Metformin enhances glucagon-like peptide 1 via cooperation between insulin and Wnt signaling

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Abstract

One aspect of the effects of metformin on glucagon-like peptide (GLP)-1 might be associated with the mechanism by which the cross talk between insulin and Wnt signaling enhances GLP1 secretion, due to the action of metformin as an insulin sensitizer. However, this remains completely unknown. In this study, we have investigated the mechanisms of the action of metformin on cross talk between insulin and Wnt signaling. GLP1 enhancement by metformin was determined in human NCI-H716 intestinal L-cells and hyperglycemic db/db mice treated with metformin (0.25 and 0.5 mM and/or 12.5 mg/kg body weight) for 24 h and 2 months. Metformin increased GLP1 secretion in L-cells and db/db mice. Metformin stimulated the nuclear translocation of β-catenin and TOPflash reporter activity, and gene depletion of β-catenin or enhancement of mutation of transcription factor 7-like 2 binding site offset GLP1. In addition, insulin receptor substrate 2 gene depletion blocked metformin-enhanced β-catenin translocation. These effects were preceded by an increase in glucose utilization and calcium influx, the activation of calcium-dependent protein kinase, and, in turn, the activation of insulin signaling, and the inhibition of glycogen synthase kinase 3β, a potent inhibitor of β-catenin. Furthermore, high blood glucose levels were controlled via GLP1 receptor-dependent insulinotropic pathways in db/db mice, which were evidenced by the increase in GLP1 and insulin levels at 30 min after oral glucose loading and pancreatic insulinotropic gene expression. Our findings indicate that the cooperation between Wnt and its upstream insulin signaling pathways might be a novel and important mechanism underlying the effects of metformin on GLP1 production.

Introduction

Glucagon-like peptide (GLP)-1 is an intestinal hormone that exerts its effects in the regulation of glucose metabolism, the stimulation of pancreatic insulin secretion, proinsulin gene expression, and the proliferation and anti-apoptosis of β-cells as well as the inhibition of glucagon release, gastric emptying, and food intake. The insulinotropic effect of GLP1 has led to the development of GLP1 analogs for the treatment of type 2 diabetes (Baggio & Drucker 2007, Kim & Lee 2010).
Metformin, a biguanide that was introduced as an anti-diabetic agent, acts through inhibition of hepatic gluconeogenesis and stimulation of glucose uptake into peripheral tissues (Sarabia et al. 1992, Radziuk et al. 1997). Recently, metformin has been increasingly used in combination with new incretin-based therapies such as GLP1 analogs and dipeptidyl peptidase (DPP)-4 inhibitors (D’Alessio 2011), but direct interaction between metformin and the incretin axis has also been suggested; the administration of metformin to obese subjects with or without type 2 diabetes or DPP4-deficient F344/DuCrj rats lead to increase in GLP1 levels (Yasuda et al. 2002, Mannucci et al. 2004). Furthermore, metformin selectively increased plasma levels of GLP1 in mice without alteration in another incretin hormone, glucose-dependent insulino- notropic polypeptide (GIP), or peptide YY, a gastrointestinal hormone that is colocalized with GLP1 in L-cells (Maida et al. 2011). Collectively, these observations indicate that the action of metformin on the gut endocrine system may be L-cell-specific and, more precisely, GLP1-specific, and may be distinct from DPP4 inhibition.

Recently, it has been reported that the insulin and Wnt signaling pathways converge their signals to produce GLP1 (Yi et al. 2008). The binding of Wnt ligands to the frizzled receptor and its coreceptor, lipoprotein-related protein (LRP)5/6, leads to the translocation of β-catenin into the nucleus and combination of β-catenin with transcription factor 7-like 2 (TCF7L2), which has its binding site in the G2 enhancer element of proglucagon (Glu), a precursor of GLP1 (Akiyama 2000). Insulin enhances GLP1 production via inhibition of glycogen synthase kinase (GSK)-3β-dependent degradation of β-catenin (Yi et al. 2008). Furthermore, in human hepatocytes, granulosa cells, and C2C12 myotubes, metformin quickly leads to insulin receptor phosphorylation and enhances insulin signaling, e.g., phosphoinositide 3 kinase (PI3K), both in the presence or absence of insulin (Kumar & Dey 2002, Gunton et al. 2003, Rice et al. 2011). We therefore hypothesized that metformin could increase GLP1 production and secretion via the bipartite transcription factor β-catenin/TCF7L2, the major effector of the canonical Wnt signaling pathway, which might be controlled by its upstream insulin signaling.

