Glucagon-like peptide 2 has limited efficacy to increase nutrient absorption in fetal and preterm pigs

Per T. Sangild,1 Christiane Malo,2 Mette Schmidt,3 Yvette M. Petersen,3 Jan Elnif,3 Jens J. Holst,4 and Randal K. Buddington5

1Department of Human Nutrition, University of Copenhagen, Frederiksberg, Denmark; 2Membrane Transport Research Group, Department of Physiology, University of Montreal, Montreal, Quebec, Canada; 3Divisions of Animal Nutrition and Reproduction, University of Copenhagen, Frederiksberg C, Denmark; 4Department of Medical Physiology, Panum Institute, Copenhagen, Denmark; and 5Department of Health and Sports Sciences, University of Memphis, Memphis, Tennessee

Submitted 7 June 2007; accepted in final form 19 September 2007

Sangild PT, Malo C, Schmidt M, Petersen YM, Elnif J, Holst JJ, Buddington RK. Glucagon-like peptide 2 has limited efficacy to increase nutrient absorption in fetal and preterm pigs. Am J Physiol Regul Integr Comp Physiol 293: R2179–R2184, 2007. First published September 26, 2007; doi:10.1152/ajpregu.00395.2007.—Exogenous glucagon-like peptide 2 (GLP-2) prevents intestinal atrophy and increases nutrient absorption in term newborn pigs receiving total parenteral nutrition (TPN). We tested the hypothesis that the immature intestine of fetuses and preterm neonates has a diminished nutrient absorption response to exogenous GLP-2. This was accomplished using catheterized fetal pigs infused for 6 days (87–91% of gestation) with GLP-2 (25 nmol·kg


The immature small intestine of premature infants has underdeveloped capacities to absorb monosaccharides, which increases the risks for malabsorption and infections (16, 23). As a consequence, parenteral nutrition [total parenteral nutrition (TPN)] is initially used for some preterm neonates before enteral nutrients are provided. However, structural and functional development of the small intestine mucosa is reduced by TPN compared with enteral nutrition (9, 19, 21). This has led to a search for therapeutic protocols that will stimulate structural and functional development of the intestine during TPN.

The intestinotropic hormone glucagon-like peptide 2 (GLP-2) is a 33-amino acid peptide that is released from intestinal L-cells in response to luminal nutrients. Exogenous administration of GLP-2 stimulates intestinal growth and functional capacities in adult laboratory rodents (8) and humans with short bowel syndrome (12). When administered to term pigs receiving TPN, GLP-2 stimulates intestinal blood flow and increases glucose uptake and the abundances of sodium-glucose cotransporter-1 (SGLT-1) mRNA and protein and the activities of brush border membrane (BBM) enzymes (6, 10, 14, 21). Providing GLP-2 to preterm and term pigs and adults of other animals receiving TPN increases intestinal mass compared with TPN alone by inhibiting apoptosis, not by stimulating a higher rate of enterocyte proliferation (4, 5, 7). These promising findings and the presence of circulating GLP-2 and intestinal GLP-2 receptors in fetuses and even higher levels in newborn premature pigs (15) led us to explore the possible benefits of using GLP-2 to stimulate development of the immature intestine in utero and in preterm pigs receiving TPN (13, 14). Although some gastrointestinal tract characteristics respond to GLP-2 administration, those of fetuses and the preterm pigs were less pronounced compared with term pigs, corresponding with a prenatal increase in the expression of GLP-2 peptide and receptors.

We hypothesized that the therapeutic efficacy of GLP-2 depends on the stage of development and varies among different intestinal characteristics. To better understand the therapeutic potential of GLP-2 and the possible limitations, we measured intestinal dimensions, rates of nutrient absorption by intact tissues and isolated BBM vesicles, and the abundance of SGLT-1 mRNA after 6 days of administering GLP-2 to fetal pigs receiving nutrients via the placenta and to preterm pigs delivered by cesarean section at 92% of gestation and receiving TPN.

MATERIALS AND METHODS

All procedures using animals were approved by the Danish National Committee on Animal Experimentation.

