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# GLUCOSE REGULATION OF DIPEPTIDYL PEPTIDASE IV GENE EXPRESSION IS MEDIATED BY HEPATOCYTE NUCLEAR FACTOR-1α IN EPITHELIAL INTESTINAL CELLS

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## SUMMARY

**1. Dipeptidyl peptidase IV (DPP-IV) is a new drug target in the treatment of Type 2 diabetes. Dipeptidyl peptidase IV enzyme activity is significantly altered in Type 2 diabetic patients with hyperglycaemia, but the underlying molecular mechanisms remain unclear.**

**2. The first aim of the present study was to clarify whether glucose regulates DPP-IV enzyme activity. To address this, DPP-IV gene expression and enzyme activity were measured in Caco2 cells cultured in the presence of low (2.5 mmol/L) or high (16.7 mmol/L) concentrations of glucose. We observed that high glucose inhibited DPP-IV gene expression and enzyme activity.**

**3. The second aim of the present study was to investigate whether hepatocyte nuclear factor (HNF)-1α contributes to glucose regulation of DPP-IV gene expression. To explore this question, associations between the gene expression of DPP-IV and HNF-1α were examined in Caco-2 cells cultured in the presence of low (2.5 mmol/L) or high (16.7 mmol/L) glucose. We found that the pattern of glucose-regulated DPP-IV gene expression is similar to that of HNF-1α. Moreover, to elucidate whether glucose regulation of DPP-IV gene expression is affected when HNF-1α is inhibited, we produced two stable cell lines in which a dominant-negative mutant HNF-1αR271G or basic vectors were stably expressed. We found that glucose regulation of DPP-IV gene expression was compromised in HNF-1αR271G cells, but was well maintained in basic vector cells.**

**4. These results suggest that glucose regulation of DPP-IV gene expression is mediated by HNF-1α.**

**Key words: Caco-2, dipeptidyl peptidase IV, glucose regulation, hepatocyte nuclear factor-1α.**

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## INTRODUCTION

Dipeptidyl peptidase IV (DPP-IV/CD26) is a multifunctional glycoprotein expressed both in soluble form and on the cell surface of various tissues, including the liver, small intestine, kidney and pancreas.[1] Dipeptidyl peptidase IV enzyme activity depends on a serine protease, which acts to remove N-terminal proline and alanine dipeptides from many biologically active polypeptides, cytokines and chemokines, such as glucagon-like peptide (GLP-1).[2] Glucagon-like peptide-1 is mainly secreted by the small intestine after ingestion of a meal, enhancing glucose-induced insulin secretion[3] and inducing satiety.[4] The action of GLP-1 is a key to normal post-prandial glucose homeostasis and constitutes the functional activity of the enteroinsular axis.[5] In Type 2 diabetic patients, DPP-IV degrades GLP-1 in response to meal ingestion,[6] resulting in a reduction of early post-prandial insulin secretion.[7] Prevention of inactivation of GLP-1 by direct inhibition of DPP-IV enzyme activity has emerged recently as a new treatment for Type 2 diabetes.[8]

Recent clinical studies have reported that DPP-IV enzyme activity is significantly altered in Type 2 diabetic patients with hyperglycaemia.[6,9] Enhanced DPP-IV enzyme activity may cause a deficiency of early post-prandial insulin secretion and aggravate the diabetic condition. However, until now, it was not known whether glucose regulates DPP-IV enzyme activity. Furthermore, it is unclear that DPP-IV enzyme activity is altered in Type 2 diabetic patients, because the molecular mechanism underlying DPP-IV gene expression has not been elucidated completely. A previous study found that hepatocyte nuclear factor (HNF)-1α contributes to the regulation of DPP-IV gene expression.[10] Hepatocyte nuclear factor-1α, the causal gene of maturity onset diabetes of the young (MODY) 3,[11] is expressed in the small intestine, pancreas, liver and kidneys.[12] Hepatocyte nuclear factor-1α is composed of three functional domains: the dimerization domain, the DNA-binding domain and the transcriptional domain.[13] Mutant HNF-1α results in synthesis of truncated proteins with simple loss of function or with dominant-negative effects.[14]

