37 °C with 25 μl of 50 mmol/l HEPES buffer (pH 7.4) containing Gly-Pro-AMC substrate. Vildagliptin/nateglinide was incorporated into the added HEPES buffer at the concentrations stated above. The reaction was stopped by addition of 70 μl of 3 mmol/l acetic acid. Free AMC was measured at excitation wavelength of 370 nm and emission wavelength of 440 nm. Blank incubations without enzyme were performed, and it was checked that the concentration-dependent effects of drugs were not due to simple interference or changes in pH in incubation buffer. The IC_{50} is defined as the concentration of inhibitor required to obtain 50% reduction in the activity under specific conditions.

Statistical analysis

Data are expressed as mean ± S.E.M. Significant differences between groups of data were assessed using the unpaired Student’s t-test, and statistical significance was assumed if P < 0.05. Area under the curve (AUC) analysis employed the trapezoidal rule (33).

Results

Metabolic effects of nateglinide in type 2 diabetic subjects

Study participants had a mean age of 57.1 ± 1.9 years, body mass index of 34.7 ± 2.6 kg/m² and diabetic duration of ~2.8 ± 1.3 years. Circulating glucose, insulin, C-peptide and DPP-IV activity were measured following a 75 g oral glucose load given 10 min after nateglinide (120 mg) or placebo (Fig. 1). Fasting glucose, insulin and C-peptide were similar for both placebo and nateglinide study mornings (data not shown).

DPP-IV activity was 27.4 ± 0.75 and 29.6 ± 2.3 nmol/ml per min in the placebo and nateglinide groups respectively, prior to any intervention (NS, P > 0.05). As shown in Fig. 1, DPP-IV activity was significantly reduced (19.9 ± 1.6 nmol/ml per min, P < 0.05) 10 min following nateglinide and reached a minimum level of 1.9 ± 0.1 nmol/ml per min at 120 min (P < 0.001). DPP-IV activity following placebo did not change, remaining at 22.5 ± 0.6 nmol/ml per min. Plasma DPP-IV activity was inhibited (twofold) in those patients receiving nateglinide as indicated by AUC values of 1362.0 ± 153.4 and 814.2 ± 108.1 for placebo and nateglinide respectively (P < 0.001). Similar inhibition of DPP-IV was observed when those patients taking metformin (n = 3) a week prior to the study were excluded from the analysis (data not shown).

Following the oral glucose load, glucose concentrations increased, reaching a maximum of 18.7 ± 1.7 and 18.2 ± 1.7 mmol/l at 90 min in the nateglinide
and placebo groups respectively ($P<0.01$; Fig. 1). Plasma glucose concentrations were lower following nateglinide from 30 to 120 min. Administration of nateglinide resulted in a rapid increase in insulin, which reached a peak level at 90 min of 637.6 ± 163.9 pmol/l ($P<0.05$, Fig. 1) and 414.3 ± 100.9 pmol/l after placebo. Overall, insulin concentrations were significantly increased at 5–120 min compared with placebo ($P<0.05$). Similarly, C-peptide levels increased to a maximum of 11.8 ± 1.35 mg/l after nateglinide levels being significantly enhanced compared with placebo at 40–90 min (Fig. 1).

**Time and concentration-dependent effects of nateglinide on DPP-IV-mediated degradation of GIP by human plasma in vitro**

Representative HPLC profiles of the time-dependent degradation of GIP in normal pooled plasma in the absence and presence of nateglinide are shown in Fig. 2. The degradation of GIP(1–42) to GIP(3–42) over the 8-h period was clearly inhibited in the presence of nateglinide (Fig. 2C). The major HPLC peptide peaks were confirmed as GIP(1–42) and GIP(3–42) using ESI–MS and spectral averaging (Fig. 3). Prominent multiple charged species ($M+3H)^{3+}$ and ($M+4H)^{4+}$ were detected, corresponding to intact $Mr$ 4981.8 and 4983.2 kDa, representing the peptide GIP(1–42). Similarly, ($M+3H)^{3+}$ and ($M+4H)^{4+}$ of 4748.7 and 4748.4 kDa, corresponded to the theoretical mass of GIP(3–42) (Fig. 3).

