GIP-Dependent Expression of Hypothalamic Genes

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Received January 14, 2011
Accepted September 1, 2011
On-line October 12, 2011

Summary
GIP (glucose dependent insulinotropic polypeptide), originally identified as an incretin peptide synthesized in the gut, has recently been identified, along with its receptors (GIPR), in the brain. Our objective was to investigate the role of GIP in hypothalamic gene expression of biomarkers linked to regulating energy balance and feeding behavior related neurocircuitry. Rats with lateral cerebroventricular cannulas were administered 10 μg GIP or 10 μl artificial cerebrospinal fluid (aCSF) daily for 4 days, after which whole hypothalami were collected. Real time Taqman™ RT-PCR was used to quantitatively compare the mRNA expression levels of a set of genes in the hypothalamus. Administration of GIP resulted in up-regulation of hypothalamic mRNA levels of AVP (46.9±4.5%), CART (25.9±2.7%), CREB1 (38.5±4.5%), GABRD (67.1±11%), JAK2 (22.1±3.6%), MAPK1 (33.8±7.8%), NPY (25.3±5.3%), OXT (49.1±5.1%), STAT3 (21.6±3.8%), and TH (33.9±8.5%). In a second experiment the same set of genes was evaluated in GIPR-/- and GIPR+/? mice to determine the effect of lack of GIP stimulation on gene expression. In GIPR-/- mice expressions of the following genes were down-regulated: AVP (27.1±7.5%), CART (28.3±3.7%), OXT (25.2±5.8%), PTGES (23.9±4.5%), and STAT3 (8.8±2.3%). These results suggest that GIP, CART, OXT and STAT3 may be involved in energy balance-related hypothalamic circuits affected by GIP.

Key words
GIP receptor knockout • ICV administration • mRNA expression • Rats • Mice

Introduction
Gastric inhibitory polypeptide (GIP) is a 42 amino acid peptide hormone, synthesized by K cells in the intestinal epithelium (Buchan et al. 1978) and released into the circulation in response to nutrient ingestion. It was renamed glucose dependent insulinotropic polypeptide, reflecting its important role as a hormone in gut physiology. GIP stimulates insulin secretion, and potentiates the action of insulin in adipose tissue and other target tissues (Moens et al. 1996, Yip and Wolfe 2000, Wideman and Kieffer 2004). GIP receptors were reported to be present in the pancreas, gut, adipose tissue, adrenal cortex and heart, and pituitary, as well as in several brain regions, including the cerebral cortex, hippocampus, hypothalamus and olfactory bulb (Usdin et al. 1993, Kaplan and Vigna 1994). Expression of GIP has also been reported in these brain regions (Nyberg et al. 2005, Sondhi et al. 2006).

The secretin family of peptides, of which GIP is a member, have been reported to be involved in a variety of brain functions such as neuro-modulation, neurogenesis, brain development, cell-cycle regulation, differentiation, and cell death as well as in regulation of food intake, body weight and body temperature (Sherwood et al. 2000, Nyberg et al. 2005). GIP also appears to have a role in bone metabolism, with GIP receptor knockout (GIPR−/−) mice exhibiting reduced bone mass, whereas transgenic mice with GIP overexpression have increased bone mass (Ding et al. 2008).

GIP regulates lipid metabolism and storage (Holst 2004) partly through its effect on insulin secretion.
and the resulting increased uptake and incorporation of glucose into lipids, as well as through direct effects in adipose tissue (Wasada 1981, Hauner et al. 1988, Yip and Wolfe 2000, Wideman and Kieffer 2004). GIPR^−/− mice were found to be resistant to diet-induced obesity and insulin resistance, and GIP has been considered as a potential pharmaceutical target in the treatment of diabetes, obesity and other metabolic disorders (Miyawaki et al. 2002, Kieffer 2003).

The objective of this study was to determine whether central administration of GIP altered expression of genes known to be involved in regulation of food intake and body energy balance and whether GIP receptor deficiency would result in opposite changes in expression of some or all of those genes. We report that GIP administered centrally to rats reduced body weight gain and induced changes in several genes involved in energy balance regulation. Furthermore, we identified a subset of those genes showing the opposite changes in expression in GIPR^−/− mice compared to GIPR^+/+ mice.

