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Biobreeding rat islets exhibit reduced antioxidative defense and *N*-acetyl cysteine treatment delays type 1 diabetes

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Abstract

Islet-level oxidative stress has been proposed as a trigger for type 1 diabetes (T1D), and release of cytokines by infiltrating immune cells further elevates reactive oxygen species (ROS), exacerbating β cell duress. To identify genes/mechanisms involved with diabeto-genesis at the β cell level, gene expression profiling and targeted follow-up studies were used to investigate islet activity in the biobreeding (BB) rat. Forty-day-old spontaneously diabetic lymphopenic BB DR*lyp/lyp* rats (before T cell insulitis) as well as nondiabetic BB DR+/+ rats, nondiabetic but lymphopenic F344*lyp/lyp* rats, and healthy Fischer (F344) rats were examined. Gene expression profiles of BB rat islets were highly distinct from F344 islets and under-expressed numerous genes involved in ROS metabolism, including glutathione S-transferase (GST) family members (*Gstm2*, *Gstm4*, *Gstm7*, *Gstt1*, *Gstp1*, and *Gstk1*), superoxide dismutases (*Sod2* and *Sod3*), peroxidases, and peroxiredoxins. This pattern of under-expression was not observed in brain, liver, or muscle. Compared with F344 rats, BB rat pancreata exhibited lower GST protein levels, while plasma GST activity was found significantly lower in BB rats. Systemic administration of the antioxidant *N*-acetyl cysteine to DR*lyp/lyp* rats altered abundances of peripheral eosinophils, reduced severity of insulitis, and significantly delayed but did not prevent diabetes onset. We find evidence of β cell

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contribution statement
M B, A M H, Å L, and M J H conceived, designed, interpreted results, and drafted/edited manuscript.; S K, J M F, R G, S J, M L K, S P, and Y-G C acquired data and conducted analyses and revised manuscript. All authors approve the content of this manuscript.

dysfunction in BB rats independent of T1D progression, which includes lower expression of genes related to antioxidative defense mechanisms during the pre-onset period that may contribute to overall T1D susceptibility.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by immunocyte infiltration of the pancreatic islets (insulitis) and destruction of the insulin-secreting β cells. Diabetes in the biobreeding (BB) rat exhibits many similarities to the human disease, including onset during puberty, insulitis, and lifelong dependency on exogenous insulin (Scott 1990, Crisa *et al*. 1992).

Diabetes in BB rats is polygenic (Wallis *et al*. 2009). The MHC (insulin-dependent diabetes mellitus locus 1, *Iddm1*) contributes the largest genetic risk as it does in humans and in the NOD mouse. In rats, this is the HLA-DQB1 homolog, RT1-B, specifically the $RT1^{u}$ haplotype (Colle 1990, Awata *et al*. 1995). The DR+/+ and DR*lyp/lyp* congenic BB rat lines differ by the *Iddm2* region (Jacob *et al*. 1992), where the T cell lymphopenia of the DR*lyp/lyp* rat arises from a single nucleotide deletion in the *Gimap5* gene (Hornum *et al*. 2002, MacMurray *et al*. 2002). T1D develops spontaneously in 100% of DR*lyp/lyp* rats at ~60 days of age and is elicited through a deficiency in $CD4^{+}CD25^{+}$ regulatory T (T_{REG}) cells, as adoptive transfer of this population prevents T1D (Lundsgaard *et al*. 2005, Poussier *et al*. 2005). DR+/+ rats possess a wild-type *Gimap5* and do not spontaneously develop T1D; however, it is possible to induce diabetes through depletion of T_{REG} cells (Mordes *et al*. 1996, Zipris *et al*. 2003). Fischer (F344) rats, which normally possess the $RT1^{lv1/lv1}$ MHC, do not develop T1D even after introgression of *Iddm1* and/or *Iddm2* (Jacob *et al*. 1992, Moralejo *et al*. 2003), indicating that they lack this additional susceptibility. Thus, predisposition for T1D independent of *Iddm1* and *Iddm2* exists in the BB rat that is phenotypically manifest upon loss of immune regulation.

Oxidative stress is associated with many pathological states and has been implicated as a trigger for T1D (reviewed in Lenzen (2008)). Compared with other tissues, such as kidney or liver, islets possess lower levels of the antioxidant enzymes superoxide dismutase (SOD) and catalase (Lenzen 2008) and may be more susceptible to redox imbalances arising from overproduction of reactive oxygen species (ROS). Furthermore, the release of pro-inflammatory cytokines by infiltrating immune cells can elevate intra-islet ROS as exposure of rodent islets to interleukin 1β (IL1β) induces nitric oxide synthase (iNOS) expression and production of the free radical nitric oxide (Corbett *et al*. 1992, Eizirik *et al*. 1993). While cultured human islets express higher levels of antioxidant enzymes than do their rodent counterparts (Welsh *et al*. 1995), inherited deficiencies in antioxidant defense mechanisms may be relevant to T1D susceptibility as strain-dependent differences in islet sensitivity to IL1β (Reimers *et al*. 1996) and lower islet levels of catalase and SOD activity have been reported in BB relative to Wistar rats (Sigfrid *et al*. 2004).

Consistent with the concept that autoimmunity involves deficiencies in self-tolerance as well as target organ-specific factors, we have discovered that β cells of DR*lyp/lyp* and DR+/+ rats share an islet-level T1D susceptibility. This is reflected by the expression of the eosinophil

and mast cell recruiting chemokine eotaxin, beginning by 40 days of age, providing a means for immunocyte recruitment and activation (Hessner *et al*. 2004, Geoffrey *et al*. 2006). Here, we further examine tissue-specific T1D susceptibility by comparing the islet transcriptomes of DR+/+ and DR*lyp/lyp* rats during the pre-onset period to those of F344 and F344*lyp/lyp* rats (*Iddm2* introgressed onto the F344 background). We find BB rat islets under-express genes involved in metabolism of ROS, in particular numerous isoforms of glutathione S-transferase (GST), an important family of enzymes that participate in the detoxification of reactive electrophilic compounds by catalyzing their conjugation to glutathione. Administration of *N*-acetyl cysteine (NAC), a thiol compound that acts directly as a free radical scavenger and a precursor in glutathione synthesis, to DR*lyp/lyp* rats, decreased abundances of peripheral eosinophils, reduced severity of insulitis, and delayed but did not prevent diabetes onset.

