OVERWEIGHT AND INCREASED DIABETES SUSCEPTIBILITY IN NEONATALLY INSULIN-TREATED ADULT RATS

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Objective. Since the offspring of gestational diabetic mothers (GD) is at increased risk to develop obesity and diabetogenic disturbances later in life, while pathophysiological mechanisms responsible are unclear, to investigate long-term consequences of neonatal hyperinsulinism occurring characteristically in GD offspring.

Methods. Newborn Wistar rats received daily subcutaneous injections of a long-acting insulin from the 8th to 11th day of life (IRI), while in controls (CO) NaCl was applied. Body weight was recorded throughout life. Glucose tolerance test was performed on the 140th day of life (1.5 g/kg glucose injected i.p. after an overnight fast and blood samples were taken up to 90 min from retroorbital plexus). On the 240th day of life, the vulnerability to a single “subdiabetogenic” dose of streptozotocin (STZ; 25 mg/kg body weight) was tested. Blood samples for estimating glucose levels were taken before STZ, and subsequently on days 2, 7, 14, 21, and 28 after STZ.

Results. IRI rats developed overweight during juvenile life until adulthood (P<0.001), characterized by a clear elevation of the Lee obesity index (P<0.005), and associated with basal hyperglycaemia (P<0.05), hyperinsulinaemia (P<0.05), as well as an increased insulin/glucose-ratio as a measure of insulin resistance (P<0.005). Impaired glucose tolerance occurred in early adulthood, and increased vulnerability to a “subdiabetogenic” dose of streptozotocin (see above), leading to significant hyperglycaemia (P<0.05), was evaluated in the 9th month of age. Accompanied by a transient reduction of hyperinsulinaemia during a period of 21 days, Lee obesity index and insulin/glucose-ratio decreased significantly after STZ treatment in IRI rats (P<0.01).

Conclusions. Overweight and increased diabetes susceptibility in adulthood due to temporary hyperinsulinism during a critical period of postnatal life are suggested to be a consequence of acquired dysregulation and overstimulation, respectively, of the pancreatic insulin secretion in rats.

Key words: Perinatal hyperinsulinism – Overweight – Insulin resistance – Streptozotocin – Diabetes susceptibility

Nearly every tenth pregnant woman is suffering from gestational diabetes, leading to fetal and neonatal hyperinsulinism (Weiss 1988). Perinatally hyperinsulinaemic offspring of mothers with diabetes during pregnancy are predisposed to later development of overweight and diabetogenic disturbances (Aerts et al. 1990; Doerner and Plagemann 1994; Plagemann et al. 1997; Kohlhoff et al. 1997), while pathophysiological mechanisms responsible are unclear. Interestingly, these patients develop early features of the metabolic syndrome, which is characterized by disturbances of carbohydrate and fat metabolism including obesity, hyperinsulinaemia, insulin resistance, and impaired glucose tolerance (Reaven 1988). In the pathogenesis of these complex alterations, a central pathogenetic role of hyperinsulinaemia is suggested (Modan et al. 1985). Therefore, in the present study we addressed the question whether a temporary hyperinsulinism during a particularly critical period of the development of neuroendocrine systems in newborn rats may lead to increased diabetes susceptibility later in life.
Materials and Methods

Animal model. The investigations were performed in the offspring, bred in our institute, of Wistar rats of an outbred colony strain (Shoe: Wist/2 (Ico)). Eight virgin female rats were time-mated at the age of three months. During pregnancy, they were singly housed under standard conditions. Newborn rats were randomly distributed among mothers on the first day of life. One newborn male rat from each mother received daily subcutaneous injections of a long-acting insulin (Berlin-Chemie, Berlin, Germany; Lot No. 081288) from the 8th to the 11th day of life (IRI group, n= 8; 0.3 IU on 8th and 9th day of life; 0.1 IU on 10th and 11th day). In eight sibling control males (CO), an appropriate volume of NaCl was applicated. No mortality occurred until the end of the study (268th day of life), neither in the IRI group nor in the controls. All animals were reared under standard conditions with a 12-hour inverse light-dark cycle (lights on from 17.00 h to 5.00 h). After weaning on the 21st day of life, rats were housed in groups of 3-4 per Plexiglass cage. All animals had free access to standard pellet diet (Altromin, Lage, Germany) and tap water was provided ad libitum. Experimental procedures were approved by the local Animal Care and Use Committee (G 0297/92).

