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Glucose-dependent insulinotropic polypeptide stimulates the release of a spectrum of biologically active proteins in human endothelial cells

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Objectives: Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) stimulate the release of insulin and exert a variety of cardiovascular effects. The beneficial effects of GLP-1 receptor agonists have been demonstrated. However, because the effects of GIP have not yet been well elucidated, its receptor agonists remain unavailable for clinical use. We investigated whether or not GIP augments the release of biologically active proteins from vascular endothelial cells and compared its effects with those of GLP-1.
Methods: GIP receptor expression and GIP-induced intracellular free calcium ($[Ca^{2+}]_i$) changes were examined in human aortic vascular endothelial cells (HAoECs). HAoECs were incubated with or without GIP or GLP-1, and the conditioned media were used for the protein array of 105 human cytokines and chemokines.
Results: HAoECs expressed GIP receptor mRNA and increased $[Ca^{2+}]_i$ in response to GIP. The presence of GIP resulted in an enhanced release of a variety of proteins, e.g., endoglin, angiogenin, SDF-1α (stromal cell-derived factor-1α), interleukin-17A, angiopoietin-2, interleukin-6, and DKK-1 (dickkopf-related protein-1). The spectra of biologically active proteins, whose release was stimulated by GIP or GLP-1, were similar; and the magnitudes of their increased release, as stimulated by GIP or GLP-1, were comparable.
Conclusions: GIP and GLP-1 stimulate the release of various cytokines, chemokines, and proangiogenic/growth-promoting factors from HAoECs suggesting their mediatory roles in incretin-induced endothelial function.

Key words: GIP, GLP-1, cytokines, chemokines, human aortic vascular endothelial cells, HAoECs

Introduction

Glucose-dependent insulinotropic polypeptide (GIP) is an incretin peptide secreted from duodenal and small intestinal K-cells upon nutrient ingestion[1,2] and promotes the glucose-dependent release of insulin after binding to its receptors on pancreatic β-cells.[3,4] Accumulating evidence has indicated the potent insulin-releasing and extra-pancreatic glucoregulatory activities of both GIP and glucagon-like peptide-1 (GLP-1), which is also an incretin.[1,2,5] The actions of GIP, via extra-pancreatic receptors,[6] mediate the development of obesity and the pathogenesis of cardiovascular disease,[7] demonstrating a potential role in the GIP/GIP receptor axis in atherosclerotic cardiovascular disease.[8-10] However, the beneficial effects of GLP-1 surpass those of GIP when administered to healthy participants and patients with type 2 diabetes.[11,12] Moreover, the clinical benefits of GLP-1 receptor agonists have already been established in a number of large-scale clinical trials,[13,14] whereas the GIP receptor agonist has not yet been marketed as a therapeutic reagent.

The expression of GLP-1 and GIP receptors are distinctly distributed in a number of extra-pancreatic organs,[6,15] suggesting that GLP-1 and GIP may have their respective physiological activities. Their cardiovascular effects are reportedly mediated via receptors expressed in vasculature.[9,16,17] The activation of the GIP receptor reportedly increases intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and cAMP (cyclic adenosine monophosphate)

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levels;[6,15] however, the direct effects of GIP on vascular endothelium remain relatively poorly understood. GIP stimulates the release of vasoactive substances in a cell-type-dependent manner.[18-21] On the other hand, GLP-1 may show counter-regulatory actions in protecting endothelial cells via nitric oxide synthase activation[16,22-24] and has been reported to upregulate vascular endothelial growth factor (VEGF).[24] The biologically active proteins involved in cardiovascular pathophysiology are induced by GIP and GLP-1 in human aortic endothelial cells (HAoECs).

We aimed to investigate the spectrum of endogenous cytokines, chemokines, and growth/angiogenesis-related factors released from human arterial endothelial cells in response to the presence of GIP and to compare the effects of GIP with those of GLP-1.

Materials and Methods

Cell culture

HAoECs purchased from Promo Cell (Heidelberg, Germany) were cultured in Endothelial Cell Basal Medium MV2 (PromoCell) supplemented with SupplementPack Endothelial Cell GM MV2 (PromoCell) in an atmosphere with 5% CO_2 at 37℃, and a passage number of P4－P5 was used.