Materials and methods

Animals and NAC treatment

BB (Bieg *et al*. 1998), F344, and F344*lyp/lyp* (Moralejo *et al*. 2003) rats were maintained at the Pacific Northwest Diabetes Research Institute, The University of Washington-Seattle, and The Medical College of Wisconsin. BB DR+/+ and DR*lyp/lyp* animals were propagated by mating DR*lyp*/+ breeders and genotyped as described (MacMurray *et al*. 2002), housed under specific pathogen-free conditions with standard light/dark cycles, and were fed a regular diet and water *ad libitum*. All federal (http://grants1.nih.gov/grants/olaw/references/phspol.htm) guidelines for use and care of laboratory animals were followed, and all protocols were approved by the respective Institutional Animal Care and Use Committees (IACUC).

DR*lyp/lyp* and DR+/+ rats were treated with NAC daily (200 mg/kg, i.p.; Sigma), dissolved in 0·9% sterile NaCl, and neutralized to pH 7 with 1 M NaOH beginning at weaning. Age-matched DR*lyp/lyp* and DR+/+ rats were sham-treated with equal volumes of 0·9% NaCl.

Animal phenotyping and tissue collection

Weight and blood glucose were measured from day 40 of age until DR*lyp/lyp* rats developed T1D (blood glucose levels ≥250 mg/dl measured with an Ascensia Elite XL glucometer; Bayer). Animals underwent weekly oral glucose tolerance tests starting from 6 weeks of age. Following a 6-h fasting, glucose solution (2 g/kg body weight) was administered by gavage, followed by blood glucose measurements at 0, 15, 30, 60, 90, and 120 min. Plasma was separated from tail vein blood and stored at −20 °C until assayed for C-peptide by RIA (Linco, St Charles, MO, USA). At onset, DR*lyp/lyp* and age-matched DR+/+ littermates were killed and pancreata were processed for histological analysis and measurement of insulin content.

Differential cell counts of peripheral blood were performed by an automated cell counter. Percentages of the different leukocyte populations were determined: 50 μl blood was incubated for 15 min in 10 ml red blood cell lysis solution, leukocytes were harvested by centrifugation, spread onto glass slides, stained with Giemsa–Grunwald solution, and

percentages of lymphocytes, polymorphonuclear cells, and monocytes were determined (800–1200 total cells per sample).

Flow cytometric analysis of peripheral blood lymphocytes was accomplished as described (Fuller *et al*. 2006). T_{REG} were identified by surface staining with anti-CD4 (clone OX-35) and anti-CD25 (clone OX-39) followed by intracellular staining with anti-Foxp3 (clone FJK-16s) using an intracellular staining kit from eBioscience (San Diego, CA, USA).

Islet gene expression profiling

Islets were isolated from normoglycemic 40-day-old rats as described (Sweet *et al*. 2004) and total RNA was extracted with Trizol (Invitrogen). RNA (~100 ng) was amplified/labeled (Affymetrix two-cycle cDNA synthesis kit, Affymetrix, Santa Clara, CA, USA) and then hybridized to the Affymetrix RG230 2.0 array as per the manufacturers' protocol. Image data were quantified with Affymetrix Expression Console Software and normalized with Robust Multichip Analysis (www.bioconductor.org) to determine signal log ratios. Data files have been deposited in The National Center for Biotechnology Information Gene Expression Omnibus (accession number GSE20214). The statistical significance of differential gene expression was derived through a Student's *t*-test and false discovery rates (FDR) were determined with Significance Analysis of Microarrays (SAM) software as described (Tusher *et al*. 2001). More stringent statistical criteria were not applied, as relevant genes and pathways would be investigated further by quantitative RT-PCR (qRT-PCR) and targeted follow-up studies. Ontological pathway analysis was performed with the Database for Annotation, Visualization, and Integrated Discovery (DAVID; Hosack *et al*. 2003). Hierarchical clustering was conducted with Genesis (Sturn *et al*. 2002).

Quantitative RT-PCR

Total RNA was extracted from brain, muscle, liver, or cultured cells using TRIzol reagent, and qRT-PCR for *Gstm2*, *Gstm7*, *Gstt1*, *Sod2*, *Sod3*, *Ccs*, *Cd68*, and *FcεR1* was performed as described (Pfaffl 2001, Wang *et al*. 2008). Primer designs and reaction performance parameters are provided in Table 1.

Rat insulinoma cell culture and cytokine exposure

RINm5f cells (ATCC, Rockville, MD, USA) were maintained in RPMI 1640 medium (Mediatech, Inc., Manassas, VA, USA) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and Pen/Strep (Lonza, Walkersville, MD, USA). Medium was changed every 2–3 days and cells were subcultured as required. Cells were allowed to adhere overnight in medium containing 10% charcoal dextran-treated serum. The medium was changed and cells were stimulated with cytokines for 24 h: IL1β (2 ng/ml), IFNγ (30 ng/ml), and TNFα (10 ng/ml) (R&D Systems, Inc., Minneapolis, MN, USA). Following treatment, medium was aspirated and cells were harvested for qRT-PCR.

Histological studies

Pancreata were processed for histology as described (Hessner *et al*. 2004, Bogdani *et al*. 2005, Geoffrey *et al*. 2006). Insulitis was evaluated in hematoxylin- and eosin-stained sections (five to seven sections per pancreas; 200–400 islets) and independently scored by

an investigator unaware of the animals' genotype as described (Fuller *et al*. 2009): Grade 0, no infiltration; Grade 1, leukocytes around ducts and vessels only; Grade 2, leukocytes around islets; Grade 3: leukocytes inside islets without change in β cell morphology; Grade 4, leukocytes inside islets with distorted β cell morphology or islets devoid of β cells.

