Increased Transcriptional Preproinsulin II β-Cell Activity in Neonatal Nonobese Diabetic Mice: In Situ Hybridization Analysis

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Abstract

In the prediabetic nonobese diabetic (NOD) mouse, a spontaneous model of type 1 diabetes, we previously reported transient postweaning hyperinsulinemia followed by progressive islet hyperplasia. A modified in situ hybridization technique was used to determine whether these effects were accompanied by changes in insulin transcriptional activity as a function of age. We found that NOD neonates express higher levels of preproinsulin II primary transcripts than age-matched C57BL/6 mice, but this difference disappeared within the first wk of age. To manipulate insulin transcriptional activity in NOD neonates, NOD mothers were treated with insulin during the last two wk of gestation. A down-regulation of β-cell hyperactivity was observed in female NOD neonates but not in male neonates. By contrast, the same insulin treatment applied to NOD.scid (severe combined immunodeficiency) mothers, devoid of functional lymphocytes but showing like NOD mice postweaning hyperinsulinemia, increased transcriptional β-cell activity in both sexes of neonates. In conclusion, NOD mice exhibit successive and transient signs of β-cell hyperactivity, reflected as early as birth by high transcriptional preproinsulin II activity and later, from weaning to around 10 wk of age, by hyperinsulinemia. Of note, when thinking in terms of in utero disease programming, the NOD neonatal transcriptional β-cell hyperactivity could be modulated by environmental (maternal and/or fetal) factors.

Keywords: NOD neonates · preproinsulin II · in situ hybridization · maternal insulin treatment

Introduction

The NOD mouse is the most widely used spontaneous animal model to study type 1 diabetes (T1D) pathogenesis [1-3]. Despite extensive research, however, the etiology of the disease is still largely unknown. In NOD mice, as in human (pre)diabetic patients, several abnormalities of immune function exist. These abnormalities, which include aberrant phenotypes and/or functions of thymocytes, antigen-presenting cells (APC), and lymphocytes, may hamper tolerance induction leading to autoimmunity [4-6]. However, similar abnormalities have been found in other autoimmune diseases and, if they explain the predisposition to autoimmunity in general, they do not explain the organ/cell-specificity of the autoimmune disease, in particular that of β-cells in T1D [2, 7, 8].

We therefore hypothesized that abnormalities of the islets of Langerhans, in addition to those in the immune system, are required for the development of the autoimmune reaction. In NOD mice, we have previously reported a transient hyperinsulinemia that co-
incidence in time with the first pancreas-infiltrating APC [8-11]. This hyperinsulinemia was followed by the formation of large islets, or mega-islets [2, 8, 12]. These mega-islets were preferentially associated with subsequent infiltrating cells (APC and lymphocytes) [8].

Hyperinsulinemia in NOD mice occurs directly after weaning. The weaning period corresponds to a dietic shift from maternal milk (rich in fatty acids and poor in carbohydrates) to laboratory chow (poor in fatty acids and rich in carbohydrates) [2, 8, 13, 14]. Even in normal rodents, this dietic shift is known to activate β-cells that have been at rest until weaning because of the low glucose-containing maternal milk. Since prediabetic NOD mice exhibit β-cell hyperactivity immediately after weaning, we questioned whether this was due to a previous glucose stimulation or so-called “glucose-priming effect” [2, 8, 14-16]. Such a priming effect may occur in NOD fetuses, due to possible abnormalities of glucose homeostasis in NOD mothers.

We therefore attempted to evaluate NOD neonatal β-cell activity. However, the measurement of circulating insulin levels in neonatal mice is technically challenging. To circumvent this problem, we determined whether β-cell stimulation is present at the transcriptional level in the pancreata of NOD neonates. For this purpose, we developed an in situ hybridization technique that assesses the rate of preproinsulin II mRNA expression. A cRNA probe complementary to the second intron of insulin II gene was constructed that specifically detects the presence of primary transcripts of this gene [17]. Primary RNA transcripts are rapidly processed to mature mRNA and transported into the cytoplasm. In vitro studies demonstrated that the half-life of a primary transcript in the nucleus is approximately 5-8 min, compared with up to 8 h for the mature spliced form of mRNA [18]. Thus increased levels of preproinsulin II primary transcripts represent increased insulin synthesis and can be used as an indicator of β-cell activity because in metabolically hyperactive β-cells, insulin production is upregulated at all points of control, including transcriptional activity.