Real-time RT-PCR

HAoECs cultured on a 6-well plate were washed twice with phosphate-buffered saline and dissolved in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted using the AGPC (acid guanidinium thiocyanate-phenol-chloroform extraction) method and reverse-transcribed into cDNA using PrimeScript RT Master Mix (Takara Bio, Shiga).[25] Human GIP receptor (GIPR) and ACTB mRNAs were amplified using synthetic oligomers as probes (GIPR forward and reverse: 5'-ACGTCTGCTGGGACTATGCT-3' and 5'-CGAAACCTGCAGCCACAT-3', respectively; Actb forward and reverse: 5'-ATTGGCAATGAGCGGTTC-3' and 5'-GGATGCCACAGGACTCCA-3', respectively, [Eurofins Genomics, Tokyo]) and were detected and quantified using C1000 Touch Thermal Cycler and CFX Real-Time PCR Detection Systems (Bio-Rad, Hercules, CA, USA) as previously described.[26] The amplification mixture contained the template cDNA and primer DNA in KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Cape Town, South Africa). After the initial denaturation of the template DNA at 94℃ for 3 minutes, the following PCR conditions were performed for a total of 40 cycles: denaturation at 94℃ for 10 seconds and annealing-extension at 60℃ for 30 seconds.

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

The $[Ca^{2+}]_i$ was measured as previously described[27,28] with the following modifications. HAoECs were seeded in a 96-well black plate with clear bottom and incubated for 48 hours at 37℃. Then, the cells were incubated for 30 minutes at 37℃ with a fluorescent loading solution prepared using Calcium Kit-Fluo 4 (Dojindo Laboratories, Kumamoto) dissolved in Hank's Balanced Salt Solution (HBSS, Nacalai Tesque, Kyoto). Afterwards, the cells were washed twice with HBSS to remove excess fluorescent dye, and the indicated concentrations of GIP were added. Fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 528 nm was measured over time using a Powerscan HT microplate reader (BioTek Instruments, Winooski, VT, USA). The overall significant difference was analyzed using repeated measures ANOVA, and multiple comparisons were performed using the Bonferroni's post hoc test and GraphPad Prism 5 (La Jolla, CA, USA). Statistical data are presented as mean ± standard error of the mean (SEM).

Antibody array analysis

The growth medium of HAoECs seeded in a 6-well plate and incubated for 48 hours at 37℃ was replaced with 2 ml fresh medium and overlaid with or without GIP or GLP-1 at a final concentration of 10-7 M. The cells were further incubated for 30 hours, and 500 μl culture supernatant was evaluated using Human XL Cytokine Array Kit (ARY022B, R&D Systems, MN, USA). The expression analysis of 105 proteins was performed according to the manufacturer's instructions. Signals were detected using ImageQuant LAS 4000 (GE Healthcare, Arlington Heights, Il, USA),[25] and signal intensity was quantified using ImageJ software (http://rsb.info.nih.gov/ij/).[29] The average intensity values of duplicate spots compared to those obtained from the supernatant of vehicle-treated cells are indicated as relative pixel density.[26]

Results

We first assessed the expression of GIP receptor gene in HAoECs. cDNA samples were prepared from six independent cell cultures and subjected to real-time RT-PCR. The target amplification was first detected at 29.91 ± 0.05 cycles and followed by exponential amplification phase, demonstrating the accumulation of PCR products (Figure 1A). To ensure assay specificity and exclude the

generation of undesired PCR products, post-amplification melting curve analysis was performed. The change in fluorescence divided by the change in temperature plotted against temperature revealed the reaction with the correct PCR product (Figure 1B). Cellular response to GIP was assessed by detecting changes in $[Ca^{2+}]_i$ in HAoECs cultured in Endothelial Cell Basal Medium MV2 supplemented with SupplementPack Endothelial Cell GM MV2 because HAoECs are vulnerable to serum and growth factor deprivation. The presence of GIP exerted a sustained increase in $[Ca^{2+}]_i$ in HAoECs, and its effects were concentration dependent (10^{-8} — 10^{-7} M) (Figure 2).

