Glucose-dependent insulinotropic peptide: differential effects on hepatic artery vs. portal vein endothelial cells

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Glucose-dependent insulinotropic peptide (GIP) has been reported to have opposing effects on splanchnic blood flow, GIP infusion in dogs results in an increase in portal vein circulation but a drop in hepatic artery blood flow. In an effort to evaluate whether these different responses were related to intrinsic differences in GIP effects, we isolated canine hepatic artery (HAEC) and portal vein endothelial cells (PVEC). We report that there are differences in GIP activation of the signal transduction pathways in these two cell types. GIP stimulates secretion of endothelin-1 (ET-1), a potent vasoconstrictor, from HAEC (EC50 0.28 nM) but not from PVEC. This effect could be abolished by preventing a rise in intracellular calcium, demonstrating the calcium dependence of GIP-induced ET-1 secretion from HAEC. The GIP effect was specific, as a GIP receptor antagonist blocked it. In contrast, GIP stimulated nitric oxide production from PVEC (EC50 0.09 nM) but not from HAEC. Taken together, our data demonstrate distinct differences in GIP effects, we isolated canine hepatic artery (HAEC) and portal vein endothelial cells (PVEC). We conclude that differences in GIP effects, we isolated canine hepatic artery (HAEC) and portal vein endothelial cells (PVEC). Our present results demonstrate that multiple GIP receptor splice variants exist, that these GIP receptor splice variants differ between endothelial cells from different vascular beds, and that these various receptor splice variants could be coupled to different signal transduction pathways (29). Binding to the GIP receptor is known to increase cAMP and intracellular calcium and more recently has also been found to increase the production of arachidonic acid derivatives (6, 26, 29).

Our laboratory was the first to report that multiple GIP receptor splice variants exist, that these GIP receptor splice variants differ between endothelial cells from different vascular beds, and that these various receptor splice variants could be coupled to different signal transduction pathways (29). Binding to the GIP receptor is known to increase cAMP and intracellular calcium and more recently has also been found to increase the production of arachidonic acid derivatives (6, 26, 29).

Our laboratory has recently reported that there are differences in functional links between the GIP receptors and secretion of bioactive peptide hormones. In particular, we found that GIP could stimulate endothelin-1 (ET-1) secretion from human umbilical vein endothelial cells but not from an immortalized endothelial cell line, ECV 304 (5).

In an attempt to shed more light on the mechanism responsible for the opposing effects on blood flow seen by GIP infusion, we made a preparation of freshly isolated canine hepatic artery endothelial cells (HAEC) and canine portal vein endothelial cells (PVEC). Our present results demonstrate that GIP stimulates ET-1 secretion from HAEC but not from PVEC, whereas GIP stimulates nitric oxide production from PVEC but not from HAEC. These opposing effects of GIP on the production of vasoactive peptides may explain the previous observations of GIP-induced vasoconstriction in one vascular bed while inducing vasodilatation in another vascular bed.

MATERIALS AND METHODS

Materials. Human GIP-(1–42) was purchased from Bachem (Torrance, CA). Fura 2-AM and EGTA-AM were purchased from Molecular Probes (Eugene, OR). Forskolin was purchased from Calbiochem (San Diego, CA). The GIP antagonist GIP-(7–30) was synthesized at the Medical College of Georgia peptide synthesis core facility.

Cell culture. For these studies, we used primary cultures of dog hepatic artery endothelial cells (HAEC) and canine portal vein endothelial cells (PVEC). Our present results demonstrate that GIP stimulates ET-1 secretion from HAEC but not from PVEC, whereas GIP stimulates nitric oxide production from PVEC but not from HAEC. These opposing effects of GIP on the production of vasoactive peptides may explain the previous observations of GIP-induced vasoconstriction in one vascular bed while inducing vasodilatation in another vascular bed.

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mg/ml dispase II and 4 mg/ml BSA. After digestion, the lumina of the vessels were flushed with HBSS, and the endothelial cells obtained were centrifuged, washed, and plated in medium 199 (M-199) supplemented with 10% fetal bovine serum, 5% bovine calf serum, 16 mg/l thymidine, 150 mg/l crude endothelial cell growth factor, 15,000 U/l heparin, 100 U/ml penicillin, and 100 mg/ml streptomycin. The vascular endothelial cells thus obtained were characterized as endothelial cells by their cobblestone monolayer appearance, angiotsin-converting enzyme activity, uptake of acetylated low-density lipoprotein, and expression of von Willebrand factor (25). Endothelial cells were seeded at passages 2–8.