Using this technique, we analyzed NOD and C57BL/6 mice at various ages and found that 1-day-old NOD neonates exhibit high levels of preproinsulin II primary transcript expression compared to age-matched C57BL/6 neonates, but the strain difference disappears within the first wk of age. In order to modulate this neonatal β-cell hyperactivity, we treated NOD mothers with insulin during the last two wk of gestation. We also tested NODscid mice, which lack functional lymphocytes and do not develop insulitis and diabetes [19] but, like NOD mice, show some degree of hyperinsulinemia and increased mega-islet formation [2, 14, 20]. Maternal insulin treatment modulates differently transcriptional β-cell activity according to strain and/or sex.

Materials and methods

Animals and treatments

NOD, NODscid and C57BL/6 mice were bred under specific pathogen-free conditions at the facilities of the Hôpital Necker, Paris, France. The animal facilities and care followed the norms stipulated by the European Community. The incidence of diabetes in the NOD colony is, by 200 days of age, 80% and 40% for females and males, respectively [10]. C57BL/6 and NOD mice were sacrificed at 1 day, and 1, 2 and 4 wk of age in the morning for pancreas sampling. Seven-wk-old NOD and NODscid females were mated, with the morning of the appearance of the vaginal plug being taken as the day zero of gestation. Then they were injected subcutaneously once a day in the late afternoon with 1.0 U human insulin/100 g body weight (Ultratard, NovoNordisk, Boulogne-Billancourt, France) or with the vehicle from day 9 to the last day of gestation [21]. Vehicle- or insulin-treated NOD and NODscid mothers were sacrificed 1 day after delivery at the same time as the neonates. Before sacrifice, NOD and NODscid mothers were rapidly bled, as previously described [10], to assess their basal nonfasting glycemia using the glucose-oxidase method (Biotrol glucose enzymatic color, Biotrol, Paris, France). Plasma insulin, glucagon and corticosterone concentrations were also determined, as described elsewhere, using standard RIAs (SB-insulin-CT, CIS Biointernational, Gif-sur-Yvette, France, Biodata, Pharmacia, St-Quentin-en-Yvelines, France and ICN Biomedicals Inc., Sorin Biomedica, Antony, France, respectively) [10, 22]. Pancreata from NOD mothers were fixed in Bouin’s solution (Sigma, Saint-Quentin-Fallavier, France), followed by 10% formalin (Merek, Paris, France) and paraffin-embedded. Sections were cut at 4 µm and stained with hematoxylin eosin (Merek) to assess the degree of insulitis.

Tissue preparation

One- and 7-day-old animals were decapitated. Their tissues were immersion-fixed overnight in freshly prepared 4% paraformaldehyde. Animals over 14 days old were anaesthetized i.p. with avertin (Sigma), flushed
with PBS containing heparin (10 U/ml) and 0.5% w/v NaNO₂ and perfused with 4% paraformaldehyde. Tissues were harvested and placed in fixative for a further 2 h. After fixation tissues were washed in 70% alcohol and embedded in paraffin using routine procedures. Sections of 4-5 µm were cut and placed on 3-aminopropyltriethoxysilane (Sigma) coated slides and stored at RT.

Construction of the murine Ins II IVS probe

A cRNA probe specific for the primary transcript of preproinsulin II was generated, based on a previous method [18]. PCR primers were designed that spanned 354 bp of the second intron (representing 72% of its sequence). The amplification product was generated by standard RT-PCR amplification from total pancreatic extracts as described previously [23], and purified on a 6% polyacrylamide gel. The resulting cDNA fragment was ligated into a pGEM-T Easy vector (Promega, Charbonnières, France) according to the manufacturer’s instructions. Plasmid DNA was prepared from recombinant clones, linearized, proteinase K digested and phenol/chloroform extracted. Digoxigenin (DIG)-labeled sense and anti-sense probes were synthesized from appropriately linearized DNA using the in vitro translation system in the presence of DIG-11-UTP, according to the manufacturer’s instructions (Boehringer Mannheim, Mannheim, Germany) with SP6 or T7 RNA polymerase. The DNA template was digested by DNase I and the RNA probe NH₄Cl precipitated and purified by spin column chromatography (Clontech, Palo Alto, Ca.). Probes were aliquoted and stored at -70°C.

