

Thymic expression of insulin-related genes in an animal model of autoimmune type 1 diabetes

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Abstract

Background Insulin and multiple other autoantigens have been implicated in the pathogenesis of autoimmune type 1 diabetes, but the origin of immunological self-reactivity specifically oriented against insulin-secreting islet β -cells remains obscure. The primary objective of the present study was to investigate the hypothesis that a defect in thymic central T-cell self-tolerance of the insulin hormone family could contribute to the pathophysiology of type 1 diabetes. This hypothesis was investigated in a classic animal model of type 1 diabetes, the Bio-Breeding (BB) rat.

Methods The expression of the mammalian insulin-related genes (*Ins*, *Igf1* and *Igf2*) was analysed in the thymus of inbred Wistar Furth rats (WF), diabetes-resistant BB (BBDR) and diabetes-prone BB (BBDP) rats.

Results RT-PCR analyses of total RNA from WF, BBDP and BBDR thymi revealed that *Igf1* and *Ins* mRNAs are present in 15/15 thymi from 2-day-old, 5-day-old and 5-week-old WF, BBDR and BBDP rats. In contrast, a complete absence of *Igf2* mRNA was observed in more than 80% of BBDP thymi. The absence of detectable *Igf2* transcripts in the thymus of BBDP rats is tissue-specific, since *Igf2* mRNAs were detected in all BBDP brains and livers examined. Using a specific immunoradiometric assay, the concentration of thymic IGF-2 protein was significantly lower in BBDP than in BBDR rats ($p < 0.01$).

Conclusions The present study suggests an association between the emergence of autoimmune diabetes and a defect in *Igf2* expression in the thymus of BBDP rats. This tissue-specific defect in gene expression could contribute both to the lymphopenia of these rats (by impaired T-cell development) and the absence of central T-cell self-tolerance of the insulin hormone family (by defective negative selection of self-reactive T-cells).
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Keywords thymus; type 1 diabetes; insulin gene family

Introduction

Type 1 diabetes is an autoimmune disease specifically directed against insulin-secreting islet β -cells and several autoantigen targets have been identified. In this cohort of autoantigens, insulin is the most specific autoantigen of the islet β -cell. Insulin has been shown to play an important role in the development of type 1 diabetes both in humans and in animal models of type 1 diabetes [1–4]. Though the autoimmune nature of type 1 diabetes is well established, animal experiments and human clinical studies have failed to determine the specific origin of the immunological

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self-reactivity against insulin-secreting islet β -cells. It has been hypothesized, however, that specific self-reactivity of the immune system may develop from a defect in the thymic establishment of central self-tolerance [5–7].

After migrating from primary hematopoietic sites (fetal yolk sac, fetal liver, and then bone marrow) into the thymus, lymphoid stem cells are induced by thymic signals to undergo a program of proliferation, gene rearrangement and differentiation. These specific thymic events result in the production of a large number of immature double-positive CD4⁺CD8⁺ thymocytes expressing a diverse repertoire of T-cell antigen receptor (TCR) combinations. This repertoire is submitted to stringent selection so that T-cells that recognize self-antigens in the context of major histocompatibility complexes (MHC) are induced to die by neglect or by negative selection [8]. This efficient deletion of self-reactive T lymphocytes is responsible for the central self-tolerance of the immune system. The tolerogenic function of the thymus is mediated by the different cellular components of the thymic parenchyme: epithelial/nurse cells (TEC/TNC), macrophages, and dendritic cells [9,10]. Central T-cell tolerance of neuroendocrine functions has been proposed to be mediated by the intrathymic expression of one dominant member of neuroendocrine families [5,7].

Insulin-like growth factor 2 (IGF-2) is the dominant polypeptide of the insulin family synthesized by TEC/TNC in the human, rat and mouse thymus. Immunoreactive (IR) IGF-1 is also detected in the thymus, but IGF-1 distribution is restricted to stromal cells with a macrophage-like morphology and distribution [11–15]. Using fetal thymic organ cultures, we demonstrated that intrathymic IGF-mediated signaling plays an active role in T-cell proliferation and differentiation during fetal development [15]. Insulin gene (*Ins*) expression has also been described in fetal and postnatal thymi from humans, rats and mice [16–20]. The primary objective of the present study was to explore whether a defect in the thymic expression of a member of the insulin family could be associated with the predisposition for autoimmune diabetes. This was investigated using a classical animal model for type 1 diabetes, the Bio-Breeding (BB) rat. BB rats are susceptible to an autoimmune diabetes syndrome that shares many characteristics with human type 1 diabetes [21].