In utero responses of fetal pigs to GLP-2. The in utero responses were studied using fetuses from six pregnant sows (Large White × Landrace) that were assigned to provide either control (n = 3 sows) or treated fetuses (remaining 3 sows). The sows were anesthetized at gestation day 99 ± 1 (87% of term). As described previously (13), a

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
midline incision was made to expose the uterus and provide access to the fetuses. A polyvinyl catheter was surgically placed into the carotid artery of three randomly selected fetal pigs that were of normal size (body mass of the fetuses was estimated based on size at the time of the procedure), after which the fetal neck and the uterus and body wall of the sow were closed. At the time of the surgery, a catheter was also placed in the uterine vein of the sow. Progestrone was administered (50 mg/day im) to the sows as a precaution against preterm labor.

All catheterized fetuses in individual sows were infused for 6 days with GLP-2 (Novo Nordisk, Bagsvaerd, Denmark) at a dose (12.5 nmol/kg in 0.9% saline) and frequency (twice each day for 2 h at 0700 and 1600) known to prevent intestinal atrophy and diminished nutrient absorptive capacities when provided to term pigs receiving TPN (21). An identical volume of 0.9% saline was provided to control pigs. The separate assignment of sows to provide saline or GLP-2 fetuses was to avoid the possible transfer of the exogenous GLP-2 among infused fetuses via the maternal circulation and possible vascular anastomoses in the placental incision site, which would compromise the use of saline fetuses from the same sow. In previous studies, we have detected litter differences for fetal, preterm, and term pigs. Therefore another 16 unoperated fetuses from the sows (n = 2–3 from each sow) were included as unoperated controls and were harvested and studied at the same time as the saline and GLP-2-infused fetuses. Data from the unoperated fetuses were used to detect if there were litter differences and, if necessary, include a litter effect in the analysis of the data. Of the 18 catheterized fetuses, 7 of the saline and 6 of the GLP-2 groups were alive and considered healthy at the time of the cesarean section.

Blood samples were collected from the sow and each fetus the day of the surgery and daily thereafter before infusion of saline or GLP-2 for measurement of pH, blood gases (PO2, PCO2), glucose, and electrolytes (Na+, Cl−, K+, Ca2+) using a blood-gas and electrolyte analyzer (NOVA Biomedical, Waltham, MA) and for hemoglobin using a hemoximeter (Radiometer, Copenhagen, Denmark). Plasma cortisol concentrations were determined by ELISA (Biomar Diagnostics, Marburg, Germany) using a method our laboratory has previously validated for use with pigs (20). Plasma GLP-2 was measured on day 0 before GLP infusion and day 6 when the fetuses were harvested 12–14 h after the last administration using a method also previously validated for pigs (11, 15). On day 4, a GLP-2 clearance study was performed by measuring plasma concentrations of GLP-2 immediately after (0 min) the 0700 infusion, and at 60, 120, and 180 min after infusion of the saline or GLP-2.

After the 6 days of infusion (at 91% gestation), the sows were anesthetized, and the fetuses were removed and euthanized (sodium pentobarbitone, 200 mg/kg iv), blood was collected by cardiac puncture, and the entire small intestine was harvested and flushed with ice-cold mamalian Ringer solution that had been aerated with a gas mixture of 95% O2 and 5% CO2. After the length and wet mass were recorded, the intestine was separated into three segments of equal length (designated as proximal, mid, and distal regions), which were weighed separately and placed in the cold aerated Ringer for evaluation of intestinal dimensions and nutrient absorption. Additional segments from each region were fixed for analysis of villus morphology (see Refs. 5, 13).

Postnatal responses of preterm pigs to GLP-2. A total of 15 pigs from three different sows (Large White × Landrace) were delivered by cesarean section at 92% of gestation (106 ± 2 days). The sows were injected with PGF2α (175 μg im cloprostenol, Estrumate; Pitman-Moore, Harefield, UK) 20 h before delivery to initiate the normal metabolic and endocrine changes that occur in fetuses and sows before spontaneous delivery (24). Immediately after delivery, each pig had catheters placed in the arterial and venous systems via the umbilicus before they were housed individually in infant incubators maintained at 34–36°C with 80–100% moisture and 1–2 liters of supplemental O2 per minute (19).

