Glucocorticoids impair fetal \( \beta \)-cell development in rats

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Received 20 March 2001; accepted in final form 4 April 2001

During the last decade, the possibility that fetal events may influence the risk of disease in adulthood has generated considerable interest. Epidemiological studies published in the early 1990s suggest strong links between fetal growth and the occurrence of degenerative diseases later in life. Individuals who were thin at birth are at increased risk for cardiovascular disease (including hypertension) and glucose intolerance or type 2 diabetes in adulthood (2, 18, 23, 34, 40). Intrauterine growth retardation (IUGR) is thus a risk factor for glucose intolerance, hypertension, and dyslipidemia, a combination called “syndrome X.” From these epidemiological findings, the idea of fetal programming, the process whereby a factor at a critical or sensitive window of development exerts effects that persist throughout life, has been advanced (3).

The detailed mechanisms by which fetal undernutrition increases the risk of syndrome X are not perfectly understood. Besides insulin resistance, which has been proposed to occur in response to undernutrition (32), a primary defect in fetal \( \beta \)-cell development has also been suggested (17). This exciting hypothesis proposes that poor nutrition in utero, at a time when \( \beta \)-cell development proceeds more rapidly, reduces this development and thereby the number of available \( \beta \)-cells later in life. Because the latter hypothesis cannot be investigated easily in clinical settings, animal models have been developed. We recently designed a rat model of undernutrition involving an overall reduction in maternal food intake during the last week of pregnancy and throughout lactation (13). In this model, fetuses with growth retardation have a decrease in pancreatic \( \beta \)-cell mass (8), which persists into adulthood (9) and ultimately causes glucose intolerance (10, 11). These findings support a role for intrauterine nutrition in programming \( \beta \)-cell development.

Another situation characterized by IUGR and subsequent glucose intolerance is fetal overexposure to glucocorticoids. Studies in humans (36) and rodents (31) have shown that maternal glucocorticoid administration during pregnancy can induce IUGR. During normal pregnancy in rats, the fetuses are protected against maternal corticosterone by a placental enzyme, \( 11 \beta \)-hydroxysteroid dehydrogenase type 2, which converts corticosterone to an inactive compound (6, 29). Inhibition of this enzyme by carbenoxolone is associated with decreased weight at birth and with glucose intolerance in adulthood (22). Similarly, administration to pregnant rats of the \( 11 \beta \)-hydroxysteroid dehydrogenase type 2-resistant synthetic glucocorticoid dexamethasone induces IUGR and programs permanent hyperglycemia and increased blood pressure in the adult offspring (30). An experimental study in rats showed that the hypertension observed in adults whose dams were fed a low-protein diet during pregnancy could be prevented by chemical blockade of ma-
ternal corticosterone production (21), suggesting that the link between maternal protein deprivation and adult-onset hypertension may be mediated by maternal glucocorticoids. In aggregate, these data support the possibility that the hypothalamo-pituitary-adrenal axis may play a role in programming the adult-onset metabolic consequences of fetal undernutrition (9, 21, 30, 33, 37).

Because maternal undernutrition and fetal overexposure to glucocorticoids lead to glucose intolerance in adulthood, a legitimate question is whether the negative effects of these two abnormalities on fetal β-cell development are linked. The present study investigated the effects of glucocorticoids on β-cell development under normal conditions and during fetal undernutrition. In normal fetuses, correlations linking fetal corticosterone to fetal weight and insulin content were evaluated. In fetuses of dams with food deprivation or decreased circulating corticosterone levels, correlations between maternal or fetal corticosterone levels and fetal β-cell mass were investigated. The results strongly support the new concept of a negative role of glucocorticoids in fetal β-cell development.

MATERIALS AND METHODS

Animals and Study Design

Animals. Female Wistar rats (200 g; Janvier Breeding Center, Le Genêt-St-Isle, France) were exposed to a 12:12-h light-dark cycle and constant temperature (22°C). They had free access to water and were fed standard laboratory rat chow (22% protein, 5% fat, 53% carbohydrates; no. 113, UAR, Villemoisson sur Orge, France). The female rats were mated, and day 0 of pregnancy was defined as the day on which a vaginal plug was expelled. The two laboratories where the study was conducted are accredited by the French Ministry of Agriculture to conduct experiments in laboratory animals (accreditation numbers 7612 and 4860).