Preparation of RNA and Southern blot. Total RNA was extracted from cells by use of TRIzol (Invitrogen Life Technologies). RNA was stored at −70 °C until use. A coupled reverse transcriptase PCR (RT-PCR) was utilized. The sense (CTGCCGTGCGCAAAGCGCCAGAT) and antisense (GCGAGCCAGCTCAGCGGTATA) oligonucleotide primers for the GIP receptor were synthesized and used as previously described (29). The primers were based on conserved sequences in the human, mouse, and rat GIP receptors. cDNA from endothelial cells was converted to cDNA via reverse transcription using the ThermoScript RT-PCR System (Invitrogen). The resulting cDNA was amplified by PCR using 0.05 U/ml Tag polymerase (Promega), 200 μM dNTP, and 0.2 μM of sense and antisense primers for the GIP receptor in a thermocycler (Perkin Elmer-Cetus). PCR products were gel-purified and transferred overnight to Hybond nylon membrane (Amersham Pharmacia Biotech). Membranes were probed for the GIP receptor by means of a cloned PCR fragment amplified with the GIP receptor primers and verified by sequence analysis. The probe was labeled with 32P by random primer labeling (Amersham Pharmacia Biotech).

Intracellular calcium measurements with fura 2. Intracellular calcium measurements using fura 2 were made as previously described (11). Briefly, endothelial cells grown in 75-cm² flasks were loaded with the calcium-sensitive dye fura 2-AM in Krebs-Ringer bicarbonate (KRB) buffer for 45 min at room temperature. The KRB composition was as follows (in mM): 118 NaCl, 25 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.25 CaCl2, and 10 glucose. The pH of the buffer was adjusted and maintained at 7.4 with 97% O2-3% CO2. The cells were then centrifuged and resuspended in KRB and placed in a cuvette in a dual-wavelength spectrophotometer (Photon Technologies International, South Brunswick, NJ). Fluorescence was measured using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Autofluorescence was measured in unloaded cells, and this value was subtracted from all the measurements.

Cellular cAMP assay. Experiments were performed as previously described (2). Briefly, confluent endothelial cells, in 6-well plates, were washed twice with M-199 and incubated in 1 ml of the same medium for 15 min at 37°C. The phosphodiesterase inhibitor IBMX (0.1 mM) was added and incubated for an additional 10 min. The test agents were then added and incubations continued for 10 min. Reactions were stopped by replacement of 1 ml of ice-cold 5% (wt/vol) trichloroacetic acid (TCA) and left on ice for 15 min, and then the cell extract was collected. A 100-μl portion of the sample was taken, and after appropriate dilution, cAMP levels were assayed by commercially available radioimmunoassay kits (Amersham Pharmacia Biotech, Piscataway, NJ).

Nitric oxide determination. Confluent endothelial cells in 6-well plates were plated in 6-well plates and placed in 6-well plates, in 6-well plates, placed in 0.1% fetal calf serum overnight before use, and then incubated for an additional 20 h with the appropriate agonist. For experiments utilizing the GIP antagonist, GIP and the antagonist were added at the same time. For experiments utilizing ET-1, AM, cells were incubated with ET-1 AM (1 or 10 μM) for 10 min, and cells were then washed and incubated with GIP for 20 h. The cell culture supernatants were measured with a commercially available RIA kit (Peninsula Labs, San Carlos, CA).

Results. Results are expressed as means ± SE. Experiments were performed in triplicate except where noted. Data were analyzed using either ANOVA with Bonferroni post hoc testing or unpaired t-tests using a commercial statistical package (Instat; Graphpad, San Diego, CA).