To investigate whether GIP stimulates the release of biologically active proteins from vascular endothelial cells, we incubated HAoECs with or without 10^{-7} M GIP, and the conditioned media were collected and used for the protein array of 105 human cytokines and chemokines (Figure 3A). The relative signal intensities of a variety of cytokines whose release was stimulated by GIP (Figure 3C) compared to those of vehicle-treated control HAoECs (Figure 3B) were quantified. The presence of GIP resulted in an increased secretion of a variety of cytokines and chemokines, including endoglin, angiogenin, stromal cell-derived factor-1α (SDF-1α), interleukin (IL)-17A, growth/differentiation factor (GDF)-15, angiopoietin-2, IL-6, dickkopf-related protein-1 (DKK-1), and

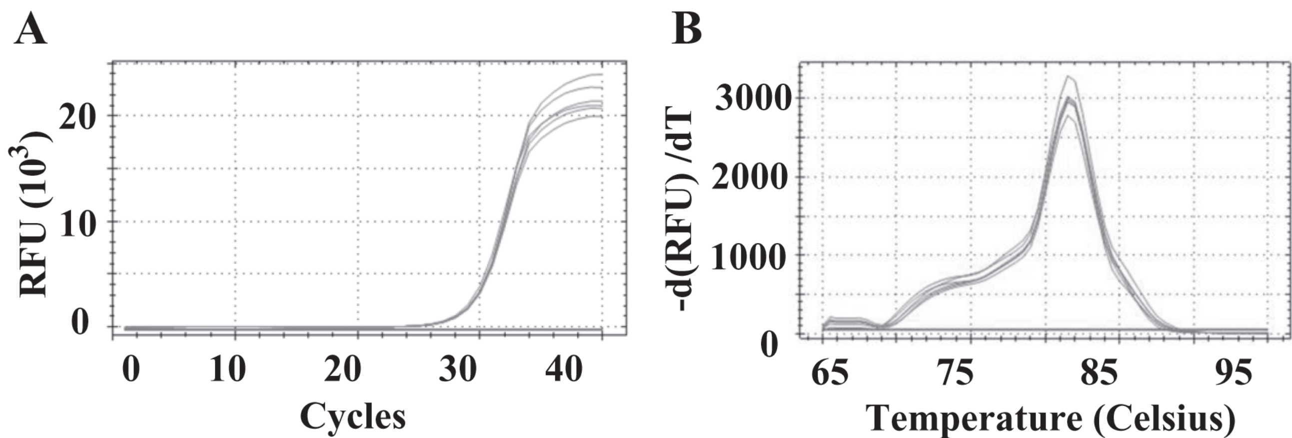


Figure 1. Real-time RT-PCR analysis of GIP receptor levels. (**A**) Amplification plot shows the accumulation of product over the duration of the real-time RT-PCR assay (n = 6). The charts were generated by plotting relative fluorescence unit (RFU) against cycle number. (**B**) Melting curve analysis. The dissociation temperature range extends from 65℃ to 95℃. The lines indicate fluorescence change with respect to the derivative of the temperature (-d[RFU]/dT) during the heating process (n = 6).