In the present study, we investigated whether glucose regulates DPP-IV gene expression and enzyme activity in Caco2 cells, which belong to an epithelial intestinal cell line[15] In addition, we investigated whether HNF-1α contributes to glucose regulation of DPP-IV gene expression in Caco-2 cells, in which a dominant-negative mutant HNF-1αR271G and basic vector (as a control) were stably expressed.

We measured DPP-IV gene expression and enzyme activity in Caco2 cells cultured in the presence of low or high concentrations of glucose.

## METHODS

### Cell culture

Caco-2 (clone TC7) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 16.7 mmol/L glucose (Sigma, St Louis, MO, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 mg/L streptomycin (Sigma) and maintained in 5% $CO_2$ at 37°C. For growth under low glucose conditions, DMEM devoid of glucose was used and was supplemented with 20% heat-inactivated FBS. Because of the serum, the glucose concentration in this solution was 2.5 mmol/L. All cells were cultured in medium containing 16.7 mmol/L glucose until the 1st day of confluence; thereafter, cells were cultured in medium containing 2.5 glucose or 16.7 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol (as an osmolality control for 16.7 mmol/L glucose) until the Day 14 of confluence.

### Plasmid constructs

For the luciferase assay, wild-type HNF-1α and mutant HNF-1αR271G cDNA, provided by Dr Jun Takeda (Gifu University, Gifu, Japan), were subcloned into pCMV6b vector. The promoter region (nucleotides (nt) –473 to +2 bp relative to the cap site) of the DPP-IV gene, including the HNF-1α-binding sites (nt –147 to –135), was subcloned into pGL3 basic reporter vector (Promega, Madison, WI, USA). For the establishment of a stable cell line, a part of the HNF-1α promoter site (nt –781 to +6 bp) was combined with mutant HNF-1αR271G cDNA and subcloned into pIRESneo2 vectors (Clontech, Mountain View, CA, USA).

### Transfection analysis

Caco-2 cells were plated 24 h before transfection and grown to 70% confluence. Transfection was performed with Lipofect AMINE Reagent (Invitrogen) according to the manufacturer's instructions. For transient transfection, cells were harvested 24 h after the start of transfection and cell extracts were prepared for luciferase and β-galactosidase assays. Luciferase activity was normalized against β-galactosidase activity. For the transfection of a stable line, all cells were harvested and subcultured into fresh complete medium 24 h after the start of transfection and 1.0 mg/mL G418 (Invitrogen) was added after 48 h to select for the expression of resistance gene. Individual clones appeared after approximately 2 weeks and selected clones were maintained in 0.5 mg/mL G418.

### RNA isolation and reverse transcription–polymerase chain reaction

Total RNA was extracted using Trizol reagents (Invitrogen). First-strand cDNA was synthesised from total RNA using Superscript-II enzyme (Invitrogen). Real-time polymerase chain reaction (PCR) was performed on the ABI7900 using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the protocol provided by the manufacturer. Respective primer sets (forward and reverse) were as follows: DPP-IV, 5'-CCTTCTACTCTGATGAGTCACTGC-3' and 5'-GTGCCACTAAGCTCCAT-CTTC-3'; HNF-1α, 5'-TACACCTGGTACGTCCGCAA-3' and 5'-CACTT-GAAACGGTTCCTCCG-3'; sucrase-isomaltase (SI), 5'-CATCCTACCAT-GTCAAGAGCCA-3' and 5'-GCTTGTTAAGGTGGTCTGGTTT-3'; β-actin, 5'-AGTACTCCGTGTGGATCGGC-3' and 5'-GCTGATCCACATCT-GCTGGA-3'. Levels of HNF-1α, DPP-IV and SI mRNA were normalized against the amount of β-actin mRNA.