To investigate our hypothesis, we treated NCI-H716 cells, a cellular model for GLP1 secretion (Reimer et al. 2001), with metformin (0.25, 0.5, 1, and 2 mM) for 24 h. We also investigated the effects of metformin in vivo using db/db mice, an animal model of type 2 diabetes, by administering metformin (12.5 and 50 mg/kg body weight, BW) for 2 months.

Materials and methods

Chemicals and reagents

Metformin, LY294002, and nifedipine were purchased from Sigma–Aldrich and Calbiochem (San Diego, CA, USA). Metformin was dissolved in 0.25% carboxymethyl-cellulose for oral administration.

Cell culture and experimental animals

NCI-H716 cells (cell lines ≤ 10 passages, ATCC, Manassas, VA, USA) were maintained in a suspension culture as recommended by the supplier. Two days before the experiment, 200 × 10⁴ cells were seeded into six-well culture plates precoated with Matrigel, as described previously (Reimer et al. 2001). On the day of the experiment, L-cells were treated with metformin (0.25 or 0.5 mM) for 24 h. Mouse studies were conducted with the approval of and in accordance with the guidelines of the Animal Care and Use Committee of the Samsung Biomedical Research Institute (SBRI No. C-A9-228-1, Seoul, Korea), which comply with national and international laws and policies (National Institutes of Health Guide for Care and Use of Laboratory Animals, 7th edition, 1996). Metformin (10 ml/kg BW) at a dose of 12.5 or 50 mg/kg BW per day was administered to 12-week-old db/db male mice (Central Lab Animal, Seoul, Korea) by oral gavage for 8 weeks. Animals were housed individually in the animal facility with a 12 h light:12 h darkness cycle.

Oral and intraperitoneal glucose tolerance tests

Oral and intraperitoneal glucose tolerance tests (OGTT and IPGTT) were performed after oral and/or i.p. administration of glucose (1 g/kg BW) as described previously (Wheatcroft et al. 2007). Blood glucose concentrations were measured using a glucose analyzer (APEC, Danvers, MA, USA) at 0, 30, 60, 90, and 120 min. Also, serum GLP1 and insulin levels were measured at 30 min after oral glucose challenge to determine the relationship between the glucose-lowering effect and GLP1 enhancement by metformin.

Biochemical assays

ELISA kits were used to quantify serum GLP1 (EDI, San Diego, CA, USA), insulin (Mercodia, Uppsala, Sweden), glucagon (R&D Systems, Minneapolis, MN, USA), C-peptide (Cusabio Biotech, Wuhan, China), and
Measurement of intracellular ATP

After stimulation with 16.7 mM glucose, cells were harvested and 2.5% trichloroacetic acid (TCA) was added to extract ATP. The TCA-containing supernatant was neutralized with Tris-acetate (pH 7.75). ATP content was measured using a luciferin/luciferase method (Promega) and determined using a Wallac Trilux 1450 Microbeta liquid scintillation counter (Wallac, Turku, Finland).

Glucose uptake assay

After treatment of cells with metformin for 24 h and glucose deprivation for 5 min, 30 μM 6-(N-(7-nitrobronazon-2-oxal-1,3-diazol-4-yl)amino)-6-deoxyglucose (2-NBDG; Molecular Probes, Eugene, OR, USA), a fluorescent and non-metabolizable glucose analog (Kumar & Dey 2002, Rice et al. 2011), in Krebs–Ringer Bicarbonate (KRB) buffer was added for 10 min. The cells were washed twice with PBS and resuspended in 1 ml PBS. Glucose uptake by L-cells was determined using a FACSort flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) at an excitation wavelength of 488 nm. Vehicle-treated cells were used as controls.

Measurement of intracellular calcium using Fluo-4 staining

After treatment with metformin for 24 h, cells were stimulated for 1 h with 16.7 mM glucose in KRB buffer and treated with 3 mM Fluo-4 (Molecular Probes) in Ca²⁺-free Krebs–Ringer–HEPES buffer supplemented with 1% BSA for 1 h without light. Cells that displayed Ca²⁺ influx were counted by using the software Image J (http://rsweb.nih.gov/ij/).