In situ hybridization

Tissue sections were deparaffinized, cleared and rehydrated, then subjected to proteinase K digestion (100 µg/ml) for 30 min at 25°C. The reaction was stopped with 0.2% glycerine and sections were re-fixed in 4% paraformaldehyde for 10 min. Sections were then rinsed in H₂O₂, dehydrated and air-dried. Sections were then covered with 30-40 µl of hybridization buffer (HB) pH 6.8, containing 100 ng/ml of DIG-labeled antisense or sense probe, 300 mM NaCl, 10 mM Na₂HPO₄, 10 mM Tris-Cl, 50 mM EDTA, 50% deionised formamide, 5% dextran sulphate, 100 µg/ml tRNA, 100 µg/ml sonicated salmon DNA. Sections were incubated overnight at 50°C in covered chambers humidified with HB without probe and 50% formamide to maintain vapor pressure. After hybridization, sections were incubated for 4 h in 2 changes of HB without probe and 50% formamide at 50°C to remove probe, washed 3x in RNase buffer (600 mM NaCl, 10 mM Tris-HCl pH 7.2, 50 mM EDTA) and incubated with 30 µg/ml RNase for 60 min at 37°C. Slides were washed in PBS and incubated in 2 changes of 2x SSC at 63°C for 60 min. Slides were then washed in Tris-buffered saline containing 0.05% Tween 20 (TNT) and incubated overnight at 4°C with anti-DIG F(ab) fragments (Boehringer) at a dilution of 1/750. At the end of the incubation slides were washed first in TNT, then in alkaline Tris-saline buffer containing 50 mM MgCl₂ pH 9.5. Slides were then placed in slide mailers containing 4.5 µg/ml nitroblue tetrazolium (NBT) (Boehringer) and 3.5 µg/ml 5-Bromo-4-chloro-3-indolyl-phosphate Na₂ (BCIP) (Boehringer) and left upright at room temperature for 4-6 h. Positive control sections were checked for staining intensity, then sections were washed in buffer containing 10 mM EDTA, dehydrated and mounted in Eukitt.

Immunohistochemistry for (pro)insulin and glucagon

Double labeling was performed on 4 µm sections cut from paraffin-embedded tissues. After dewaxing and rehydrating, the tissues were washed in Tris-saline buffer (0.1M Tris-HCl, pH 7.5, 0.15M NaCl, 0.05% Tween 20). Sections were incubated with optimally diluted guinea pig anti-insulin serum (Linco Research Inc., St. Charles, MO) and rabbit anti-glucagon serum (Chemicon International Inc, Temecula, CA) overnight at 4°C. Slides were thoroughly washed in Tris-saline buffer and incubated first with swine anti-rabbit IgG conjugated with alkaline phosphatase (DAKO, Trappes, France) for 30 min at room temperature, rinsed and then incubated with rabbit anti-guinea pig IgG conjugated with horseradish peroxidase (DAKO, Trappes, France) for a further 30 min. Sections were rinsed first in Tris-saline buffer (pH 7.5), then briefly in alkaline Tris-saline buffer (pH 9.5 containing 50 mM MgCl₂). Immunoreactive glucagon was revealed using a substrate consisting of 0.34 mg/ml NBT and 0.18 mg/ml BCIP. Slides were protected from light and periodically checked for staining intensity. The reaction was stopped by returning sections to neutral pH in Tris-saline buffer containing 10 mM EDTA. Slides were washed thoroughly for at least 30 min, then reacted with the peroxidase chromogen diaminobenzidine (Sigma) in PBS with 0.02% H₂O₂ for 1-2 min. Slides were finally rinsed in water counterstained with methyl green (Fluka, Buchs, Switzerland), dehydrated through alcohol, cleared and mounted.
Image analysis