All of the preterm pigs were maintained on TPN using an elemental nutrient solution consisting of free amino acids (45.5 g amino acids/l; Vamin 18F), glucose (72.5 g/l), lipid (30.7 g/l; Intralipid), minerals, and vitamins (2% Calcium-Sandoz, 0.6% Addiphos, 0.6% Peditrace, 1.0% Vitalipid, and 0.1% Soluvit; KABI Pharma). The TPN solution was administered continuously via the venous catheter to 15 of the pigs at a rate of 170 ml·kg−1·day−1, which provided 550 kJ·kg−1·day−1 and 8 g amino acids·kg−1·day−1. The nutrient solution was supplemented with 2-h infusions twice each day (at 0700 and 1600) of either human GLP-2 (12.5 nmol/kg, GLP-2; n = 8) or an identical volume of 0.1% porcine serum albumin in 0.9% buffered saline (controls; n = 7).

Blood samples were drawn from the arterial catheter each morning to monitor blood gases and metabolites. Concentrations of GLP-2 and cortisol were measured on days 2, 4, and 6 before the morning GLP-2 infusion. In addition, on the morning of day 6, we collected plasma samples at 0, 60, and 120 min after the completion of the 2-h GLP-2 infusion. The pigs were killed and processed as described above for the fetal pigs.

Measurement of intestinal dimensions. A 10-cm segment was removed from the middle of each of the three regions and opened along its length. The circumference of a segment was used to calculate nominal surface area of the segment (cm2; not accounting for surface area amplification due to villi and microvilli). The percentage of mucosa for each segment was determined on a dry matter basis by gentle scraping with a plastic slide and drying both the mucosa and the underlying tissues (50°C for 72 h). The 10-cm segments were assumed to be representative of each region and were used to calculate regional values, which were summed to provide estimates of the surface area and mucosal mass for the entire small intestine.

Measurement of nutrient absorption by intact tissues. An adjacent segment from each region was used to prepare everted sleeves for measuring initial rates of carrier-mediated glucose transport and rates of absorption (carrier mediated and carrier independent) for leucine, lysine, and proline, which are substrates for the neutral, basic, and imino amino acid transporters (2). The tissues were incubated for 2 min in Ringer with tracer concentrations of D-[14C]glucose with L-[1-14C]glucose or L-[1-14C]leucine with [14C]polyethylene glycol (mol wt = 4,000). Rates of glucose transport and amino acid absorption were measured in the presence and absence of 50 nmol/l of the same, but unlabeled nutrient and uptake values were normalized to tissue mass.

The capacity of each region to transport glucose and absorb the amino acids was calculated as the product of rates of uptake times regional mass. The capacities of the entire small intestine, which were estimated by summing the regional values, were normalized to body weight to account for individual variation.

Ratios were calculated for the accumulation of tracer nutrient by the tissues in the absence relative to the presence of 50 nmol/l unlabeled nutrient. These accumulation ratios were used to determine whether a saturable component of absorption was present (2). Specifically, accumulation ratios > 1.0 were considered to be indicative of competition between tracer and unlabeled nutrient for a limited number of transporters, whereas values not differing from 1.0 suggest the transporters were present in very low densities and absorption was largely occurring by simple diffusion.

Measurement of nutrient transport by BBMVs. A 2- to 3-cm piece was removed from each of the three regions, immediately frozen in liquid nitrogen, and stored at −70°C until used to measure BBMV nutrient uptake. A standard protocol was used to prepare and evaluate the purity of the BBMV and to measure rates of nutrient accumulation (26). Briefly, the frozen tissues were homogenized, and the BBMV were isolated using MgCl2 precipitation and resuspended in 50 mM Tris-HEPES buffer (pH = 7.5) with 0.1 mM MgSO4, 200 mM KCl, and 125 mM mannitol. Maltase activity in the homogenates and BBMV was assayed to assess enrichment. Aliquots of the final BBMV preparation were added to an uptake medium consisting of 50 mM
Tris-HEPES buffer (pH = 7.5) with 0.1 mM MgSO₄, 192 mM NaCl, 8 mM KCl, 125 mM mannitol, and tracer concentrations of glucose or amino acid (4 μM). Accumulation of the tracer nutrient by the BBMV at 37°C was measured during the linear phase using a fast sampling, rapid filtration device programmed to collect nine samples over the first 2.7 s of incubation for leucine and the first 4.5 s for lysine, proline, and glucose. Due to the limited amounts of tissue available, uptake by BBMV prepared from fetuses was limited to glucose and leucine. The initial rates of BBMV uptake were calculated from the relationship between incubation time and accumulation of radioactivity using linear regression analysis. If a curve deviated from linearity, the first-degree coefficient of the second-degree polynomial was instead used.