RESULTS

GIP receptor is present in canine HAEC and PVEC. Although we had previously documented the presence of the GIP receptor in all endothelial cells tested (29), dog endothelial cells were not among those we had examined. Initial experiments focused on whether canine HAEC and PVEC do in fact contain the GIP receptor. As shown in Fig. 1, GIP receptor mRNA could be demonstrated in both HAEC and PVEC by coupled reverse transcription-PCR and confirmed by sequence analysis and Southern analysis. Shown as a control is the human GIP receptor from the immortalized human umbilical vein endothelial cell line ECV 304. Even though the dog GIP receptor has not been cloned, it appears to be similar to the human GIP receptor; thus the RT-PCR assay was based on the sequence of the human GIP receptor. Sequence analysis of the amplified canine GIP receptor fragment shows 98% homology between the two receptors in the region of the RT-PCR assay. As previously described (29), a number of additional splice variants were observed in addition to the predicted wild-type receptor. The specific alternatively spliced products obtained varied on occasion from sample to sample, but the most representative pattern is depicted in Fig. 1. It is as yet unclear to what extent the alternative splicing contributes to the observed effects on GIP action. To determine whether the signal transduction pathways for GIP were different in these two cell types, we first examined the effect of GIP on intracellular calcium.

GIP stimulates an increase in intracellular calcium in HAEC but not PVEC. As a G protein-coupled seven-transmembrane receptor, the GIP receptor can be coupled to both the phosphoinositol-intracellular calcium and the cAMP signal transduction pathways. Previous experiments from our laboratory (29) showed that there were differences in the magnitude of change of intracellular calcium between endothelial cells from umbilical vein and pulmonary artery in response to GIP. As shown in Fig. 2, GIP at concentrations between 10−11 and 10−7 M increased intracellular calcium over baseline. In con-
Fig. 1. Glucose-dependent insulinotropic peptide (GIP) receptor is present in both hepatic arterial (HAEC) and portal vein endothelial cells (PVEC) by Southern blot analysis. mRNA was extracted from dog PVEC and HAEC. Samples were PCR amplified and reverse transcribed (RT-PCR) and analyzed by Southern blot. As shown, both PVEC and HAEC contained a transcript of the appropriate size compared with a positive control (human umbilical vein endothelial cell line ECV 304). Shown is a representative blot of 3 different experiments.

Fig. 2. GIP increases intracellular calcium in HAEC but not in PVEC. HAEC or PVEC were loaded with the calcium-sensitive probe fura 2. Cells were stimulated with increasing concentrations of GIP and changes in the fluorescence ratio (340/380) measured using a dual-photon spectrofluorometer (A). GIP increased intracellular calcium in HAEC (B) but not in PVEC (C). Results are expressed as means ± SE of the 340/380 ratio of 3 different experiments/data points.
GIP has no effect on cAMP production in either HAEC or PVEC. Because GIP has been reported to increase cAMP in various different cell types, we examined GIP’s effects on cAMP production. As shown in Fig. 3, GIP did not significantly increase cAMP levels. Interestingly, basal levels of cAMP were higher in PVEC. As a positive control, we used forskolin, an activator of adenylate cyclase, which increased cAMP levels between 30- and 50-fold, demonstrating the viability of these cells. In an attempt to determine whether the differential response to GIP in the contractile state of the portal vein vs. the hepatic artery related to differences in GIP stimulation of vasoactive substances, we examined GIP’s effect on the production of nitric oxide.

GIP stimulates nitric oxide production in PVEC but not HAEC. Nitric oxide is a potent vasodilator released from the intact endothelium to induce relaxation in the underlying smooth muscle (22). We examined the ability of GIP to stimulate nitric oxide production from HAEC or PVEC. As seen in Fig. 4, GIP stimulated nitric oxide production from PVEC but not HAEC. GIP’s effect on nitric oxide production

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**Fig. 3.** GIP has no effect on changes in cellular cAMP content in either HAEC or PVEC. HAEC or PVEC were stimulated with increasing concentrations of GIP. There was no statistically significant change in cAMP in either HAEC or PVEC in response to GIP. In contrast, the positive control forskolin (10 μM) significantly increased cAMP in both HAEC and PVEC. Results are expressed as cellular cAMP content and are means ± SE of 3 different experiments (*P < 0.0001 vs. control).

