Fetal and neonatal exposure to AZT and low-protein diet affects glucose homeostasis: a model with implications for AIDS prevention

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Morten, K., P. Field, N. Ashley, K. A. Williams, D. Harris, M. Hartley, A. Clark, and J. Poulton. Fetal and neonatal exposure to AZT and low-protein diet affects glucose homeostasis: a model with implications for AIDS prevention. Am J Physiol Endocrinol Metab 289: E1115–E1118, 2005. First published July 12, 2005; doi:10.1152/ajpendo.00226.2005.—Zidovudine (AZT) lowers the perinatal transmission of HIV but can impair mitochondrial function by depleting mitochondrial DNA (mtDNA). AZT therapy and perinatal nutritional deprivation affect the body fat distribution, which influences glucose tolerance. We sought to model intrauterine exposure to AZT in humans to determine whether it interacts with low-protein diet (LPD) to impact on birth weight and glucose homeostasis in the offspring. Pregnant dams and their offspring were given AZT, an LPD, or AZT and an LPD (LPD + AZT). AZT reduced mtDNA copy number in liver and birth weight in the offspring and increased their fasting glucose and insulin (P = 0.021, 0.03, 0.001, and 0.011 respectively) at 6–8 wk of age. LPD decreased litter size and birth weight (P = 0.01 and 0.012). In the LPD + AZT group, birth weight and litter size were reduced compared with untreated controls, and fasting blood glucose and insulin were raised. There was a significant interaction between LPD and AZT on fasting insulin levels (P = 0.025). Islet size was not significantly affected, but the mean β-cell area/islet was reduced in the LPD + AZT group compared with controls (P < 0.05). Early exposure to AZT interacts with LPD to impair fetal development in this model. This combination appeared to impair the supply of insulin and, hence, glucose homeostasis, perhaps as a result of impaired mitochondrial function. Although it is not certain that this can be extrapolated to humans, maternal nutritional deprivation combined with AIDS therapy could influence both birth weight and onset of diabetes.

Sayre syndrome due to mtDNA rearrangements is associated with the development of small islets (20) and diabetes (19). Impaired insulin secretion is thus a major factor in mitochondrial diabetes (7) as in type 1 diabetes (T1D).

Defective maternal nutrition, in the form of a low-protein diet (LPD), is associated with low birth weight in humans and in animal models (5), and this is associated with decreased insulin sensitivity, central obesity (12), and type 2 diabetes (T2D) developing later in life (6). This combination of factors predisposing to T2D is exacerbated by a common mtDNA variant (4). In addition, an LPD has been shown to reduce mtDNA copy number in mice (8). In the developing fetus and neonate, these factors might interact by influencing the number of copies of mtDNA in tissues that are critical for intrauterine metabolic programming. This study aimed to determine the effects of fetal and neonatal exposure of mice to AZT, with or without maternal LPD, on birth weight and glucose homeostasis.

MATERIALS AND METHODS

Animal studies. All animals were housed and managed in accordance with the United Kingdom’s Home Office protocols (17). The treatment groups consisted of virgin female C57BL/6 mice that were allotted to one of four different regimens after timed matings. The first group (AZT; n = 8 mothers) was treated with AZT (0.15 mg/ml from GlaxoSmithKline) in their drinking water. A second group (LPD; n = 15) was given ad libitum access to a reduced protein diet (5% total protein by weight). A third group (LPD + AZT; n = 12) was given both AZT in their drinking water and the LPD. Untreated animals were used as controls (C; n = 15).

The regimens were maintained following delivery of the pups. After weaning (21 days postpartum), pups were maintained on their allotted regimens until 28 or >42 days of age, when they were fasted overnight and blood was taken for glucose and insulin assay. The pancreas was removed for histological examination and quantitative morphometry of islets and endocrine cells. Liver samples were snap frozen in liquid N2 for determination of mtDNA content. Plasma glucose was determined using a glucose analyzer (Glucose Meter, Abbot Laboratories). Plasma immunoreactive insulin concentration was assayed with an RIA kit (Linco Research, St. Charles, MO). The lower limit of the assay is 0.02 ng/ml with a coefficient of variation within and between assays of <10%.

