Insulinotropic actions of nateglinide in type 2 diabetic patients and effects on dipeptidyl peptidase-IV activity and GIP degradation

Aine M. McKillop, Nicola A. Duffy, John R. Lindsay, Brian D. Green, Patterson S, Finbarr P.M. O’Harte, Patrick M. Bell, Peter R. Flatt.

1 School of Biomedical Sciences, University of Ulster, Coleraine, N. Ireland, UK.
2 Endocrinology and Diabetes, Altnagelvin Hospital, Londonderry, N. Ireland, UK.
3 School of Biological Sciences, Queens University Belfast, N. Ireland, UK
4 Regional Centre for Endocrinology and Diabetes, Royal Victoria Hospital, Belfast, N. Ireland, UK.

Running title: Nateglinide, DPP-IV and GIP

Keywords: diabetes, nateglinide, incretin

Word Count: 5067

Corresponding author:
Dr Aine M. McKillop
School of Biomedical Sciences
University of Ulster
Coleraine BT52 1SA
Northern Ireland, U.K.
Tel: +44-28-70-323066
Fax: +44-28-70-324965
E-mail: am.mckillop@ulster.ac.uk
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 (120mg) 10 min prior to a 75g oral glucose load in a randomised cross-over design.

Results: Plasma glucose reached 18.2 ± 1.7 mmol/l 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/min, P<0.05), reaching a minimum of 1.9 ± 0.1 nmol/ml/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 ± 163.9pmol/l (P<0.05) and 11.8 ± 1.4mg/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) to GIP(3-42) in vitro.

Comparison of in vitro inhibition of DPP-IV by nateglinide and vildagliptin revealed IC50 values of 17.1 µM 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 mediated indirectly through DPP-IV inhibition and increased bioavailability of GIP and other incretins merits consideration.
Introduction

Development of type 2 diabetes and the transition from normal glucose tolerance to impaired glucose tolerance is 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 beta 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 antihyperglycaemic 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 beta-cells via the closure of the \( K_{\text{ATP}} \) channels by binding to sulfonylurea receptor (SUR) subunits resulting in inhibition of ATP sensitive \( K^+ \) channels causing cell depolarisation, \( Ca^{2+} \) influx and insulin release (7,8). Notably nateglinide inhibits \( K_{\text{ATP}} \) channels more rapidly and with a shorter duration of action than sulfonylurea 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 approx 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 insulinotropic 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, Tyr\textsuperscript{1}-Ala\textsuperscript{2} and His\textsuperscript{7}-Ala\textsuperscript{8} from the N-terminus of GIP and GLP-1, respectively (10). Due 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 anti-diabetic 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. (777 E. Evelyn Avenue Sunnyvale, CA 94086). RPMI-1640, penicillin, streptomycin, foetal bovine serum, Hanks’ balanced saline solution, trypsin/EDTA were purchased from Gibco Life Technologies Ltd. (Paisley, Strathclyde, UK). Gly-Pro-MCA, 7-amino-4-methylcoumarin-AMC, N,N-dimethyl-formamide (DMF) and purified porcine kidney (DPP IV) (EC 3.4.14.5) were supplied by Sigma (Poole, Dorset, UK). All water used was purified using a Milli-Q Water Purification System (Millipore Corporation, Milford, MA, USA). All other chemicals were of analytical grade.

Subjects and study design

Equal numbers of male and female subjects participated in this study (Table 1). All ten were outpatients, being treated by diet therapy alone (n=6), metformin (n=3) or gliclazide (n=1), with normal renal function (serum creatinine <115 µmol/l), who agreed to be hospitalised for 4 h to take part in the study. Glucose, HbA1c, insulin and C-peptide (mean ± SEM) of the subjects were 7.3 ± 0.6%, 99.8 ± 17.7 pmol/l and 3.3 ± 0.4 ug/l, respectively. All drugs were stopped one week prior to the study period. An intravenous cannula was inserted into the antecubital vein, which was kept patent by means of heparinised saline. The subjects were given oral nateglinide (120mg) or placebo at time t –10 min followed by a 75g oral glucose tolerance test at t0 min in a
randomised cross-over design. Plasma DPP-IV activity, glucose, insulin and C-peptide were determined at a number of time points between t -15 and 210 min. Informed consent was obtained from all participants and the studies were approved by the Ethics Committee of The Queen’s University of Belfast.