**Fig. 4.** GIP increases nitric oxide (NO) production from PVEC but not from HAEC. HAEC or PVEC were stimulated with increasing concentrations of GIP, and NO production was measured. GIP at a concentration of 10^{-10} M and above had a statistically significant effect on NO production from PVEC but not from HAEC. Shown are means ± SE of 9 different experiments (*P < 0.01 vs. control).

**Fig. 5.** GIP stimulates endothelin (ET)-1 secretion from HAEC but not from PVEC. HAEC (A) or PVEC (B) were stimulated with increasing concentrations of GIP and ET-1 release into the medium measured after 20 h of stimulation. Shown are means ± SE of 9 different experiments (*P < 0.05; **P < 0.01 vs. control).
from PVEC became statistically significant at GIP concentrations of \(10^{-10}\) M or higher. In contrast, even at concentrations as high as \(10^{-7}\) M, GIP had no effect on nitric oxide production from HAEC. These experiments examined GIP’s effects on the vasodilator nitric oxide. Thus we next wished to examine GIP effects on ET-1, a potent vasoconstrictor.

**GIP increases ET-1 secretion in HAEC but not PVEC through a calcium-mediated mechanism.** ET-1 is a potent and long-lasting vasoconstrictor secreted from endothelial cells in response to a large number of factors, including cytokines, peptide hormones, stretch, and shear stress (20). Both HAEC and PVEC were stimulated with increasing concentrations of GIP (Fig. 5). As shown, GIP significantly stimulated ET-1 secretion from HAEC at concentrations of \(10^{-10}\) M or higher (Fig. 5A). The effect of GIP on ET-1 secretion at the higher concentrations was comparable to that of leptin (\(10^{-7}\) M GIP: 3.05 ± 0.23 ng/ml; 500 ng/ml leptin: 3.18 ± 0.33 ng/ml), a known stimulator of ET-1 secretion from endothelial cells. In contrast, GIP had no significant effect on ET-1 secretion from PVEC (Fig. 5B). To demonstrate that GIP’s effect on ET-1 secretion from HAEC was a specific effect, we added GIP (100 nM) to HAEC in the presence or absence of a GIP receptor blocker, GIP-(7–30). As shown in Fig. 6A, GIP-(7–30) at a concentration of \(10^{-6}\) M completely abolished the GIP effect on ET-1, demonstrating the specificity of the effect.

To further define the underlying mechanism for the GIP effect on ET-1 secretion, we utilized the cell-permeant form of the calcium-binding agent EGTA. Upon entering the cell, the ester form of EGTA is cleaved and trapped inside the cell. EGTA-AM abolishes the GIP-induced elevation in intracellular calcium and, at both \(10^{-6}\) and \(10^{-5}\) M, significantly inhibits GIP-induced secretion of ET-1 from HAEC (Fig. 6B). These data suggest that GIP-induced elevations in intracellular calcium are responsible for GIP-stimulated ET-1 secretion.

We (5) have previously shown that GIP stimulates \(^3\text{H}\)thymidine incorporation in human umbilical vein endothelial cells through both ET-1-dependent and -independent mechanisms. Thus we next examined GIP’s effects on the proliferative response of both HAEC and PVEC.

**GIP stimulates \(^3\text{H}\)thymidine incorporation in both HAEC and PVEC.** For these experiments, \(^3\text{H}\)thymidine incorporation was used as an index of the proliferative response. HAEC and PVEC were stimulated with increasing concentrations of GIP, and \(^3\text{H}\)thymidine incorporation was measured. Shown are means ± SE of 9 different experiments. (*\(P < 0.01\) vs. control).

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**Fig. 7. GIP stimulates \(^3\text{H}\)thymidine incorporation in both HAEC and PVEC.** HAEC or PVEC were stimulated with increasing concentrations of GIP, and \(^3\text{H}\)thymidine incorporation was measured. Shown are means ± SE of 9 different experiments. (*\(P < 0.01\) vs. control).
HAEC. For both HAEC and PVEC, GIP’s effect reached a plateau and did not increase further with higher doses of GIP.

**DISCUSSION**

The data presented here demonstrate that GIP differentially regulates the signal transduction pathways and hormonal secretion from two different endothelial cell types. We report that GIP stimulates intracellular calcium release and ET-1 secretion from HAEC but not from PVEC. In contrast, GIP was found to stimulate nitric oxide production from PVEC but not from HAEC.

