Leucine (Leu) is an essential branched-chain amino acid, which activates the mammalian target of rapamycin (mTOR) signaling pathway. The effect of Leu on cell differentiation during embryonic development is unknown. Here, we show that Leu supplementation during pregnancy significantly increased fetal body weight, caused fetal hyperglycemia and hypoinsulinemia, and decreased the relative islet area. We also used rat embryonic pancreatic explant culture for elucidating the mechanism of Leu action on β-cell development. We found that in the presence of Leu, differentiation of pancreatic duodenal homeobox-1-positive progenitor cells into neurogenin3-positive endocrine progenitor cells was inefficient and resulted in decreased β-cell formation. Mechanistically, Leu increases the intracellular levels of hypoxia-inducible factor 1-α, a repressor of endocrine fate in the pancreas, by activating the mTOR complex 1 signaling pathway. Collectively, our findings indicate that Leu supplementation during pregnancy could potentially increase the risk of type 2 diabetes mellitus by inhibiting the differentiation of pancreatic endocrine progenitor cells during a susceptible period of fetal life.

Type 2 diabetes mellitus (T2DM) is a progressive disease in which glucose homeostasis is compromised as a result of impaired insulin secretion. Results from human and animal studies support the idea that the β-cell mass in T2DM cannot adapt insulin secretion in spite of increased insulin needs (1). Thus, understanding the underlying mechanisms that regulate β-cell mass could be used to develop new strategies to treat T2DM. T2DM is frequently associated with body weight gain and obesity and a high nutritional state, and it is now well established that nutrients regulate β-cell mass and function (2). Nutrients not only provide energy but also function as signaling molecules that directly influence feeding behavior, energy production, cell growth, and differentiation (3). For example, nutrients, such as folate and taurine, are now used as dietary supplements during gestation to protect the fetus from developing birth defects (4,5). Leucine (Leu), an essential branched-chain amino acid (BCAA), is a nutrient that increases the birth weight of newborns and prevents the development of a fetal growth defect in pregnancies where fetal growth is abnormal (6,7). In addition to being a crucial amino acid for protein synthesis, Leu is a potent activator of the mammalian target of rapamycin (mTOR), a Ser/Thr kinase, which is involved in many cellular processes that include protein synthesis, cell growth, and metabolism (8). In mature pancreatic β-cells, Leu has many functions, such as stimulating insulin release and regulating gene expression and protein synthesis (9). However, the function of Leu in pancreatic cell development has not been elucidated.

The mature pancreas contains exocrine acinar cells that secrete digestive enzymes into the intestine and endocrine islets that synthesize hormones, such as insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells), and pancreatic polypeptide (PP cells). The pancreas originates from the dorsal and ventral regions of the foregut endoderm directly posterior to the stomach. Signals that originate from adjacent mesodermal structures control pancreatic development and the formation of endocrine and exocrine tissue (10). Results from studies in genetically engineered mice have identified a hierarchy of transcription factors that regulate pancreatic specification, growth, and differentiation (11). First, the pancreas-committed endodermal region of the foregut expresses the homeodomain factor pancreatic duodenal homeobox-1 (PDX-1) (12). Next, the basic helix-loop-helix transcription factor, neurogenin3 (NGN3), initiates the endocrine differentiation program in epithelial pancreatic progenitor cells. Indeed, Ngn3-deficient mice fail to generate any pancreatic endocrine cells (13), and the results from lineage-tracing experiments have provided direct evidence that NGN3-expressing cells are islet progenitors (14). Subsequently, additional transcription factors then determine the specific endocrine cell fate (15).

Here, we evaluated the effect of dietary Leu supplementation on the regulation of β-cell mass during pancreatic development. We report that increasing Leu intake in pregnant rats resulted in hyperglycemic hypoinsulinemic fetuses with increased body weight without an adaptation in fetal β-cell mass. To elucidate the effect of Leu on β-cell mass regulation, we used an in vitro bioassay that mimics the major steps that occur during β-cell development from fetal pancreatic progenitor cells (16). Our data demonstrate that Leu increased the expression of the hypoxia-inducible factor 1-α (HIF-1α), a repressor of the development of NGN3-positive pancreatic endocrine progenitor cells (17), by activating the mTOR pathway, which, in turn, prevented β-cell development.

Previous results demonstrate that mTOR has a crucial role in adult β-cell proliferation (18). The results of the current study delineate a new role for the mTOR signaling pathway in the control of β-cell differentiation during prenatal life. Our results also indicate that dietary Leu supplementation and other mTOR activators during a short sensitive period of fetal pancreatic development can increase the risk of developing T2DM in later life.