Materials and methods

Animals and tissue isolation

Thymus, brain and liver of inbred Wistar-Furth (WF) rats, diabetes-resistant (BBDR) and diabetes-prone (BBDP) BB rats (2 days to 5 weeks of age) were taken from animals obtained from the BB rat colony maintained at the University of Massachusetts Medical School, Worcester, MA. The animals in this colony have been inbred for more than 50 generations and are housed in viral antibody free (VAF) conditions. The overall incidence of diabetes in the BBDP strain of BB rats is 86% between 56 and 120 days of

age and 0% in the BBDR strain. The experimental procedures were carried out in accordance with the Ethical Committee on Animal Experimentation at the University of Liège and the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School.

RNA isolation and RT-PCR analyses

Tripure (Roche Diagnostics, Basel, Switzerland) was added to tissue fragments, and RNA was extracted according to the manufacturer's instructions. Samples were treated with RNase-free DNase I (Roche Diagnostics) to remove potentially contaminating DNA. Each time point was repeated three times.

Reverse transcription (RT) was performed using 3 μ g total RNA in the presence of 1 mM dNTP (Amersham Pharmacia Biotech, Uppsala, Sweden), 50 mM Tris-HCl pH 8.3, 75 mM KCl, 5 mM MgCl₂, 30 IU avian reverse transcriptase (Promega, Madison, WI, USA) and 500 nM random hexamer primers (Amersham Pharmacia Biotech) in a final volume of 25 μ l. Samples were incubated at 70°C for 10 min and 1 h at 42°C.

The PCR reaction was carried out in a final volume of 50 μ l using the equivalent of 600 ng total RNA (5 μ l of the RT reaction). The RNA was amplified in the presence of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M each of dATP, dCTP, dGTP, dTTP, 1.5 U TaKaRa Taq™ DNA polymerase (all reagents from Takara Shuzo Co. Ltd, Shiga, Japan) and 0.5 μ M of each primer (Life Technologies, Merelbeke, Belgium). The newly synthesized cDNAs were denatured at 94°C for 3 min and amplified by 30 PCR cycles at 94°C for 90 s, annealed for 90 s at the indicated temperature (55°C for IGF-1, 54°C for IGF-2, 57°C for insulin and 54°C for actin), 72°C for 90 s, followed by 72°C for 10 min. The sequences of oligonucleotide primers were as follows: *Igf1* (upstream: ATCTCTTCTACCTGGCAGTC, downstream: TACATCTCAGCCTCCTCAG); *Igf2* (upstream: CACGCTTCAGTTTGTCTGTT, downstream: CGGGTCTTTGGGTGGTAAC); *Ins* (upstream: ATGGC CCTGTGGATGCGCTTC, downstream: GTTGCAGTAGTTCTCCAGTTG; these primers amplify transcripts from both *Ins1* and *Ins2* genes) [22]; β *actin* (upstream: GATGGTGGGTATGGGTCAGAAGGA, downstream: GCTCATTGCCGATAG TGATGACCT) [23]. To exclude the possibility of amplifying genomic DNA, primers were chosen in different exons of IGF genes. Control PCRs have also been performed in the absence of RT. PCR products were fractionated by electrophoresis on 1% agarose gel. The identity of the RT-PCR-products amplified with *Igf1*, *Igf2* and *Ins* primers was confirmed by Southern blotting.

Immunoradiometric assay of thymic IGF-1 and IGF-2

The procedure used a two-site immunoradiometric assay (IRMA) principle described by Miles *et al.* [24]. The DSL-9100 ACTIVETM IGF-1 and IGF-2 IRMA (Diagnostic

Systems Laboratories, Inc., Webster, TX, USA), included a simple extraction step in which IGF-1 and IGF-2 were separated from their binding proteins. Thymic proteins purified from 15 BBDR and 15 BBDP samples after RNA isolation with tripure reagent were collected individually, neutralized and submitted to the assay procedure. The IRMA was performed in anti-IGF-1 or anti-IGF-2-coated tubes. Aliquots (50 μ l) of the reconstituted standard, control and extracted proteins were incubated for 3 h at room temperature on a shaker set at 180 rpm, then the pellets were washed three times with 2 ml deionized water. Tubes were incubated for 1 h at room temperature with 100 μ l anti-IGF-1 or anti-IGF-2 labeled antibodies, decanted, washed three times, and bound radioactivity was quantified using a gamma counter.