Consecutive sections were immunostained using antibodies targeting CD45 (leukocyte common antigen), CD3, CD4, CD8 (T cell subsets), CD45R (B-cells), and CD68 (macrophages/monocytes); >1000 cells/pancreas were counted; and percentages were determined. Pancreatic sections were also stained with anti-glucagon (Sigma) and anti-GST-mu (GSTM, Abcam, Cambridge, MA, USA) antibodies as described (Hessner *et al*. 2004, Geoffrey *et al*. 2006). Slides were imaged on a Nikon E600 system (Nikon USA, Melville, NY, USA). MetaMorph version 6.3r3 software (Universal Imaging, Buckinghamshire, UK) was used to trace the islet and to capture the fluorescence intensity within the tracing for a minimum of ten islets per section, one section per rat and $n \geq 4$ rats per strain.

GST, glutathione reductase, and glutathione measurements

Assays for measurement of plasma GST activity, glutathione reductase levels, and glutathione levels were conducted as per the manufacturers' instructions (Arbor Assays, Ann Arbor, MI, USA). All samples were analyzed in duplicate and results were analyzed using four-parameter logistic curve fitting.

Statistical analysis

Data are expressed as mean±S.D. of n independent experiments unless otherwise noted. The statistical significance of differences in glycemic levels between experimental and control groups was calculated by the Kruskal–Wallis test. Changes in percentage of different cell populations and insulitis score were tested by the Mann–Whitney *U* test. $P<0{\cdot}05$ was considered significant. Survival was analyzed with the Kaplan–Meier method.

Results

Islet gene expression profiling

Given that β cells of 40-day-old BB rats show evidence of eotaxin expression, before detection of insulitis in DR*lyp/lyp* rats (Hessner *et al*. 2004, Geoffrey *et al*. 2006), we compared the islet transcriptomes of normoglycemic 40-day-old DR*lyp/lyp* and DR+/+ rats vs F344 and F344*lyp/lyp* rats with the goal of identifying pathways/mechanisms associated with T1D susceptibility common to BB rats, independent of *Gimap5* status. Hierarchical clustering of the significantly regulated probe sets illustrates the distinctiveness between these lineages at 40 days of age (Fig. 1A). Genes over-expressed and under-expressed in BB islets (n=2084 and 2545 probe sets respectively; Supplementary Table 1, see section on supplementary data given at the end of this article) were independently evaluated for biological pathway enrichment using the DAVID annotation tool (Supplementary Table 2, see section on supplementary data given at the end of this article). Selected pathway terms that functionally discriminate the islet expression profiles between the BB and the F344 strains are tabulated in Table 2.

Genes related to antioxidant activity and the glutathione pathways are under-represented in islets of BB compared with F344 rats

Glutathione metabolism and antioxidant activity were identified as enriched pathway terms when analyzing genes under-expressed in DR*lyp/lyp* and DR+/+ relative to F344 and F344*lyp/lyp* islets (Table 2). Within these terms were 27 unique genes related to oxidative defense mechanisms. Among these were members of the GST superfamily (*Gstk1*, *Gstp1*, *Gstt1*, *Mgst3*, *Gstm2*, *Gstm4*, *Gstm5*, and *Gstm7*), which function in the detoxification of electrophilic compounds through their conjugation to reduced glutathione. BB rat islets showed lower expression of γ-glutamyltransferases (*Ggt1*, *Ggt5*, and *Ggt7*) and 5-oxoprolinase (*Oplah*) important in glutathione synthesis/metabolism. Also under-expressed in BB islets were glutathione peroxidase 4 (*Gpx4*), a phospholipid hydroperoxidase that protects cells against membrane lipid peroxidation through the reduction of hydrogen peroxide, organic hydroperoxides, and lipid peroxides; peroxiredoxin 4 (*Prdx4*) and *Prdx5*, peroxidases that respectively control oxidative stress within the endoplasmic reticulum and mitochondria by reducing hydrogen peroxide; as well as superoxide dismutase 2 (*Sod2*, mitochondrial MnSOD) and *Sod3* (extracellular Cu-ZnSOD). Apolipoprotein E (*Apoe*), which possesses antioxidant properties and can be downregulated under conditions of oxidative stress (Espiritu & Mazzone 2008), was under-expressed by BB rat islets. Ornithine decarboxylase (*Odc1*) and spermidine synthase (*Srm*), both important in the synthesis of the antioxidant polyamine, spermidine, were also under-expressed in BB islets. The overall pattern of reduced expression levels of these and other genes related to ROS defense/metabolism are illustrated in Fig. 1B.

Examination of GST expression in islets and other tissues

qRT-PCR of the independent islet RNA isolations from each rat used to generate the islet RNA pools for each strain in the array studies confirmed under-expression of *Gstm2*, *Gstm7*, *Gstt1*, *Sod2*, *Sod3*, and copper chaperone for SOD (*Ccs*, a metallochaperone required for incorporation of copper into *Sod1*) in BB rat islets. Smaller expression differences were observed in day 40 liver, brain, and skeletal muscle of DR*lyp/lyp* and DR+/+ vs F344*lyp/lyp* and F344 rats (Fig. 2A).

Immunofluorescent staining was used to localize and examine relative GST protein levels in pancreatic sections of 5-week-old DR*lyp/lyp*, DR+/+, F344, and F344*lyp/lyp* rats. Consistent with the array and RT-PCR expression measurements, significantly lower fluorescence indicative of reduced protein GST protein levels was observed in islets of DR*lyp/lyp* and DR +/+ rats (Fig. 2B).

Reduced plasma GST activity in BB rats

Significantly lower GST activity levels were detected in plasma of DR*lyp/lyp* and DR+/+ rats compared with F344 and F344*lyp/lyp* rats (Fig. 3A). To exclude the possibility that lower GST activity was due to a reduced ability to convert oxidized glutathione to reduced glutathione or a lower level of substrate, plasma glutathione reductase activity as well as total and free glutathione levels were measured. Neither glutathione reductase activity nor the percentage of free glutathione was significantly different between strains (Fig. 3B and C).