The effects of nateglinide (62 μmol/l–1 mmol/l) on the degradation of GIP after incubation with purified DPP-IV at 0, 2, 4 and 8 h are summarised in Fig. 4. Nateglinide significantly inhibited the degradation of GIP(1–42) to GIP(3–42) in both a time- and concentration-dependent manner. Even the lowest concentration of nateglinide tested (62.5 μmol/l) inhibited degradation by 48–79%.

**Inhibitory effect of vildagliptin on DPP-IV-mediated degradation of GIP**

As shown in Fig. 5, the inhibitory effects of vildagliptin on DPP-IV activity were notable, giving an $IC_{50}$ of 2.1 μM, as observed in previous studies (34). Nateglinide inhibition of DPP-IV activity in vitro resulted in an $IC_{50}$ of 17.1 μM, demonstrating less of an inhibitory effect than vildagliptin.

**DPP-IV-mediated peptide degradation in plasma from type 2 diabetic subjects given nateglinide**

In vitro investigations of DPP-IV-mediated peptide degradation of diabetic subjects taken at 150 min following oral administration of nateglinide or placebo were carried out using RP–HPLC (Fig. 6). Degradation of GIP(1–42) to the truncated fragment GIP(3–42) was decreased in plasma taken from type 2 diabetic subjects treated with nateglinide (120 mg), compared with placebo control ($P<0.01$). Percentage intact GIP(1–42) remaining in plasma after nateglinide was 93 ± 2.2%, 83 ± 0.0% ($P<0.05$) and 64 ± 1.0% ($P<0.01$), and was higher in 2, 4 and 8 h incubations compared with placebo (91 ± 0.4%, 72 ± 1.4% ($P<0.05$) and 38 ± 0.7% ($P<0.01$)) respectively (Fig. 6).

![Figure 3 ESI–MS confirmation of (A) GIP(1–42) and (B) GIP(3–42) in HPLC separated peaks. Samples were derived from HPLC peaks shown in Fig. 2. Molecular masses were determined using prominent multiple charged ions and the equation: $M_r = iM_z - IM_z$, where $M_z$ is molecular mass, $M$ is $m/z$ ratio, $i$ is number of charges and $M_z$ is mass of a proton.](www.eje-online.org)
The beneficial effects of nateglinide on β-cell function and blood glucose control are clearly documented in type 2 diabetic patients (4, 6). The key actions of nateglinide concern improvement of β-cell function and restoration of first-phase insulin release with reduced postprandial hyperglycaemia. The drug also seems to preserve β-cell function over time and has been linked to alleviation of insulin resistance, possibly by decreasing glucotoxicity (4, 6, 35). In this study, the insulin-releasing and glucose-lowering effects of nateglinide in type 2 diabetic subjects were accompanied by a rapid inhibition of DPP-IV activity.

Several studies have investigated the activity of DPP-IV in healthy subjects and in diabetes (21, 25–28). Although it remains unclear whether enzyme activity is increased, unchanged or decreased, promising clinical data emerging from the use of DPP-IV inhibitors confirm DPP-IV as a therapeutically useful target in type 2 diabetes (14, 16–20). Indeed, Januvia (Sitagliptin) was launched in October 2006 as the first DPP-IV inhibitor for use in the clinical treatment of type 2 diabetes (18, 19, 36).

The idea that some of the established antidiabetic drugs may have effects on DPP-IV activity as an unsuspected component of their spectrum of actions was seeded by observations with the biguanide metformin. Thus, although hotly disputed and so far unexplained (37), metformin has been shown by several laboratories to inhibit DPP-IV activity in type 2 diabetic subjects (24, 38). Further, clinical and animal studies have demonstrated that metformin increased circulating concentrations of active GLP-1 (22, 23, 39). Interestingly, pioglitazone (a thiazolidinedione) has also been reported to lower DPP-IV activity (24). This has recently been confirmed (29), but the significance of this and the possible effects of other antidiabetic drugs at relevant therapeutic concentrations needs more attention (40).