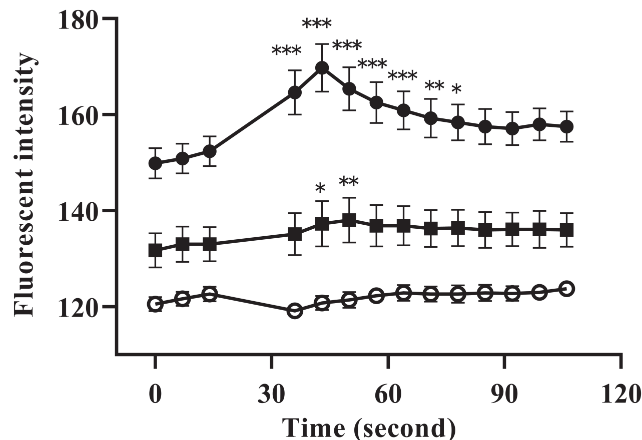


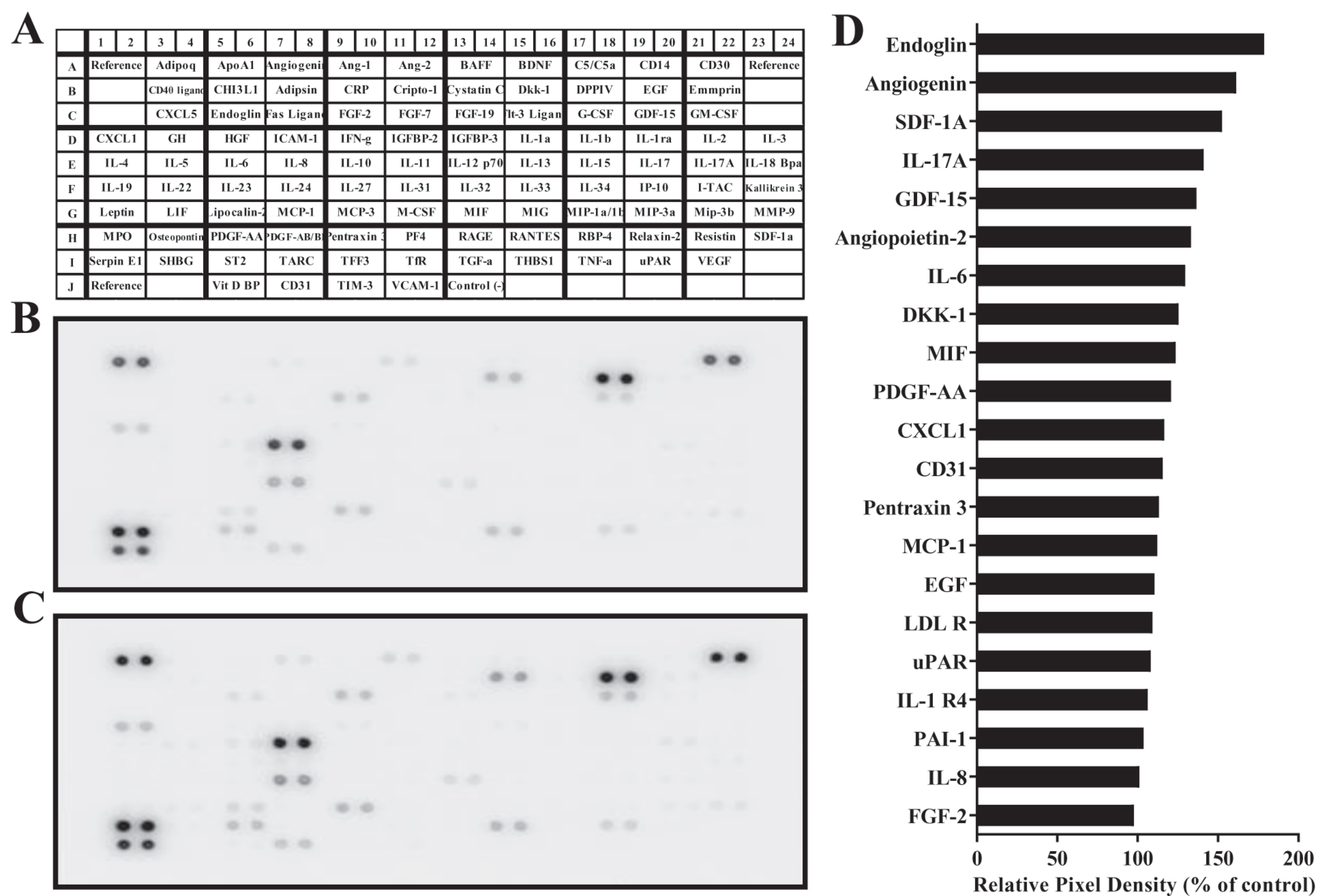
Figure 2. GIP induces changes in $[Ca^{2+}]_i$. Subconfluent culture of Fluo 4-loaded HAoECs were incubated with (closed circles: 10^{-7} M GIP, closed squares: 10^{-8} M GIP) or without (open circles) GIP, and changes in the fluorescence intensity were monitored (n = 8). Statistical data are presented as mean ± standard error of the mean (SEM). *P < 0.05, **P < 0.01, ***P < 0.001, vs. baseline.