Material and Methods

Male Sprague-Dawley rats (250-274 g) purchased from Harlan, Inc. (Indianapolis, IN) were used in the experiments 1 and 2. Ten week old C57BL6 GIPR^+/+ (n=20) and GIPR^−/− mice (n=18) were obtained from Dr. Carlos Isales (Medical College of Georgia). Two pairs of homozygous mice (GIPR^−/−) with targeted disruptions of exon 4 and 5 of the GIPR gene were originally obtained from the laboratory of Dr. Yuichiro Yamada (Department of Metabolism and Clinical Nutrition, Kyoto University Graduate School of Medicine, Kyoto, Japan), from which a breeding colony was established. The GIPR knockout mice were generated, bred, and maintained in the C57BL/6 line (Miyawaki et al. 1999). These mice were bred and maintained on a C57Bl/6 genetic background in Dr. Isales’ facility. Mice were kept four animals/cage, at 25 °C with a 12/12 h light/dark cycle. They had free access to a standard diet (Harlan TakLad Rodent Diet (W) 8604) and water ad libitum. Rats were housed singly in a light (12 h on/12h off) – and temperature controlled environment (22±1 °C, humidity 50 %). Rats were fed standard rodent chow and provided with water ad libitum.

Effect of icv injection of GIP on food intake and weight gain in rats

Rats were implanted with chronic lateral cerebroventricular cannulas as previously described (Choi et al. 2003), and after recovery from surgery, they were randomly assigned to four groups (n=8). Control rats were administered 10 μl artificial cerebrospinal fluid (aCSF) at 24-hour intervals for 4 days. The GIP-treated rats were administered 0.1, 1.0 or 10 μg/day GIP in 10 μl aCSF for 4 days. The injections were carried out using an injector cannula (C313I, Plastics One, Roanoke, VA) that protruded 1.1 mm below the tip of the guide cannula and that was connected to a Gilmist microsyringe by way of polyethylene tubing (PE20 Intramedic, Cat. #427406, BD, Sparks, MD). Food and water were available ad libitum. Food intake was measured daily and rats were weighed daily. All experiments and surgical procedures were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of the University of Georgia. Twenty-four hours after the last icv injection, rats were killed by decapitation after a brief exposure to carbon dioxide.

Effect of icv injection of GIP on gene expression in rats

Rats were implanted with chronic lateral cerebroventricular cannulas as previously described (Choi et al. 2003), and after recovery from surgery, they were randomly assigned to four groups (n=8). Control rats were administered 10 μl artificial cerebrospinal fluid (aCSF) at 24-hour intervals for 4 days; the GIP-treated rats were administered 10 μg/day GIP in 10 μl aCSF for 4 days. The dose used was selected based on the results of the previous experiment. Food and water were available ad libitum. Twenty-four hours after the last injection rats were killed by decapitation after a brief exposure to carbon dioxide. The brains were removed rapidly after decapitation and immediately frozen by placing them on plastic cassettes on top of powdered dry ice. Once completely frozen, they were stored at −80 °C. The brains were thawed to −20 °C before the hypothalamic dissection.

Hypothalamic gene expression in GIPR^−/− mice

In this experiment the hypothalamic gene expression profile of GIPR^−/− mice was compared to that from GIPR^+/+ mice. Mice were deeply anesthetized with CO2 before decapitation. The brains were removed rapidly after decapitation and immediately frozen on powdered dry ice. They were stored at −80 °C. The brains were thawed to −20 °C before the hypothalamic dissection. Hypothalamic blocks from two mice of the same group were randomly pooled together for RNA extraction.
Extraction of total RNA, reverse transcription (RT) and real-time PCR