### Preparation of protein and immunoblots

Protein was prepared as described by Oliver *et al.*[10] Protein samples (50 μg) were electrophoresed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) by electroblotting. For immunological blots, membranes were incubated with anti-HNF-1α antibody (N-19; Santa Cruz, Santa Cruz, CA, USA) and anti-actin (Sigma). Bands were visualized with ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA).

### Determination of DPP-IV and SI activity

All cells were washed twice with phosphate-buffered saline (PBS) and then lysed in M-Per Mammalian Protein Extraction Regent (Pierce, Rockford, IL, USA). In the present study, DPP-IV activity was assayed using the DPPIV-Glo™ Protease Assay Kit (Promega), according to the instructions provided by the manufacturer. Sucrase-isomaltase activity was assayed as described previously.[16] Protein contents were determined using the BCA protein assay kit (Pierce).

### Statistical analysis

Statistical analyses were performed by Student's *t*-test using the Statview Statistical Package (SAS Institute, Cary, NC, USA). All data are shown as the mean±SEM of six independent experiments and statistical significance was set at $P < 0.05$.

## RESULTS

### Effects of glucose concentration on DPP-IV and HNF-1α gene expression

We observed that DPP-IV gene expression and enzyme activity were lower on Day 1 of confluence in Caco-2 cells (Fig. 1). With differentiation of Caco-2 cells, DPP-IV gene expression and enzyme activity were significantly increased in the presence of both concentrations of glucose (Fig. 1). However, DPP-IV gene expression was markedly increased in Caco-2 cells cultured in the presence of 2.5 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol (as an osmolality control for 16.7 mmol/L glucose) and twofold higher than that in cells cultured in the presence of 16.7 mmol/L glucose on Day 14 of confluence (Fig. 1a). Moreover, this increased DPP-IV gene expression in Caco-2 cells cultured in the presence of 2.5 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol resulted in a threefold increase in DPP-IV activity (Fig. 1b). This indicates that high concentrations of glucose suppress DPP-IV gene expression, resulting in decreased DPP-IV activity in Caco-2 cells.

In addition, HNF-1α gene expression was increased with the differentiation of Caco-2 cells in the presence of both low and high concentrations of glucose (Fig. 2). On Day 14 of confluence, HNF-1α gene expression was higher in cells cultured in the presence of 2.5 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol than in cells cultured in the presence of 16.7 mmol/L glucose (Fig. 2). The pattern of gene expression of DPP-IV and its regulation by glucose is similar to that for HNF-1α, suggesting that HNF-1α may be involved in glucose regulation of DPP-IV gene expression.

### Effects of mutant HNF-1αR271G on wild-type HNF-1α

Mutant HNF-1αR271G has been identified as a causal mutation for MODY3 subjects.[11] In the present study, we tested whether

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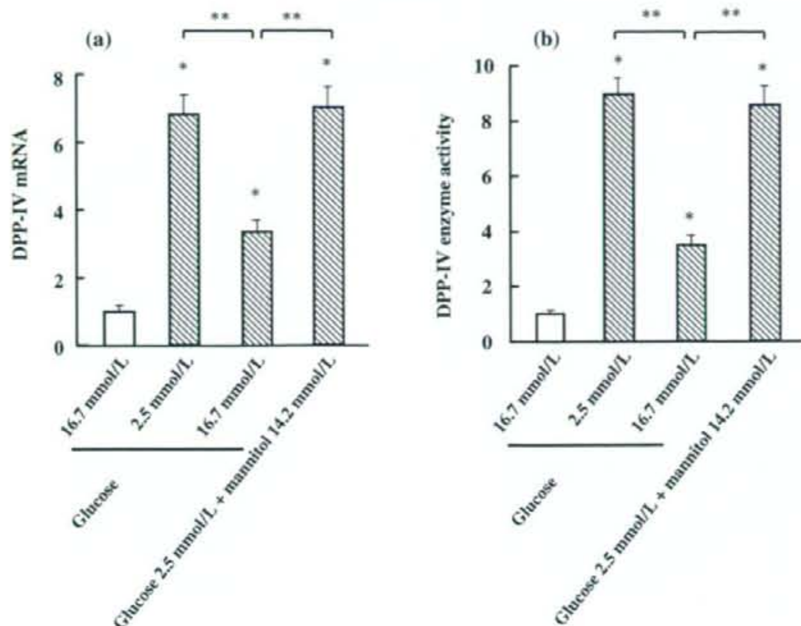