Enzyme activity assay

Mitochondrial complex I activities were expressed by measuring kinetic substrate (NADH) oxidation (MitoScience, Eugene, OR, USA). The mitochondria of cells and intestinal tissue were extracted by lysis and homogenization followed by low- (600 g) and high-(11 000 g) speed centrifugation using a mitochondria isolation kit (Sigma–Aldrich). DPP4 activity was determined by fluorometrically monitoring the conversion of Gly-Pro-4-oMe-2-NA into 7-amino-4-methylcoumarin for 1 min (Calbiochem).

TOP/FOPflash transfection

cDNA TOPflash and FOPflash reporter plasmids were purchased from Millipore (Billerica, MA, USA). In these plasmids, expression of a luciferase reporter gene is driven by a minimum TK promoter fused with three copies of the TCF7L2-binding site and its mutant-binding site. The L-cells (50×10⁴) were seeded into 24-well plates for 24 h and transfected with 100 ng TOPflash or FOPflash using Lipofectamine (Invitrogen). After transfection, cells were treated with metformin for 24 h and harvested for luciferase reporter gene analysis (Promega).

RNA interference

siRNA against human β-catenin, insulin receptor substrate 2 (IRS2), and scrambled RNA (as control) were purchased from Dharmacon Research (Lafayette, CO, USA). The L-cells (200×10⁴) were seeded into six-well plates for 24 h and then transfected with 50 nM siRNA using Lipofectamine 2000 (Invitrogen). After transfection, cells were treated with metformin for 24, 48, or 72 h.

Immunohistochemical and immunofluorescent staining

For immunohistochemistry (IHC), isolated mouse pancreas and intestinal tissue were fixed with 10% formalin, embedded in paraffin, mounted on slides, deparaffinized in xylene and ethanol, and blocked with 10% normal horse serum, as detailed previously (Brown et al. 1996). Tissues were incubated with primary antibodies (1:200) overnight at 4 °C followed by the addition of biotinylated anti-rabbit IgG and then avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Nuclei were stained with hematoxylin (Sigma–Aldrich). Tissues for immunofluorescence were incubated with primary antibody (1:200) followed by secondary antibody (1:1000) labeled with FITC (Invitrogen). Nuclei were stained with Hoechst 33258 (0.5 mM in PBS; Sigma–Aldrich). Images were captured using an Eclipse TE 200 confocal microscope (Nikon, Tokyo, Japan) and quantified by using the software Image J (http://rsweb.nih.gov/ij/) for the measurement of GLP1R fluorescence intensity (Fig. 1B) or islet area (Supplementary Figure S7, see section on supplementary data given at the end of this article).

Western blotting

Cells were lysed in lysis buffer (20 mM Tris–HCl; pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton
X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM β-glycerophosphate containing NaF, phenylmethanesulfonylfluoride, and Na3VO4, and centrifuged at 12,000 g for 10 min at 4°C. Twenty micrograms of protein were separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes. Proteins were visualized with primary antibody (1:1000), followed by HRP-labeled secondary antibody (1:5000). Western blot bands were quantified using the software Image J (http://rsbweb.nih.gov/ij/) and corrected by β-actin (Supplementary Figure S1, see section on supplementary data given at the end of this article). Relative activity of each protein was calculated by the formula: phosphorylated protein band/total protein band (Supplementary Figure S2).

Total RNA extraction and real-time PCR

Total RNA was isolated and reverse-transcribed using TRIzol reagent and a reverse transcriptase kit (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was conducted using a Biosystem 7500 real-time PCR system (ABI, Foster City, CA, USA). Five hundred nanograms of cDNA were amplified in a 25 μl SYBR Green PCR master mix (ABI) with specific primer pairs (Supplementary Table S1, see section on supplementary data given at the end of this article). The relative expression levels of target genes were calculated from the change in the threshold cycle (ΔCt) as 2^−ΔΔCt, where ΔΔCt = ΔCtreatment − ΔCtcontrol and ΔCt = ΔCttarget gene − ΔCtβ-actin.
Microarray analysis

