Digestion and absorption of nutrients subsequent to food intake is associated with increased secretion of multiple gut peptides that are synthesized by specialized enteroendocrine cells located in the epithelium of the stomach, distal ileum and colon, and in pancreatic islets (1) (Figure 1).

A number of peptide hormones are encoded by the single proglucagon gene. In the pancreas, proglucagon is cleaved to glucagon, glicentin-related pancreatic peptide (GRPP) and a major proglucagon fragment. In the intestinal L-cells, the molecule is processed to GLP-1 (glucagon-like peptide-1), GLP-2 (glucagon-like peptide-2), and glicentin, which is further broken down to oxyntomodulin (2) (Figure 2 on next page).

Two hormones, glucagon-like peptide-1 (GLP-1), and glucose dependent insulinotropic polypeptide (GIP) are responsible for the incretin effect (3). Incretin increases the magnitude of meal-stimulated insulin secretion from islet cells in a glucose-dependent manner. Incretin action facilitates the uptake of glucose by muscle tissue and liver while simultaneously suppressing glucagon secretion by the cells of the islets, leading to reduced endogenous production of glucose from hepatic sources. Preclinical studies indicate that both GLP-1 and GIP increase levels of cAMP leading to expansion of cell mass and resistance to cell apoptosis (4, 5).

GLP-1 is a 30 amino acid peptide hormone produced in the intestinal epithelial endocrine L-cells. In addition to stimulating insulin secretion and to inhibiting glucagon secretion, GLP-1 also inhibits gastrointestinal motility and secretion and thus acts as an enterogastrone and part of the “ileal brake” mechanism. GLP-1 also appears to be a physiological regulator of appetite and food intake. Decreased secretion of GLP-1 may contribute to the development of obesity, and exaggerated secretion may be responsible for postprandial reactive hypoglycemia (2, 6).

GIP is a 42 amino acid peptide hormone synthesized in and secreted from K-cells in the intestinal epithelium. Unlike GLP-1, which exerts multiple non-incretin activities in the regulation of blood glucose, the primary action of GIP is stimulation of glucose-dependent insulin secretion.

GIP receptors are expressed on adipocytes. Experimental data derived from studies of the GIP receptor knockout mice strongly suggests a role for the GIP receptor in the regulation of body weight. Emerging evidence suggests that gastric bypass surgery rapidly cures diabetes in grossly obese subjects, at least in part as the result of surgical bypass of GIP-secreting K-cells in the upper small intestine. GIP, in addition to its insulinotropic effects, may promote fat storage and obesity,
either by direct insulin-mimetic effects in adipose tissue or via enhancement of resistin-mediated stimulated lipoprotein lipase activity. Thus, blocking these effects pharmacologically could be a strategy for treatment of obesity (3, 7).

Both GLP-1 and GIP are inactivated by the enzyme dipeptidyl peptidase-4 (DPP-4) (8). In circulation, the first two N-terminal amino acids of active form of GLP-1, GLP-1 (7-36) amide and GLP-1 (7-37), are extremely rapidly cleaved by DPP-4, which leads to the formation of major circulating forms of GLP-1 in peripheral plasma, GLP-1 (9-36) amide and GLP-1 (9-37).

In fact, inhibitors of DPP-4 have been approved as therapeutic agents for treatment of type 2 diabetes (e.g. sitagliptin). While measurement of total GLP-1, i.e. the sum of intact hormone and its metabolites, reveals total L-cell secretion, the level of the intact GLP-1 reflects endocrine actions of the peptide. The biologically active form of GIP (amino acids 1-42) is also rapidly inactivated to biologically inactive GIP (3-42). GIP infused into human subjects is rapidly degraded, with a T1/2 of ~7 min (9, 10) (Table 1).

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In addition to GLP-1, distal-intestinal L-cells postprandially secrete oxyntomodulin and peptide tyrosine-tyrosine (PYY). These two hormones are considered to be satiety signals decreasing food intake, and body weight (11, 12).