**RESEARCH DESIGN AND METHODS**

Pregnant Wistar rats were purchased from CERJ (Le Genest, France). The first day post coitum was taken as embryonic day 0.5 (E0.5). The pregnant rats were...
supplementation (data not shown). On the other hand, Leu supplementation increased fetal body weight (5.9 ± 0.08 vs. 4.8 ± 0.12 g; P < 0.005) (Fig. 1A) and fetal pancreatic weight (408.7 ± 17.62 vs. 317.3 ± 24.53 mg; P < 0.005) (Fig. 1B). Leu supplementation had no effect on the fetal β-cell mass in absolute terms but resulted in a reduction in the relative fetal β-cell mass, as measured by the decreased β-cell area–to–pancreatic area and β-cell mass–to–fetal body weight ratios (Fig. 1C and D). This relative defect in the β-cell mass in pups of the Leu-supplemented dams was associated with an increase in fetal whole blood glucose levels (213 ± 6.4 vs. 114.3 ± 9.4 mg/dL; P < 0.005) (Fig. 1E) and a decrease in fetal plasma insulin levels (2.90 ± 0.05 vs. 4.12 ± 0.11 ng/mL; P < 0.005) (Fig. 1F).

We next asked whether pups of Leu-fed experiments have the ability to compensate for the low β-cell mass at birth. We first assessed β-cell proliferation in 2-week-old rats under Leu treatment during prenatal life. The proliferative rate measured by bromodeoxyuridine incorporation was unchanged compared with 2-week-old control rats (Supplementary Fig. 1A). After aged 4 weeks, body weight and glycemia measured after an overnight fast were similar in rats under Leu treatment during prenatal life compared with controls (Supplementary Fig. 1B; some data not shown). However, these rats showed impaired glucose tolerance compared with controls, while insulin resistance was not observed (Supplementary Fig. 1C and D). Finally, in 4-week-old rats under Leu treatment during prenatal life, β-cell mass defects persisted (data not shown).
prenatal life, β-cell mass remained lower compared with 4-week-old control rats (Supplementary Fig. 1E).

Since Leu supplementation of the pregnant dams resulted in a β-cell mass defect in the pups, we decided to investigate the mechanism of Leu action on β-cell development.

**In vitro Leu treatment impairs β-cell development.** To determine the role of Leu in pancreatic development, we cultured E13.5 pancreata for 7 days under conditions that allowed endocrine and acinar cell development (16). Leu treatment (10 mmol/L) did not modify pancreatic shape and growth (Fig. 2A and C for quantification), or acinar cell development, as measured by amylase immunostaining (Fig. 2B and C for quantification). On the other hand, Leu treatment sharply decreased the β-cell number, as measured by insulin immunostaining (Fig. 2B and C for quantification). Since Leu is a potent insulin secretagogue, the expressions of four other β-cell markers were determined by immunohistochemistry to rule out the possibility that the decreased insulin immunostaining after Leu treatment was due to its potency as an insulin secretagogue. The expressions of prohormone convertase 1/3 (Supplementary Fig. 2A), PDX-1 (Supplementary Fig. 2B), the transcription factor MafA, and the zinc transporter Slc30a8 (Supplementary Fig. 2C) were all reduced. Finally, the effects of l-Leu on β-cell development were not mimicked by d-Leu (data not shown). Such data indicate that l-Leu treatment represses β-cell development.

**Leu treatment impairs endocrine progenitor cell development.** To determine how Leu treatment impairs β-cell development, we cultured E13.5 pancreata with or
without 10 mmol/L Leu for 1, 3, 5, and 7 days. Leu treatment did not modify amylase mRNA levels but decreased insulin mRNA levels (Fig. 3). We next determined the expression levels of Ngn3 mRNA, a specific pancreatic endocrine progenitor marker (13), in cultured E13.5 pancreata. In E13.5 rat pancreatic explants, as previously shown, in absence of added Leu, Ngn3 mRNA levels increased after 1 day of culture, peaked on day 3, and then decreased (16). When Leu was added, we observed a dramatic decrease in Ngn3 mRNA levels (Fig. 3A). This decrease in Ngn3 mRNA levels was paralleled by a decrease in the number of Ngn3-expressing cells (4728 ± 6408 vs. 959 ± 28; P < 0.01) (Fig. 3B and C). Finally, Leu also caused a dose-dependent repressive effect on the mRNA levels of the three genes, namely Ngn3, its target Insm1 (23), and insulin (Supplementary Fig. 3). Collectively, these results indicate that Leu treatment decreased both the number of endocrine progenitor cells that express NGN3 and the number of β-cells.