Gene expression profiles were assessed using a Ref-8 v3 Expression BeadChip (Illumina, San Diego, CA, USA), which contains more than 46,000 individual human genes per piece. Each gene was derived from the NCBI Human Reference Sequence (RefSeq) database. For microarray analysis, the isolated RNA were subjected to cDNA synthesis and transcription-biotinylated cRNA synthesis; they were then hybridized, scanned, and quantified using an Illumina bead array reader and software (Illumina Beadstation 500; Illumina) as detailed previously (Kim et al. 2010). Target sequence signals with detection \( P \) values of \( \leq 0.05 \) were quantile normalized using the program R (version 2.4.1). Biological processes were analyzed via Protein Analysis Through Evolutionary Relationships (PANTHER; http://www.pantherdb.org). The results of two-way hierarchical clustering analysis of gene expression profiles are shown in Supplementary Figure S6, see section on supplementary data given at the end of this article.

Statistical analyses

All experiments were conducted at least in triplicate \((n = 3)\), and the results were expressed as the mean ± S.E.M. Statistical analyses were conducted via ANOVA, followed by Duncan’s multiple range test or Student’s \( t \)-test using SAS Proprietary Software Release 8.2 (SAS Institute, Cary, NC, USA). Data with \( P \) values \(<0.05\) were considered significant.

Results

Metformin increases expression and content of GLP1 as well as Glu

Metformin increased both the contents and the protein expression levels of GLP1, as well as the expression of Glu in L-cells compared with controls \((P<0.05, \text{Fig. 2A, C, and D})\). Also, \(db/db\) mice treated with metformin had higher contents and expression levels of GLP1, as well as higher expression of Glu in serum and the intestines, than mice not treated with metformin \((P<0.05, \text{Fig. 2B, C, and D})\). The increment in serum GLP1 was larger in the postprandial state (90 min) than in the fasting state in \(db/db\) mice \((P<0.05, \text{Fig. 2B})\). IHC revealed that the expression of GLP1 (green color) was more abundant in sections of the ileum from metformin-treated mice than controls \((\text{Fig. 2E})\). Interestingly, at low concentrations of metformin \((0.25\) and \(0.5\) mM), GLP1 production was increased but the phosphorylation of AMPK and its upstream liver kinase B (LKB) was not stimulated, whereas at high concentrations \((1\) and \(2\) mM and/or \(50\) mg/kg BW), the phosphorylation of AMPK and LKB was increased without increase in GLP1 production \((\text{Fig. 2D and Supplementary Figure S1A and S2, see section on supplementary data given at the end of this article})\). The latter might be attributable to an inhibitory effect by the excessive amount of metformin, which was proven by MTT assay \((\text{Fig. 2F})\). Thus, we chose the doses \((0.25\) and \(0.5\) mM and/or \(12.5\) mg/kg BW) of metformin, which did not decrease cellular viability or BW \((\text{Supplementary Figure S3})\) and enhanced GLP1 production, to investigate the mechanisms. These findings indicate that the stimulatory effects of metformin on GLP1 production are not mediated through an AMPK-dependent pathway.

Metformin enhances GLP1 levels via the Wnt signaling pathway which is controlled by upstream insulin signaling

In L-cells treated with metformin, the expression and nuclear translocation of \(\beta\)-catenin were increased compared with the controls \((\text{Fig. 3A and Supplementary Figure S1B})\). GLP1 enhancement by metformin was closely associated with \(\beta\)-catenin nuclear translocation \((\text{Fig. 3B})\). In the absence of \(\beta\)-catenin by using siRNA, the stimulatory effects of metformin on GLP1 secretion disappeared, but were later restored concomitantly with increased \(\beta\)-catenin expression \((72–96\) h post-transfection of \(\beta\)-catenin siRNA into L-cells, \(\text{Fig. 3B})\). Metformin also increased the transcription of a luciferase reporter gene containing three copies of the TCF7L2-binding sites \((\text{TOPflash})\) in \(Glu\), but did not alter the transcription of a luciferase reporter gene containing the mutant TCF7L2-binding sites in L-cells \((\text{FOPflash, P}<0.05, \text{Fig. 3C})\). In this regard, GLP1 enhancement by \(0.5\) mM metformin was offset by transfection of \(\text{FOPflash, but not TOPflash, P}<0.05, \text{Fig. 3D})\. The microarray data for L-cells also demonstrated that metformin increased the expression of cyclin D1, a Wnt-responsive gene, but decreased the expression of \(GSK3\beta\), a \(\beta\)-catenin inhibitor, compared with controls \((\text{Table 1 and Supplementary Figure S5})\). Interestingly, depletion of IRS2 by siRNA blocked metformin-induced translocation of \(\beta\)-catenin into the nucleus and enhancement of GLP1 in the L-cells \((\text{Fig. 3E and F and Supplementary Figure S1C})\). In this regard, these findings indicate that \(\beta\)-catenin/TCF7L2 may play a primary role in GLP1 enhancement by metformin, and the insulin signaling pathway is upstream of Wnt signaling. In the intestines of metformin-treated mice, both expression and nuclear translocation of...
β-catenin were increased in a similar pattern to that observed in L-cells (Fig. 3A and Supplementary Figure S1B).