Determination of mitochondrial copy number by real-time PCR. Single-color molecular beacon PCR reactions were used to quantify mtDNA levels relative to nuclear DNA. All primers and probes were
from MBG Biotech. Primer sequences were as follows: forward primer 12993F, ATAACAGCTATGTACACGC, and reverse primer 13092R, TGAAGTCGGGTGTCATTTTGTT, for the mitochondrial fragment; MDGmouseF, GGCTCTTCTACTCTGATGTTCT, and reverse primer MDGmouseR, CTGCAAGCTATGGCCATAG, for the nuclear fragment (within the 3-methyladenine DNA glycosylase gene). Probe designs were performed with Primer Express 2.0 software (Applied Biosystems). Each probe was labeled with FAM at the 5′ end and TAMRA at the 3′ end. The probe oligonucleotide sequences were as follows: probe mitmo1302P, ACTTCGTAA-CAATAAACAAACCCGTTTTC, targeting the mitochondrial fragment; and MDGmouse(probe), ACCCAAGGCTAGTACAGGCGCAAGAT, targeting the nuclear fragment. Real-time PCR amplifications were performed with triplicate 25-μl reactions, containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 250 nM each of either mitochondrial or nuclear primers, 100 nM of the corresponding nuclear or mitochondrial probe, and 4 ng of target DNA. Amplification reactions were performed in a GeneAmp 5700 sequence detection system (Applied Biosystems) with the following thermal profile: 95°C denaturation and enzyme activation step for 10 min followed by 40 cycles of 95°C denaturation for 15 s and 60°C annealing for 60 s.

Threshold cycle number (Ct) was calculated with SDS software v.1.7 (Applied Biosciences) and a manual setting of the baseline. Ct values were used for the relative copy number calculations by relating them to the standard curve generated from a reference DNA. Serial dilutions of the reference DNA forming a series of standards spanning a 10-fold range of mtDNA concentrations of genomic DNA were included in each experiment to generate the standard curves. In addition, rho zero (mtDNA-free) DNA and three “no DNA” controls included in each experiment to generate the standard curves. In v.1.7 (Applied Biosciences) and a manual setting of the baseline. CT was no significant interaction between AZT and LPD on litter size (Fig. 1A and Table 1). Significantly reduced body weights of mothers on LPD (P < 0.001) were consistent with reduced protein intake.

**RESULTS**

**mtDNA copy number.** Pups with early exposure to AZT had a significantly lower mtDNA copy number in liver than controls [average mtDNA copy number was 74% (95% CI 58–89%)] taking the average value for untreated controls as 100%, P = 0.021]. There was no significant effect of LPD on offspring mtDNA content.

**Pre- and postbirth development.** Early exposure to AZT reduced birth weight (P = 0.03) but not subsequent weight gain (Fig. 1 and Table 1). A reduced-protein diet significantly lowered litter size as well as birth weight compared with the control group (P = 0.01 ANOVA on pups per mother averaged over cages and P = 0.012, respectively). There was also a significant reduction in birth weight due to AZT (P = 0.025; Fig. 1B), and the effect of this was additive with LPD. There was no significant interaction between AZT and LPD on litter size (Fig. 1A and Table 1). Significantly reduced body weights of mothers on LPD (P < 0.001) were consistent with reduced protein intake.

**Morphology and distribution of pancreatic islets.** Insulin- and glucagon-positive cells were present in islets of all animals. Pancreatic islets from animals exposed to a combination LPD + AZT (n = 15) had significantly lower insulin-positive β-cell area/islet (1.719 ± 297 μm²) compared with controls (932 ± 223 μm², P < 0.05, Wilcoxon rank sum test). Neither AZT nor LPD alone had a significant effect. There were no significant differences in islet size or the pancreatic proportion of endocrine cells between any of the groups studied, and no difference in α-cell distribution.

**Measurements of glucose and insulin.** Both the AZT and LPD + AZT pups (n = 12 and 14, respectively) had significantly higher fasting blood glucose (Z, 7.2 ± 0.3 mmol/l, P < 0.05; LPD + AZT, 7.5 ± 0.4 mmol/l, P < 0.001) compared with controls (6.1 ± 0.3 mmol/l n = 23; Fig. 1 and Table 1). Similarly, the AZT pups had a higher fasting insulin (P = 0.011), both off and on LPD (AZT, 78 ± 1 nmol; LPD+AZT, 52 ± 3 nmol vs. controls 38 ± 4 nmol; Fig. 1 and Table 1). The effect of AZT on insulin was reduced by the addition of LPD (significant interaction, P = 0.025), but LPD alone had no significant effect on either fasting glucose or insulin. Fasting insulin was measured in eight of the AZT mothers and found to be raised relative to control mice (n = 20; 58.0 nmol vs. 37.0 nmol, respectively, P < 0.05).