To confirm our in vitro finding that Leu decreases the number of NGN3-positive endocrine progenitors, we then determined NGN3 expression in pancreatic cells of E16 fetuses from the pregnant dams that drank Leu-supplemented water for 3 days, starting on E13.5. At E16, Leu supplementation reduced the number of NGN3-positive cells (Fig. 4A and B), the number of β-cells (Supplementary Fig. 4A and B), and mRNA expression levels of Ngn3 and its target gene, Insm1 (Fig. 4C). Leu supplementation had no effect on amylase mRNA levels in E16 pancreata (Fig. 4C).

**Leu decreases the number of Ngn3-positive endocrine progenitor cells via mTOR complex 1.** In many tissues, Leu activates the mTOR signaling pathway (24,25). This
activation also occurs in the fetal pancreas where Leu treatment enhanced phosphorylation of the p70 S6 kinase substrate, 40S ribosomal protein S6 kinase (rpS6), and 4E-BP1 (Fig. 5A and Supplementary Fig. 5 for quantification). We next used rapamycin, a specific inhibitor of mTOR complex 1 (mTORC1) to interfere with this process. At 10 nmol/L, rapamycin treatment blocked rpS6 phosphorylation in E13.5 rat fetal pancreata without affecting Akt phosphorylation on Ser473 (Fig. 5B). At a higher concentration (100 nmol/L), rapamycin was less specific: it repressed both rpS6 and Akt phosphorylation (Fig. 5B) and decreased global pancreatic cell growth (data not shown). Thus, 10 nmol/L concentration of rapamycin was used in subsequent experiments. It is important that rapamycin (10 nmol/L) inhibited Leu-induced rpS6 phosphorylation in cultured E13.5 rat pancreatic buds (Fig. 5C).

We then asked whether rapamycin treatment could reverse the effects of Leu on pancreatic β-cell development in cultured E13.5 rat pancreatic buds. Rapamycin (10 nmol/L) alone did not modify pancreatic growth or β-cell and acinar cell differentiation (Fig. 5D and E). Leu caused a dramatic decrease in insulin expression (Fig. 5F) and β-cell development (Fig. 5D and E), whereas rapamycin reversed Leu-induced repression of β-cell development in the fetal pancreas (Fig. 5D–F). Although rapamycin alone did not modify Ngn3 expression, which was used as a marker of the endocrine progenitor cells, rapamycin did reverse the repressive effects of Leu on Ngn3 expression. (Fig. 5G). These results suggest that Leu activates an mTOR-dependent signaling pathway to repress the development of Ngn3-expressing pancreatic endocrine progenitor cells and inhibit β-cell development.

Other BCAAs, such as Val, also have been shown to activate the mTOR signaling pathway in different cell types (8,27). Supplementary Fig. 6A–C shows the effect of Val on cultured E13.5 rat pancreata. Val mimicked the effects of Leu. In fact, Val treatment (10 nmol/L) for 24 h induced rpS6 phosphorylation (Supplementary Fig. 6A). Val also decreased both Ngn3 and insulin mRNA levels in the cultured E13.5 rat pancreata after 3 and 7 days of exposure to Val (Supplementary Fig. 6B and C), while Ala, one of the simplest amino acids, did not affect mTOR signaling and β-cell differentiation (Supplementary Fig. 6D–F). These findings indicate that BCAAs suppress Ngn3 expression and β-cell development.

**Leu induces HIF-1α, an inhibitor of β-cell differentiation.** We next determined how Leu, via the mTORC1 pathway, repressed Ngn3 expression. We previously demonstrated that HIF-1α is a repressor of Ngn3 expression (17). Western blot analysis of the protein lysates from pooled E13.5 fetal pancreata indicates that Leu treatment for 1 or 7 days increases HIF-1α expression and mTOR signaling (Fig. 6A). It is interesting that this induction of HIF-1α expression was partly reduced by 10 nmol/L rapamycin, which inhibited the phosphorylation of rpS6 (Fig. 6B and C). We then measured the effect of Leu on the expression of the HIF-1α targets. Leu increased the expression of Glut1 and Redd1 (Fig. 6D), Vegf, Ldhα, and Pkg1 mRNA (Supplementary Fig. 7). Rapamycin alone did not modify Glut1 and Redd1 mRNA levels, whereas it reversed the inductive effect of Leu on HIF-1α targets (Fig. 6B).