Body weight and metabolic parameters. Body weight was recorded throughout life. Additionally, in adult life the Lee obesity index was calculated by dividing the cube root of body weight by body length (BERNARDIS and PATTERSON 1968). Daily mean food intake was measured from the 110th to 115th day of life, housing the animals individually in feeding cages. For glucose tolerance tests on the 140th day of life, 1.5 g glucose per kg body weight (20 % glucose solution) were injected intraperitoneally (i.p.) after an overnight fast (16 h). Blood samples for determination of glucose (glucoseoxidase-peroxidase method; Dr. Lange GmbH, Berlin, Germany) were taken prior to, and 15, 30, and 90 min post injection (p.i.) by puncture of the retroorbital plexus under light ether anesthesia.

Application of a "subdiabetogenic" dose of streptozotocin. In adult life, the vulnerability to a single "subdiabetogenic" dose of streptozotocin (STZ) was tested (WEGNER et al. 1985). After taking a basal blood sample, as described above, on the 240th day of life (day 0), animals received a single injection of 25 mg STZ per kg body weight i.p. (Serva/ Biochemica, Heidelberg, Germany; lot No. 35 503). Further blood samples were taken on days 2, 7, 14, 21, and 28 after STZ treatment under basal conditions (non-fasting). From measurements of blood glucose the area under the curve of glucose against time (AUCG) was calculated over the observation period (day 0-28 after STZ treatment).

Insulin estimation. Immunoreactive plasma insulin before and after STZ treatment was determined by a modified commercial radioimmunoassay (BioChem ImmunoSystems, Freiburg, Germany), using rat insulin (Novo Nordisk, Copenhagen, Denmark) as standard preparation (biological potency: 21.3 IU/mg). Intraassay coefficient of variation was 4.5 % to 7.4 % in a concentration range of 9.1 to 94.2 mIU/l (n = 10). The interassay coefficient ranged between 11.9 to 16.2 % (concentration range 7.8 to 51.0 mIU/l; n = 22-31).

Statistical evaluation. Data are expressed as means ± S. E. ANOVA, followed by paired or unpaired t-test (with or without Welch correction), or Mann-Whitney U-test, respectively, were used to determine significant differences between the groups. Relations between variables were analyzed by Spearman’s rank correlation test. All statistical evaluations were performed using the SPSS-for-windows statistical package (SPSS software, Munich, Germany).

Results

Development of body weight. Neonatal application of insulin resulted in the development of clear overweight in IRI rats, with a significantly increased body weight starting on the 21st day of life and persisting through juvenile life until adulthood (Fig. 1). In adult life, the Lee obesity index was found to be significantly increased in IRI rats (Tab. 1). Mean food intake showed a nonsignificant elevation in the IRI group on the 110th-115th day of life, housing the animals individually in feeding cages.

Glucose tolerance in adulthood. Fasting blood glucose on the 140th day of life did not differ between the groups (CO: 3.7 ± 0.7 g/day (n = 8) vs IRI: 24 ± 0.8 g/day (n = 8); difference n.s.).

Glucose tolerance in adulthood. Fasting blood glucose on the 140th day of life did not differ between the groups (CO: 3.7 ± 0.2 mmol/l (n = 8) vs IRI: 3.7 ± 0.2 mmol/l (n = 8)). Glucose tolerance test, however, revealed an impaired glucose tolerance (IGT) in IRI rats. At 30 min after glucose loading,
blood glucose levels (expressed as mean±S.E.) were significantly increased (P<0.037 by U test) in the IRI group (CO: 7.96 ± 0.17 mmol/l vs. IRI: 8.97 ± 0.47 mmol/l), while the 15-min value (CO: 9.7 ± 0.7 mmol/l vs. IRI: 11.3 ± 0.7 mmol/l; difference n.s.) and 90-min value (CO: 6.8 ± 0.6 mmol/l vs. IRI: 8.4 ± 1.0 mmol/l; difference n.s.) showed a nonsignificant elevation in the IRI group.

Blood glucose, plasma insulin and body weight before and after application of a "subdiabetogenic"