**DISCUSSION**

We have confirmed that early exposure of mice to AZT reduces mtDNA copy number in the liver to 73% of normal levels. Furthermore, we have shown that the effects of early exposure to either AZT or LPD lowered birth weight compared with untreated controls (P = 0.03 and 0.012, respectively; Fig. 1) and that these effects were additive. LPD also reduced litter size (P = 0.01; Fig. 1A). Fasting blood glucose and insulin concentrations were raised in the LPD + AZT and AZT groups compared with controls (P = 0.001 and 0.011 for AZT on fasting blood glucose and insulin, respectively; Fig. 1, C and D), consistent with impaired glucose homeostasis. Islet size was not significantly affected, but the mean insulin-positive cell area/islet was reduced in the LPD + AZT group compared with control mice. This is consistent with increased insulin requirement resulting in degranulation (and therefore no recognition) of β-cells in these animals, as there was no change in islet size or proportion of glucagon-positive cells. This also implies some impairment of insulin granule synthesis in these animals. Consistent with earlier studies (14), LPD alone reduced both birth weight and litter size but not glucose homeostasis. This suggests that AZT combined with LPD affects both intrauterine development and glucose homeostasis. This combination has the potential to decrease the ability of β-cells...
to supply sufficient insulin; hence, it may increase the susceptibility to diabetes later in life.

The raised fasting insulin concentrations probably indicate increased insulin resistance in the AZT pups. Insulin resistance is an important feature of T2D, but although fasting glucose was elevated, no animal was diabetic. Pups exposed to AZT, LPD, or both were smaller at birth than controls. Both fasting insulin and birth weight were higher in the AZT group than in either the LPD or the LPD + AZT group. Fasting insulin levels were not significantly increased in the LPD group. This supports previous studies that suggested that low birth weight resulting from LPD does not cause insulin resistance in mice (14). Our data are compatible with the suggestion that insulin resistance is a cause of low birth weight. However, it does not suggest that low birth weight causes insulin resistance, because the LPD + AZT mice were smaller at birth than the AZT mice, whereas their increase in fasting insulin level was less extreme. This suggests that LPD + AZT mice are less insulin resistant than the AZT group, reflecting these combined influences. Formal glucose tolerance testing would clarify this. The changes in birth weight and fasting glucose were significant in the LPD + AZT group (Fig. 1, B and C). These are potentially linked to mtDNA copy number, as both nucleoside reverse transcriptase inhibitor (NRTI) treatment (16, 21) and LPD (8, 14) in the perinatal period cause depletion of mtDNA. It is clear that reduced mtDNA copy number could impair insulin supply and sensing because of the importance of mitochondrial function to both insulin secretion and islet development (20, 22). Insulin supply is an important determinant of fetal growth. Hence, low birth weight may be causally related to altered glucose homeostasis in our model. These data suggest that mitochondrial function has a substantial effect on thinness at birth in pups given an LPD and are consistent with our observation that a common human mtDNA variant is associated with thinness at birth (4). Hence, mtDNA variation may also make a significant contribution to the inheritance pattern of maternal constraint and, thence, glucose homeostasis in adult life (6).

Recent studies of pregnant women and animal models have raised concerns regarding potentially serious mitochondrial

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Table 1. Summary of P values calculated in ANOVA

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>LPD</th>
<th>AZT</th>
<th>LPD + AZT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litter size</td>
<td>0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Birth weight</td>
<td>0.012</td>
<td>0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>NS</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>NS</td>
<td>0.011</td>
<td>0.025</td>
</tr>
</tbody>
</table>

For each dependent variable, factors considered in the model were effects of low-protein diet (LPD), zidovudine (AZT), and interaction between LPD and AZT (LPD + AZT). Nos. (n) in each group used for significance testing are shown in Fig. 1, except for no. of pups per litter. In this case, statistical testing was carried out on pups per mother averaged over cages (7 control, 6 LPD, 3 AZT, and 4 LPD + AZT cages), but Fig. 1 shows pups averaged over all mothers. This ANOVA was repeated using a model where LPD and AZT were considered separately without an interaction, and this did not materially affect the significance.
toxicity-related side effects in infants born to mothers who received NRTIs during their pregnancy to prevent HIV-1 perinatal transmission (3). Small-scale studies in humans have demonstrated that the offspring from mothers receiving NRTIs for HIV may have reduced mtDNA copy number in their placentas (21) and blood (16). Similarly, infants exposed to perinatal antiretroviral therapy may have lactic acidemia persisting for months after the therapy has been discontinued (1), but there are insufficient published data to definitively link the use of this therapy with effects on mitochondrial function (9). Our study provides the first evidence of a link between mitochondrial function manifesting as an effect on glucose homeostasis and intrauterine exposure to AZT. Consistent with other studies, we suggest that mitochondrial function may be central to metabolic programming (4) and glucose homeostasis (10, 15).

In conclusion, exposure of mice to AZT and LPD during their development resulted in impaired glucose homeostasis in later life. The effect of AZT treatment on fetal size was exacerbated by poor nutrition, and this combination appeared to impair the supply of insulin. Because birth weight is an important determinant of infant mortality (24), this is a potential disadvantage of administration of NRTIs in pregnancy. Although it is not certain that this can be extrapolated to humans, the risk of increasing susceptibility to T2D in adult life has potential implications for management of the world epidemics of both AIDS and T2D.

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GRANTS

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