macrophage migration inhibitory factor (MIF) (Figure 3D). To compare the spectra of proteins and magnitudes of protein release implicated by the presence of GLP-1 or GIP, we treated HAoECs with 10^{-7} M GLP-1 or GIP and assessed the release of cytokines and chemokines. GLP-1 stimulated the release of endoglin, IL-17A, angiogenin, SDF-1α, IL-6, DKK-1, GDF-15, CXCL1 (C-X-C motif chemokine ligand 1), angiopoietin-2 and other proteins (Figure 4D). The spectra of cytokines and chemokines whose release from HAoECs was stimulated by GIP and GLP-1 were highly similar despite the two peptides having different amino acid sequences and acting on different cell surface receptors.

Discussion

Because the GIP receptor has been shown to be expressed in many endothelial cells,[30] we first confirmed whether primary HAoECs, originally isolated from the thoracic and abdominal aorta, express the GIP receptor and show typical intracellular signaling that endothelial cells display in response to GIP. Real-time RT-PCR analysis revealed the expression of GIP receptor mRNA in HAoECs. The melting curve analysis showed that the probe we used bound to a complementary target, whereas the threshold cycles of 29.91 ± 0.05 were comparable with many other functioning receptor genes in a number of cultured cells.[31,32] Addition of GIP to cultured HAoECs resulted in an increased $[Ca^{2+}]_i$. The effect was evident at 10^{-8} and 10^{-7} M of GIP even though the experiments were performed using growing HAoECs supplemented with full growth media, in which conditions mitogenesis-related receptors were not maximally upregulated. Because HAoECs are vulnerable to serum/growth factor deprivation, we performed all experiments using growing HAoECs. These results suggest the expression of GIP receptor and presence of downstream intracellular signaling cascade in HAoECs.

A growing body of evidence indicates the beneficial role of GLP-1 receptor agonists in treating type 2 diabetes



	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18	19 20	21 22	23 24
A	Reference	Adipoq	ApoA1	Angiogeni	Ang-1	Ang-2	BAFF	BDNF	C5/C5a	CD14	CD30	Reference
B		CD40 ligand	CHI3L1	Adipsin	CRP	Cripto-1	Cystatin C	Dkk-1	DPPIV	EGF	Emmprin	
C		CXCL5	Endoglin	Fas Ligand	FGF-2	FGF-7	FGF-19	Flt-3 Ligan	G-CSF	GDF-15	GM-CSF	
D	CXCL1	GH	HGF	ICAM-1	IFN-g	IGFBP-2	IGFBP-3	IL-1a	IL-1b	IL-1ra	IL-2	IL-3
E	IL-4	IL-5	IL-6	IL-8	IL-10	IL-11	IL-12 p70	IL-13	IL-15	IL-17	IL-17A	IL-18 Bpa
F	IL-19	IL-22	IL-23	IL-24	IL-27	IL-31	IL-32	IL-33	IL-34	IP-10	I-TAC	Kallikrein 3
G	Leptin	LIF	Lipocalin-2	MCP-1	MCP-3	M-CSF	MIF	MIG	MIP-1a/1b	MIP-3a	Mip-3b	MMP-9
H	MPO	Osteopontin	PDGF-AA	PDGF-AB/BB	Pentraxin 3	PF4	RAGE	RANTES	RBP-4	Relaxin-2	Resistin	SDF-1a
I	Serpin E1	SHBG	ST2	TARC	TFF3	TfR	TGF-a	THBS1	TNF-a	uPAR	VEGF	
J	Reference		Vit D BP	CD31	TIM-3	VCAM-1	Control (-)					