Statistical methods

Values obtained from multiple experiments are reported as mean \pm SEM. Significance levels were determined by Mann-Whitney *U*-test.

Results

Expression of *Ins* and *Igfs* in WF and BB rat thymi

RT-PCR analyses of total RNA from BBDP and BBDR rat thymi revealed that *Igf1* and *Ins* mRNAs are present in 15/15 thymi from 5-day-old BBDR and BBDP (Figures 1 and 2). In contrast, no detectable *Igf2* mRNA is observed in most of the BBDP thymi (11/15). Since *Igf2* mRNA in rodents is known to decline after birth, we investigated whether an earlier decrease in *Igf2* mRNA expression leads to the observed defect of *Igf2* in BBDP rat thymi.

Igf2 transcripts were identified in the thymus of 10/10 5-week-old and 2-day-old BBDR and WF thymi, but *Igf2* mRNAs were not detectable in more than 80% of the thymi of 5-week-old and 2-day-old BBDP rats (Figures 3 and 4). The absence of detectable *Igf2* transcripts in the thymus of BBDP rats is tissue-specific, since *Igf2* mRNAs were readily detected in the brain and liver of these animals (data not shown). Amplification of β *actin* cDNA (600 bp) was used as an internal standard (Figures 1–4). Southern blotting was used to confirm that the RT-PCR products observed at the predicted sizes on the gels were in fact the cDNAs of *Ins*, *Igf1* and *Igf2* (data not shown).

Characterization of IR IGF-1 and IGF-2 in BB rat thymus

Immunoreactive (IR) IGF-1 and IGF-2 in thymi of BBDP and BBDR rats are shown in Figure 5. A marked difference in the concentration of thymic IR IGF-2 concentrations was observed between the two groups of rats (BBDP 11.2 ± 0.91 ng/mg protein vs BBDR 30.7 ± 4.17 ng/mg protein; mean \pm SEM of 15 thymic extracts). As assessed by Mann-Whitney *U*-test, this difference was highly significant ($p < 0.01$) (Figure 5). There was no significant difference in IR IGF-1 concentrations in BBDP (12.1 ± 0.21 ng/mg protein) and BBDR (11.3 ± 0.47 ng/mg protein) thymi.

Discussion

Autoimmune type 1 diabetes in humans and animal models results from the destruction of insulin-secreting islet β -cells by self-reactive T-cells [25]. The mechanisms leading to the generation of these self-reactive T-cells are still unknown. Among a number of identified islet β -cell

