**Abstract**

**Background:** Glucagon-like peptide 1 (GLP-1) is an incretin hormone that stimulates glucose-induced insulin secretion, increases β-cell proliferation, neogenesis and β-cell mass. In adults, plasma concentrations of amidated GLP-1 are typically within the 5–10 pmol/l range in the fasting state and increases to ~50 pmol/l after ingestion of a mixed meal.

**Research design and methods:** We measured plasma glucose, insulin and amidated forms of GLP-1 prefeed and then at 20 and 60 min post-feed following ingestion of a 60–70 ml of standard milk feed in preterm (n = 10, 34–37 weeks) and term newborn infants (n = 12, 37–42 weeks). Reverse-phase fast protein liquid chromatography was used to characterise the molecular nature of the circulating GLP-1.

**Results:** Mean birth weight was 3.18 kg and mean age at sampling for GLP-1 was 7.7 days. The mean basal GLP-1 concentration was 79.1 pmol/l, which increased to 156.6 pmol/l (G70.9, P<0.001) and 121.5 pmol/l (G59.2) at 20 and 60 min respectively. Reverse-phase chromatography analysis suggested that the majority of GLP-1 immunoreactivity (~75%) represented GLP-1 (7–36) amide and (9–36) amide.

**Conclusions:** Basal and post-feed amidated GLP-1 concentrations in neonates are grossly raised with the major fractions of circulating GLP-1 being (7–36) amide and (9–36) amide. Elevated GLP-1 concentrations in the newborn period may have a role in regulating maturation of enteroendocrine system and also of increasing pancreatic β-cell mass and regeneration. The high levels of GLP-1 may be due to immaturity of the dipeptidyl peptidase IV and or lower glomerular filtration rate in the neonatal period. Further studies are required to understand the role of GLP-1 in the neonatal period.
β-cell growth (13). Given the role of GLP-1 in regulating these processes, it would be important to understand the GLP-1 response to meal ingestion and the role of GLP-1 in newborns. Hence, in this study, we measured basal and post-feed amidated GLP-1 levels in response to milk ingestion in newborn infants and compared these values with those from adult control patients.

**Patients and methods**

**Study population**

A total of 22 appropriately grown preterm and term newborn infants admitted to the neonatal unit at the Basildon University Hospital, Essex, UK, were prospectively recruited during a 12-month period. Multicentre ethical approval for the study was obtained from the Institute of Child Health, University College London and from South Essex ethical committee. The gestational age of the infant was assessed by the mother’s last menstrual period and/or early ultrasound dating. Infants whose mothers had pregestational and gestational diabetes mellitus or were receiving hormonal therapy such as thyroxine during pregnancy were excluded. Preterm infants whose mothers received antenatal corticosteroids any time during pregnancy or prior to delivery were also excluded from the study.

**Blood samples**

Blood samples were collected before feeds (time 0 sample 1) and then at 20 (sample 2) and 60 (sample 3) min after feeds. Samples were collected at an age when babies were on 4-hourly feeds of 60–70 ml of standard formula feeds. The blood collection was scheduled to coincide with the clinical blood sampling procedure in order to minimise any unnecessary disturbance to the infants. Most of the babies spend 15–20 min to complete the feed volume. All the babies were haemodynamically stable with no respiratory support. They were also not on any medications. All the babies were admitted in the neonatal unit for establishing feeds. The i.v. access was established in all the babies 20 min prior to venous sampling. A dummy was used as a pacifier to control the pain. If the venous cannula was not functioning, venous sampling was done at the appropriate times with a pacifier to control the pain. Venous blood samples were collected into: i) a prechilled lithium heparin bottle for serum insulin measurements and ii) a prechilled lithium heparin vial containing tryosolol for GLP-1 measurement. After the collection of blood, serum was immediately separated by cold centrifuge and stored at −40 °C for subsequent analyses.

Adult overnight fasting control samples were obtained from 15 healthy subjects aged 22–38 years with a BMI range of 20–26.5 kg/m².