**Fig. 1** Dipeptidyl peptidase IV (DPP-IV) gene expression (a) and enzyme activity (b) in Caco-2 cells cultured in the presence of 2.5 or 16.7 mmol/L glucose on Day 1 (□) or 14 (▨) of confluence. Data are the mean±SEM from six independent experiments. *$P < 0.01$ compared with 16.7 mmol/L glucose on Day 1 of confluence. **$P < 0.01$. The DPP-IV gene expression and enzyme activity were normalized against that of cells cultured in the presence of 16.7 mmol/L glucose on Day 1 of confluence, which was defined as one unit.

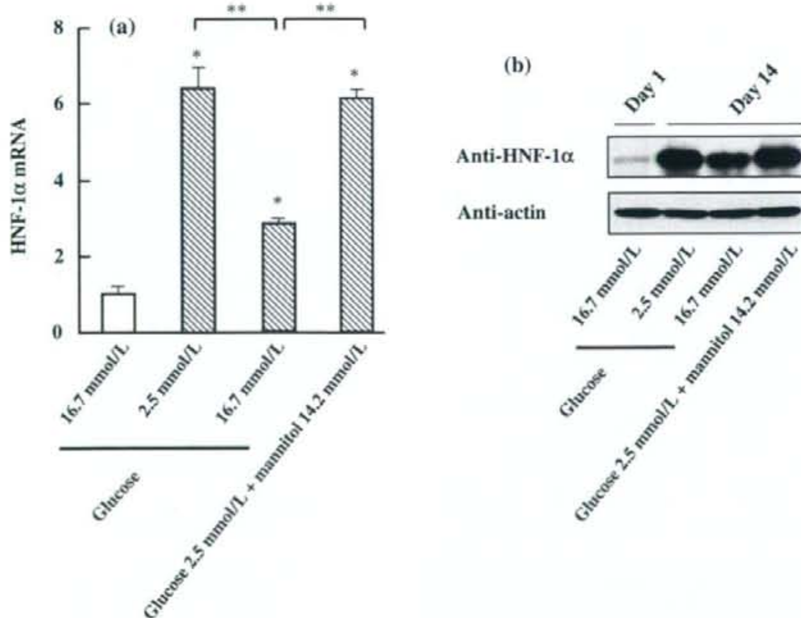


**Fig. 2** Hepatocyte nuclear factor (HNF)-1α mRNA expression (a) and protein levels (b) in Caco-2 cells cultured in the presence of 2.5 or 16.7 mmol/L glucose on Day 1 (□) or 14 (▨) of confluence. Data are the mean±SEM from six independent experiments. *$P < 0.01$ compared with 16.7 mmol/L glucose on Day 1 of confluence. **$P < 0.01$. The mRNA expression of HNF-1α was normalized against that in cells cultured in the presence of 16.7 mmol/L glucose on Day 1 of confluence, which was defined as one unit.

HNF-1αR271G has a dominant negative effect on wild-type HNF-1α by a promoter assay using DPP-IV promoter luciferase construct in Caco-2 cells. Varying amounts of mutant HNF-1αR271G constructs (0–1.0 μg) were cotransfected with a constant amount of wild-type HNF-1α (0.25 μg) into Caco-2 cells. There were no significant differences in the transactivation of DPP-IV promoter, regardless of whether the HNF-1αR271G construct was transfected (Fig. 3a). The HNF-1αR271G mutant dose-dependently reduced transactivation of the DPP-IV promoter, showing a strong dominant-negative effect on wild-type HNF-1α (Fig. 3a). These findings indicate that HNF-1αR271G not only has lesser transactivation activity on the DPP-IV promoter, but also has a dominant-negative effect on wild-type HNF-1α.