Collectively, our data indicate that Leu increases intracellular HIF-1α levels and activates the HIF-1α signaling pathway, and these two effects are mediated by the mTOR signaling pathway. This process results in Ngn3 repression and, consequently, decreases β-cell differentiation.

**DISCUSSION**

In this study, we provide both in vivo and in vitro evidence that Leu impairs β-cell development without compensatory process after birth. We also demonstrate that Leu represses β-cell development in the developing pancreas by activating the mTOR signaling pathway and increasing HIF-1α levels, a repressor of pancreatic endocrine progenitor cells (17).

In mature pancreatic β-cells, Leu has many functions. Leu can stimulate insulin release (28). Long-term Leu treatment of islets from T2DM patients improves the insulin secretory function of pancreatic β-cells (29), and Leu administration improves glycemic control in humans and rodents with T2DM (30,31). In pancreatic β-cells, Leu regulates gene transcription and protein synthesis (27,29). Leu also...
regulates β-cell proliferation, and this effect is mediated by the mTOR signaling pathway (32). Specifically, adult mice with a β-cell–specific deletion of the tuberin/TSC2 protein, the main inhibitor of mTOR activity, have increased β-cell proliferation and are hyperinsulinemic and hypoglycemic (18). Rapamycin, an mTORC1 inhibitor, has been reported as being a repressor of mature β-cell proliferation (33). Thus, the role of Leu and the mTOR signaling pathway in
mature β-cell proliferation is now well established. Although it is known that the mTOR signaling pathway regulates the differentiation of many cell types, such as adipocytes or hematopoietic stem cells (34,35), its role in regulating pancreatic cell differentiation still remains undefined.

Our first set of data indicated that Leu supplementation of pregnant dams caused an increase in fetal body weight and fetal hyperglycemia without an adaptive response in fetal β-cell mass. To understand the mechanism of Leu action on fetal pancreatic development, we investigated the actions of Leu in an E13.5 pancreatic explant assay, which we have previously used for defining the different factors and conditions that regulate β-cell development from PDX-1–positive pancreatic progenitors (16,17,22). Using this assay, we demonstrated that Leu, a known activator of the mTOR signaling pathway (24,25), does not modify pancreatic shape and growth or acinar cell development. On the other hand, we demonstrated that Leu regulates a highly specific step in β-cell development, namely, the formation of NGN3-positive endocrine progenitor cells from PDX-1–positive pancreatic progenitor cells. This is a crucial step in endocrine cell development in the pancreas because NGN3-deficient mice lack pancreatic endocrine cells (13). The signals that control the development of NGN3-positive endocrine progenitor cells from PDX-1–positive pancreatic progenitor cells are poorly defined. Specifically, the signals and conditions that underlie the differentiation of embryonic stem cells into PDX-1–positive pancreatic progenitor cells are now well established (36,37). On the other hand, much remains to be discovered regarding the signals that regulate the next steps in the development of the endocrine pathway (i.e., differentiation of PDX-1–positive pancreatic progenitor cells into endocrine progenitor cells and mature endocrine cells) (38). Here, we report that Leu is an inhibitor of β-cell differentiation by repressing Ngn3 induction in pancreatic progenitor cells, and this action is mediated by the mTOR signaling pathway.

mTOR protein exists in two functionally distinct complexes: mTORC1 and mTORC2 (39,40). mTORC1 is sensitive to rapamycin. This complex contains raptor, the regulatory-associated protein of mTOR, which interacts with mTOR, as well as modulates the phosphorylation of rpS6 and 4E-BP1, both of which are key regulators of protein translation (39). In contrast, the rapamycin-insensitive mTORC2 contains rictor, which is involved in Akt phosphorylation at Ser473 (40,41).

Our data demonstrate that Leu triggers the phosphorylation of both rpS6 and 4E-BP1 in the pancreas and that this action is blocked by rapamycin at a concentration (10 nmol/L) that does not affect Akt phosphorylation. We found that Leu can influence the fate of endocrine progenitor cells by inhibiting the formation of NGN3-positive endocrine progenitor cells from PDX-1–positive pancreatic progenitor cells. Since this inhibition can be prevented by 10 nmol/L rapamycin, it is reasonable to assume that the activity of mTORC1 and the actions of Leu are closely linked. Of interest, rapamycin at a concentration that selectively inhibits activity of mTORC1 did not modify pancreatic development, and this result suggests that basal