**Activation of the insulin signaling pathways occurs through the increase in glucose utilization and calcium mobilization by metformin**

In the L-cells, metformin increased the expression of insulin signaling molecules such as IRS2, phospho-PI3K, and phospho-protein kinase B (PKB) and its upstream genes including calcium-calmodulin-dependent kinase 2 (CAMK2) and cAMP response element binding protein (CREB), and the phosphorylation of its downstream gene GSK3β at serine residue 9 as compared with controls (Fig. 4A and Supplementary Figure S1, E and F, and S2). Interestingly, this insulin signaling axis was regulated by glucose utilization and calcium mobilization. FACS and luciferin/Luc analyses, the methods for glucose uptake and ATP contents, revealed that metformin increased the uptake of 2-NBDG, a non-matabolizable glucose analog, into the L-cells, and increased intracellular ATP levels, an indicator of glucose utilization in L-cells, as compared with controls (P < 0.05, Fig. 4B and C), which were followed by an increase in the influx of calcium (stained by Fluo-4 dye) through L-type calcium channels, which was evidenced by the addition of 10 nM nifedipine, an L-type calcium channel blocker. The addition of nifedipine resulted in a decrease in intracellular calcium levels as compared with metformin (P < 0.05, Fig. 4D and E).
alone in L-cells ($P < 0.05$, Fig. 4D). Taken together, the glucose utilization and a subsequent calcium mobilization are thought to stimulate the phosphorylation of CAMK2 and CREB, calcium-dependent protein kinases (Fig. 4A and Supplementary Figure S1, E and F, and S2).

Enhancement of glucose uptake into L-cells was associated with an increased expression of sodium/glucose cotransporter 1 (SGLT1) and its upstream molecule Hur ($P < 0.05$, Fig. 4E and Supplementary Figure S1D).

GLP1 enhancement by metformin occurs through the Wnt signaling pathway, which is controlled by insulin signaling. Human NCI-H716 intestinal L-cells and hyperglycemic db/db male mice were treated with metformin (0.25, 0.5 mM, and/or 12.5 mg/kg body weight) for 24 h and 2 months, respectively. (A) Protein expression levels and nuclear translocation of $β$-catenin in L-cells and intestinal tissue of db/db mice. (B) mRNA levels of $β$-catenin and GLP1 content in L-cells. L-cells were transfected with scramble RNA or $β$-catenin siRNA for 24 h and then treated with metformin for 48–96 h. (C) Relative transcription levels of a luciferase reporter gene containing three copies of the TCF7L2-binding sites (TOPflash) or mutant TCF7L2-binding sites (FOPflash) in L-cells. L-cells were transfected with plasmid for 24 h and then treated with metformin for 24 h.

(D) Changes in GLP1 contents after transfection of TOPflash or FOPflash. L-cells were transfected with plasmid for 24 h and then treated with metformin for 24 h. (E) $β$-catenin protein expression levels in the cytosol and nuclear extracts from L-cells treated with metformin after transfection of IRS2 siRNA. The stimulatory effect of metformin on $β$-catenin nuclear translocation was blocked at 48 h post-transfection with IRS-2 siRNA. (F) Change in GLP1 contents after transfection of IRS2 siRNA. Data are expressed as mean ± S.E.M. (n = 3–5). *$^a$Mean values with dissimilar superscript letters are significantly different between the groups (Duncan’s test; $P < 0.05$). †Significant differences between two groups (Student’s t-test; $P < 0.05$).