Inflammatory processes as a basis for reduced islet GST expression

As the under-expression of genes related to antioxidant defense was observed only in islets, we hypothesized that innate inflammatory processes may underlie reduced GST expression. DR+/+ rats do not develop insulitis nor T1D rats. By histological examination, DR*lyp/lyp* islets are free of immune cell infiltrates at day 40 but not at time points after 60 days (Hessner *et al*. 2004). To exclude the possibility that macrophages and/or mast cells, at abundances difficult to capture in routine histological studies, were present in the day 40 islets used for the gene expression studies, we respectively performed qRT-PCR for CD68 and FcεR1. Islets of 40-day-old DR*lyp/lyp*, DR+/+, F344*lyp/lyp*, and F344 islets expressed less CD68 and FcεR1 transcript compared with normoglycemic day 60 DR*lyp/lyp* islets. These differences reached statistical significance ($P<0{\cdot}05$) in comparisons between day 60 DR*lyp/lyp* islets vs day 40 DR*lyp/lyp* and DR+/+ islets. While not of the level observed in day 60 DR*lyp/lyp* islets, significantly higher levels ($P<0{\cdot}05$) of CD68 transcript were observed when comparing day 40 F344 islets to day 40 DR*lyp/lyp* and DR+/+ islets (Fig. 4A). These data are consistent with previous studies showing islets of day 40 DR*lyp/lyp* and DR+/+ rats free of immune infiltrates (Hessner *et al*. 2004). Further, these results support that the observed gene expression differences were not a consequence of the activity of infiltrating immune cells. However, it cannot be excluded that the activity of resident islet mast cells and macrophages may differ between the strains.

Our previous studies on BB rats revealed the presence of inflammatory processes in both DR+/+ and DR*lyp/lyp* rats at day 60 as well as evidence these processes begin as early as day 40 in DR*lyp/lyp* rats (Kaldunski *et al*. 2010). To examine the influence of inflammatory cytokines and cytokine-induced oxidative stress on the expression of GSTs, RINm5f rat insulinoma cells were treated with IL1β, TNFα, and IFNγ. Relative to untreated cultures, a 24-h cytokine treatment resulted in significant decreases in *Gstm2* (−3·3-fold, $P<0{\cdot}001$), *Gstm7* (−3·6-fold, $P<0{\cdot}001$), and *Sod3* (−4·3-fold, $P<0{\cdot}05$) expression while *Sod2* expression was increased (+13·1-fold, $P<0{\cdot}01$, Fig. 4B). Similar results were obtained when using the rat INS-1 cell line (data not shown). Given our previous early detection of inflammation in the BB rat (Kaldunski *et al*. 2010) and the sensitivity of *Gst* gene expression to cytokine exposure in RINm5f cells, it cannot be excluded that under-expression of GSTs in BB rat islets is the consequence of early pre-onset inflammatory processes. However, increased expression of *Sod2* in response to IL1β, TNFα, and IFNγ suggests that β cell intrinsic factors also contribute to the overall under-expressed antioxidant genes.

Delay of BB rat T1D through treatment with NAC

Given the evidence for deficiencies in islet antioxidant capacity in BB rats during the pre-onset period relative to Fischer rats, we tested the effectiveness of the glutathione precursor NAC in delaying diabetes onset in DR*lyp/lyp* rats. Treatment with NAC resulted in a modest but significant delay in onset relative to sham-treated controls ($P=0{\cdot}033$, Fig. 5A).

No differences in growth rate, weight, or organ weight (kidney, spleen, thymus, adrenal gland, and thyroid; $P>0{\cdot}05$) were observed between NAC- and sham-treated DR*lyp/lyp* rats. NAC- and sham-treated DR*lyp/lyp* rats showed similar progressive weight gain that abruptly

ceased a few days before diabetes onset; both groups exhibited a slight increase in blood glucose levels in the 2 days preceding the disease onset, being between 150 and 200 mg/dl, followed by levels >250 mg/dl (Fig. 5B). NAC treatment did not influence blood glucose levels in DR+/+ rats, which remained within the normal range values as in nontreated DR +/+ rats (data not shown).

As under-expression of antioxidant genes is evident in both BB sub-strains, OGTT, at days 42, 49, 56, and 63, was used to assess the effect of NAC treatment on islet function in DR*lyp/lyp* and DR+/+ rats. While DR+/+ animals remained glucose tolerant at all time points, NAC-and sham-treated DR*lyp/lyp* rats gradually became glucose intolerant. These data, aligned to diabetes onset (Fig. 5C), show that all DR*lyp/lyp* rats were glucose tolerant at −4 to −2 weeks. At −1 week, both NAC- and sham-treated DR*lyp/lyp* rats exhibited higher blood glucose levels than DR+/+ rats ($P<0{\cdot}05$). While blood glucose levels were lower in NAC- vs sham-treated DR*lyp/lyp* rats at 30 through 90 min after the glucose challenge at −1 week, the difference did not reach statistical significance ($P>0{\cdot}5$). OGTT did not reveal any change in glucose tolerance when comparing NAC- vs sham-treated DR+/+ rats.

At onset, circulating C-peptide levels and pancreatic insulin content in DR*lyp/lyp* rats were <10% of those of age-matched DR+/+ rats ($P<0{\cdot}05$), and these parameters were not influenced by NAC treatment (data not shown). NAC treatment did not affect peripheral GST levels in DR*lyp/lyp* or DR+/+ rats. A trend of increased free glutathione levels (~20%), while not reaching statistical significance, was observed in NAC-treated vs sham-treated DR*lyp/lyp* and DR+/+ rats (data not shown).