Total hypothalamic RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s protocol. The integrity of the total RNA obtained from all the samples were verified using the RNA 6000 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The RT reaction was performed on 100 ng of total RNA per sample in a 20 µl reaction mixture using the cDNA Archive Kit with MultiScribe™ Reverse Transcriptase (Applied Biosystems, Inc., ABI, Foster City, CA) according to the manufacturer’s instructions. Reactions were incubated initially at 25 °C for 10 min and subsequently at 37 °C for 120 min. Quantitative PCR (Taqman™) assays chosen for the transcripts to be evaluated were from Assays-On-Demand™ (ABI), a pre-validated library of QPCR assays and were incorporated into 384-well MicroFluidic™ cards. All of the oligonucleotide primer and fluorogenic probe sets for Taqman™ real time PCR in rats and mice were from ABI (Table 1). Two µl of the cDNA along with 50 µl of 2 x PCR master mixes were loaded into respective channels on the microfluidic card followed by a brief centrifugation. The card was then sealed and real-time PCR and relative quantification were carried out on the ABI PRISM 7900 Sequence Detection System. The cycle conditions were: 94.5 °C for 15 min, followed by 40 cycles of 97 °C for 30 s, 59.7 °C for 1 min. Data were analyzed using Sequence Detection Systems software (Applied Biosystems) and the Relative Quantification (RQ) method, which represents the fold difference of mRNA level in rats treated with GIP relative to rats treated with control or the GIPR-/- mice relative to GIPR+/? mice. Preliminary experiments were carried out to determine the most appropriate endogenous control gene to use to correct for differences in the amounts of total RNA added to each reaction. For the rat experiment, mRNA expressions were normalized with 18 S as an endogenous control to correct  the differences in the amount of total RNA added to each reaction. β-ACT was used as endogenous control in the mouse experiment. The ∆C_T values were calculated initially using C_T for a specific gene mRNA minus C_T for 18 S mRNA (in rats) and β-ACT (in the mice) in the sample. The mean mRNA expressions from the rats treated with GIP were compared with those from the cCSF treated rats and mRNA expressions from the GIPR+/- mice were compared to those from the GIPR+/- mice using the formula: Relative Quantification (RQ) = 2^(-∆∆C_T) (∆∆C_T is the average control group ∆C_T values minus the average experimental group ∆C_T values, and ∆∆C_T of 1 equates to a two-fold difference in starting amount of cDNA). The relative quantification values from each gene were used to compare the hypothalamic gene expression between two groups.

Statistical analysis

Food intake and body weight gain data were analyzed using the general linear model ANOVA (Statistica v. 7.1; StatSoft, Inc., Tulsa, OK). Fishers LSD test was used to determine significance among means, with p<0.05 considered significant. Data are expressed as means ± S.E.M.

Gene expression data are shown as means ± S.E.M. of relative quantification (RQ) values from the Sequence Detection System (SDS, ABI-Perkin Elmer, Foster City, CA) files for all the genes. Statistical significance was assessed by general linear model ANOVA for multiple comparisons between the means for the different treatment/genotype groups. The Benjamini-Hochberg procedure was used to control for false discovery rate. A p<0.05 was considered to be significant. Multivariate analysis between genes was performed with Pearson correlation.

Results

Effect of icv administration of GIP on food intake and body weight of rats

GIP administered icv for 4 days in rats had no effect on food intake (data not shown). However, 10 µg/day GIP caused a significant loss of body weight compared to control and the other GIP treatments (Fig. 1). This dose was then selected for determination of effects on hypothalamic gene expression.

Effect of icv administration of GIP on hypothalamic gene expression in rats

Increased hypothalamic mRNA expression levels were found for AVP (46.9±4.5 %), CART (25.9±2.7 %), CREB1 (38.5±4.5 %), GABRD (67.1±11 %), JAK2 (22.1±3.6 %), MAPK1 (33.8±7.8 %), NPY (25.3±5.3 %), OXT (49.1±5.1 %), STAT3 (21.6±3.8 %), and TH (33.9±8.5 %) after 4 days of icv injections of 10 µg/day GIP in rats (Table 2).
Table 1. Probes used for real time PCR.