Food restriction. Maternal undernutrition was achieved as described previously (12). Briefly, the dams were fed 50% of the daily ad libitum intake (i.e., 12 g/day) from day 14 to day 21 of pregnancy. Control dams were fed ad libitum. All animals were fed every day at 1800.

To determine the effect of undernutrition on maternal corticosterone levels, blood samples were collected on days 15, 17, and 19 of pregnancy (between 1000 and 1200) from the tail vein in tubes containing 5% EDTA. After centrifugation, the plasma was separated and stored at −20°C until use for corticosterone assays. On day 21 of pregnancy, the dams were killed by decapitation, and blood flowing from the open necks was collected and processed as described above. Fetuses (10–12/litter) were collected by cesarean section, weighed, and immediately killed by decapitation. Blood samples were processed for corticosterone determination as described above, and the fetal pancreases were dissected and fixed for immunohistochemistry. This experimental protocol was used to investigate the impact of corticosterone overexposure on the alteration of fetal β-cell mass observed during undernutrition.

Metyrapone treatment. Another means of investigating the role of glucocorticoids on fetal β-cell development is exposure of the fetuses to low circulating corticosterone levels while the dams are fed a normal diet. In this study, a decrease in corticosterone levels was obtained by performing maternal adrenalectomy on day 14 of pregnancy (ADX group) or by combining this procedure with administration of metyrapone (ADX-Mety group). Metyrapone, which crosses the placental barrier and inhibits fetal steroid production (1), was injected subcutaneously into the dams at a dose of 25 mg (dissolved in 200 μl of 0.9% NaCl) twice daily (0900 and 1900) from day 16 to day 21 of pregnancy. The ADX dams received injections of the vehicle alone. The fetuses were collected on day 21 of pregnancy, and their pancreases were excised. Inasmuch as fetal rats do not produce corticosterone until day 16 (8), metyrapone treatment was not given before this time. Cross-reactivity of the anti-corticosterone antibody with 11-deoxy-corticosterone, which accumulates during metyrapone treatment, did not allow the measurements of fetal corticosterone levels; however, the twofold increase in fetal adrenal weight compared with that of fetuses from sham-operated dams indicated the efficiency of the adrenal blockade.

Fixation and processing for immunohistochemistry. For immunohistochemical studies, the pancreases were fixed in a 3.7% formalin solution, dehydrated in 100% ethanol and 100% toluene using an automatic tissue processor (model TP1020, Leica, Rueil Malmaison, France), and embedded in paraffin using the Paraffin-Embedding Center (model EG 1160, Leica). A rotary microtome (model RM 2145, Leica) was used to cut the entire pancreases into 6-μm-thick sections, which were collected on gelatin-coated slides. The slides were left at 37°C overnight and then stored at 4°C until they were processed for immunohistochemical studies.

Morphometry measurements. β-Cells were detected using a polyclonal guinea pig anti-insulin antibody (Dako, Trappes, France) revealed by incubation with an alkaline phosphatase-conjugated anti-rabbit antibody and stained blue by nitro blue tetrazolium (Vector, Bioys, Compiègne, France).
The β-cell fraction was measured using a Leica DMRB microscope equipped with a color videocamera coupled to a Quantimet 500MC computer (screen magnification ×24), as described previously (12). Briefly, the β-cell fraction was measured as the ratio of the insulin-positive cell area to the total tissue area on the entire section. Five sections taken at 150-μm intervals throughout the pancreas were analyzed from five fetuses in each group. The β-cell mass was obtained by multiplying the β-cell fraction by the weight of the pancreas. The number of islets (defined as insulin-positive aggregates ≥25 μm diameter) per square centimeter was determined.

**Insulin content determination.** After sonication of the pancreas in 4 ml of cold acidified ethyl alcohol (1.5% HCl-75% ethyl alcohol), the insulin was extracted overnight at −20°C and the pancreatic remnants were centrifuged. The supernatant was kept at −20°C until use.