Effect of NAC on circulating leukocytes and insulitis

Flow cytometry and differential counts found DR*lyp/lyp* rats characteristically lymphopenic (Crisa *et al*. 1992, Groen *et al*. 1996, Greiner *et al*. 1997), possessing <15% the normal T cell count relative to DR+/+ rats. NAC treatment did not alter the abundances of T cells nor the relative abundances of lymphocytes, neutrophils, basophils, and monocytes (data not shown). It is well documented that DR*lyp/lyp* rats exhibit eosinophilia and islet eosinophil infiltration before diabetes onset (Kurner *et al*. 1986, Maruta *et al*. 1989, Eastman *et al*. 1991, Hessner *et al*. 2004). The eosinophilia is one of the several DR*lyp/lyp* phenotypes that parallels immunodysregulation poly-endocrinopathy enteropathy X-linked syndrome (IPEX), a human disorder that arises through mutations to *FoxP3*, a transcription factor required for T_{REG} cell development, and is characterized by autoimmunities that include diabetes (Geoffrey *et al*. 2006). An increase was observed in the proportion and the number of circulating eosinophils in both the NAC- and the sham-treated DR*lyp/lyp* rats in the 4 weeks before diabetes onset; however, it was significantly less in the NAC-treated group (Fig. 6A).

Pancreata of NAC- and sham-treated DR*lyp/lyp* rats at onset, as well as age-matched NAC- and sham-treated DR+/+ rats, were histologically evaluated. Inflammatory cells were scarce or absent in DR+/+ pancreata but were present in both the exocrine and the islet tissue of NAC-and sham-treated DR*lyp/lyp* rats. Nearly all islets of NAC-and sham-treated DR*lyp/lyp* rats exhibited insulitis; however, the severity differed between the two groups. Grade 4

insulitis was observed in 58 and 82% of NAC- and sham-treated DR*lyp/lyp* islets (Fig. 6B) respectively. More islets (34%) in the NAC-treated DR*lyp/lyp* rats were of Grade 2 or 3 inflammation compared with only 9% of the islets in the sham-treated DR*lyp/lyp* rats. The effect of NAC on the circulating eosinophil cell abundance was not associated with a reduction in the proportion of islets possessing eosinophils in the peri-islet inflammation nor with the number of eosinophils present in these infiltrates (data not shown).

At diabetes onset, macrophages (CD68+) made up the majority (>90%) of the inflammatory cells present in the exocrine tissue and penetrating the NAC- and sham-treated DR*lyp/lyp* islets. T cells (CD3+, CD4+, and CD8+) were present but were very few in number while B-cells (CD45R+) were not detected. The percentage of CD68+ cells in the pancreas was lower in NAC-treated DR*lyp/lyp* rats (Fig. 6C), being ~50% that observed in the exocrine and endocrine tissues of sham-treated DR*lyp/lyp* rats. These data suggest that NAC may attenuate the severity of innate pancreatic inflammation, thereby reducing the rate of disease progression in BB rats.

Discussion

β Cells of lymphopenic and non-lymphopenic BB rats exhibit eotaxin expression by 40 days of age, before development of insulitis in DR*lyp/lyp* rats (Geoffrey *et al*. 2006). Eotaxin expression by β cells is a means of recruiting eosinophils, mast cells, and other immune cells that bear the receptor CCR3 to the islet. Given that, generally, healthy tissues do not express immune cell recruiting chemokines that promote inflammation, we have considered eotaxin expression a sign of underlying pathology in BB rats. Furthermore, β cell expression of eotaxin correlates with the ability of the BB lineage to develop T1D (either spontaneously in the DR*lyp/lyp* rat or through induction protocols in the DR+/+ rat). Therefore, we examined islet transcriptomes at day 40 to capture a time after evidence of islet dysfunction, but before evidence of insulitis and/or robust adaptive immunity, to identify factors in the BB rat that may contribute to T1D susceptibility at the β cell level.

Comparison of the day 40 islet transcriptomes of DR*lyp/lyp* and DR+/+ to F344*lyp/lyp* and F344 rats found the two lineages highly distinct. Confirming our previous studies, eotaxin transcript (*Ccl11*) was found more abundant in BB vs F344 islets (1·7-fold, P=0·033). Other transcripts consistent with immune cell recruitment/activation were differentially regulated in BB rat islets, including the neutrophil chemoattractant *Cxcl1* (2·6-fold, P=0·016) and the IL22 antagonist *Il22ra2* (−2·74-fold, P=0·002). Extending observations of lower catalase and SOD expression in BB rat islets (Pisanti *et al*. 1988, Sigfrid *et al*. 2004), gene expression related to multiple ROS defense mechanisms was significantly under-represented in BB vs F344 islets, in particular members of the GST superfamily. This raises the question whether the lower antioxidant gene expression is a consequence of islet-level pathological/inflammatory processes and/or genetic control.

CD68 and FcεR1 transcript levels were not different between day 40 DR*lyp/lyp* and DR+/+ islets and found lower in day 40 BB islets compared with day 40 F344 and F344*lyp/lyp* islets. Thus, greater numbers of infiltrated macrophages and mast cells were not co-isolated with the day 40 BB rat islets. While these and histological studies are consistent with an

absence of insulitis at day 40, we cannot exclude that co-isolated resident myeloid cells, if any, possessed different activities between the two lineages. Further, we cannot exclude the possibility that activity of immunocytes at the islet periphery, lost during isolation, may be responsible for under-expression of antioxidant genes in BB islets. We found that cytokine treatment of RINm5f cells reduced expression of *Gstm2*, *Gstm7*, and *Sod3*, but the level of *Gstt1* was not altered. Consistent with other reports, we also found that cytokine treatment of RINm5F cells increased *Sod2* expression (Borg *et al*. 1992, Rieneck *et al*. 2000, Cardozo *et al*. 2001, Sigfrid *et al*. 2004). Taken together, these results suggest that both β cell intrinsic and extrinsic factors (such as innate immune cell-mediated inflammation) contribute to reduced expression of antioxidant genes in BB vs F344 islets.