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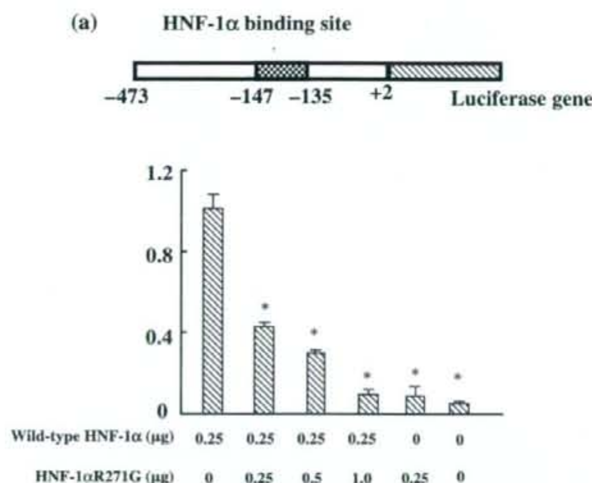


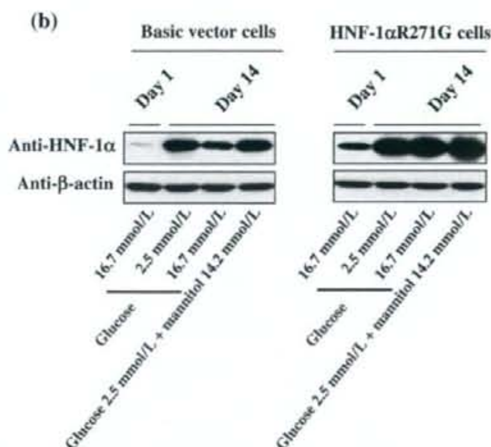


**Fig. 3** Effects of mutant HNF-1αR271G on wild-type hepatocyte nuclear factor (HNF)-1α (a) and detection of mutant HNF-1αR271G gene expression in stably transfected cells by immunoblot (b). Wild-type HNF-1α (0.25 μg) and β-galactosidase construct (0.25 μg) were cotransfected with increasing amounts (0, 0.25, 0.5, 1.0 μg) of mutant HNF-1αR271G in Caco-2 cells. The total amount of plasmid was adjusted to 2.0 μg using pCMV6b vector. Data are the mean±SEM from six independent experiments. $^{*}P < 0.01$ compared with wild-type HNF-1α (0.25 μg) alone.