**Hormone Assays**

**Corticosterone assay.** Plasma corticosterone was assayed after delipidation in isooctane followed by extraction in ethyl acetate. Experiments with known amounts of corticosterone showed that recovery exceeded 95%. Corticosterone levels were determined using an RIA with a highly specific corticosterone antiserum (UCB Bioproducts), as previously described (5). The detection threshold was 1 ng/ml. The intra- and interassay variations were 2.4 and 4.4%, respectively.

**Insulin assay.** Immunoreactive insulin was measured using an RIA with moniodized 125I-labeled porcine insulin (Sorin Biomedica, Salligia, Italy) as the tracer, guinea pig anti-insulin antibody (kindly provided by Dr. Van Schravendijk, Brussels, Belgium), and purified rat insulin (Novo, Boulogne, France) as the standard. Charcoal was used to separate free from bound hormone. The sensitivity of the assay was 0.25 ng/ml (6 μU/ml).

**Statistical Analysis**

Values are means ± SD. Statistical analysis was performed using multiple analysis of variance followed by Fisher’s protected least significant difference post hoc test. Unpaired Student’s t-test was also used when appropriate. Correlations between variables were studied by standard linear regression and confirmed by the nonparametric Spearman’s rank correlation coefficient (Spearman’s ρ). P < 0.05 was considered statistically significant. All statistical tests were performed using the StatView 4.0 package.

**RESULTS**

**Body Weight and Pancreatic Insulin Content Were Negatively Correlated With Corticosterone in 21-Day-Old Fetuses of Dams Fed a Normal Diet**

Linear regression analysis showed that fetal weight and pancreatic insulin content were negatively correlated to fetal corticosterone levels \( r^2 = 0.323, P = 0.005 \) (Fig. 1A) and \( r^2 = 0.190, P = 0.03 \) (Fig. 1B), respectively. There was also a significant rank correlation when the data were analyzed using the nonparametric Spearman’s ρ \( ρ = -0.411, P = 0.049 \) (Fig. 1A) and \( ρ = -0.582, P = 0.006 \) (Fig. 1B). No significant correlation was found between fetal weight and insulin content (not shown). These results suggested that corticosterone levels in the fetal circulation influenced body weight and β-cell development in fetuses with normal nutrition. Consequently, we sought to determine whether undernutrition modifies maternal and fetal corticosterone levels and whether these modifications affect fetal pancreatic development.

**Effects of Undernutrition**

In the pregnant control dams, corticosterone levels were significantly higher on day 21 than earlier in the pregnancy \( P < 0.01 \); Fig. 2), in keeping with previous data (10). However, corticosterone levels on days 19 and 21 were significantly increased in the food-restricted pregnant dams compared with the control dams \( P < 0.01 \) on day 19 and \( P < 0.05 \) on day 21; Fig. 2).

In the fetuses from food-restricted dams, corticosterone levels were elevated by 30% \( P < 0.001 \); Table 1). Fetal adrenal weight was reduced, indicating that the fetal corticosterone increase was due, at least in part, to maternal corticosterone overproduction (Table 1). In
addition, 21-day-old fetuses from food-restricted dams had significant decreases in body weight (P < 0.001) and pancreatic weight (P < 0.01) compared with fetuses of control dams (Table 1). Total insulin content per pancreas and relative insulin content per gram of body weight were decreased by one-half in these fetuses (P < 0.001; Table 1).

Normalization of Maternal Corticosterone Restored β-Cell Mass in the Fetuses With Undernutrition

To investigate the possible role of glucocorticoid production on the decreased insulin content noted in fetuses from food-restricted dams, we designed the ADX-Cort-R model, in which maternal corticosterone levels remain normal, despite food restriction. As expected,

Table 1. Characteristics of control fetuses and fetuses from food-restricted dams at 21 days gestation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Food Restricted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal weight, g</strong></td>
<td>5.9 ± 0.2</td>
<td>4.8 ± 0.2‡</td>
</tr>
<tr>
<td><strong>Pancreatic weight, mg</strong></td>
<td>28.9 ± 1.9</td>
<td>23.1 ± 4.4†</td>
</tr>
<tr>
<td><strong>Total insulin content, μg</strong></td>
<td>1.93 ± 0.49</td>
<td>0.95 ± 0.40‡</td>
</tr>
<tr>
<td>Relative insulin content, ng/g body wt</td>
<td>327 ± 82</td>
<td>196 ± 84‡</td>
</tr>
<tr>
<td><strong>Relative adrenal weight, mg/g body wt</strong></td>
<td>0.72 ± 0.1</td>
<td>0.62 ± 0.07*</td>
</tr>
<tr>
<td><strong>Corticosterone level, ng/ml</strong></td>
<td>23.4 ± 5.6</td>
<td>30.6 ± 5.4‡</td>
</tr>
</tbody>
</table>