Lower total plasma antioxidants have been reported in first-degree relatives of T1D patients (Rocic *et al*. 1997), supporting a possible genetic basis for under-expression of antioxidant genes that remains to be elucidated in humans and BB rats. *Gstm2*, *Gstm4*, and *Gstm7* are targets of NRF2 (NFE212) (nuclear factor E2 p45-related factor 2) (Malhotra *et al*. 2010), a transcription factor that activates genes in response to oxidative stress (Chen & Kong 2004). Although we did not observe differential expression of *Nrf2* or its negative regulator *Keap1* in the array studies, *Park7*, a positive regulator of Nrf2 (Clements *et al*. 2006), was significantly under-expressed in BB rat islets. It is noteworthy that the four mu-class GSTs (*Gstm2*, *Gstm4*, *Gstm5*, and *Gstm7*) map to *Iddm3* (cytoband 2q34 (Jacob *et al*. 1992, Klaff *et al*. 1999)), a region syntenic to *Idd18.2* in mouse and the *1p13.3* T1D susceptibility region in human, and in crosses between DR*lyp/lyp* and F344 rats, a single F344 allele at *Iddm3* has been sufficient for T1D protection, suggesting the presence of a recessive diabetes susceptibility trait at this locus.

Consistent with other animal studies that associate augmented antioxidant reserve/defense with amelioration of T1D (Prasad 2000, Piganelli *et al*. 2002, Szabo *et al*. 2002, Delmastro & Piganelli 2011), treatment of DR*lyp/lyp* rats with NAC delayed diabetes onset. Reported dosages of NAC vary widely, with our literature searches revealing dosages as high as 1 g/kg per day being administered to adult Wistar rats (Ozaras *et al*. 2003). Our optimization studies revealed that a dosage 400 mg/kg per day was not well tolerated by weanling rats, which showed slower weight gain during the first week of the NAC administration. While mild toxicity and/or off target effects cannot be completely excluded, at the 200 mg/kg per day NAC dosage, DR*lyp/lyp* rats were not different from the controls from 21 days of age, when the treatment was started, for either blood glucose or weight gain through at least 46 days of age. The NAC and saline-treated nonlymphopenic/nondiabetic BB control rats were not different in any of the parameters analyzed at any age (45–230 days). As reviewed (Delmastro & Piganelli 2011), ROS potentially acts at multiple levels of diabetogenesis: an islet-level autoimmune trigger, activating innate immune cells and initiating insulitis, promoting T cell-mediated adaptive immune responses, and impairing FOXP3 expression, thereby suppressing T_{REG} activity in autoimmune disorders (Brahmachari & Pahan 2010). NAC treatment reduced the severity of the insulitic lesion in DR*lyp/lyp* rats but did not increase the frequency of T_{REG} cells, nor did it alter the level of *Foxp3* expression in these immune regulators (data not shown). NAC treatment significantly reduced the eosinophilia that develops in DR*lyp/lyp* rats during the weeks immediately preceding diabetes onset.

NAC inhibits apoptosis of many cell types; however, it exerts a pro-apoptotic effect on human peripheral blood eosinophils exposed to inflammatory cytokines by inhibiting NF-κB activation and blocking production of survival factors (Martinez-Losa *et al*. 2007). Therefore, it is plausible that eosinophil survival was similarly modulated in the NAC-treated DR*lyp/lyp* rats. While a direct role for eosinophils remains to be proven in both BB rat and human T1D, recently eosinophils of T1D patients were found to express high levels of myeloid α-defensins and myeloperoxidase, suggesting that eosinophils could contribute to the innate inflammatory state that may underlie the development of diabetes (Neuwirth *et al*. 2012). In our study, it is possible that the modest benefit of systemic NAC administration observed here arose through action at the level of the β cell or immunomodulation or both.

Notably, in the array studies, trypsin 1 (*Prss1*) and chymotrypsin (*Cela2a* and *Ctrb1*) transcripts exhibited decreased abundance in the DR*lyp/lyp* and DR+/+ compared with the F344 and F344*lyp/lyp* islet pools, possibly indicating different exocrine contamination in the preparations. Islets of Langerhans consist of α cells (15–20% of total cells, produce glucagon), β cells (65–80% of total cells, produce insulin and islet amyloid polypeptide), delta cells (3–10% of total cells, producing somatostatin), pancreatic polypeptide cells (3–5% of total cells, producing pancreatic polypeptide), and epsilon cells (<1% of total cells, producing ghrelin) (Elayat *et al*. 1995). Although interrogated by the array, transcripts for glucagon, insulin 1, insulin 2, islet amyloid polypeptide, somatostatin, pancreatic polypeptide, and ghrelin were not differentially expressed. The concordant results of the follow-up RT-PCR studies on antioxidant enzymes (which utilized the six independent islet RNA isolations for each strain), the GST protein staining and plasma GST studies, and the NAC administration to DR*lyp/lyp* rats suggest that potentially modest differences in exocrine contamination of the islet preparations did not negatively influence the outcome of the study and each result independently supports that deficiencies in antioxidative defense exist in the BB rat.

While day 40 BB rat islets under-express genes related to ROS defense, they express a broader transcriptional signature consisting of both pro-survival/repair and apoptotic pathways (such as under-expression of *Akt1*, *Pdx1*, *Akt2*, and *Perp*; and overexpression of *Hsf2*, *Ccar1*, and *Bclaf*) suggestive of underlying dysfunction. In pursuing the hypothesis that β cell dysfunction is a prerequisite for T1D in BB rats, these day 40 analyses, before insulitis in DR*lyp/lyp* rats, serve as justification for longitudinal studies aimed at defining how and when this state arises and determining its mechanistic basis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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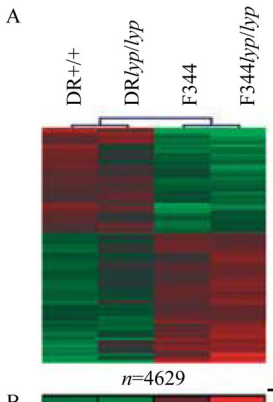