**Abundance of SGLT-1 mRNA.** Full-thickness pieces of proximal small intestine were snap frozen in liquid nitrogen and stored at −70°C until used to isolate total cellular RNA. The abundance of SGLT-1 mRNA was quantified by relative reverse transcription and polymerase chain reactions (PCR), as described previously (13, 21). The oligonucleotide primers for the detection of cDNA were specific for porcine SGLT-1 (sense, 5′-CGAAGTAGTGGTGGTGCGCC-3′, annealing temperature of 55°C), and expression was evaluated relative to an internal 18S ribosomal RNA standard, with competitor primers included in the reaction mixture (at a ratio of 3:7). PCR amplification products were electrophoresed on 1% agarose gels and visualized with 0.1% elidinium bromide. The abundances of SGLT-1 PCR products were related to those for 18S RNA based on optical densitometry readings.

**Data analysis.** Values presented in Tables 1 and 2 and Figs. 1–3 are means (or least squares means when a litter effect was detected) and standard errors. The effects of treatment and intestinal region (proximal, middle, and distal) were analyzed by analysis of variance using a linear model. Our previous studies have revealed significant variation in intestinal structure and functions among fetuses and preterm newborn pigs that originate from different sows. Therefore, a litter effect was included in the model for evaluation of treatment and region effects. For the fetal studies, values from unoperated fetuses were included in the statistical analyses to account for sow (litter) effects in the evaluation of treatment effects. When a treatment (or region) effect was detected, the least significant difference test was used to detect differences between individual means. Rates of nutrient uptake by intact tissues and BBMV were analyzed across the three regions. The univariate procedure (SAS, 1998) was used to detect accumulation ratios that differed from 1.0. A probability value of $P < 0.05$ was used as the critical level of significance for all statistical evaluations.

**RESULTS**

**In utero responses of pigs to GLP-2.** Although concentrations of GLP-2 in the fetuses at the time of surgery were below the limit of detection (<5 pM), plasma samples collected on day 6 from saline-infused fetuses averaged 14 ± 2 pM GLP-2 (controls, Fig. 1). This was comparable to the 15 ± 4 pM measured in unoperated fetuses when they were harvested on day 6, but much lower than values for the seven GLP-2 fetuses (252 ± 98 pM, $P < 0.05$ basals, Fig. 1). Immediately after the morning 2-h GLP-2 infusion on day 4, fetal GLP-2 levels were orders of magnitude higher than basal levels (190,000 ± 72,000 pM at 0 min; Fig. 1). The concentrations declined thereafter, although they remained significantly higher at 180 min postinfusion (2,093 ± 1,416 pM) relative to values before infusion ($P < 0.05$). Values in the maternal (sow) plasma over the 6 days of the experiment averaged 28 ± 9 pM and did not differ between sows with saline or GLP-2-infused fetuses.

**Plasma cortisol levels in blood samples collected immediately after delivery were similar between fetuses receiving GLP-2 and saline, with both tending to be higher compared with unoperated pigs (58 ± 11 ng/ml for pooled data vs. 35 ± 7 ng/ml, $P = 0.10$). Body weight did not differ among the unoperated pigs and those receiving GLP-2 or saline (mean 1.17 ± 0.04 kg). Administering GLP-2 in utero did not increase intestinal dimensions compared with saline controls, except for the greater circumference ($P < 0.05$; Table 1).

**Administration of GLP-2 in utero did not increase rates of carrier-mediated glucose uptake by intact tissues (Fig. 2) and BBMV (Table 2), or increase the glucose uptake capacities of the entire small intestine compared with control fetuses receiving saline and unoperated controls (data not presented). The accumulation ratios for glucose transport were also similar for both groups (46 ± 4) and were indicative that a saturable component of glucose absorption was present. The undeveloped response of glucose transport to GLP-2 was corroborated by the lack of a significant increase in the abundance of SGLT-1 mRNA (Fig. 3).