**Hormone assays**

All the samples were measured together centrally to avoid inter-assay variation. Insulin was measured using radio immunoassay by automated immunolite machine. Glucose was measured by glucose oxidase method. GLP-1-like immunoreactivity was measured by a specific and sensitive RIA, previously established (13, 15). The antibody was produced in rabbits against GLP-1 coupled to BSA. The antibody cross-reacted 100% with all amidated forms of GLP-1 but did not cross-react with glycine-extended forms (GLP-1 (1–37) and GLP-1 (7–37)) or any other known pancreatic or gastrointestinal peptide. 125I-GLP-1 was prepared by the iodogen method (16) and purified by HPLC. The specific activity of the 125I-GLP-1 label was 48 Bq/fmol. The assay was performed in a total volume of 0.7 ml of 0.06 M sodium barbitone buffer (pH 8) containing 0.3% BSA. The assay was incubated for 3 days at 4 °C before separation of the free and antibody-bound label by charcoal absorption. The limit of detection was 7.5 pmol/l with an intra-assay variation of 5.4%.

**Reverse-phase fast protein liquid chromatography**

Reverse-phase fast protein liquid chromatography (FPLC) was used to further characterise the molecular form of GLP-1 immunoreactivity detected in the samples. Peptide was extracted from plasma using Sep-Pak C18 cartridges (Waters, Hertfordshire, UK), as previously described (17). The extracts were dissolved in 1 ml distilled water plus trifluoroacetic acid (TFA) 0.05% (v/v), then filtered through 0.2 μm hydrophilic membranes (Satorius, Gottingen, Germany). Of this volume, 0.5 ml was fractionated by FPLC on a high-resolution reverse-phase (Pep RPC 1 ml HR) column (GE Healthcare, Life Sciences, Amersham). The column was eluted with a 10–45% gradient of acetonitrile (AcN)/water 0.05% (v/v) TFA over 60 min. Fractions were collected at 1-minute intervals. Fractions from all runs were dried in a Savant vacuum centrifuge, reconstituted in GLP-1 assay buffer and GLP-1 immunoreactivity content determined by RIA. The remaining sample was used to calculate the percentage recovery. Recovery was calculated as GLP-1 immunoreactivity (fmol) recovered from each sample, compared with GLP-1 immunoreactivity loaded on to the FPLC column (fmol), multiplied by 100, and expressed as a percentage. Synthetic human GLP-1 (7–36) amide and GLP-1 (9–36) amide were run on the same gradient for comparison.

**Statistics**

All results are expressed as mean ± 2 s.d. Statistical analyses were carried out as one-way analyses of variance for correlated sample (one-way repeated ANOVA test). Tukey’s multiple comparison test was used to test the statistical significance between the samples. The software package used was PRISM.
Results

Patient characteristics
Mean gestational age (GA) of the infants was 37.18 weeks. Ten neonates were preterm with GA of 34–37 weeks, rest were term. At the time of sample collection, corrected GA of four babies was less than 37 weeks while the rest were greater than term. Mean birth weight was 3.18 kg with a range of 2.2–4.2 kg. All the neonates were appropriately grown for GA. Blood sampling for the neonates were done while they fed 60–70 ml per feed (standard milk formula), which corresponded with 100–160 ml/kg per day of total feed volume. Average age at sampling was 7.7 days with a range of 4–10 days. Table 1 summarises clinical profile and anthropometric indexes of the study population.

Basal and post-feed GLP-1 concentration
The mean GLP-1 concentration before the feeds was 79.1 pmol/l (± 52.1). This basal GLP-1 value is markedly elevated in comparison with the adult basal GLP-1 concentration of 19 pmol/l (Fig. 1). The mean GLP-1 value increased to 156.6 (± 70.9) and 121.5 (± 59.2) pmol/l at 20 and 60 min post-feed (Fig. 2). The post-feed GLP-1 concentrations were also markedly elevated in comparison with the adults. The GLP-1 value at 20 and 60 min post-feed was significantly increased (P<0.0001) in comparison with the basal value. Both the blood glucose and serum insulin concentrations rose appropriately in response to feeding. Plasma GLP-1 levels did not correlate with anthropometry, GA and or birth weight and serum insulin concentration.

Reverse-phase FPLC was used to further analyse GLP-1 immunoreactivity extracted from plasma by Sep-Pak cartridge. All FPLC columns had a recovery O 65%. The major peak of GLP-1 immunoreactivity ( > 75% on all columns) eluted in the position corresponding to synthetic GLP-1 (7–36) amide and (9–36) amide. Both GLP-1 (7–36) amide and GLP-1 (9–36) amide eluted at the same position. A representative profile is shown in Fig. 3.