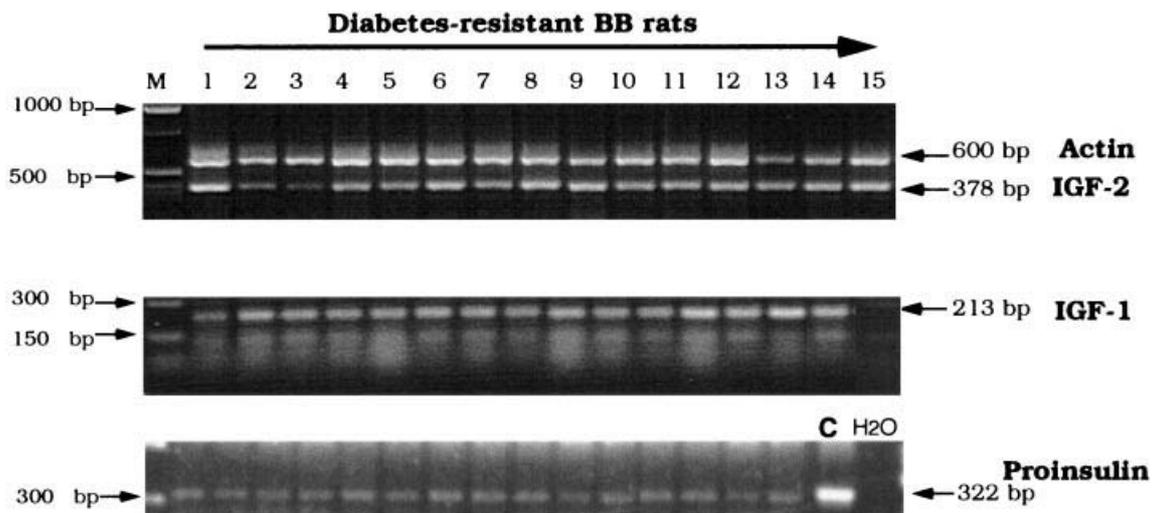


Figure 1. Amplification of *Igf1*, *Igf2*, *Ins* and β *actin* mRNAs from 5-day-old diabetes-resistant (BBDR) rat thymi by RT-PCR. RT-PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. The predicted sizes for the amplification bands using primers specific for *Igf1*, *Igf2* and *Ins* were 213, 378 and 322 bp, respectively. M1, 100 bp markers; C, brain or pancreas as positive controls; H₂O, negative control (no RNA). Amplification of β *actin* cDNA (600 bp) was used as an internal standard

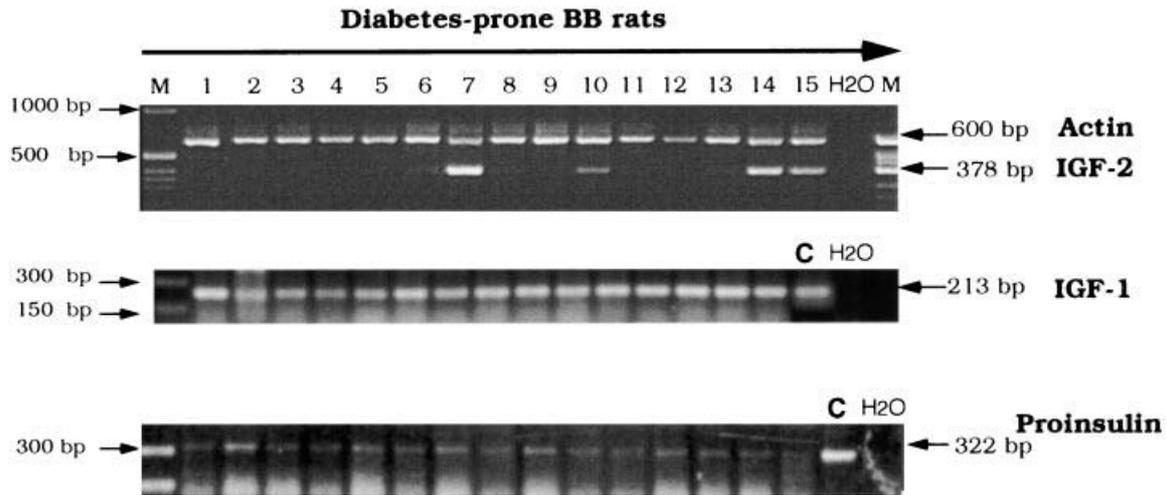


Figure 2. Amplification of *Igf1*, *Igf2*, *Ins* and β *actin* mRNAs from 5-day-old diabetes-prone (BBDP) rats thymi by RT-PCR. RT-PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. M1, 100 bp markers; C, brain or pancreas as positive controls; H₂O, negative control (no RNA). Amplification of β *actin* cDNA (600 bp) was used as an internal standard

autoantigens, insulin is considered to be a critical target and the most specific autoantigen of β -cells [2–4]. Insulin as an immune target has been associated with the development of autoimmune diabetes in humans and NOD mice [1–4], though its role as an autoantigen in the BB rat remains to be clarified. Based on the data implicating autoreactivity to insulin in the pathogenesis of type 1 diabetes, both ourselves and others have suggested that intrathymic expression of *INS* and *IGF* could induce the central T-cell tolerance of the insulin family [11,16–18,20].