**Table 1**

Basal plasma insulin, body weight, Lee obesity index, and insulin/glucose-ratio before (day 0) and after (day 7-28) injection of a "subdiabetogenic" dose of streptozotocin (STZ; 25 mg/kg body weight) in adulthood (9th month of life) of neonatally insulin-treated rats (IRI) as compared to control rats (CO).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day</th>
<th>CO (n = 8)</th>
<th>IRI (n = 8)</th>
<th>CO vs IRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (mIU/l)</td>
<td>0</td>
<td>9.3 ± 1.3</td>
<td>27.8 ± 3.8</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>16.6 ± 2.1</td>
<td>18.7 ± 1.4</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>16.8 ± 2.0</td>
<td>21.1 ± 2.4</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>15.6 ± 1.4</td>
<td>16.8 ± 2.2</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>13.1 ± 0.6</td>
<td>28.8 ± 3.5</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>0</td>
<td>426 ± 7.0</td>
<td>508 ± 13</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>408 ± 14</td>
<td>478 ± 16</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>Lee obesity index (x 10^-3)</td>
<td>0</td>
<td>300 ± 2.0</td>
<td>314 ± 3.0</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>299 ± 3.0</td>
<td>306 ± 3.0^a</td>
<td>n.s.</td>
</tr>
<tr>
<td>Insulin/glucose-ratio (IU/mol)</td>
<td>0</td>
<td>2.3 ± 0.3</td>
<td>6.1 ± 1.0</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>2.3 ± 0.1</td>
<td>4.7 ± 0.6^a</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

day 28 vs day 0: * p < 0.01.
prior to application of STZ on the 240th day of life (day 0), IRI rats showed basal hyperglycaemia (Fig. 2), hyperinsulinaemia (Tab. 1) and an elevated insulin/glucose-ratio (Tab. 1). Application of a "subdiabetogenic" dose of STZ resulted in increased blood glucose levels in the IRI group throughout the whole observation period, reaching significance on day 2 and 14 after STZ injection. Permanent elevation of blood glucose levels until the 28th day p.i. resulted in a significantly increased AUCG in the IRI group (Fig. 2). This was accompanied by near normalization of plasma insulin in IRI rats as compared to controls from the 7th to 21st day after STZ treatment (Tab. 1). Prior to STZ treatment, absolute body weights were elevated in IRI rats as compared to controls, accompanied by a highly significantly increased Lee obesity index prior to STZ in the IRI group. On the 28th day, however, the Lee index did not show a significant group difference, resulting from a significant decrease of Lee obesity index in IRI rats after STZ treatment (Tab. 1).

Correlations. Lee obesity index on the 240th day of life (day 0) was significantly correlated to basal insulin levels (r = 0.56; p < 0.025; n = 16). Furthermore, the insulin/glucose-ratio on day 0 showed a positive correlation to the Lee index (r = 0.51; p < 0.05; n = 16), while no significant correlation on day 28 after STZ was observed (r = 0.35; n.s.; n = 16). The AUCG over the observation period (day 0-28) was positively correlated to the Lee obesity index prior to STZ treatment (r = 0.58; p < 0.05; n = 16).

Discussion

In the presented study, by peripheral application of insulin hyperinsulinism was induced in newborn
rats during a limited time period of neuroendocrine development, which corresponds approximately to the last trimester of pregnancy in humans (Doerner 1976). In adult life, overweight, hyperinsulinaemia, signs of insulin resistance, IGT, and increased diabetes susceptibility were observed in neonatally insulin-treated rats. Obesity and insulin resistance in adulthood were significantly reduced by a single "subdiabetogenic" dose of STZ, accompanied by transient reduction of hyperinsulinaemia.

Characterized by a clearly elevated Lee obesity index as a measure of increased body fat (Bernardis and Patterson 1968; Dubuis et al. 1996), neonatally insulin-treated rats developed overweight from juvenile life until adulthood. This was not associated with a significant increase of food intake in IRI rats. Nevertheless, it cannot be excluded that the small enlargement of food intake over a longer time period may have contributed cumulatively to the increased body weight in IRI rats. On the other hand, IRI rats showed basal hyperinsulinaemia, probably substantially contributing to the development of obesity (Modan et al. 1985). This was associated with an increased insulin/glucose-ratio, which was shown in experimental as well as clinical studies to be a good indicator of insulin resistance in vivo (Deutsch et al. 1993; Legro et al. 1998). Remarkably, a decreased insulin sensitivity already at weaning as well as in adult age was also observed in another animal model of perinatal hyperinsulinism, i.e., in neonatally overfed rats (Macho et al. 1997; Plagemann et al. 1998a). In adulthood, impaired glucose tolerance occurred in the neonatally insulin-treated rats. This is in parallel with clinical observations in perinatally hyperinsulinaemic offspring of gestational diabetic mothers who develop overweight and impaired glucose tolerance already in juvenile life and early adulthood (Doerner and Plagemann 1994; Plagemann et al. 1997). Unfortunately, for the present experiment insulin profiles during glucose tolerance tests cannot be provided and should therefore be determined in future studies in this animal model. However, the basal insulin/glucose-ratio was found to be highly significantly correlated to insulin resistance measured by minimal modelling, a highly sensitive method to determine insulin sensitivity, in a recent clinical study (Legro et al. 1998). Furthermore, it was recommended by these investigators as useful for screening for insulin resistance. Therefore, an increased insulin/glucose-ratio in IRI rats accompanied by increased basal insulin levels might be interpreted as a sign of insulin resistance in our animal model.