We performed a semi-quantitative analysis of preproinsulin II expression via a VIDAS-RT image analysis system (Kontron Elektronik GmbH/Carl Zeiss, Weesp, The Netherlands). Measurements were made at 200x magnification. Islets were identified under phase contrast and circled. Positively stained nuclei inside individual islets were enumerated at four thresholds of staining intensity. The number of positive nuclei was divided by the area of the islet. Data are expressed as the mean of positive nuclei/islet area (x10^-3).

Statistical analysis

Values are mean ± SEM of 4 to 10 neonates per age group in each strain depending on the experiment. For each animal 3 noncontiguous sections were analyzed. Data were analyzed with Statistica software (Statsoft, Tulsa, OK). Possible effects of strain, age and treatment were analyzed using ANOVA for each variable investigated. Post-hoc analysis, using the Tukeys HSD method (in situ hybridization) or the Neuwman-Keuls method (insulitis and endocrine parameters), was performed when effects and interactions were significant (p < 0.05), as assessed by ANOVA.

Results

The islet distribution of preproinsulin II primary transcript expression is heterogeneous

To determine whether β-cell hyperactivity was present in NOD neonatal pancreata, we utilized an intervening sequence assay that exploited the short half-life of primary transcripts in the nucleus as an indirect measure of transcriprional activity. In serial sections stained for the primary transcript of preproinsulin II (Figure 1a) or for proinsulin and glucagon protein to visualize β- and α-cells, respectively (Figure 1b), a specific signal was localized to the β-cell area of a subpopulation of islets when compared to proinsulin and glucagon staining. Figure 1c shows a high power view β-cell nuclei stained for preproinsulin II primary transcripts. Staining was restricted to the nucleus although not all nuclei are stained in a given islet. Sections hybridized with sense probes had a complete absence of staining (Figure 1d), similar to sections preincubated with RNase before hybridization (data not shown).

NOD neonates have high levels of preproinsulin primary transcript expression

Using a semi-quantitative approach to gauge the level of transcriptional activity, we measured the number of positive nuclei per islet area at 4 different thresholds (100, 75, 50, 25) of staining intensity. Threshold values were arbitrarily assigned to encompass the range of staining intensities. Of note, in both strains at all ages, smaller islets tended to contain the most strongly stained nuclei (data not shown), possibly reflecting strong transcriptional β-cell activity during islet neogenesis [22]. In addition, 1-day-old NOD neonates had a significantly greater number of stained nuclei when compared to age-matched C57BL/6 mice.
β-Cell Hyperactivity in NOD Mice at Birth

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Figure 2. Transcriptional activity is increased in neonatal NOD mice. In situ hybridization for the ppIns II was carried out on paraffin-embedded sections as described in the materials and methods. A VIDAS image analysis system was used to semi-quantify the number and intensity of stained nuclei. The number of positive nuclei per islet area was measured at 4 different thresholds (25, 50, 75, 100) of staining intensity. These threshold values were arbitrarily assigned to encompass the range of staining intensities. The graph shows the number of nuclei stained per islet area at different arbitrarily assigned thresholds of intensity in NOD (●●) and C57Bl/6 (○○) mice during the early developmental period. Means ± SEM of 10 mice per age group in each strain, p-values from 0.02 to 0.00003.

Maternal insulin treatment decreases the level of preproinsulin primary transcript expression in female NOD neonates, but not in males

We then tried to modulate the transcriptional β-cell hyperactivity observed in NOD neonates. We therefore treated NOD mares with insulin (1U/100g bw) during the last two wk of gestation. Mean values of basal nonfasting glycemia, insulinemia, glucagonemia and circulating corticosterone in NOD mothers, 1 day post-partum, are given in Table 1. Vehicle- and insulin-treated NOD mice show similar values for all parameters investigated. We also checked the efficacy of insulin treatment on the degree of insulitis (Figure 3). As expected, insulin treatment significantly decreased the percentage of islets with insulitis (p < 0.028) and, by contrast, significantly increased that of unaffected islets (p < 0.025). For in situ hybridization, we analyzed here female and male neonates separately. As can be seen from Figure 4, levels of transcriptional β-cell activity were similar in female and male NOD neonates from vehicle-treated NOD mothers. However, maternal insulin treatment significantly decreased the level of preproinsulin transcriptional activity in female NOD neonates (p < 0.006, post-hoc analyses), but not in males. This sexual dimorphism in insulin response was observed in two separate experiments.