| Gene symbol | Gene name                               | Rat ABI assay ID | Mice ABI assay ID | Function                                                                                           |
|-------------|-----------------------------------------|-----------------|------------------|---------------------------------------------------------------------------------------------------|
| 18S         |                                        | 4342379-18S     | ------           | House-keeping gene                                                                                 |
| β-ACT       | actin, beta                             | ------          | Mm00607939_s1   | House-keeping gene                                                                                 |
| AVP         | arginine vasopressin                    | Rn00566449_m1   | Mm00437761_g1   | Neuroendocrine and feeding regulation (Bray 2000, Landgraf 2006)                                    |
| CART        | cocaine and amphetamine regulated transcript | Rn00567382_m1   | Mm00489086_m1   | Energy balance, bone metabolism, anxiety (Asakawa et al. 2001, Kong et al. 2003, Tian et al. 2004, Wortley et al. 2004, Elefteriou et al. 2005) |
| CREB1       | cAMP responsive element binding protein 1 | Rn00578829_g1   | Mm00501607_m1   | Learning, memory, anxiety, feeding related circuits (Carlezon et al. 2005, Duman and Duman 2005, Georgescu et al. 2005, Altarejos and Montminy 2011) |
| MAPK1       | mitogen activated protein kinase 1       | Rn00587719_m1   | Mm00442479_m1   | Sympathetic activation, leptin and insulin feeding circuits, circadian rhythm (Coogan and Piggins 2004, Hegyi et al. 2004, Rahmouni et al. 2004, Ambati et al. 2007) |
| OXT         | oxytocin                                | Rn00564446_g1   | Mm00726655_s1   | Social behaviors, anxiety, feeding behavior (Bale et al. 2001, Sabatier et al. 2007, Veenema and Neumann 2008) |
| SCT         | secretin                                | Rn00575360_g1   | Mm00441235_g1   | Feeding behavior, autism (Ambati et al. 2007, 2009, Lee et al. 2010, Cheng et al. 2011)              |
| TH          | tyrosine hydroxylase                    | Rn00562500_m1   | Mm00447546_m1   | Feeding, reward (Ambati et al. 2007, 2009, Lee et al. 2010)                                        |
| GABRD       | gamma-aminobutyric acid A receptor, delta | Rn00568740_m1   | Mm00433476_ml   | Inhibitory neurotransmitter, cognition, speech, feeding behavior (Windpassinger et al. 2002, Ambati et al. 2009) |
| JAK2        | Janus kinase 2                          | Rn00580452_ml   | Mm00434561_ml   | Leptin signaling (Sandberg et al. 2004, Villanueva and Myers 2008)                                 |
| NPY         | neuropeptide Y                          | Rn00561681_ml   | Mm00445771_ml   | Feeding behavior (Kalra et al. 1991, Kalra and Kalra 2003)                                         |
| POMC        | pro-opiomelanocortin                    | Rn00595020_ml   | Mm00435874_ml   | Feeding behavior (Boston 2001, Li et al. 2003)                                                      |
| PTGES       | prostaglandin E synthase                | Mm00456961_ml   | Mm00452105_ml   | Inflammation, feeding behavior (Turnbull and Rivier 1996, Ohinata et al. 2008, Ambati et al. 2009) |
| SOCS3       | suppressor of cytokine signaling 3      | Rn00585674_sl   | Mm00545913_sl   | Leptin signaling, neuroimmuneendocrine modulator (Auernhammer et al. 2000, Morris and Rui 2009)     |
| STAT3       | signal transducer and activator of transcription 3 | Rn00562562_ml   | Mm00456961_ml   | Signal transduction in feeding related circuits (Carvalheira et al. 2001, Zhao et al. 2002, Hommel et al. 2006, Gao et al. 2007) |

*POMC1, mouse; POMC2, rat.
Fig. 1. Weight gain/loss in rats treated daily for 4 days with control (aCSF) or 10 µg/d GIP administered into the lateral cerebral ventricle. Data are means ± S.E.M. Means not denoted with a common superscript are different, *<p<0.05.

Correlation between CREB1 and MAPK1 mRNA expression levels in GIP-treated rats

The mRNA levels of both CREB1 and MAPK1 showed a proportional increase with a positive correlation (Correlation coefficient, r=0.87, p<0.001) in the hypothalamus of GIP-treated rats.

Hypothalamic gene expression in GIPR-/- and GIPR+/? mice

The hypothalamic mRNA levels of some biomarkers were lower in hypothalami of GIPR-/- mice when compared with those of wild-type controls. Relative to GIPR+/? mice, expression levels of hypothalamic mRNAs of AVP (27.1±7.5 %), CART (28.3±3.7 %), OXT (25.2±5.8 %), PTGES (23.9±4.5 %), and STAT3 (8.8±2.3 %) in GIPR-/- mice were down-regulated (Table 2).