Figure 3. GIP induces the release of biologically active proteins. Subconfluent cultures of HAoECs were incubated with GIP, and the culture supernatant was used for protein antibody array. (**A**) Reference key for Human Cytokine Array Kit adapted from the manufacturer's information. (**B**, **C**) Chemiluminescent array images after probing with conditioned media incubated in the absence (**B**) or presence of 10^{-7} M GIP (**C**). (**D**) Relative immunoreactivities of proteins whose release was stimulated by GIP in HAoECs and 2-spot mean values relative to those pertaining to untreated HAoECs.

and in reducing poor cardiovascular outcomes.[13,33] GLP-1 related therapy has beneficial pleiotropic effects on cardiovascular disease beyond the glycemic control; however, its direct action on endothelial production and release of biologically active proteins remains unclear.[24] GLP-1 directly promoted angiogenesis in an in vitro model utilizing human umbilical vein endothelial cells,[34] and upregulated VEGF generation to stimulate the proliferation and differentiation of endothelial progenitor cells.[35] However, the spectra of cytokines, chemokines, and growth/angiogenesis-related proteins released from HAoECs and stimulated by GIP and GLP-1 remains to be investigated.

In the present study, we examined the proteins released from HAoECs using a human cytokine antibody array that detects 105 cytokines, chemokines, and growth/angiogenesis-related proteins and identified and compared the spectra of proteins whose release is stimulated by GIP and GLP-1. The presence of GIP resulted in an increased release of multiple proangiogenic proteins, including endoglin, angiogenin, SDF-1α, and angiopoietin-2, proinflammatory cytokines, including IL-17A, GDF-15, IL-6, and MIF, and potent growth factors, such as PDGF-AA (platelet derived growth factor-AA). The presence of GLP-1 strongly enhanced the release of endoglin, IL-17A, angiogenin, and SDF-1α among other proteins. The spectra of proteins whose release was stimulated by GIP and GLP-1 are similar even though the two incretins act on different receptors. To evaluate magnitude of protein release from incretin-treated and vehicle-treated cells, we assessed 500 μl conditioned media for 30 hours. Because HAoECs are sensitive to growth factor deprivation, the cells were incubated in full-growth media; therefore, possibly, cell surface receptors were not upregulated. The cytokines, chemokines, and other soluble pro-angiogenesis/growth-promoting factors that were released following the addition of and stimulation by GIP or GLP-1 were likely diluted in the conditioned media, and the released proteins were considered to represent the potent effects of GIP or