Values are means ± SD from 21–34 fetuses. Fetal body and pancreatic weight and weight of the 2 adrenals were determined just after cesarean delivery in control fetuses and fetuses from food-restricted dams. Pancreatic insulin contents were determined by RIA after insulin extraction. Plasma corticosterone was determined by RIA. Statistical analysis was performed using Student's t-test; *P < 0.05; †P < 0.01; ‡P < 0.001 vs. controls.

Values in the Sham-R group had high corticosterone levels (P < 0.01; Fig. 3A) compared with the Sham-C group. The Sham-R fetuses had a 35% decrease in β-cell fraction (P < 0.01) and a 45% decrease in relative β-cell mass per gram of body weight (P < 0.01; Table 2)
GLUCOCORTICOIDS IMPAIR FETAL \( \beta \)-CELL DEVELOPMENT

Table 2. Fetal body and pancreatic weights after maternal corticosterone normalization

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham-C</th>
<th>ADX-Cort-C</th>
<th>Sham-R</th>
<th>ADX-Cort-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>5.3 ± 0.1</td>
<td>5.3 ± 0.02</td>
<td>4.7 ± 0.1†</td>
<td>4.6 ± 0.05†</td>
</tr>
<tr>
<td>Pancreatic weight, mg</td>
<td>27.7 ± 2.5</td>
<td>27.1 ± 3.7</td>
<td>20.5 ± 3.4†</td>
<td>19.1 ± 1.0†</td>
</tr>
<tr>
<td>( \beta )-Cell fraction, %</td>
<td>1.29 ± 0.21</td>
<td>1.23 ± 0.19</td>
<td>0.84 ± 0.08†</td>
<td>1.43 ± 0.08§</td>
</tr>
<tr>
<td>Individual ( \beta )-cell size, ( \mu m^2 )</td>
<td>90.2 ± 4.9</td>
<td>84.6 ± 3.0</td>
<td>89.2 ± 10.3</td>
<td>91.7 ± 3.7</td>
</tr>
<tr>
<td>Relative ( \beta )-cell mass, ( \mu g/g )</td>
<td>67.3 ± 10.1</td>
<td>63.8 ± 17.5</td>
<td>37.0 ± 9.3†</td>
<td>58.7 ± 3.1‡</td>
</tr>
</tbody>
</table>

Values are means ± SD. Fetal body and pancreatic weights were determined immediately after delivery by cesarean section. \( \beta \)-Cell fraction in the pancreas is the ratio of insulin-positive area to total pancreatic area measured by morphometry (see MATERIALS AND METHODS). Individual \( \beta \)-cell area was manually measured after light counterstaining in \( \geq 50 \) insulin-positive cells per animal (n = 5/group). Values of \( \beta \)-cell mass described in Fig. 3 were used to calculate the relative \( \beta \)-cell mass per gram of body weight (n = 5 fetuses/group). Statistical analysis was performed using ANOVA followed by Fisher’s protected least significant difference: †P < 0.05; §P < 0.01 in fetuses from food-restricted dams vs. corresponding group fed ad libitum; ‡P < 0.05 vs. Sham-W; ††P < 0.01 in fetuses from food-restricted adrenalectomized dams with normalized corticosterone levels (ADX-Cort-R) vs. fetuses from food-restricted sham-operated dams (Sham-R).