Maternal insulin treatment increased preproinsulin primary transcript expression in both sexes of NODscid neonates

NODscid mice are lymphocyte-deficient controls of NOD mice that show, like NOD mice, postweaning hyperinsulinemia compared to various control strains [2, 14, 19]. We therefore treated NODscid mothers with insulin during the last two wk of gestation and analyzed the pancreata of their progeny by in situ hybridization. As shown in Table 1, mean basal nonfasting glycemia of vehicle- and insulin-treated NODscid mothers, 1 day post-partum, were similar but tended to be higher than corresponding NOD mother glycemia (p = 0.09 and 0.06 for vehicle and insulin-treated mice, respectively). Circulating NODscid insulin and corticosterone levels were not affected by previous maternal insulin treatment and were similar to those observed in NOD mice. However, 1 day post-partum, glucagonemia showed trends toward higher values in vehicle- or insulin-treated NODscid mothers than corresponding NOD mothers (p = 0.05 and 0.08, respectively). As can be seen in Figure 5, maternal insulin treatment increased preproinsulin transcriptional activity levels in both female and male NODscid neonates (p < 0.04 and p < 0.003 respectively, post-hoc analyses).

Discussion

We previously demonstrated, starting after weaning, a transient hyperinsulinemia in prediabetic NOD mice compared to various control strains (C57BL/6, DBA/2 and BALB/c) [2, 8, 19, 11, 14]. Because of possible maternal abnormalities of glucose homeostasis, we tried to analyze here whether this hyperinsu-
linemia could result from an in utero “glucose-priming” effect on fetal β-cells [2, 15, 16, 24]. Indeed, if most of the NOD mothers are not diabetic at the beginning of gestation, some of them become diabetic during gestation or in the post-partum period. Due to the difficulty of measuring circulating insulin in mouse neonates, we set up an in situ approach to assess β-cell activity in their pancreata. We showed that NOD neonates exhibit increased transcriptional β-cell activity at birth compared to age-matched C57BL/6 neonates. However, it should be underlined that the distribution of preproinsulin II primary transcripts is highly heterogeneous. Indeed, not all islets express preproinsulin II primary transcripts and not all nuclei in a given islet. Moreover, the difference between the two strains disappears within the first wk of age. At this age and regardless of the strain, pups are fed from birth onwards by low glucose-containing maternal milk that induces a β-cell rest lasting until weaning.

Then we tried to modulate the β-cell hyperactivity observed in NOD neonates. We therefore treated NOD mothers during the last two wk of gestation at a dose of insulin that lowers glycemia by 50% 2 h after injection (data not shown). Of note, this prenatal insulin treatment decreases maternal insulitis by about 50%, as checked 1 day post-partum. While β-cell activity levels are similar in female and male NOD neonates from vehicle-treated NOD mothers, maternal insulin treatment exclusively down-regulates it in female neonates. The reason for the sexual dimorphism in β-cell reactivity of NOD neonates remains to be determined. It is possible that androgens, produced by male fetuses before birth [25], induce a state of insulin resistance [2, 8, 10, 20, 26, 27], thereby leading to insulin-resistant hyperstimulated β-cells in males. Moreover, insulin does not practically cross the placental barrier unless insulin antibodies are present [28]. Indeed, insulin antibodies have been described in NOD mice [29, 30]. In such a case, insulin might control its own secretion at different levels in opposite ways: at the level of the central nervous system, particularly, during the neonatal period, and at the pancreas level [31-34]. The sexual dimorphism might therefore take place at both levels. Finally, we expected a down-regulation of β-cell activity in neonates by treating the mothers with insulin, due to a better control of glucose metabolism during gestation. Because it was impossible to measure repeatedly maternal glycemia during gestation (in order to avoid stressful manipulation), we assessed maternal glycemia 1 day post-partum. However, after mating at 7 wk of age and at the end of the first gestation, none of the NOD mothers became diabetic, suggesting that, at least during the first gestation, a “glucose-priming” effect might not be responsible for the subsequent postweaning hyperinsulinemia.