Evidence for the possible significance of the presently observed inhibitory effect of nateglinide on DPP-IV activity and the enhancement of incretin action in humans comes from various sources. Thus, nateglinide has been found to be most effective in reducing postprandial hyperglycaemia when administered immediately before meals (4). This appears to correspond with the window of GIP secretion from the upper small intestine (41, 42), and the very rapid and pronounced inhibition of DPP-IV activity. Furthermore, recent animal studies have shown that nateglinide significantly enhanced the insulin-releasing and glucose-lowering actions of GLP-1, being associated with DPP-IV inhibition, diminished GLP-1 degradation and increased circulating concentrations of the active form of GLP-1 (29). Also notable is the observation that the β-cell sensitivity to GIP is increased by treatments such as DPP-IV inhibition, which improve glycaemic control (43).

Growing evidence suggests that the extrapancreatic mechanisms of DPP-IV inhibition may have a role to play in the improvement of glycaemic control in patients with type 2 diabetes mellitus. As the GIP receptor is...
GIP and GLP-1 may have a number of other extrapancreatic functions (44, 45). This has prompted renewed awareness of GIP-mediated effects including its role in bone deposition, cognitive function and lipid metabolism (45, 46). Further studies are also required to ascertain the possible biological activity of GIP and GLP-1 degradation products. For example, the metabolite, GLP-1(9–36) amide, has been shown to have important extrapancreatic effects particularly with regard to the cardiovascular system and insulino-mimetic effects (47–49).

Unfortunately, we could not measure circulating concentrations of the active form of the major incretin GIP at the time of this study due to lack of availability of appropriate antibody or commercial assay. However, we were able to clearly demonstrate that plasma taken from diabetic subjects administered nateglinide was significantly less able to degrade GIP(1–42) to GIP(3–42) in vitro than plasma from placebo-treated controls. Additionally, in vitro studies demonstrated that nateglinide caused a concentration-dependent inhibition of DPP-IV activity in a plasma pool from normal human subjects (50). A 35% inhibition was observed at 25 μmol/l, which is within the therapeutic range, and estimated at 10–27 μmol/l (51). Further studies using pooled plasma or purified DPP-IV to exclude possible involvement of other circulating peptidases, indicated that nateglinide caused a concentration- and time-dependent decrease in conversion of GIP to the truncated metabolite GIP(3–42) as confirmed by ESI–MS. Notably, previous studies have shown that even partial inhibition of DPP-IV can reduce peptide degradation and potentiate the insulinotropic effect of incretins (52).

The inhibitory potency of nateglinide on DPP-IV-catalysed degradation of GIP was investigated in a comparative study with vildagliptin, which is a well-known potent and selective DPP-IV inhibitor that possesses anti-hyperglycaemic activity (53). The extent of DPP-IV inhibition exhibited by nateglinide was in the 20 μM range as determined by IC50 values and was less than the considerable inhibitory power of vildagliptin. IC50 values were used merely to compare the relative inhibitory potency of vildagliptin and nateglinide, and no assumptions are being made concerning the mechanism of inhibition.

In conclusion, this study has revealed that nateglinide exerts prompt inhibitory effects on DPP-IV activity following administration to type 2 diabetic patients. We propose that this is linked to enhanced bioavailability of secreted incretin hormones, specifically GIP. Incretin hormones have many therapeutically useful anti-diabetic effects as also exploited by clinical emergence of DPP-IV inhibitors (16–20). The principal antidiabetic action of nateglinide is undoubtedly mediated through inhibition of β-cell K-ATP channel activity and consequent stimulation of insulin secretion. However, the possibility that some part of the actions of nateglinide are indirectly mediated through increased bioavailability of GIP and GLP-1 merits consideration.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

These studies were supported by the Research and Development Office of the Department for Health and Personal Social Services for Northern Ireland and University of Ulster Research Strategy Funding.

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Received 12 August 2009
Accepted 15 September 2009