compared with Sham-C fetuses, in line with the decreased insulin content described above. Normalization of maternal corticosterone levels (Fig. 3A) in the ADX-Cort-R dams restored \( \beta \)-cell fraction, \( \beta \)-cell mass, and the number of islets per square centimeter to nearly normal levels in their fetuses (Table 2, Fig. 3, B and C, respectively), despite the food restriction. Fetal adrenal weight was also restored (data not shown). Individual \( \beta \)-cell size did not vary in any experimental conditions (Table 2). However, it is important to note that, in ADX-Cort-R fetuses, maternal adrenalectomy and corticosterone supply did not compensate for the negative effect of food restriction on fetal body and pancreatic weight (Table 2). The relative \( \beta \)-cell mass per gram of body weight in these fetuses reached the levels observed for control fetuses (Table 2). Fetal body and pancreatic weights, \( \beta \)-cell fraction, \( \beta \)-cell mass, or islet number per square centimeter was not significantly different between the two control groups, namely, Sham-C and ADX-Cort-C (Table 2, Fig. 3, B and C). Taken together, our findings strongly suggested a major effect of corticosterone on fetal \( \beta \)-cell development during undernutrition and led us to study the effect of corticosterone levels on \( \beta \)-cell mass in animals with normal nutrition.

\( \beta \)-Cell Mass in Fetuses From ADX Females Given Metyrapone

The impact on fetal \( \beta \)-cell development of low fetal corticosterone levels was investigated in fetuses from adrenalectomized dams given metyrapone to inhibit fetal steroid production. Fetal adrenal weight was 2.9 ± 0.4 mg in fetuses from sham-operated dams (Sham), 4.1 ± 0.4 mg in fetuses from adrenalectomized dams without metyrapone treatment (ADX, \( P < 0.05 \) vs. Sham), and 5.5 ± 1.4 mg in fetuses from adrenalectomized dams treated with metyrapone (Mety, \( P < 0.01 \) vs. Sham, \( P < 0.05 \) vs. ADX). Fetal weight did not vary in any experimental conditions (not shown). Fetal \( \beta \)-cell mass increased from 355 ± 48 \( \mu g \) in Sham to 516 ± 160 \( \mu g \) after maternal adrenalectomy (ADX) and rose further after metyrapone treatment to 757 ± 125 \( \mu g \) (\( P < 0.05 \) vs. ADX, \( P < 0.01 \) vs. Sham; Fig. 4A).

Interestingly, the metyrapone-induced \( \beta \)-cell mass increase was associated with increases in the number of islets per square centimeter (Fig. 4B) and in mean islet size (Fig. 4C).

DISCUSSION

The present study was designed to investigate the effects of glucocorticoids on fetal \( \beta \)-cell development and to determine whether overexposure to glucocorticoids contributes to the decrease in \( \beta \)-cell mass observed during fetal undernutrition. Our results make a strong case for a key role of corticosterone in \( \beta \)-cell development. In animals with normal nutrition, pancreatic insulin content was negatively correlated to corticosterone levels, and \( \beta \)-cell mass increased when fetal steroid production was impaired. In fetuses with undernutrition, in contrast, \( \beta \)-cell mass was decreased and corticosterone levels increased.

Glucocorticoid overexposure during pregnancy has been reported to cause IUGR in humans (36) and animals (4), as well as glucose intolerance later in life in rodents (30). We tested the hypothesis that glucocorticoids may affect fetal \( \beta \)-cell development, not only in fetuses with normal nutrition, but also in fetuses with IUGR. In fetuses with normal nutrition, we found a negative correlation between fetal corticosterone levels and fetal weight. Similarly, increased cortisol levels have been reported in human neonates with IUGR (7, 11, 14). Our finding that insulin content was correlated with corticosterone levels but not with fetal weight in normal rat fetuses suggests that insulin content may be more heavily dependent on glucocorticoid exposure than on nutritional status. The negative correlation between fetal corticosterone and insulin content in our study supports a negative effect of glucocorticoids on \( \beta \)-cell development. In an earlier study (11), we found that glucose intolerance occurred as a result of a primary defect in \( \beta \)-cell development in a rat model of perinatal undernutrition, and we hypothesized that this defect might be due to glucocorticoid overexposure in utero. The present study showed clearly that maternal food restriction increased maternal and fetal corticosterone levels and decreased fetal pancreatic insulin.
content and β-cell mass. Preventing the corticosterone increase in the food-restricted dams restored the fetal β-cell mass. Thus food restriction caused the corticosterone elevation, which in turn caused the β-cell mass decrease. Interestingly, restoration of the fetal β-cell mass was associated with correction of the decrease in the islet number per square centimeter, the main neonatal abnormality induced by undernutrition in this rat model (12). Although β-cell proliferation was not measured in fetuses at 21 days gestation, the fact that it was not decreased at birth (i.e., 12 h later) in undernourished neonates does not favor this hypothesis (12). Besides, the corticosterone elevation or normalization observed during malnutrition was not associated with a different β-cell size. On the other hand, increased apoptosis might contribute to the decreased β-cell mass observed during overexposure to glucocorticoids. Indeed, it has been shown recently by Weinhaus and co-workers (41) that dexamethasone inhibited the islet cell proliferation induced by prolactin while increasing apoptosis. Whether similar alterations of β-cell proliferation and/or apoptosis occur in utero during overexposure to glucocorticoids deserves further investigations.