![Figure 3](image-url)  
**Figure 3.** Maternal insulin treatment during the two last weeks of gestation decreases the degree of insulitis. In both vehicle- or insulin-treated groups, islets were examined and the respective percentages of intact, peri-infiltrated and severely damaged islets were determined. Values are means ± SEM (n = 951 and n = 984 islets in vehicle- and insulin-treated NOD mothers, respectively, p = 0.025 and p = 0.028 for unaffected islets and islets showing insulitis, respectively).

We then used the NODscid model, devoid of functional lymphocytes but also exhibiting post-weaning hyperinsulinemia, as a lymphocyte-deficient control of
the NOD mouse, which can also overcome the possible problem of insulin transfer through the placental barrier. In the progeny of insulin-treated NODscid mothers, we do not observe a sexual dimorphism in transcriptional β-cell activity to insulin, but in contrast an up-regulation in both sexes. An explanation could be the existence of abnormalities of glucose homeostasis in mice with the scid mutation, and in particular in NODscid mice, but not in males. However, we show here that nonfasting basal glycemia of NODscid mothers, whether they have been vehicle- or insulin-treated, are slightly higher 1 day after delivery, compared to those of NOD mice, in relationship with slightly higher circulating levels of glucagon, a well known counterregulatory hormone [35]. Also, we recently described a state of insulin resistance in NOD and NODscid mice during the first gestation, possibly linked to abnormal maternal HPA axis regulation [36]. Finally, we published that 7-day-old NODscid neonates show an unexpected pattern of higher basal glycemia than NOD and C57BL/6 neonates [22]. Of note in this regard, nude BALB/c mice, which are deprived of a thymus, exhibit several endocrine effects including impaired glucose tolerance at a very young age and peripheral insensitivity to insulin [37, 38]. Therefore, subtle differences in the regulation of glucose metabolism at the maternal and fetal levels during NOD and NODscid gestation might trigger the peculiar β-cell response of their female and male progeny.

In conclusion, NOD mice exhibit successive signs of transient β-cell hyperactivity that take place, as shown here around birth, and afterwards at weaning. This neonatal β-cell hyperactivity completes the list of events that happen during the perinatal period in NOD mice. First, and in agreement with our data, NOD fetal pancreatic organ cultures produce more insulin that those from C57BL/6 or BALB/c mice [39]. Second, prenatal stress increases diabetes incidence in the female progeny, possibly through hyper-stimulation of the HPA axis and induction of insulin resistance leading to overworked β-cells [40]. Third, a higher number of some type of APC are present at birth in NOD and NODscid pancreata compared to those of C57BL/6, DBA/2 and BALB/c [41]. Fourth, neonatal injection of glucose and arginine leads to diabetes enhancement and increased islet autoantigen expression (ICA and GAD 67) in NOD females only [42]. Fifth, neonatal separation of NOD neonates from their mothers increases diabetic risk probably in association with augmented peripheral insulin need [43]. After weaning, APC infiltration is accentuated when β-cell hyperactivity reappears, thus suggesting a close relationship between islet hyperactivity and immune cell infiltration [2, 8]. As discussed elsewhere, β-cell hyperactivity might be of importance in the pathogenesis of T1D: hyperactive endocrine cells are more prone to autoimmune reactions because of higher levels of autoantigens, adhesion and MHC molecules and because of a higher sensitivity to cytokine-induced damage [2, 7, 8, 11]. Of note, in human T1D, the existence of such a transient β-cell hyperactivity, triggered by a
state of insulin resistance that might accelerate disease progression, has recently been emphasized [44-47].

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