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. TFA-H$_2$O (10% v/v) was added to terminate the reaction after which samples were frozen (-20°C) until 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 (62.5 µmol/l-1mmol/l). Concentration-dependent effects of nateglinide on GIP degradation were studied using 5 µl purified porcine kidney DPP-IV. Peptide samples were separated using a Vydac C-18 (4.6 x 250 mm) column (The Separations Group, Hesperia, CA, USA) equilibrated with 0.12% (v/v) TFA-H$_2$O at a flow rate of 1.0 ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile-H$_2$O, 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, U.K.). HPLC fractions were applied at a flow rate of 10 µl/min by syringe injection to the electrospray ionization 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 $M_r = iM_i - \frac{iM_h}{i}$ (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 (7-amino-4-methyl-coumarin) liberated from the DPP IV substrate, Gly-Pro-AMC. Plasma (20 µl), 50 mmol/l HEPES (N-[2-Hydroxyethyl] piperazine-N’-[2-ethanesulfonic 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 3.0 mol/l acetic acid (pH 2.6) to terminate DPP-IV enzyme activity. Free AMC was measured at excitation wavelength 370 nm and emission wavelength 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 of AMC of plasma in 1 min. Intra- and interassay CVs were 2.1% and 6.9%, respectively.

HbA$_{1c}$ 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 (MEIA; Abbott Laboratories Ltd, Berkshire, UK) which has a sensitivity of 6 pmol/l and an intra-assay coefficient of variance 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, Cambridgeshire, UK).

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 anti-diabetic drugs, vildagliptin (0-10 µM) and nateglinide (0-500 µM). As above, GIP was incubated for 60 min at 37°C with 25µl of 50mmol/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 70µl of 3 mmol/l acetic acid. Free AMC was measured at excitation wavelength 370 nm and emission wavelength 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 of the activity under specific conditions.
**Statistical analysis**

Data are expressed as mean ± SEM. 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 y, body mass index of 34.7 ± 2.6 Kg/m$^2$ and duration of diabetes of approximately 2.8 ± 1.3 y. Circulating glucose, insulin, C-peptide and DPP-IV activity were measured following a 75g oral glucose load given 10 min after nateglinide (120mg) or placebo (Figure 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/min in the placebo and nateglinide groups, prior to any intervention (NS, *P*>0.05). As shown in Figure 1, DPP-IV activity was significantly reduced (19.9 ± 1.6 nmol/ml/min, *P*<0.05) 10 min following nateglinide, and reached a minimum level of 1.9 ± 0.1 nmol/ml/min at 120 min (*P*<0.001). DPP-IV activity following placebo did not change, remaining at 22.5 ± 0.6 nmol/ml/min. Plasma DPP-IV activity was inhibited (2-fold) 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 mmol/l and 18.2 ± 1.7 mmol/l at 90 min in the nateglinide and placebo groups, respectively ($P<0.001$) (Figure 1). Plasma glucose concentrations were lower following nateglinide from 30-120 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 ($P<0.05$, Figure 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 (Figure 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 Figure 2. The degradation of GIP(1-42) to GIP(3-42) over the 8 h period was clearly inhibited in the presence of nateglinide (Figure 2C). The major HPLC peptide peaks were confirmed as GIP(1-42) and GIP(3-42) using ESI-MS and spectral averaging (Figure 3). Prominent multiple charged species ($M+3H$)$^{1+}$ and ($M+4H$)$^{4+}$ were detected, corresponding to intact Mr 4981.8 and 4983.2 Da, representing the peptide GIP(1-42). Similarly, ($M+3H$)$^{3+}$ and ($M+4H$)$^{4+}$ of 4748.7 and 4748.4 Da, corresponded to the theoretical mass of GIP(3-42) (Figure 3).
The effects of (62 µmol/l - 1 mmol/l) nateglinide on the degradation of GIP after incubation with purified DPP-IV at 0, 2, 4 and 8 h are summarised in Figure 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 Figure 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 (33). 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 (Figure 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 $93 \pm 2.2 \%$, $83 \pm 0.0 \%$ ($P<0.05$) and $64 \pm 1.0\%$ ($P<0.01$), and was higher in 2, 4 and 8 h incubations compared with placebo ($91 \pm 0.4 \%$, $72 \pm 1.4 \%$ ($P<0.05$) and $38 \pm 0.7 \%$ ($P<0.01$)), respectively (Figure 6).
Discussion
The beneficial effects of nateglinide on beta cell function and blood glucose control are clearly documented in type 2 diabetic patients (4,6). The key actions of nateglinide concern improvement of beta cell function and restoration of first phase insulin release with reduced postprandial hyperglycaemia. The drug also seems to preserve beta 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 thus enhancement of incretin action in humans comes from various sources. Thus nateglinide has been found to be most effective in reducing post-prandial hyperglycaemia when administered immediately before meals (4). This appears to correspond with the window of GIP secretion from 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 beta cell sensitivity to GIP is increased by treatments such as DPP-IV inhibition that 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 widely distributed in peripheral organs such as the pancreas, gut, bone, adipose tissue, heart and brain, GIP like 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 insulinomimetic
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, 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 IC\textsubscript{50} values, and was less than the considerable inhibitory power of
vildagliptin. IC\textsubscript{50} 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 antidiabetic
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 beta cell K-ATP channel activity and consequent stimulation of insulin secretion.
However, the possibility that some part of the actions of nateglinide are mediated
indirectly through increased bioavailability of GIP and GLP-1 merits consideration.