Table 1 Clinical characteristics of neonates included in the study, n=22.

<table>
<thead>
<tr>
<th>Mean (± s.o.)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>37.18 (2.28)</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.14 (0.55)</td>
</tr>
<tr>
<td>Weight at sampling (kg)</td>
<td>3.2 (0.55)</td>
</tr>
<tr>
<td>Male: female</td>
<td>12:10</td>
</tr>
<tr>
<td>Apgar score</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Age of sampling (days)</td>
<td>7.68 (1.86)</td>
</tr>
<tr>
<td>Feed volume – 4 h (ml)</td>
<td>66.1 (4.1)</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>33.68 (0.96)</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>49.65 (1)</td>
</tr>
</tbody>
</table>

Figure 1 Comparison of fasting GLP-1 concentration in adults and neonates.

Plasma glucose and insulin response to feeding
The prefeed blood glucose concentration was 3.9 mmol/l (± 0.7), with 20- and 60-minute values of 4.8 (± 0.99) and 4.6 (± 0.78) mmol/l respectively. The corresponding serum insulin concentrations were 9.2 (± 13.36), 21.80 (± 21.67) and 18.37 (± 23.81) mU/l respectively. No hypoglycaemia was documented in any infant post-feed. Figures 4 and 5 show the insulin and glucose concentrations at baseline and 20 and 60 min post-feed. No correlation of GLP-1, glucose and insulin concentrations were found in individual newborns. The hormones also did not correlate with feed volume, anthropometry, GA and birth weight.

Discussion
In this study, we have demonstrated grossly elevated basal and post-feed amidated GLP-1 concentrations in neonates. Reverse-phase chromatography analysis suggested that GLP-1 (7–36) amide and GLP-1 (9–36) amide were the major forms of GLP-1 immunoreactivity present. In comparison with the adult levels of amidated forms of GLP-1, the basal GLP-1 level in the newborn...
period was ten times higher. GLP-1 is rapidly degraded by the enzyme DPP-IV thus limiting its metabolic stability and eliminating its insulinotropic activity. In adults, the half-life of intact GLP-1 has been calculated as less than 2 min and less than 5 min for the metabolite (5). However, the pharmacokinetics of GLP-1 is not known in newborn infants.

Only one study has so far measured GLP-1 concentration in preterm neonates (18). They have also demonstrated high concentration of GLP-1 before (14.8 ± 4.0) and 50–60 min after feeds (69 ± 28). Preterm neonates in this study were on a combination of 2- to 3-hourly enteral and continuous parenteral feeds. Previous studies have shown that other gut hormones (such as motilin, gastrin, enteroglucagon, neurotensin, gastric inhibitory polypeptide and pancreatic polypeptide) exert important effects on gut growth, secretion and motility and on intermediary metabolism (19). The post-natal hormonal surges observed in the newborn period may play a key role in the post-natal adaptations to enteral feeding. In previous studies in the neonatal period, it has been shown that GIP is elevated in the basal state on day 6 of life with marked postprandial elevations observed on day 24 of life (20). Although we were not able to measure GLP-1 concentrations on different days, the GLP-1 responses observed in our patients are similar to those reported for studies on GIP. Hence, elevated plasma GLP-1 concentrations in the neonatal period like the other gut hormones may have beneficial effects on gut growth maturation and motility.

Based on mostly rodent studies the neonatal period is characterised by marked changes in pancreatic β-cell proliferation, neogenesis and apoptosis (13, 14). The biochemical and molecular mechanisms regulating β-cell proliferation and mass are not known, but GLP-1 has potent effects on β-cell proliferation and mass (13). GLP-1 promotes the proliferation and neogenesis of β-cells, increases β-cell mass, reduces β-cell apoptosis, and increases differentiation of exocrine-like cells towards a more differentiated β-cell phenotype (13). Therefore, it is possible that elevated GLP-1 levels in the newborn period may have a role in regulating β-cell mass and inducing resistance to apoptosis.