Previously, defects in the cytoarchitecture of thymic epithelium have been observed in BB rats and were proposed to contribute both to the impairment of T-cell differentiation and in the susceptibility to autoimmune diabetes of these animals [26]. The present study shows a defect of *Igf2* expression in the thymus of more than 80% of BBDP rats, while *Igf1* and *Ins* are expressed in the thymi of the three strains analyzed (BBDR, BBDP and WF). This defect is tissue-specific, since BBDP rat brains and livers express readily detectable *Igf2* mRNA. There is a concordance in the proportion of thymi that have defects

in *Igf2* expression and the incidence rate of diabetes in BBDP rats (86%). Since thymic IGF-mediated signaling plays an active role in the early steps of T cell-differentiation [15], the defect of thymic *Igf2* expression could also contribute with genetic factors (like *lyp*) to the lymphopenia of these animals [27,28]. It will be of interest to investigate whether the thymic *Igf2* defect contributes to the lack of regulatory RT6⁺ T-cells in BBDP rats.

The molecular mechanisms responsible for the defect of *Igf2* expression in the thymus of BBDP rats remain to be determined. Rat *Igf2* contains six exons, is transcribed from three distinct promoters (P1, P2 and P3), and is expressed in a tissue-specific and developmentally regulated manner [29–32]. In the rat fetus, *Igf1* and *Igf2* are expressed in different tissues with a marked predominance of *Igf2* [33–35]. During the first 3 weeks of postnatal life, *Igf2* transcripts gradually decline to very low levels except in organs such as the brain, spinal cord, striated muscle and thymus. Within these organs, *Igf2* transcripts and IGF-2 protein are also detected at adulthood [11,33,34,36,37]. In the present study,

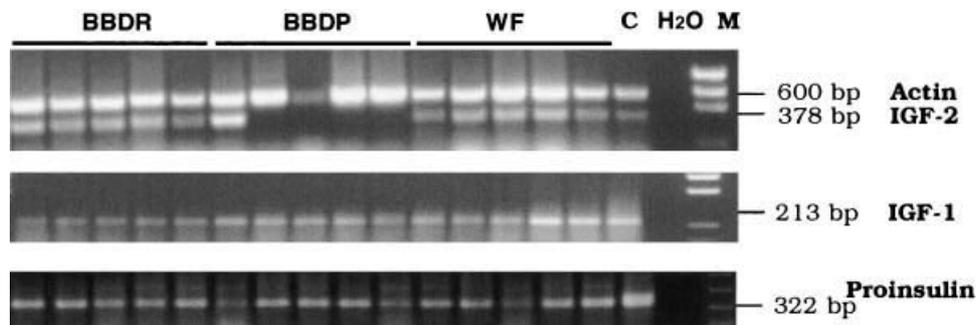


Figure 3. Amplification of *Igf1*, *Igf2*, *Ins* and β *actin* mRNAs from 2-day-old BBDP, BBDR and WF rat thymi by RT-PCR. RT-PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. M1, 100 bp markers; C, brain or pancreas as positive controls; H₂O, negative control (no RNA). Amplification of β *actin* cDNA (600 bp) was used as an internal standard

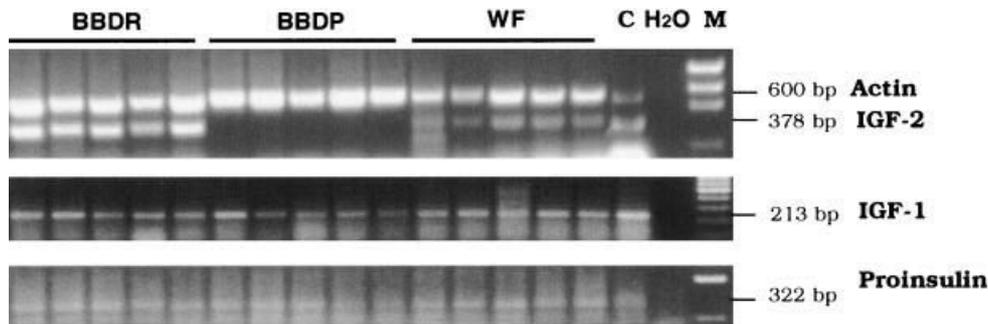


Figure 4. Amplification of *Igf1*, *Igf2*, *Ins* and β *actin* mRNAs from 5-week-old BBDP, BBDR and WF rat thymi by RT-PCR. RT-PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. M1, 100 bp markers; C, brain or pancreas as positive control; H₂O, negative control (no RNA). Amplification of β *actin* cDNA (600 bp) was used as an internal standard

Igf2 transcripts and IGF-2 proteins were present both in adult and neonatal thymi from BBDR and WF rats. Thymic *Igf2* expression in BB rats is also under the control of promoters P2 and P3 (data not shown). Since *Igf2* transcripts are detected in brain and liver of BBDP rats, a tissue-specific mechanism may be hypothesized to explain the defect of *Igf2* expression in the thymus of BBDP rats.