Declarations 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.
References


evidence for independence from metabolic control and three key enzymes in
hemorphin metabolism, cathepsin D, ACE and DPP. *Peptides* 2009 30 256-61.

27. Vilsboll T, Krarup T, Deacon CF, Madsbad S, Holst JJ. Reduced postprandial
countentration of intact biologically active glucagon-like peptide 1 in type 2

Ognibene A, Rotella CM. Hyperglycaemia increases dipeptidyl peptidase IV

29. Duffy N, Green BD, Irwin N, Gault VA, McKillop AM, O’Harte FP, Flatt PR.
Effects of antidiabetic drugs in dipeptidyl peptidase IV: Nateglinide is an inhibitor
of DPPIV and augments antidiabetic activity of glucagon-like peptide-1. *Eur J

30. Fujiwara K, Tsuru D. New chromogenic and fluorogenic substrates for

1971 32 199-201.


34. Brandt I, Joossens J, Chen X, Maes M-B, Scharpé S, De Meester I, Lambeir A-M.
Inhibition of dipeptidyl-peptidase IV catalyzed peptide truncation by Vildagliptin
*Biochemical Pharmacology* 2005 70 134-143.


42. Falko JM, Crockett, SE, Cataland S, Mazzaferri EL. Gastric inhibitory polypeptide (GIP) stimulated by fat ingestion in man. *J Clin Endocrinol Metab* 1975 **41** 260-265.


45. McIntosh CH, Widenmaier S, Kim SJ. Glucose-dependent insulinotropic polypeptide (Gastric Inhibitory Polypeptide; GIP). *Vitam Horm* 2009 **80** 409-71.


50. McKillop AM, Duffy NA, Lindsay JR Green BD, O’Harte FPM, Bell PM, Flatt PR. Insulinotropic actions of nateglinide are accompanied by inhibition of dipeptidyl peptidase-IV activity *Diabetologia* 2006 **49**(Suppl 1) 482.


Legends for figures

Figure 1: Effects of nateglinide on circulating DPP-IV activity and circulating concentrations of glucose, insulin and C-peptide in type 2 diabetic subjects. Nateglinide (120 mg) or placebo tablet, was taken 10 min before an oral (75g) glucose load. Values are mean ± SEM of 5 observations. *P<0.05, **P<0.01 and ***P<0.001, compared to placebo at the same time point.