Oxyntomodulin, a 37 amino-acid peptide (the first 29 amino-acids contain the glucagon structure), delays gastric emptying and decreases gastric acid secretion. In rodents, exogenous administration of oxyntomodulin decreases food intake while increasing energy expenditure, and chronic injections reduce body-weight gain. Although the mechanisms are not entirely understood, these effects most probably are mediated via GLP-1 receptor, since oxyntomodulin does not alter feeding in GLP-1 receptor-deficient mice, and the GLP-1-receptor antagonist, exendin9–39, blocks oxyntomodulin-induced anorexia. Oxyntomodulin is a candidate substrate for DPP-4. The midsection of oxyntomodulin may also be a target for degradative enzymes such as the ectopeptidases and neutral endopeptidase 24.11 (NEP) (13).

PYY, the other gut hormone, increases ileal absorption and inhibits gastric emptying along with inhibition of gallbladder and pancreatic secretion (14). Both isoforms of the PYY, PYY (1-36), and the DPP-4 cleaved PYY (3-36), have anorexigenic effects. However the truncated form is more potent. In adults, PYY (3-36) accounts for 37% and 54% of total PYY immunoreactivity in basal and postprandial plasma, respectively. Anorectic effects of PYY (3-36) appear to be mediated by the Y2 receptor, thus Y2 receptor agonists are considered in the treatment of obesity.

Ghrelin, discovered as a natural ligand for the growth hormone (GH) secretagogue receptor, is produced predominantly by enteroeendocrine cells in oxyntic glands of the stomach. In addition to potently stimulating secretion of GH from the pituitary, ghrelin, which increases preprandially and drops rapidly after meals, acts as an orexigenic peptide increasing appetite, food intake, body weight, and adipogenesis (15).
During synthesis, ghrelin is acylated on 3-serine with an ester-linked fatty acid group. Acylation is essential for ghrelin activity at the GH secretagogue receptor, but is readily cleaved by endogenous esterase activity (16). Both active acyl-ghrelin and des-acyl ghrelin are found in circulation. Recent studies suggest that des-acyl ghrelin has multiple biological activities as well. Since some of these actions of des-acyl ghrelin oppose those of acyl-ghrelin, the ratio of acyl- to desacetyl ghrelin may determine overall physiological response. In circulation, ghrelin, but not des-acyl ghrelin, may be predominantly bound to carrier proteins. Ghrelin has specific lipoprotein interactions not seen with des-acyl ghrelin, and antisera to ghrelin differ in ability to detect these bound forms (16, 17).

**PRE-ANALYTICAL CONSIDERATIONS**

Plasma levels of most gut hormones rise shortly after a meal and fall rapidly thereafter, mainly because they are eliminated by the kidneys, but also because they are enzymatically inactivated.

Testing for incretins, and other gut hormones, presents numerous challenges because of their instability. Degradation and modification of gut hormones caused by proteolytic enzymes, especially DPP-4 and NEP, occur not only in vivo, but also during and after blood collection. Therefore, proper sample collection and meticulous pre-analytical and analytical sample handling are crucial for successful quantification of these biomarkers.

The best practice for minimizing preanalytical variability associated with blood collection, processing and storage is to collect samples directly into tubes containing protease inhibitors. Mixing blood with protease inhibitors during collection instantly protects plasma proteins, which enhances analyte recovery and long-term preservation.

To measure the active forms of GLP-1 and GIP, blood must be immediately preserved with DPP-4 inhibitor. Tubes containing DPP-4 inhibitor suitable for the measurements of active forms of incretins are commercially available (BD Diagnostics P700 tubes). BD has introduced P800 tubes that contain a protease inhibitor cocktail in addition to DPP-4 inhibitor. P800 tubes are recommended not only for active forms of incretins, but also for other gut hormones. The addition of a protease inhibitor cocktail to inhibit proteases that may act on the peptides during long-term storage (even in the frozen state) is important.

As an alternative to using collection tubes containing the appropriate inhibitors, blood samples may be spiked with a protease inhibitor cocktail within 30 seconds of blood collection. An appropriate cocktail should contain in addition to a DPP-4 inhibitor, a broad spectrum of inhibitors including EDTA, inhibiting metalloproteinases, serine protease inhibitors and cysteine protease inhibitors.

Additional pre-analytical steps should be considered when samples are obtained for quantification of active acylated ghrelin. Pacific Biomarkers (PacBio) has developed a process that will stabilize the analyte. Please contact PacBio for specific details.