A balance of positive and negative influences between different genes determines the susceptibility of BB rats to autoimmune diabetes. The main diabetes susceptibility locus in humans, mice and rats is the MHC class II gene. In the BB rat, this susceptibility locus is the RT1^u haplotype on chromosome 20 (*iddm2*) [38]. A second non-MHC locus strongly associated with diabetes susceptibility present on chromosome 4 has been termed *iddm4* [39]. The homozygous presence of the lymphopenia gene (*lyp*) on chromosome 4 (*iddm1*) appears to remove regulatory T-cells, thus permitting the susceptibility for diabetes determined by *iddm2* and *iddm4* to be expressed [39,40]. In the absence of regulation due to the homozygous presence of *lyp*, T-cell-mediated β -cell destruction and overt hyperglycemia ensues. With regard to diabetes resistance, a locus close to *Igf2* has been described recently [41].

The present study suggests an association between the defect of *Igf2* expression in the thymus and the emergence of autoimmune diabetes in BBDP rats, but the extrapolation of these findings to type 1 diabetes in humans is premature. In humans, the *IDDM2* type 1 diabetes

susceptibility locus maps to a variable number of tandem repeat (VNTR) minisatellite upstream of *INS* [42]. Protective VNTR class III alleles (140 to >200 repeats) are associated with higher levels of *INS* mRNA levels in human thymus [17,18]. No significant difference in human thymic or pancreatic *IGF2* mRNA levels can be observed in association with the protective class of VNTR alleles [43]. However, VNTR also has effects on *IGF2* expression since, in placenta at term and HepG2 hepatoma cell line, higher *IGF2* mRNA levels are associated with shorter VNTR class I alleles than longer VNTR class III alleles [44]. From these data, it clearly appears that the mechanisms controlling the intrathymic expression of insulin and insulin-related genes are deserving of further investigation. Additional studies are also required to distinguish the type of the immune response (immunogenic or tolerogenic) elicited by oral and parenteral administration of insulin and IGFs to pre-diabetic and new-onset diabetic individuals. Preliminary reports suggest that oral insulin is unable to promote immunological tolerance to the residual β -cells in overtly diabetic type 1 individuals [45,46]. However, the outcome of the oral insulin arm of the diabetes prevention trial in the DPT study in the United States has not yet been reported. The available data suggest, however, that investigation into alternative approaches to insulin for immunomodulatory intervention in pre-diabetic and new-onset diabetic individuals must continue.

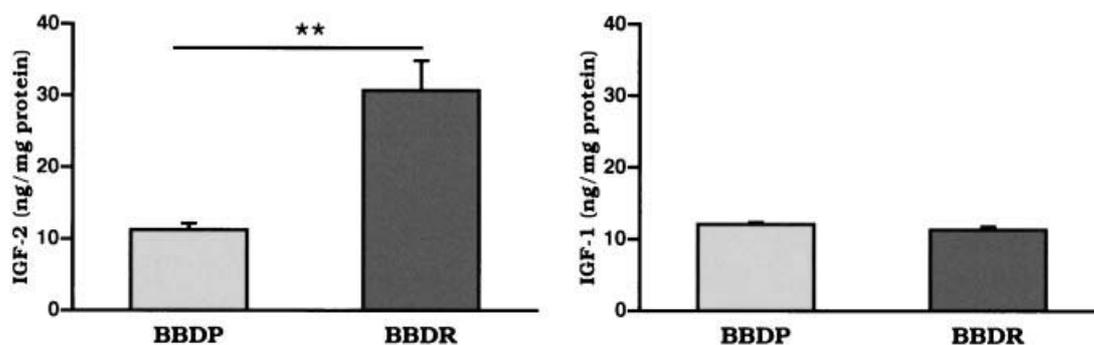


Figure 5. IR IGF-1 and IGF-2 in thymi of BBDP and BBDR rats. Proteins of 15 individual thymi from BBDR and BBDP rats were extracted and IR IGF-1 and IGF-2 were quantified with specific IRMAs (** $p < 0.01$)

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