Furthermore, increased ATP content in L-cells was associated with an increase in gene expression, including expression of the genes encoding the enzymes required for glycolysis and the tricarboxylic acid (TCA) cycle, such as phosphofructokinase, isocitrate dehydrogenase 3, and malate dehydrogenase 1 (Table 1 and Supplementary Figure S5), as well as mitochondrial complex 1 activity ($P < 0.05$, Fig. 4F and Supplementary Figure S4). The participation of these signaling factors in GLP1 production and secretion was
demonstrated by a variety of inhibitors, including nifedipine, LY294002, and SGLT1 siRNA (P < 0.05, Fig. 4G). SGLT1 expression was dramatically decreased by 48 h-post transfection of SGLT1 siRNA into L-cells.

Animal study also revealed increases in the expression of genes involved in insulin signaling axis (Fig. 4A), intracellular levels of ATP (Fig. 4C), the expression of SGLT1 and Hur (Fig. 4E), and the activity of complex I (Fig. 4F) by metformin administration.

**Elevated GLP1 levels and its incretin effect contribute to the glucose-lowering effect of metformin**

Mice treated with metformin exhibited lower blood glucose levels and AUC values after oral and/or intraperitoneal glucose compared with mice that had not received metformin (P < 0.05) (Fig. 1A and Supplementary Figure S6). To determine whether this glucose-lowering effect might be related to the elevated GLP1 levels and a subsequent improvement in insulin secretory response, serum GLP1 and insulin level were measured at 30 min after glucose challenge. As expected, the metformin-treated mice showed higher GLP1 and insulin levels in treated mice showed higher GLP1 and insulin levels in serum than controls (Fig. 1A middle and right panel) in contrast to blood glucose levels. These results were associated with higher expression of GLP1 receptor in islets and the increase in molecules and/or genes involved in GLP1-dependent insulinotropic mechanisms, such as cAMP, phospho-CREB, and pancreas/duodenum homeobox 1 (PDX1; Fig. 1B, C, D, and E and Supplementary Figure S1G and S2) in the pancreas. Activation of this insulinotropic mechanism led to the elevation of serum insulin and C-peptide levels but not glucagon level (P < 0.05, Table 2) and the increase in number and mass of β-cells of metformin-treated mice compared with controls (Fig. 1F and Supplementary Figure S7, see section on supplementary data given at the end of this article). Collectively, the elevated levels of GLP1 might be one of the important mechanisms underlying the glucose-lowering effects of metformin (Fig. 5).

### Discussion

We observed that the glucose-lowering effects of metformin may be at least associated with the elevated GLP1 levels and incretin effect on the pancreas, which is supported by the increase in serum GLP1 and insulin levels and the activation of pancreatic insulinotropic mechanisms after glucose challenge. Until recently, this stimulatory effect of metformin on GLP1 was reported to be associated with the inhibition of DPP4, an enzyme that abolishes the biological activity of GLP1 (D’Alessio 2011). However, in our study we identified a novel mechanism by which metformin directly stimulated GLP1 production and secretion through cross talk between the insulin and Wnt signaling pathways. Indeed, metformin increased the expression and contents of GLP1 as well as the activation of the insulin and Wnt signaling pathways in L-cells and in the intestines of db/db mice. However, this effect of metformin on GLP1 was blocked by β-catenin and Irs2 gene depletion, and/or PI3K inhibitor, and interestingly β-catenin nuclear translocation by metformin was offset by Irs2 gene depletion. In this regard, GLP1 enhancement by metformin occurred through β-catenin and its upstream insulin signaling, and metformin increased β-catenin nuclear translocation and the transcription of