The GIP receptor belongs to the seven-transmembrane domain family of receptors and is widely distributed in the body (26). The GIP receptor is linked to both the cAMP and the phosphoinositol signal transduction pathways (8, 21). However, we (29) have previously shown that the GIP receptor can be differentially linked to one signal transduction pathway or the other. In the present study, we find that GIP increases intracellular calcium in HAEC but not in PVEC. Curiously, however, GIP did not increase cellular cAMP content in either endothelial cell type. This may be related to a coupling of the GIP receptor in PVEC different from the traditional signaling pathways.

GIP is known to play a major role in modulating insulin secretion (8). However, it is clear that GIP has multiple other effects, since its receptor is present in many tissues, including brain, endothelium, intestine, pancreas, adrenal, and bone (3, 26). GIP levels rise rapidly after a meal and remain above fasting values throughout the day. Basal GIP concentrations vary between 0.06 and 0.1 nM and increase to between 0.2 and 0.5 nM postprandially (9, 10, 12, 17, 18, 23). These concentrations are in the range of GIP concentrations found to activate the GIP receptor. GIP probably plays a role as an integrative hormone: it coordinates nutrient absorption and utilization in different tissues in the body (4, 8).

In a study by Kogire et al. (16), the authors demonstrated that infusion of GIP in conscious dogs (at concentrations of 1, 100, or 500 pmol/kg) resulted in a dose-dependent increase in portal vein blood flow (7, 15, and 46% of basal blood flow). The increased mesenteric arterial blood flow was associated with a decrease in vascular resistance. In contrast, hepatic artery blood flow was decreased in a dose-dependent manner (17, 21, and 35% of basal blood flow) at the same GIP concentrations. These results on blood flow with GIP infusion are distinct from the effects of glucagon infusion. Even though the glucagon receptor belongs to the same receptor family as GIP, activation of this receptor in the gut leads to an increase in blood flow in the portal vein but does not change hepatic artery blood flow (16). These findings highlight the fact that GIP plays a very specific and distinct role in modulating postprandial intestinal blood flow.

A key element in nutrient utilization is changes in splanchnic blood flow. Nitric oxide is a potent, short-lived vasodilator that clearly plays an important role in regulating vascular tone. Physiologically, intestinal blood flow is known to change in response to a meal. To maximize nutrient absorption, splanchnic blood flow increases. Nitric oxide appears to play an important role in regulating both basal and postprandial splanchnic blood flow (1). In contrast to the vasodilatory effect of a meal ingestion on splanchnic blood flow, blood flow in some vascular bed decreases after a meal. ET-1 is a potent vasoconstrictor released from endothelial cells in response to multiple signals. ET-1 levels appear to be increased after a meal; thus ET-1 is the most likely hormone responsible for hepatic artery vasoconstriction (19). Interestingly, changes in intracellular calcium are known to stimulate ET-1 release. Therefore GIP’s ability to increase intracellular calcium in HAEC but not in PVEC (Fig. 2) may account for the fact that GIP stimulates ET-1 release in HAEC but not in PVEC.

The seemingly disparate effects of GIP on vasodilator release from PVEC and vasoconstrictor release from HAEC would be consistent with what occurs postprandially in vivo, where splanchnic blood flow increases while the blood flow in other vascular beds decreases. The mechanism responsible for the different effects of GIP on these two endothelial cell types is not clear. However, in view of the different effects of GIP on intracellular calcium release in HAEC vs. PVEC, there may be differences in G protein coupling of the receptor to the signal transduction pathways. There is a precedent for this in another member of this receptor family, the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor. Splice variants of the PACAP receptor have been found to be differentially linked to either the cAMP or the phosphoinositol signal transduction pathways (24).

In summary, the present study demonstrates that GIP stimulates release of the vasoconstrictor ET-1 from HAEC while simultaneously stimulating release of the vasodilator nitric oxide from PVEC. The differential effects of GIP on these two endothelial cell types appear to account for previous observations where GIP infusion resulted in increased blood flow in one vascular bed but not another (16).

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