Hypothalamic mRNA expression of MAPK1 (38.2±4.0 %, p<0.01) and NPY (31.6±8.5 %, p<0.01) was increased in GIPR-/- mice relative to GIPR+/? mice.

Discussion

GIP is considered to be one of the principle incretin factors of the enteroinsular axis system which has been implicated to play a critical role in the pathogenesis of diabetes and obesity (Wideman and Kieffer 2004, Yip and Wolfe 2000). The current study was designed to investigate the alterations in expression of hypothalamic genes involved in feeding behavior and energy balance regulation in rats treated icv with GIP and in GIPR deficient mice.

AVP is suggested to trigger a variety of effects relating to regulation of water, glucose and electrolytes and neuroendocrine functions and may also play a role in energy balance regulation (Bray 2000, Landgraf 2006). The structurally-related neuropeptide oxytocin (OXT) has also been implicated in feeding, complex social behaviors and anxiety-associated behaviors (Bale et al. 2001, Sabatier et al. 2007). Higher levels of AVP and OXT mRNA expression were found following icv GIP treatment in rats. Furthermore, both AVP and OXT expressions were lower in GIPR-/- mice, thus suggesting that these hypothalamic neuropeptides may mediate GIP-induced behavioral changes related to energy balance regulation.

Cocaine- and amphetamine-regulated transcript (CART) is highly expressed in the hypothalamus, particularly in the paraventricular (PVN) and arcuate (ARC) nuclei (Couceyro et al. 1997), which are critical in the regulation of energy homeostasis. CART was originally reported to be an anorectic neuropeptide, based on icv administration of CART(55-102) (Kristensen et al. 1998, Stanley et al. 2001) and on overexpression of CART after administration of recombinant adeno-associated virus (rAAV)-CART into the third ventricle in diet-induced obese rats (Qing and Chen, 2007). However, icv administration of CART is also associated with motor disturbances which may interfere with feeding (Aja et al. 2001). In contrast, direct administration of CART (55-102) into discrete hypothalamic nuclei (Abbott et al. 2001) and over-expression of CART in the ARC (Kong et al. 2003) and PVN (Smith et al. 2008) have been shown to increase food intake and weight gain.

In our study hypothalamic CART expression was increased in rats treated icv with GIP and decreased in mice deficient in GIPR. Although GIP did not affect food intake in the rats, it did cause weight loss, and a previous study has shown that overexpression of GIP in mice resulted in enhanced locomotor behavior and no increase in anxiety behavior (Ding et al. 2006). Thus, the changes in whole hypothalamic CART expression may be related to the effect of GIP on energy balance regulation, but probably not through changes in anxiety or locomotor behavior.

STAT proteins are a family of cytoplasmic transcription factors activated by tyrosine phosphorylation and dimerization in response to cytokines, growth factors and other extracellular ligands (Dandoy-Dron et al. 1995, Sano et al. 1999). In particular, STAT3 has been shown to play an important role in the regulation of energy balance. STAT3 is a known downstream component of both leptin and ciliary neurotrophic factor (CNTF) action (Hubschle et al. 2001, Lambert et al. 2001). Mice with STAT3 deletion...
in leptin receptor positive neurons of the hypothalamus become hyperphagic after weaning, have increased body weight and body fat, and exhibit impaired glucose tolerance (Cui et al. 2004). While phosphorylation levels of STAT3 are believed to be more critical than variations of mRNA level, our previous study showed that mRNA levels of STAT3 in the arcuate nucleus/median eminence of the hypothalamus were significantly up-regulated in rats with ICV treatment of leptin or CNTF (Ambati et al. 2007). The results from the present study show increased expression of STAT3 in the hypothalamus of rats treated icv with GIP and decreased expression of hypothalamic STAT3 in mice deficient in GIPR. Thus, STAT3 may also be a downstream transcription factor involved in the central effect of GIP on energy balance regulation.