<table>
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<th>Description</th>
<th>Fold change</th>
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<td>2.22 1.42</td>
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Table 1 Gene expression profiles in L-cells exposed to 0.5 or 2 mM metformin. The fold changes are represented as the mean gene expression ratios from each of two independently repeated microarray experiments comparing cells treated with metformin (0.5 or 2 mM).
Figure 4
Glucose utilization and calcium mobilization play primary roles in activated insulin signaling by metformin. Human NCI-H716 intestinal L-cells and hyperglycemic db/db male mice were treated with metformin (0.25, 0.5 mM, and/or 12.5 mg/kg body weight) for 24 h and 2 months, respectively. (A) Protein expression levels of insulin signal molecules and phosphorylation levels of GSK3β and CAMK2 and CREB in L-cells and intestinal tissue of db/db mice treated with metformin. (B) Glucose uptake using the non-metabolizable glucose analog, 2-NBDG, into L-cells treated with metformin. 2-NBDG is a fluorescent glucose analog that has been used to monitor glucose uptake in live cells and the fluorescence intensity was determined by FACS analysis. (C) Intracellular ATP concentrations using a luciferin/luciferase method. ATP was extracted from TCA-lysed L-cells and intestinal tissue of db/db mice treated with metformin. (D) Influx of calcium into L-cells treated with metformin and inhibition of the flow of calcium by nifedipine, an L-type calcium channel blocker. Intracellular calcium was stained with green fluorescent Fluo-4 dye (green arrow); original magnification, ×400. The number of cells, stained with Fluo-4 or Dapi, was quantified using the Image J program. Calcium influx was expressed as the ratio (as a percentage) of the number of cells stained with Fluo-4/the number of cells stained with Dapi. (E) Relative mRNA expression levels of SGLT1 and protein expression levels of Hur protein and SGLT1 in L-cells and intestinal tissue of db/db mice treated with metformin. (F) Activity of complex I in L-cells and intestinal tissue of db/db mice treated with metformin. (G) GLP1 levels after application of nifedipine, LY294002, and SGLT1 siRNA and the magnitude of SGLT1 knockdown after 48 h transfection of SGLT1 siRNA. Data are expressed as mean ± S.E.M. (n = 3–5). *Mean values with dissimilar superscript letters were significantly different between the groups (Duncan’s test; P < 0.05). †Significant differences between two groups (Student’s t-test; P < 0.05).
production through cooperation with Wnt signaling pathways (Yi et al. 2008) and metformin activates the insulin signaling pathways such as IRS2 and PI3K in the hepatocytes, granulosa cells, and C2C12 myotubes (Kumar & Dey 2002, Gunton et al. 2003).

On the other hand, research into the action of metformin on the insulin signaling pathway has been focused on IRS1/2 and/or PI3K. However, we found a new pathway, i.e., glucose utilization and calcium mobilization. Metformin therapy has long been known to lower post-prandial blood glucose concentrations via inhibition of intestinal glucose absorption, but this hypothesis has been disputed (Jackson et al. 1987). In our study, metformin increased the intestinal absorption of glucose by increasing the expression of SGLT1, an energy-dependent glucose transporter in the brush border membrane of the lumen. Increased SGLT1 expression may be associated with an

**Table 2** Biochemical parameters involved in glucose homeostasis. Insulin, C-peptide, and glucagon were measured in sera from db/db mice (n=3) after overnight fasting. Data are expressed as mean ± S.E.M. (n=3–5)

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<th>C-peptide (ng/ml)</th>
<th>Glucagon (ng/ml)</th>
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<tr>
<td>Met (12.5 mg/kg BW)</td>
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*Significant differences between two groups (Student’s t-test; P < 0.05).

the Wnt-responsive gene Glu, a precursor of GLP1, through the activation of the insulin signaling pathway (IRS2/phospho-PI3K/phospho-PKB) followed by the inhibition of GSK-3β.

These findings are consistent with recent studies, which showed that insulin enhances intestinal GLP1