Figure 2: Representative HPLC profiles of the degradation of GIP(1-42) to the major degradation fragment, GIP(3-42) by DPP-IV in the absence of nateglinide at (A) 0 h, (B) 8 h and (C) at 8 h in the presence of 1 mmol/l nateglinide. GIP(1-42) and GIP(3-42) were separated using a Vydac C-18 (4.6 x 250 mm) analytical column.

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 Figure 2. Molecular masses were determined using prominent multiple charged ions and the equation: $M_r = iM_i - iM_h$ where $M_r$ is molecular mass, $M_i$ is m/z ratio, $i$ is number of charges and $M_h$ is mass of a proton.

Figure 4: Concentration-dependent inhibition of DPP-IV-mediated degradation of GIP by nateglinide. The effects of nateglinide (6.25 µmol/l – 1 mmol/l) on the degradation of GIP were studied after incubation with purified DPP IV for 8h. Reaction products were separated by HPLC on a Vydac (4.6 x 250 mm) analytical column and results expressed as percentage of intact peptide. Values are mean ± SEM of 2 observations. *P<0.05, **P<0.01 and ***P<0.001, compared to control.
Figure 5: Inhibitory effect of vildagliptin and nateglinide on DPP-IV mediated degradation of GIP in vitro. Effect of increasing concentrations of (A) nateglinide and (B) vildagliptin on endogenous DPP-IV activity are shown. Values are mean ± SEM (n=3). *P<0.05, **P<0.01 and ***P<0.001, compared to control.

Figure 6: Percentage intact GIP relative to the major degradation fragment GIP(3-42) after 0 - 8 h incubation with plasma from type 2 diabetic subjects taken after 150 min either nateglinide or placebo as shown in Figure 1. Values are mean ± SEM of 5 observations. DPP IV activity in plasma after nateglinide or placebo was 2.1 ± 0.2 and 20.9 ± 0.4 nmol/ml/min respectively *P<0.05 and **P<0.01, compared to placebo at the same incubation time.
Table 1: Characteristics of type 2 diabetic subjects (Data are expressed as Mean ± SEM).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo Group (n=10)</th>
<th>Nateglinide Group (n=10)</th>
</tr>
</thead>
<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>
</tbody>
</table>
Figure 1:

- **Nateglinide**
- **Control**

A. DPP-IV activity (nmol/ml/min)
- **OGTT**
- **OGTT**

B. Plasma glucose (mmol/l)
- **OGTT**
- **OGTT**

C. Serum insulin (pmol/l)
- **OGTT**
- **OGTT**

D. Plasma C-peptide (mg/l)
- **OGTT**
- **OGTT**
Figure 2:

A

B

C

0h

8h

8h + Nateglinide

GIP(1-42)

GIP (3-42)

Absorbance (206nm)

Retention time (min)
Figure 3:

A  GIP(1-42)  4982.5 Da

B  GIP(3-42)  4746.4 Da
Figure 4:

![Graph showing the effect of nateglinide on intact GIP(1-42) percentage over time.](image-url)

- Control
- Nateglinide
  - 62.5 µmol/l
  - 125 µmol/l
  - 250 µmol/l
  - 500 µmol/l
  - 1 mmol/l

**Note:** Graph indicates significant differences at various time points (0, 2, 4, 6, 8 hours) with asterisks (*, **, ***).
Figure 5

**Panel A:**
- DPP-IV activity (%)
- Nateglinide concentration (Log (M))
- $IC_{50} = -4.768 (17.1 \mu M)$

**Panel B:**
- DPP-IV activity (%)
- Vildagliptin concentration (Log (M))
- $IC_{50} = -5.677 (2.1 \mu M)$
Figure 6:

- **Subjects given placebo**
- **Subjects given nateglinide**

The graph shows the percentage of intact GIP(1-42) over different incubation times (h) for subjects given placebo and nateglinide. The data points are marked with asterisks (*) and double asterisks (**) indicating statistical significance.