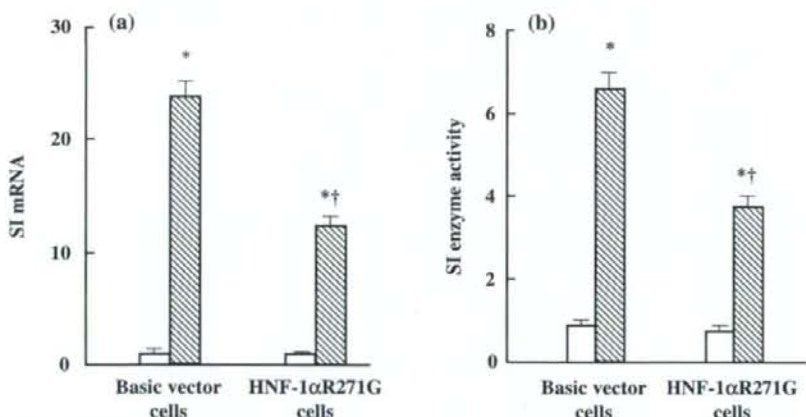


**Fig. 4** Sucrase-isomaltase (SI) gene expression (a) and enzyme activity (b) in basic vector- and HNF-1αR271G-transfected cells cultured in the presence of 16.7 mmol/L glucose on Day 1 (□) or 14 (▨) of confluence. Sucrase-isomaltase gene expression and enzyme activity were normalized against that in basic vector-transfected cells on Day 1 of confluence, which was defined as one unit. Data are the mean±SEM from six independent experiments. $^{*}P < 0.01$ compared with Day 1 of confluence; $^{\dagger}P < 0.01$ compared with basic vector cells on Day 14 of confluence.

## Dominant-negative mutant HNF-1αR271G gene expression in stable cells

We planned to clarify whether glucose regulation of DPP-IV gene expression was affected when the function of HNF-1α was inhibited by dominant-negative mutant HNF-1αR271G. In preconfluent undifferentiated Caco-2 cells, endogenous DPP-IV gene expression and enzyme activity were not altered, despite transient overexpression of the mutant HNF-1αR271G gene (data not shown). To resolve this question, we produced two cell lines in which the dominant-negative mutant HNF-1αR271G and basic vector (as a control) were transfected. Stable constitutive expression of the mutant HNF-1αR271G gene was confirmed in protein level on Days 1 and 14 of confluence (Fig. 3b). Because the HNF-1α promoter site was used in the HNF-1αR271G expression vector, the expression pattern of the HNF-1αR271G gene in HNF-1αR271G cells was similar to that of HNF-1α in basic vector cells (Fig. 3b). With differentiation of HNF-1αR271G cells, an increased expression of the HNF-1αR271G gene was observed in protein level (Fig. 3b), and presumably inhibited endogenous HNF-1α function.

## Confirmation of endogenous HNF-1α dysfunction in HNF-1αR271G cells

To confirm whether endogenous HNF-1α function is specifically inhibited by transfection of dominant-negative mutant HNF-1αR271G, we examined SI gene expression and enzyme activity in HNF-1αR271G cells. On Day 1 of confluence, there were no differences between basic vector and HNF-1αR271G cells in terms of SI gene expression or enzyme activity (Fig. 4). However, SI gene expression and enzyme activity were significantly lower in HNF-1αR271G cells than basic vector cells on Day 14 of confluence (Fig. 4). This indicates that endogenous HNF-1α function was specifically inhibited by transfection of the dominant-negative mutant HNF-1αR271G in HNF-1αR271G cells.

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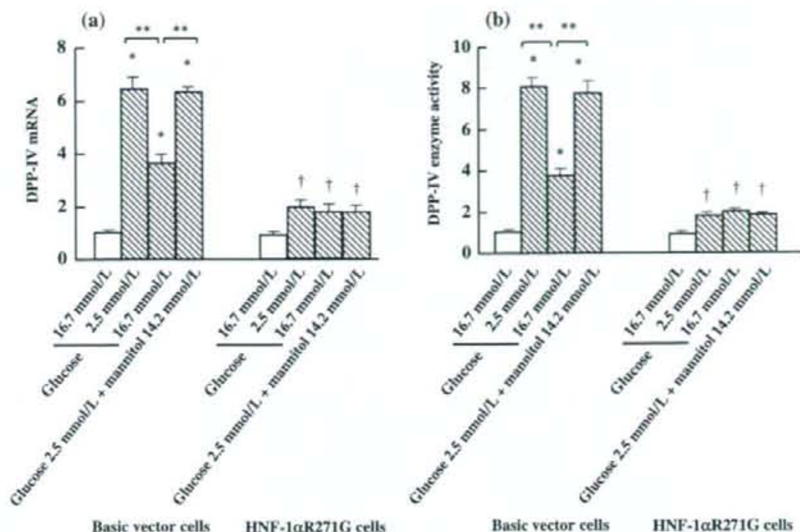