The observation that pancreatic weight was not restored in fetuses from food-restricted dams with normalized corticosterone levels suggested in the pancreas a more specific and negative role of corticosteroids on the β-cells. To confirm these results, we studied the effects of fetal glucocorticoid underexposure on β-cell mass in normal rats. To reduce fetal corticosterone levels to a very low level, adrenalectomy was performed in the dams and followed by administration of metyrapone, a drug that inhibits fetal steroid production (1). In the fetuses, β-cell mass increased twofold compared with the controls. Increases in mean islet size and islet number per square centimeter were noted also. Thus glucocorticoid underexposure may promote islet neogenesis, whereas overexposure may have the opposite effect. The increase in islet size in the fetuses with glucocorticoid underexposure may reflect increased β-cell proliferation and/or β-cell hypertrophy. Taken together, our experiments demonstrate that β-cells do develop during the time window investigated in our study and that this development is sensitive to glucocorticoids. Whether glucocorticoids play a similar role earlier in fetal life remains to be determined. There is some evidence that maternal glucocorticoids may be required to maintain early β-cell development between days 12 and 15 of pregnancy (20). The mechanisms by which glucocorticoids modulate β-cell development deserve further investigation. Whether glucocorticoids affect β-cells directly or influence the differentiation of precursor cells into exocrine or endocrine cells remains to be determined. Several studies suggest a direct effect of glucocorticoids on β-cells. It has been shown that β-cells express the glucocorticoid receptor (26) as early as day 13 in rat fetuses (19, 20). The identification of a negative glucocorticoid response element in the human insulin promoter (15), together with reports of decreased insulin or GLUT-2 mRNA levels in β-cell lines or adult islets exposed to dexamethasone (16, 38, 41), also supports a direct effect of glucocorticoids on β-cells. Alternatively,
glucocorticoids may influence the development or the maturation of the exocrine pancreas. Positive regulation of the mouse amylase gene by glucocorticoids through a glucose response element has been reported (39). The AR42J cell line, which shares the multipotency of pancreatic precursor cells, has been shown to differentiate into acinar cells in vitro when exposed to dexamethasone (24) and into insulin-secreting cells when exposed to activin and β-cellulin (25). Moreover, early in vitro studies demonstrated that glucocorticoids inhibited insulin content and islet mass in cultured explants while enhancing the accumulation of exocrine enzymes and the acinar mass (27, 35). Taken together, these data suggest that glucocorticoids may favor development of the exocrine pancreas and inhibit development of the endocrine pancreas, possibly by guiding pancreatic precursor cells toward the exocrine differentiation pathway.

Experimental studies in animals have documented many examples of fetal programming of chronic degenerative diseases. Several showed that the development of hypertension in adults is linked to alterations in glucocorticoid levels during fetal life (37). Another study demonstrated that treatment of pregnant rats with dexamethasone induced glucose intolerance later in life in the offspring. Associations have been reported between these disorders and increased hepatic expression of the glucocorticoid receptor and of phosphoenolpyruvate carboxykinase (30). To our knowledge, the present work is the first evidence of a link between glucocorticoid levels during fetal life (37). Another example of fetal programming of chronic degenerative disease is the guidance pancreatic precursor cells toward the exocrine differentiation pathway.

We thank Belinda Duchene for excellent technical assistance.

This work was funded by the Institut National de la Santé et de la Recherche Médicale. B. Blondeau has been awarded a doctoral fellowship by the Ministère de l’Éducation Nationale, de la Recherche, et de la Technologie.

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