Gene symbol	Probe set ID	UniGene ID	Fold of change	*P* value
Gstm2 *	1370952_at	Rn. 625	–3.1	3.1×10^{-4}
Ggtl *	1368374_a_at	Rn. 10010	–4.1	1.6×10^{-3}
Anpep *	1386938_at	Rn. 11132	–3.4	1.4×10^{-3}
Prdx4 †	1387891_at	Rn. 17958	–3.8	1.5×10^{-3}
Oplah *	1368091_at	Rn. 3066	–2.7	7.3×10^{-5}
Cyba	1370219_at	Rn. 5856	–2.5	4.5×10^{-4}
Gstpl *	1388122_at	Rn. 87063	–2.1	6.3×10^{-3}
Apoe †	1370862_at	Rn. 32351	–4.4	7.3×10^{-5}
Srm *	1367834_at	Rn. 22258	–3.1	1.3×10^{-2}
Gstm7 *	1387023_at	Rn. 6036	–3.0	1.2×10^{-3}
Mgst3 * †	1388300_at	Rn. 1916	–3.4	6.9×10^{-5}
Gstt1 * †	1368354_at	Rn. 11122	–2.2	6.8×10^{-3}
Gpx4 * †	1386871_at	Rn. 3647	–2.1	1.4×10^{-2}
Ccs	1387038_at	Rn. 12311	–2.4	6.4×10^{-3}
Gstm4 *	1375909_at	Rn. 198607	–2.0	5.5×10^{-6}
Prdx5 †	1367677_at	Rn. 2844	–1.8	2.7×10^{-5}
Ggt5 *	1387729_at	Rn. 44367	–1.7	5.5×10^{-4}
Txndc17 †	1388555_at	Rn. 18447	–2.1	1.2×10^{-4}
Nxn †	1375980_at	Rn. 105982	–1.8	6.5×10^{-3}
Pdlim1	1367946_at	Rn. 11170	–1.7	1.1×10^{-3}
Sod3 †	1368322_at	Rn. 10358	–1.8	1.2×10^{-3}
Ggct *	1392541_at	Rn. 129060	–2.0	1.4×10^{-4}
Sepp1	1387339_at	Rn. 1451	–1.5	1.9×10^{-2}
Msra	1387531_at	Rn. 163306	–1.5	1.3×10^{-2}
Foxm1	1369018_at	Rn. 10665	–1.6	4.3×10^{-3}
Sod2 †	1370172_at	Rn. 10488	–1.5	4.8×10^{-4}
Gstm 5 *	1370813_at	Rn. 9158	–1.6	4.3×10^{-2}
Rrm2 *	1389408_at	Rn. 144946	–1.5	3.0×10^{-3}
Cygb †	1367913_at	Rn. 105938	–1.5	2.0×10^{-3}
Nudt1	1368917_at	Rn. 10669	–1.5	2.1×10^{-3}
Mt3	1370124_at	Rn. 11325	–1.6	7.7×10^{-3}
Ggt7 *	1368307_at	Rn. 15462	–1.6	5.6×10^{-3}
Srxn1	1372510_at	Rn. 2835	–1.6	3.3×10^{-3}
Sels †	1387915_at	Rn. 4197	–1.6	3.0×10^{-2}
Pnkp	1393367_at	Rn. 45061	–1.5	1.9×10^{-2}
Atox1	1367945_at	Rn. 11992	–1.9	1.2×10^{-2}
Odc1 *	1370163_at	Rn. 874	–2.3	7.2×10^{-4}
Gstk1 *†	1398378_at	Rn. 109452	–1.8	8.4×10^{-3}

Figure 1.

Gene expression profiling studies on BB vs Fischer islets at 40 days of age. Individual RNA extractions were prepared from islets of six rats per strain and a pool was created for each strain by an equal RNA contribution from each rat. Each RNA pool was then subjected to duplicate array analysis. 4629 regulated probe sets were identified (DR+/+ and DR*lyp/lyp* vs F344 and F344*lyp/lyp*; $|\log_2 \text{ratio}| > 0.5$, +/− 1.4-fold; $P<0.05$ Student's *t*-test), all of which were found in the estimates of differential expression obtained through a permutation test using SAM (FDR<10%) (Tusher *et al*. 2001). (A) Hierarchical clustering of 4629 probe sets that exhibited significant differences. (B) Expression levels for selected genes related to pathway terms 'glutathione metabolism' (indicated by *) and 'antioxidant activity' (indicated by †) as well as other genes (unmarked) related to ROS defense mechanisms.

Tabulated are the gene symbol, Affymetrix probe set identifier, UniGene identifier, fold of change (mean DR*lyp/lyp* and DR+/+ vs mean F344*lyp/lyp* and F344), and *P* value (Student's *t*-test). The data presented in heatmaps A and B are expressed as a ratio relative to the mean of all conditions.