**Fig. 5** Dipeptidyl peptidase IV (DPP-IV) gene expression (a) and enzyme activity (b) in basic vector- and HNF-1αR271G-transfected cells cultured in the presence of 2.5 and 16.7 mmol/L glucose on Day 1 (□) or 14 (▨) of confluence. Data are the mean±SEM from six independent experiments. †$P < 0.05$, *$P < 0.01$ compared with the corresponding cells cultured in the presence of 16.7 mmol/L glucose on Day 1 of confluence; **$P < 0.01$. Dipeptidyl peptidase IV gene expression and enzyme activity were against that of cells cultured in the presence of 16.7 mmol/L glucose on Day 1 of confluence, which was defined as one unit.

## Effects of glucose on DPP-IV gene expression in HNF-1αR271G cells

To clarify changes in glucose regulation of DPP-IV gene expression in HNF-1αR271G cells, basic vector and HNF-1αR271G cells were cultured in the presence of 2.5 or 16.7 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol until Day 14 of confluence. On Day 1 of confluence, no differences were observed in DPP-IV gene expression and enzyme activity between basic vector and HNF-1αR271G cells (Fig. 5). On Day 14 of confluence, DPP-IV gene expression was markedly decreased, resulting in reduced DPP-IV enzyme activity in HNF-1αR271G cells compared with that in basic vector cells (Fig. 5). The glucose regulation of DPP-IV gene expression was confirmed in basic vector cells on Day 14 of confluence. However, it was compromised in HNF-1αR271G cells (Fig. 5). No differences were detected in DPP-IV gene expression and enzyme activity in HNF-1αR271G cells, regardless of glucose concentration (Fig. 5). This suggests that endogenous HNF-1α dysfunction compromises the glucose regulation of DPP-IV gene expression in Caco-2 cells.

## DISCUSSION

Degradation of GLP-1 by DPP-IV causes a deficiency of early post-prandial insulin secretion and aggravates the diabetic condition. Previous clinical studies have reported that, among Type 2 diabetic patients, reduced DPP-IV activity has been found in subjects with poor glycaemic control and elderly obese subjects.[9,17] Although high glucose concentrations and hyperosmolarity induced by high glucose concentrations regulate the expression of a number of genes *in vivo* and *in vitro*,[18,19] it is not known whether high glucose concentrations and high glucose-induced hyperosmolarity regulate DPP-IV gene expression and enzyme activity. In the present study, we found that the transcription level of DPP-IV gene expression was lower in Caco-2 cells cultured in the presence of 16.7 mmol/L glucose compared with cells cultured in the presence of either 2.5 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol, resulting in reduced DPP-IV activity. This suggests that, in Caco-2 cells, high glucose concentrations, but not high glucose-induced hyperosmolarity, suppress DPP-IV gene expression, resulting in decreased DPP-IV enzyme activity. An electrophoretic mobility shift assay (EMSA) study has shown that HNF-1α has a binding site in the (−147/−135) fragment of the DPP-IV gene promoter.[20] In a previous study, we reported that HNF-1α is able to promote DPP-IV gene expression in Caco-2 cells.[10] In the present study, HNF-1α gene expression was also lower in Caco-2 cells cultured in the presence of 16.7 mmol/L glucose compared with 2.5 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol. The pattern of gene expression pattern of DPP-IV and its regulation by glucose is similar to that for HNF-1α in Caco-2 cells. Therefore, we hypothesize that glucose regulation of DPP-IV gene expression is mediated by HNF-1α.