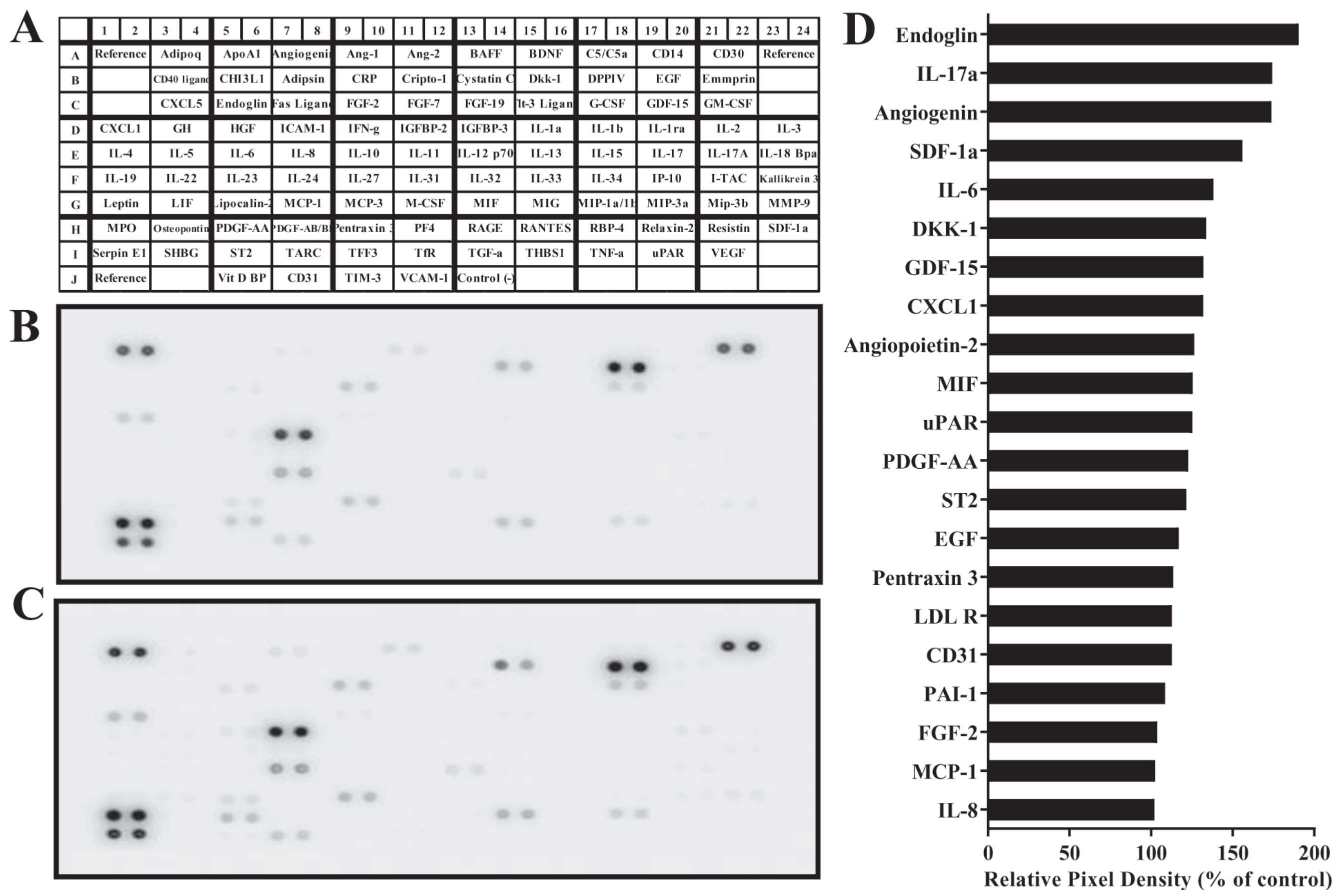


Figure 4. GLP-1 induces the release of biologically active proteins. Subconfluent cultures of HAoECs were incubated with GLP-1, and the culture supernatant was used for protein antibody array. (**A**) Reference key for Human Cytokine Array Kit adapted from the manufacturer's information. (**B**, **C**) Chemiluminescent array images after probing with conditioned media incubated in the absence (**B**) or presence of 10^{-7} M GLP-1 (**C**). (**D**) Relative immunoreactivities of respective proteins whose release was stimulated by GLP-1 in HAoECs and 2-spot mean values relative to those pertaining to untreated HAoECs.

GLP-1.

Despite the comparable stimulatory effects of GIP and GLP-1 on human endothelial cells in the present study, and a number of previous experimental studies, demonstrating their similarly potent insulinotropic and extra-pancreatic effects, the effects of GIP administered to healthy participants and patients with type 2 diabetes were diminished compared to those of GLP-1.[11,12] GIP readily binds to albumin, IgG (immunoglobulin G), and transferrin, which are found in abundance in the human peripheral circulation, whereas the binding of GLP-1 to abundant proteins in the plasma is minimal.[36] It is plausible that the reduced biological effects elicited by exogenously administered GIP in comparison to GLP-1 may be due not to their difference in biological potency but to other reasons, including diminished concentration owing to their high binding affinity with plasma proteins.

In conclusion, both GIP and GLP-1 stimulate the release of a variety of cytokines, chemokines, and proangiogenic/growth-promoting factors from cultured human aortic endothelial cells. The spectra of proteins released in response to the presence of GIP and GLP-1 are highly similar and consist of various proangiogenic factors and cytokines suggesting their mediatory roles in incretin-induced endothelial function.

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Conflicts of Interest: None

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