In addition to applying appropriate protease inhibitors, blood samples should be kept on ice at all times and processed in a refrigerated centrifuge, aliquoted immediately, and stored frozen at -70°C or below.

Minimizing time at ambient temperature during testing helps maintain sample integrity. Immediate snap freeze on dry-ice after use permits the same aliquot of sample to be used at a later time.

It is important to recognize that sample stability tolerance usually applies to total time. Users need to take under consideration the cumulative effect of all handling and storage. For example, samples that have undergone a number of freeze thaw cycles verified during assay validation as being safe for analyte integrity (i.e. % mean difference was not higher that 10% comparing to baseline values) cannot be stored for the same time at -70°C or lower as samples that were not thawed at all before use. Similarly, samples that were not processed immediately after blood collection, or were spun in the centrifuge without refrigeration will not tolerate well a short storage at 4°C compared to samples handled appropriately. It is important to minimize exposure of the samples to sub-optimal conditions at every step of sample handling or storage.
ANALYTICAL CONSIDERATIONS

Appropriate sample handling is key to successful laboratory testing. Another significant challenge in the measurement of incretins and gut hormones is the performance of available assays. Experience at PacBio indicates that commercial assays are often inadequate for clinical studies. However, modification of manufacturer’s recommendations and careful user-oversight can achieve acceptable assay performance.

The major concerns with commercial assays are sensitivity and among-lot consistency. In order to remedy this, PacBio has introduced specific preanalytical and analytical processes as well as data analysis to improve sensitivity. In addition, PacBio has extensive experience in controlling for lot-to-lot variation and assuring reagent consistency. Please contact PacBio for further details.

Lastly, it is important to note that current incretin and gut hormone assays are not standardized in reference to both quantification and specificity. Thus, values using different assays may not be directly compared.

CONCLUSION

Reliable quantification of incretins and gut hormones in human plasma is essential in clinical studies leading to development of therapeutics for diabetes and obesity treatment, and depends heavily on appropriate blood sample collection, selection of appropriate assays and careful monitoring of performance.
<table>
<thead>
<tr>
<th>HORMONE</th>
<th>SOURCE</th>
<th>ACTIONS</th>
<th>BIOACTIVE FORMS</th>
<th>INACTIVATING ENZYME</th>
<th>T½ OF ACTIVE BIOFORM (IN VIVO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1</td>
<td>Distal ileal L cells</td>
<td>Insulinotropic, decreases glucagon secretion, regulates gastro-intestinal motility and appetite</td>
<td>7-36 amide, 7-37</td>
<td>DPP-4</td>
<td>1½ to 2 min</td>
</tr>
<tr>
<td>GIP</td>
<td>Duodenal K cells</td>
<td>Insulinotropic, increases glucagon secretion, stimulates beta-cells growth</td>
<td>1-42</td>
<td>DPP-4</td>
<td>~7 min</td>
</tr>
<tr>
<td>PYY</td>
<td>Colorectal L cells</td>
<td>Decreases food intake, inhibits gastric acid secretion and gastric emptying</td>
<td>3-36*</td>
<td>DPP-4</td>
<td></td>
</tr>
<tr>
<td>Glucagon</td>
<td>α-cells of the islets of Langerhans</td>
<td>Increases blood glucose, stimulates the release of insulin</td>
<td>1-29</td>
<td>DPP-4, NEP</td>
<td>~6 to 7 min</td>
</tr>
<tr>
<td>Oxyntomodulin</td>
<td>Colorectal L cells</td>
<td>Decreases food intake, inhibits gastric acid secretion, no effect on gastric emptying</td>
<td>1-37</td>
<td>DPP-4, NEP</td>
<td>~6 to 7 min</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>Enteroendocrine cells in the oxyntic glands of the stomach</td>
<td>Increases appetite, food intake, body weight, and adipogenesis</td>
<td>N-octanoylated (Ser3)</td>
<td>Esterase</td>
<td>~9 to 13 min</td>
</tr>
</tbody>
</table>

* Both isoforms are bioactive, however PYY (3-36) is more potent than PYY (1-36)
REFERENCES