In our study, we have measured GLP-1 concentration in neonates while they have been fed 60–70 ml of standard formula feeds in every 4 h. Frequent feeding and presence of nutrients in the gut could continuously trigger GLP-1 secretion in neonates. In adults, fasting studies on GLP-1 secretion have been performed after a period of fasting greater than 8 h (4, 21, 22). In our study, the increment on GLP-1 concentration after feeds in the neonates is, however, much higher than those seen in the adult studies (4, 6–8); this suggests that feeds stimulate greater GLP-1 secretion in the neonates than in adults.

GLP-1 is also cleared by the kidneys and studies in rat kidney have shown that GLP-1 is removed from the peripheral circulation, by a mechanism that involves glomerular filtration and tubular catabolism (23). Hence, high plasma GLP-1 levels in newborn infants may reflect the immaturity of the DPP-IV enzyme
system or reduced clearance by the kidney. The glomerular filtration rate (GFR) at birth is low in full-term infants (typically 10–15 ml/min per m²) and doubles by 1 week of age. Adult values are reached by about 6 months of age (24). In patients with impaired GFR (due to chronic renal insufficiency), intact GLP-1 is not significantly elevated compared with the healthy subjects (25). This is because the kidney plays an important role in eliminating the metabolites of GLP-1 rather than intact GLP-1 (25). The plasma levels of metabolite GLP-1 (9–36) amide are elevated in chronic renal insufficiency suggesting that the kidney is a major site for its extraction. In our study, we have measured amidated GLP-1 and were not able to distinguish between the intact GLP-1 molecule and the metabolite GLP-1 (9–36) amide. It is possible that the metabolite GLP-1 (9–36) amide is present in high concentrations and that this may be due to the reduced GFR.

One could argue that immaturity of the DPP-IV enzyme complex may potentially increase the serum concentration of the intact GLP-1 and the biologically inactive metabolite GLP-1 (9–36 amide). However, since we were measuring metabolites of GLP-1 (9–36 and 7–36 amides) and their concentrations were high, we suggest that DPP-IV enzyme is well matured in neonates. Recent study has shown that DPP-IV inhibitors leads to nonlinear increase in DPP-IV and GLP-1 possibly either due to decreased secretion of GLP-1 by reverse feedback mechanism, or by metabolism of GLP-1 through as yet unknown alternative pathways (25, 26).

Plasma levels of total GLP-1 are elevated in adult patients undergoing gastric bypass surgery and postprandial elevations in levels of the GLP-1 have been described in patients after Roux-en-Y gastric bypass surgery (27, 28). Markedly elevated postprandial GLP-1 levels leading to the syndrome of postprandial hyperinsulinaemic hypoglycaemia (associated with pancreatic nesidioblastosis) have been postulated in several patients after gastric bypass (29, 30). Shorter length of gut in neonates with faster transit time could mimic dumping syndrome; however, despite the markedly elevated plasma GLP-1 concentrations and raised serum insulin concentrations in our study population, there was no postprandial hyperinsulinaemic hypoglycaemia at 20 or 60 min post-feed. This suggests that besides stimulating pancreatic β-cells to secrete insulin and maintaining blood glucose, GLP-1 has a further role in the maturation of enteroendocrine system and pancreatic β-cell proliferation.

Unlike adults, newborn babies and children require frequent feeding. This helps in maintaining blood glucose in the normal range and provides extra calories for growth. Increased frequency of feeding could help in maturation of gut and may provide excess incretins for development and maturation of the enteroendocrine system. High increment of GLP-1 after feeds could also protect the integrity of maturing neonatal enteric system from feed overload by reducing gastric emptying and functioning as an ileal break (31). The protection is probably further enhanced by the role of GLP-1 in increasing satiety and decreasing appetite through its effect on the hypothalamus (32).

In conclusion, this study has shown that total GLP-1 is markedly elevated in the basal and postprandial state in newborn infants. The markedly elevated GLP-1 levels are not associated with postprandial hyperinsulinaemic hypoglycaemia. The high GLP-1 levels may be due to immaturity of the DPP IV and the lower GFR in the neonatal period. The elevated GLP-1 levels in the newborn period may also have a role in regulating pancreatic β-cell mass and regeneration. Further studies are required to understand the role of GLP-1 in the neonatal period.

Declaration of interest
All authors declare that they do not have any financial or other potential conflict of interest and also there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements
We are grateful to the Biochemistry Department at Basildon, Great Ormond Street and Hammersmith hospital for processing the blood samples.

References