GABRD is a receptor for gamma-aminobutyric acid, an inhibitory neuropeptide involved in a number of hypothalamic and higher brain functions, including neuronal network development, cognition and speech (Windpassinger et al. 2002). CREB1 protein is a transcription factor and usually considered a marker of neuron activation (Zachariou et al. 2001). In rats treated icv with GIP, GABRD and CREB1 mRNA expression was increased, suggesting elevated neuronal activity as a response to GIP treatment. Interestingly, proportionate increase in mRNA expression levels of CREB1 and MAPK1 in the hypothalamus of GIP-treated rats indicated a possible MAPK-1 signaling-dependent neuron activation by CREB1 (Pandey 2003, Euskirchen et al. 2004). CREB1 plays a regulatory role in the nervous system and is widely believed to promote neuronal survival, precursor proliferation and other neuronal-related functions (Rodriguez and Ferrer 2007). Phosphorylation of CREB through MAPK signaling is a critical event in the network of neuronal activation, neuronal proliferation, differentiation and synaptic function. Activation of this network also stimulates neurite outgrowth in the central nervous system and also in neuronal cell-lines (Ma’ayan et al. 2009). Furthermore, there is evidence that phosphorylation of CREB regulates NPY expression in the brain (Pandey 2003).

In conclusion, our data suggest that GIP and its receptor may be involved in energy balance regulation by the hypothalamus as evident by the changes in expression levels of genes such as AVP, CART, OXT, and STAT3.

**Conflict of Interest**

There is no conflict of interest.

**Acknowledgements**

This work was supported in part by Georgia Research Alliance Eminent Scholar endowment held by CAB.

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**Table 2.** Hypothalamic mRNA expression (mean ± S.E.M.) of hypothalamic genes in GIPR+/+ and GIPR−/− mice* and in rats** after 4 days ICV injection of 0 or 10 μg/d GIP.

| Gene | GIPR+/+ mice | GIPR−/− mice | Rats (0 μg/d GIP ICV) | Rats (10 μg/d GIP ICV) |
|------|--------------|--------------|-----------------------|------------------------|
| AVP  | 0.85 ± 0.09a | 0.62 ± 0.08b | 0.78 ± 0.06a          | 1.14 ± 0.03y           |
| CART | 0.7 ± 0.05a  | 0.50 ± 0.03b | 0.97 ± 0.07a          | 1.22 ± 0.02b           |
| CREB1| 1.08 ± 0.02  | 1.04 ± 0.02  | 0.81 ± 0.04a          | 1.12 ± 0.03y           |
| GABRD| 1.44 ± 0.1   | 1.52 ± 0.05  | 0.89 ± 0.03a          | 1.49 ± 0.1y            |
| JAK2 | 1.16 ± 0.04  | 1.2 ± 0.03   | 0.84 ± 0.06a          | 1.03 ± 0.03b           |
| MAPK1| 1.31 ± 0.06a | 1.82 ± 0.07y | 0.85 ± 0.06a          | 1.14± 0.06b            |
| NPY  | 1.29 ± 0.06a | 1.7 ± 0.11b  | 0.75 ± 0.06a          | 0.94 ± 0.05b           |
| OXT  | 0.8 ± 0.06a  | 0.6 ± 0.06b  | 0.85 ± 0.09a          | 1.27 ± 0.04y           |
| POMC | 0.85± 0.09   | 0.66 ± 0.14  | 0.75 ± 0.07           | 0.85 ± 0.04            |
| PTGES| 1.17 ± 0.07a | 0.89 ± 0.04b | 0.93 ± 0.06           | 1.08 ± 0.06            |
| SCT  | 1.1 ± 0.15   | 0.83 ± 0.10  | 0.85 ± 0.08           | 1.14 ± 0.01            |
| SOCS3| 1.32 ± 0.15  | 0.84 ± 0.06  | 0.90 ± 0.07           | 0.95 ± 0.04            |
| STAT3| 1.15 ± 0.03a | 1.05 ± 0.03b | 0.79 ± 0.06a          | 0.96 ± 0.03b           |
| TH   | 0.85 ± 0.07  | 0.7 ± 0.04   | 0.76 ± 0.09a          | 1.01 ± 0.06b           |

*Data are normalized to β-ACT values; **Data are normalized to the 18S values; means with different superscripts are different ap<0.05; bp<0.01.
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