FIG. 6. Leu induces HIF-1α, a repressor of β-cell differentiation. A: Representative immunoblot for HIF-1α expression in protein lysates from E13.5 pancreatic buds that were treated with Leu for 1 or 7 days. Immunoblot for phospho (P)-rpS6 indicates that Leu treatment induced mTOR activity. B: Representative immunoblot for HIF-1α in protein lysates from E13.5 pancreatic buds that were treated for 24 h with rapamycin (Rapa), Leu, or Leu and rapamycin. Immunoblot for P-rpS6 indicates that Leu treatment induced mTOR activity. The quantified results are presented in (C). D: Real-time PCR quantification of two HIF-1α target genes, Glut1 and Redd1 mRNA in pancreata after 0, 1, 3, 5, and 7 days in culture without Leu (white bars), with rapamycin (gray bars), with Leu (black bars), or with both rapamycin and Leu (dashed bars). Data are mean ± SEM of at least three independent experiments. *P < 0.05. (A high-quality color representation of this figure is available in the online issue.)
mTORC1 activity is not required for β-cell differentiation from pancreatic progenitor cells. This lack of need for mTORC1 in cell development has also been described for other cell types, such as hematopoietic stem cells (42). In these cells, activation of mTORC1 leads to loss of stem cell quiescence and stem cell exhaustion, and rapamycin inhibits mTORC1 signaling without affecting cell count or differentiation (42). Rapamycin-insensitive mTORC2 is reported to be sensitive to rapamycin at the 100 nmol/L concentration (26). Since we found that the 100 nmol/L concentration of rapamycin decreased global pancreatic cell growth and inhibited Akt phosphorylation on Ser473, this result supports the notion that mTORC2 regulates pancreatic growth by activating Akt signaling (43).

Recent studies establish an essential role of the Rag (Ras-related GTPase) family of GTPase in the regulation of mTORC1 activity by BCAAs (44), suggesting that Rag GTPases could be involved in the effects of BCAAs on pancreatic development observed here. To the best of our knowledge, information is not available concerning the role of Rag GTPases during pancreatic development. It will now be interesting to define whether Rag GTPases are implicated in the effects of BCAAs on pancreatic endocrine cell development.

We also demonstrate that Leu prevents Ngn3 induction by increasing intracellular HIF-1α levels after activation of the mTOR signaling pathway. HIF-1α is a transcription factor that regulates many hypoxic responses in cells and tissues. HIF-1α is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions and is stabilized by hypoxia (45). It was recently reported that HIF-1α levels are regulated by the mTORC1 pathway under normoxic conditions (46). Specifically, HIF-1α levels are increased when the mTORC1 signaling pathway is activated and reduced when this pathway is inhibited by rapamycin (46). We previously reported that HIF-1α represses β-cell differentiation by inhibiting Ngn3 expression during pancreatic development under hypoxic conditions (17). Thus, our results show that Leu can be added to the list of signals that repress pancreatic β-cell development by increasing HIF-1α levels after activation of the mTOR signaling pathway.

The results of epidemiological studies in humans indicate the existence of a strong association between maternal nutrition (either under- or overnutrition) and the risk of developing chronic diseases, such as T2DM, in the offspring (47). The importance of the relationship between pancreatic β-cell development and nutritional status is highlighted in reports, which have shown that undernutrition of fetal rodents results in a decreased β-cell mass when they become adults (48). Thus, pancreatic β-cell development is highly sensitive to nutritional status. Our findings demonstrate that maternal supplementation during pregnancy with a BCAA, such as Leu, impairs β-cell development in the fetal pancreas by repressing a highly specific step during β-cell differentiation. This new information is important because the results of recent studies indicate that an early defect in β-cell development is not subject to growth compensation and, thus, can lead to the development of T2DM in adults (49).

Finally, nutrient supplementation during pregnancy is an appealing strategy for conferring short- and long-term benefits to the offspring (50). Our findings that BCAA supplementation during pregnancy activates the mTOR signaling pathway and impairs β-cell differentiation in the developing pancreas suggest that a careful evaluation of the potential for adverse consequences of dietary supplementation during pregnancy is now needed.

ACKNOWLEDGMENTS

This work was supported by grants from INSERM ("Junior 5-year Contract" to L.R.), the CODDIM Region Ile de France (to R.S.), and the Beta Cell Biology Consortium (Grant U19-DK-072495 to R.S.).

No potential conflicts of interest relevant to this article were reported.

L.R. designed and performed experiments, analyzed data, and wrote the manuscript. V.A. performed experiments. B.D. provided expertise and reagents. R.S. designed the experiments and wrote the manuscript. L.R. serves as the guarantor for the article.

The authors thank Dr. Arieh Bonzon, (Consulwrite, Jerusalem, Israel) for his comments and editorial assistance in preparing this manuscript.

REFERENCES