In neonatally insulin-treated rats, a clearly increased vulnerability to a single 'subdiabetogenic' dose of streptozotocin (STZ) was observed. STZ is known to induce a selective destruction of pancreatic beta-cells, dose-dependently. While doses of 30 to 200 mg/kg body weight are used to induce overt diabetes in rats, multiple- or single-low-dose models were developed to investigate a possible predisposition to diabetes under several experimental conditions (Shafirir 1990). A dose of 25 mg/kg body weight is known to be 'subdiabetogenic' in normal adult rats (Junod et al. 1969), confirmed in our study by only minor and transient changes of blood glucose after STZ application in control rats. In contrast, immediately after STZ treatment, neonatally insulin-treated rats displayed significantly increased glucose concentrations, thereby pointing to an increased diabetes susceptibility. Hyperinsulinaemia was clearly reduced in the IRI rats until the 21st day after STZ injection. Simultaneously, Lee obesity index and insulin/glucose-ratio displayed a reduction from day prior until the 28th day after STZ treatment. Remarkably, a significant decrease of both parameters only occurred in the overweight, hyperinsulinaemic IRI rats. Since hyperinsulinaemia promotes the development of obesity (Modan et al. 1985), and, on the other hand, a reduction of plasma insulin levels is known to restore peripheral insulin sensitivity (Soll et al. 1975), our results might indicate a pathogenetic role of persisting hyperinsulinaemia in the development of overweight and insulin resistance in this animal model. Thus, 'subdiabetogenic' STZ treatment may offer a new model for studies on overweight and insulin resistance, beyond the "classical" applications of this substance in immunological research.

However, the question arises why insulin levels rose again, at the end of the observation period after near normalization of insulinaemia and despite falling blood glucose. In our opinion, this might point to an extrapancreatic dysregulation and overstimulation, respectively, of the pancreatic insulin secretion. Note-worthy, hypothalamic nuclei are decisively involved
in the regulation of insulin secretion, body weight and metabolism (for review see Bray et al. 1990; Kalra and Kalra 1996). In rats, the differentiation of these hypothalamic nuclei lasts until the 15th-20th day of postnatal life (Doerner 1976; Pozzo-Miller and Aoki 1992). The period around the 10th day of age represents a particularly critical time point in development of rats, with a particularly increased vulnerability of neuroendocrine systems to permanent malorganization (Doerner 1976; Erikson et al. 1992). Remarkably, permanent, i.e., lifelong malformation of hypothalamic regulation centres of body weight and metabolism was observed in perinatally hyperinsulinaemic offspring of gestational diabetic mother rats, which displayed similar disturbances of body weight gain and metabolism as described here (Doerner and Plagemann 1994; Plagemann et al. 1998b, 1998c). Furthermore, a selectively intrahypothalamically localized hyperinsulinism induced during the same time period (8th-11th day of life) as insulin treatment lasted in the present experiment resulted in the development of hyperinsulinaemia, obesity, IGT, and an increased vulnerability to STZ in later life of rats, associated with dysorganization of the VMN (Plagemann et al. 1992; Doerner and Plagemann 1994; Plagemann et al. 1998d). Since insulin is capable to cross the blood-brain barrier (Banks et al. 1997), which is characterized by an increased permeability during perinatal life (Ugrumov et al. 1983), it seems possible that overweight and increased diabetes susceptibility in neonatally insulin-treated rats may result, at least in part, from a disturbed organization and function, respectively, of hypothalamic regulation centers of metabolism and body weight, induced and acquired due to hyperinsulinism during a critical period of hypothalamic development.

Since the induction of gestational diabetes in rats frequently does not lead to fetal and neonatal macrosomia but to underweight and retardation in the offspring (Golob et al. 1970), possibly limiting the value of this animal model for pathophysiological research which tries to compare to the situation in humans (Herrera and Zorzano 1984), neonatal insulin application may offer an alternative model for the study of consequences of hyperinsulinism during critical periods of development in rats.

In conclusion, our findings suggest overweight, signs of insulin resistance and increased diabetes susceptibility as a consequence of basal pancreatic hyperactivity and hyperinsulinaemia, respectively, in adult rats neonatally exposed to elevated insulin levels.

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