**Figure 5**
Schematic of potential GLP1 enhancement by metformin. Metformin (<1 mM and/or 12.5 mg/kg body weight) enhances glucose uptake via the SGLT1 transporter and concomitantly increases glucose utilization via upregulation of genes involved in glycolysis, the TCA cycle, and the electron transfer system, which closes the ATP-sensitive K⁺ channel and opens the L-type Ca²⁺ channel by membrane depolarization. Increased intracellular Ca²⁺ activates CAMK2 and CREB, thereby phosphorylating IRS2, and, in turn, PI3K, and PKB. These events lead to β-catenin nuclear translocation through inactivation of GSK3β. Nuclear combination of β-catenin with TCF7L2 stimulates transcriptional activity of Glu, a precursor of GLP1, and increases GLP1 production. SGLT1, sodium/glucose cotransporter; CAMK2, calcium-calmodulin dependent kinase 2; CREB, cAMP response element binding protein; IRS2, insulin receptor substrate 2; PI3K, phosphoinositide 3 kinase; PKB, protein kinase B; APC, adenomatous polyposis coli; GSK3β, glycogen synthase kinase 3; TCF7L2, transcription factor 7-like 2; β-TrCP, β-transducin repeat containing protein; LEF/TCF4, lymphoide enhancer factor/T-cell factor 4.
increase in Hur protein, as Hur protein has been demonstrated to bind to the UTR of SGLT mRNA and upregulate SGLT1 expression (Loflin & Lever 2001). In addition, our microarray data showed that metformin increased the expression of sodium–potassium ATPase, which might lead to a lower gradient of sodium ions in enterocytes and consequently stimulate the uptake of sodium ions by upregulating the expression of SGLT1. Interestingly, metformin increased the intestinal utilization of absorbed glucose, which was estimated by measuring intracellular ATP levels, through the upregulation of genes involved in glycolysis, the TCA cycle, and mitochondrial complex I. These results were in agreement with other studies which showed that metformin significantly increased SGLT1 (SLC5A1) gene expression as well as glucose utilization in the duodenum and jejunum (Lenzen et al. 1996) and metformin administration reversed the decreased activity of PFK, a key enzyme of glycolysis, in the skeletal muscle, liver, and adipose tissue of diabetic mice (Da Silva et al. 2010). However, metformin has been reported to suppress NADH-dependent dehydrogenase complex I activity, because it induces NADH-dependent lactic acidosis in cases of excessive dosage (Batandier et al. 2006, Piwkowska et al. 2010). Indeed, higher concentrations (1 and/or 2 mM) of metformin suppressed complex I activity (Supplementary Figure S4) but lower concentrations of metformin (0.5 mM and 12.5 mg/kg BW) did not. This discrepancy between dosages warrants further study.

An increase in ATP could close ATP-sensitive potassium channels and a subsequent membrane depolarization leads L-type calcium channels to open followed by calcium mobilization (Reimann et al. 2005, Bailey et al. 2008). The opening of L-type calcium channels was determined using nifedipine, an L-type calcium channel blocker. Addition of nifedipine to metformin reversed the increase in calcium levels in L-cells treated with metformin. Also, the calcium mobilization stimulated the phosphorylation of CAMK2 and CREB and the induction of IRS2 as shown in previous studies (Sheng et al. 1991, Hook & Means 2001). In this regard, glucose utilization and calcium mobilization might be the primary mechanism by which metformin activates insulin signaling pathways in the intestinal cells.

Metformin decreased blood glucose levels concurrently with the elevation of GLP1 and insulin levels. In addition, mice treated with metformin showed higher expression of GLP1 receptor in islets, and higher cAMP levels and phospho-CREB and PDX1 expression in the pancreas than mice not treated with metformin. These results are consistent with the evidence of other studies which showed that metformin enhanced the expression of the genes encoding the receptors for both GIP and GLP1 in mouse islets and also increased the effects of GIP and GLP1 on insulin secretion from β-cells (Maida et al. 2011). Thus, these results indicate the possibility that the blood glucose-lowering effects of metformin are associated with the elevated GLP1 levels, because GLP1 binds to the N-terminal extracellular region of the GLP1 receptor in β-cells (Montrose-Rafizadeh et al. 1999) increasing intracellular cAMP levels via adenylylate cyclase, and subsequently inducing the phosphorylation of CREB (Holz 2004) and the expression of PDX1 (Wang et al. 1999). Activation of this insulinotropic mechanism led to the elevation of insulin and C-peptide levels during fasting as well as glucose loading in mice treated with metformin twice daily for 4–5 days. Our findings could be interpreted as relevant to humans, as intestinal and/or oral metformin concentrations (0.25, 0.5 mM, and/or 12.5 mg/kg BW) are within the range recorded in the jejunum, which had about 30–300 times higher concentrations than plasma (8–24 μM), in patients who took metformin at the dosage of 850 mg once daily for 2–3 weeks (Tucker et al. 1981, Bailey et al. 2008) and the db/db mice have similar phenotypes to human type 2 diabetic patients.

Taken together, these results indicates that metformin seems to enhance GLP1 production and secretion via the cooperation between Wnt and its upstream insulin signaling pathways, and that glucose utilization and calcium mobilization play a prime role in the activation of insulin signaling pathways. Furthermore, metformin could exert glucoregulatory actions via the modulation of the incretin axis, which provides the novel function of metformin as a mediator of endocrine communication between the gut and pancreatic islets, and which could be a novel target for drug development.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-13-0381.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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