**Rates of amino acid absorption by intact tissues (analyzed across regions) after 5 days of GLP-2 did not differ among groups for leucine and proline, but were higher for lysine (Fig. 2), resulting in higher absorption capacities for lysine ($P < 0.05$). The lack of a GLP-2 response for leucine uptake was also evident from the BBMV uptake measurements (Table 2); lysine and proline were not studied in the fetuses). Accumulation ratios for leucine were similar among groups (32 ± 3; pooled data for control, GLP-2, and unoperated fetuses) and were indicative that a saturable component of absorption was present. The accumulation ratios for lysine were lower, and the response to GLP-2 did not reach significance (3.3 ± 0.4 vs. 4.9 ± 0.4; $P = 0.06$). Accumulation ratios were lowest for proline and particularly for fetuses receiving GLP-2 (1.3 ± 0.2 vs. 2.2 ± 0.2; $P < 0.01$ for the comparison of GLP-2 pigs with pooled data for control and unoperated fetuses, which were similar).

**Responses of preterm pigs to postnatal GLP-2.** Compared with newborn term pigs delivered by cesarean section (17), the
Premature pigs were hypoxic and had hypercapnia and acidemia for the first 24 h after birth. This was evident from the lower blood pH (7.31 ± 0.01 vs. 7.47 ± 0.01) and oxygen saturation (67 ± 5 vs. 98 ± 2%), and higher PCO₂ values (65 ± 3 vs. 39 ± 1 Torr). After 24 h, the values were comparable to those of newborn term pigs and did not differ between the groups.

In all pigs, intact tissue absorption declined significantly from the proximal to the distal small intestine for all four nutrients, but the gradient was more pronounced for glucose compared with the three amino acids (data not presented).
GLP-2 infusion did not increase rates of absorption by intact tissues (nmol·mg⁻¹·min⁻¹) averaged across the three regions for glucose and the amino acids (Fig. 2) and by BBMV (Table 2), nor did it increase the abundance of SGLT-1 mRNA (Fig. 3). However, because of 31% more intestinal mass, absorptive capacities for the entire small intestine (μmol·kg⁻¹·min⁻¹) of the GLP-2 pigs tended to be higher (P < 0.1 for comparisons of all 4 nutrients) compared with those of pigs receiving saline.

Similar to fetal pigs, accumulation ratios for glucose indicated a saturable component of uptake was present. Among the amino acids, the ratios for leucine were higher (P < 0.05) than for lysine, with both exceeding a value of 1.0. The accumulation ratio for proline (0.7 ± 0.1) did not differ from 1.0, and the apparent lack of a saturable component of uptake corresponded with low to negligible accumulation of proline by BBMV.

DISCUSSION

The final 10% of gestation is critical for the maturation of essential internal organs, including the gastrointestinal tract, and pigs that are delivered before 90% of gestation have very limited viability, even with intensive care. Exemplifying this critical period of prenatal development are the dramatic increases during the final 10% of gestation in the capacities of the pig small intestine to absorb nutrients due to a combination of rapid increases in intestinal size and mass-specific rates of nutrient absorption (3, 19). Corresponding with this, rates of glucose uptake by intact tissues, BBMV, SGLT-1 mRNA abundance, and glucose uptake capacities of the entire small intestine for preterm pigs exceeded corresponding values for fetuses. The impaired glucose absorption typical of preterm infants (16, 23) has led to the consideration that maturation of the pig intestine during the final 10% of gestation is comparable to what occurs during the third trimester of human fetal development (20, 21). The present findings provide additional evidence of the complex interactions that exist among the genetic determinants and signaling molecules that drive perinatal intestinal development. Moreover, our findings address a critical need for more information about ways to accelerate structural and functional development of the immature small intestine of preterm infants to improve clinical outcomes.

In utero responses of fetuses to exogenous GLP-2. Circulating levels of endogenous GLP-2 increase before birth, and the highest concentrations are measured shortly after the onset of suckling, although the response to feeding may be slightly delayed if pigs are delivered preterm (15). Infusion of 25 nmol·kg⁻¹·d⁻¹ GLP-2 into fetuses resulted in circulating concentrations that were three orders of magnitude higher than those measured in the unoperated and saline-infused fetuses (present study) and the concentrations we have measured in the blood of fetal and preterm pigs after enteral feeding (1, 15). These findings indicate the amount of GLP-2 administered in utero significantly exceeded endogenous production and represents a pharmacological dose. Moreover, the nearly 70-fold higher concentrations 1 h after infusion in the GLP-2 fetuses than in the GLP-2 preterm pigs, despite the same dosage, suggest the fetuses had a reduced rate of clearance. To avoid anemia in the fetal and preterm pigs, the blood sample collection was restricted to few time points that did not allow for an accurate evaluation of GLP-2 clearance rates following exogenous infusions.