To elucidate the hypothesis mentioned above that glucose regulation of DPP-IV gene expression is mediated by HNF-1α, we investigated whether glucose regulation of DPP-IV gene expression in Caco-2 cells was affected when the function of endogenous HNF-1α was inhibited. Specific inhibition of endogenous HNF-1α by transfection of a dominant-negative mutant HNF-1αR271G was confirmed by downregulation of SI gene expression and enzyme activity in HNF-1αR271G cells. Hepatocyte nuclear factor-1α directly binds to two sites (SIF2, SIF3) of the promoter of the SI gene and upregulates SI gene expression.[21] A reduction in SI gene expression and enzyme activity has been observed in Caco-2 cells in which a dominant-negative mutant of HNF-1α was stably expressed.[22]

Although HNF-1α promotes DPP-IV gene expression,[10] no significant differences were observed in DPP-IV gene expression and enzyme activity in stably transfected cells on Day 1 of confluence. At the beginning of differentation, little DPP-IV gene expression was found because expression of the DPP-IV gene in Caco-2 cells is closely associated with their differentiation state.[23] This may be one reason why expression of the HNF-1αR271G mutant did not affect DPP-IV gene expression and enzyme activity on Day 1 of confluence. However, on Day 14 of confluence, DPP-IV gene expression and enzyme activity in Caco-2 cells were lower in HNF-1αR271G-transfected cells compared with those transfected with basic vector

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and cultured in the presence of the same concentration of glucose. This indicates that transfected HNF-1αR271G had a dominant-negative effect on endogenous HNF-1α in the stable cells. The glucose regulation of DPP-IV gene expression was confirmed in basic vector-transfected cells. However, glucose regulation of DPP-IV gene expression was compromised in HNF-1αR271G-transfected cells. No differences were observed in DPP-IV gene expression and enzyme activity in HNF-1αR271G cells on Day 14 of confluence, regardless of the concentration of glucose in which the cells were cultured. Therefore, it is thought that inhibition of HNF-1α function by the dominant-negative mutant HNF-1αR271G is responsible for attenuation of glucose regulation of DPP-IV gene expression in HNF-1αR271G cells. These results support our hypothesis that glucose regulation of DPP-IV gene expression in Caco-2 cells is mediated by HNF-1α.

There is little information on DPP-IV enzyme activity in patients with Type 2 diabetes. Of patients with Type 2 diabetes, a reduction in DPP-IV enzyme activity has been observed in patients with poor glycaemic control, as well as in elderly obese patients;[9,17] however, another report has suggested that patients with Type 2 diabetes have increased DPP-IV enzyme activity.[6] The results of the present study do not rule out the possibility that DPP-IV enzyme activity may be increased in Type 2 diabetes, but hyperglycaemia seems not to be a causative factor for increased DPP-IV enzyme activity. In patients with Type 2 diabetes, glucose tolerance and insulin resistance may contribute to altered DPP-IV enzyme activity;[24,25] in addition, the effect of therapeutic agents on DPP-IV enzyme activity should not be overlooked.[26,27] Prevention of the inactivation of GLP-1 by direct inhibition of DPP-IV enzyme activity has emerged recently as a new treatment for Type 2 diabetes.[8] Although the results of the present study suggest that hyperglycaemia may reduce DPP-IV enzyme activity in patients with Type 2 diabetes, there is no doubt that DPP-IV inhibitors are useful therapeutic agents for Type 2 diabetes. Many clinical studies have reported that DPP-IV inhibitors significantly augment exogenously administered GLP-1, resulting in increased endogenous GLP-1 concentrations in the plasma and stimulating glucose-induced insulin secretion in Type 2 diabetes patients.[28,29] There are no inhibitors of DPP-IV gene expression available, because the molecular mechanism of DPP-IV gene expression has not yet been fully uncovered. The results of the present study indicate that HNF-1α is apparently an effective target in the development of DPP-IV inhibitors and its inhibition may lead to the suppression of DPP-IV gene expression.

In the present study, we found that glucose regulation of DPP-IV gene expression in Caco-2 cells is mediated by HNF-1α. This finding may help elucidate the molecular mechanism of glucose regulation of DPP-IV gene expression. The glucose-responsive element has not been identified in the HNF-1α gene promoter. In addition, the signalling pathways involved in glucose regulation of DPP-IV gene expression have not been fully elucidated and further detailed studies are required.

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