Despite the presence of GLP-2 receptor mRNA (15), the structural responses of the fetal intestine between 87 and 91% of gestation to the elevated plasma concentrations of GLP-2 were limited to the increase in small intestinal circumference (present study), but without an increase in villus height (15). Moreover, the increased absorption of lysine, but not of the other amino acids and glucose, is similar to the increase in activity of aminopeptidase N, but not of four other BBM enzymes (13). The restricted response of the fetal intestine to GLP-2 implies the receptors are not expressed, are not functional, or the downstream signaling pathway is not fully developed. Alternatively, the selective responses of some, but not all, intestinal characteristics to GLP-2 may reflect different trajectories of development for the various intestinal characteristics. Hence, if GLP-2 accelerates intestinal maturation, characteristics that mature earlier in gestation are more likely to exhibit a greater magnitude of response.

Postnatal responses of preterm neonates to GLP-2. The intestines of newborn term pigs receiving enteral nutrients or TPN with GLP-2 grow faster, have greater hydrolytic capacities, and higher rates and capacities of nutrient absorption than those of littermates receiving TPN alone (14, 19–21). Newborn preterm pigs receiving enteral nutrients or GLP-2 during TPN also have larger intestines relative to pigs receiving TPN alone (Refs. 5, 19, present study). However, rates of nutrient absorption by intact tissues and BBMV are not increased by administration of GLP-2 to preterm pigs receiving TPN (present study) or by providing enteral nutrients (19). The lack of response of the nutrient transporters to GLP-2 was corroborated by an insignificant increase in the abundance of SGLT-1 mRNA. As a result, even though the GLP-2 pigs had larger intestines, the capacities of the entire small intestine to absorb nutrients were only 10–20% higher than those of TPN pigs. These findings suggest that GLP-2 administration to preterm pigs receiving TPN elicits only slight and nonsignificant increases in the abundances and activities of BBM transporters. In contrast, providing GLP-2 to pigs delivered at term and receiving TPN resulted in carrier-mediated glucose absorptive capacities that were almost 2.5-fold higher than those of pigs receiving only TPN due to a combination of increases in intestinal dimensions and the expression of SGLT-1 (21).

It is uncertain if and how different stages of development might respond to the infusion of pharmacological doses of GLP-2. The reduced response elicited in the fetal and preterm pigs may have been caused by downregulation of the GLP-2 receptors, especially in the fetal pigs. However, administration of a similar dose to neonatal term pigs receiving TPN elicited pronounced morphological, histological, and functional responses by the small intestine (5, 13, 14, 21). An alternative explanation is that, despite the presence of GLP-2 receptor mRNA receptors in fetuses and preterm pigs (15), the associated signaling pathways may not be fully developed, thereby restricting the range and magnitude of responses to exogenous GLP-2.

Perspectives. Development of nutritional and therapeutic regimens for preterm infants with immature gut functions will be facilitated by a better understanding of the ontogenetic development of the signaling networks that regulate gastrointestinal tract growth and maturation. Our present findings with fetal and preterm pigs, in conjunction with our laboratory’s previous studies using term pigs (21), indicate that, despite the
production of endogenous GLP-2 and the presence of GLP-2 receptors during the last 10% of gestation, the responses of the pig intestine to administration of exogenous GLP-2 are reduced. Regulation of intestinal maturation by glucocorticoids also develops during late gestation in pigs (18, 22, 25). However, even though cortisol levels increase considerably during the period of fetal development examined, this did not correspond with an increased responsiveness to GLP-2. Hence, the therapeutic efficacy of GLP-2, glucocorticoids, and other molecules that regulate GIT development are restricted to specific stages of development.

ACKNOWLEDGMENTS

The technical assistance of Merete Stubgaard, Bente Synnether, and Inger Heintze is gratefully acknowledged. The GLP-2 was a generous gift from L. Thim of Novo Nordisk, Bagsvaerd, Denmark.

GRANTS

The work presented here was supported by the Danish Research Councils.

REFERENCES