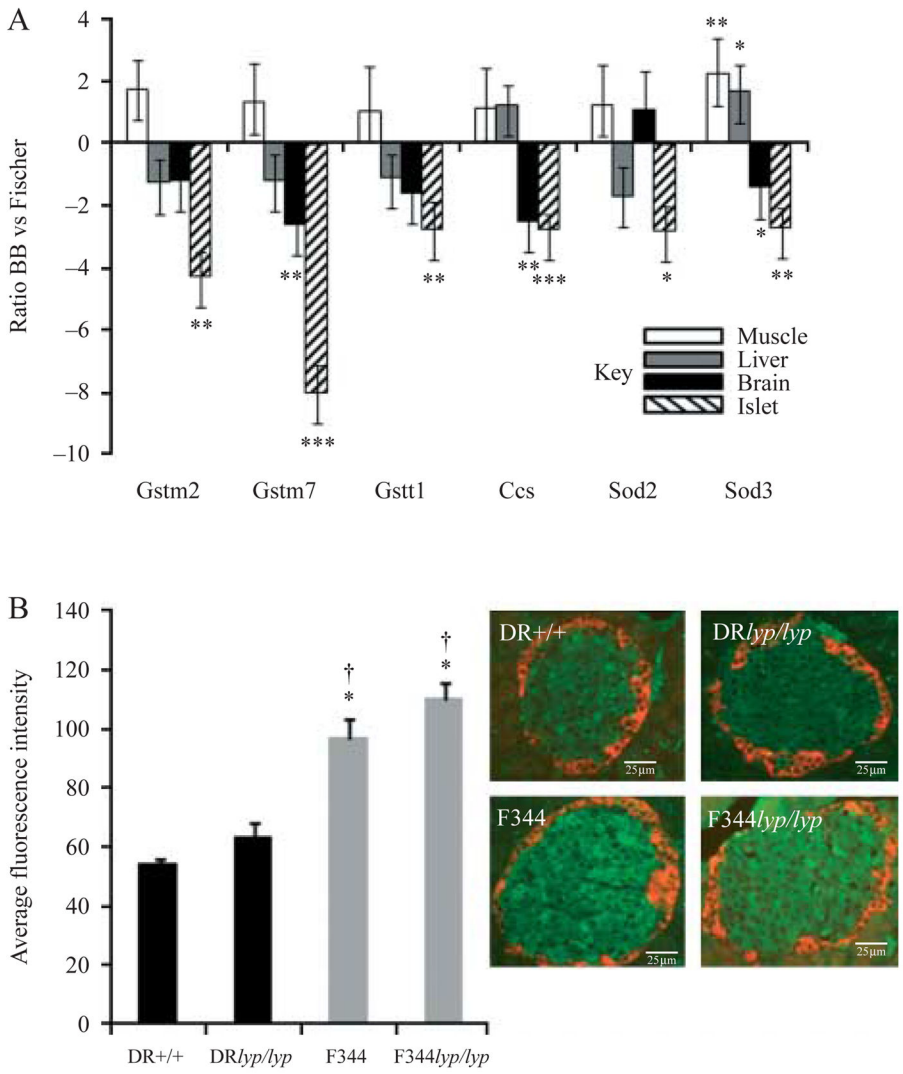

Figure 2.

Antioxidant gene expression in BB (DR+/+ and DR*lyp/lyp*) vs Fischer (F344 and F344*lyp/lyp*) rat tissues. (A) Gene expression of *Gstm2*, *Gstm7*, *Gstt1*, *Ccs*, *Sod2*, and *Sod3* were examined by quantitative RT-PCR in the six individual day 40 islet RNA samples that comprised each of the pools analyzed in the microarray studies, as well as individual RNAs extracted from day 40 liver, brain, and muscle of DR*lyp/lyp*, DR+/+, F344*lyp/lyp*, and F344 rats (n=4 rats per strain) to determine whether under-expression of antioxidant genes was only limited to islets. Bar graph represents the ratio of BB vs Fischer (mean±S.D.), white column=muscle, gray column=liver, black column=brain, striped column=islet. *P<0·05, **P<0·01, ***P<0·001 (Mann–Whitney rank sum). (B) To examine GST protein levels, pancreatic sections of 5-week-old DR*lyp/lyp*, DR+/+, F344, and F344*lyp/lyp* rats were stained with anti-mu-class GST antibodies (FITC). A dual immunofluorescence protocol was employed, using an anti-glucagon antibody (Texas Red) to stain α cells. At least three animals were evaluated for each strain/time point and between 18 and 51 islets per animal

were imaged. Average FITC fluorescence intensity is plotted (mean±S.E.M.). †P<0·05 vs DR+/+, *P<0·05 vs DR*lyp/lyp* (one-way ANOVA).

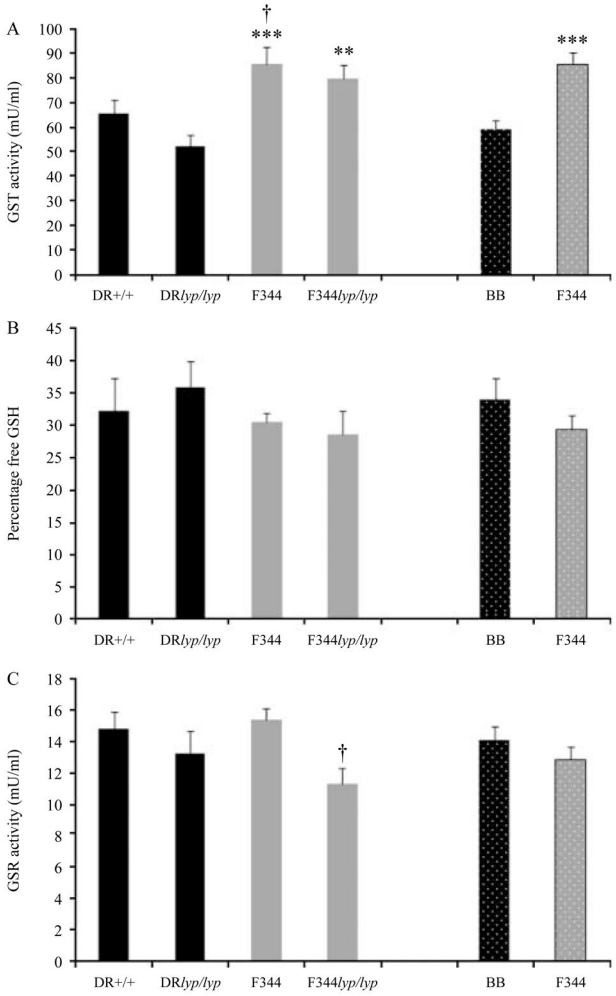

Figure 3.

Plasma GST activity, plasma glutathione levels, and plasma glutathione reductase activity in BB (DR+/+ and DR*lyp/lyp*) vs Fischer (F344 and F344*lyp/lyp*) rats. Plasma of 5-week-old rats for each strain was analyzed (*n*=9–18 rats per strain). (A) Significantly lower GST activity levels were detected in plasma of DR*lyp/lyp* and DR+/+ rats compared with F344 and F344*lyp/lyp* rats. (B) Differences in percentage of free glutathione (available for oxidation) were not significant between strains. (C) Differences in plasma glutathione reductase (GSR) were not detected. Mean values ±S.E.M. are plotted. ***P*<0·01 vs DR*lyp/lyp*, ****P*<0·001 vs DR*lyp/lyp*, †*P*<0·05 vs DR+/+ (Student's *t*-test).

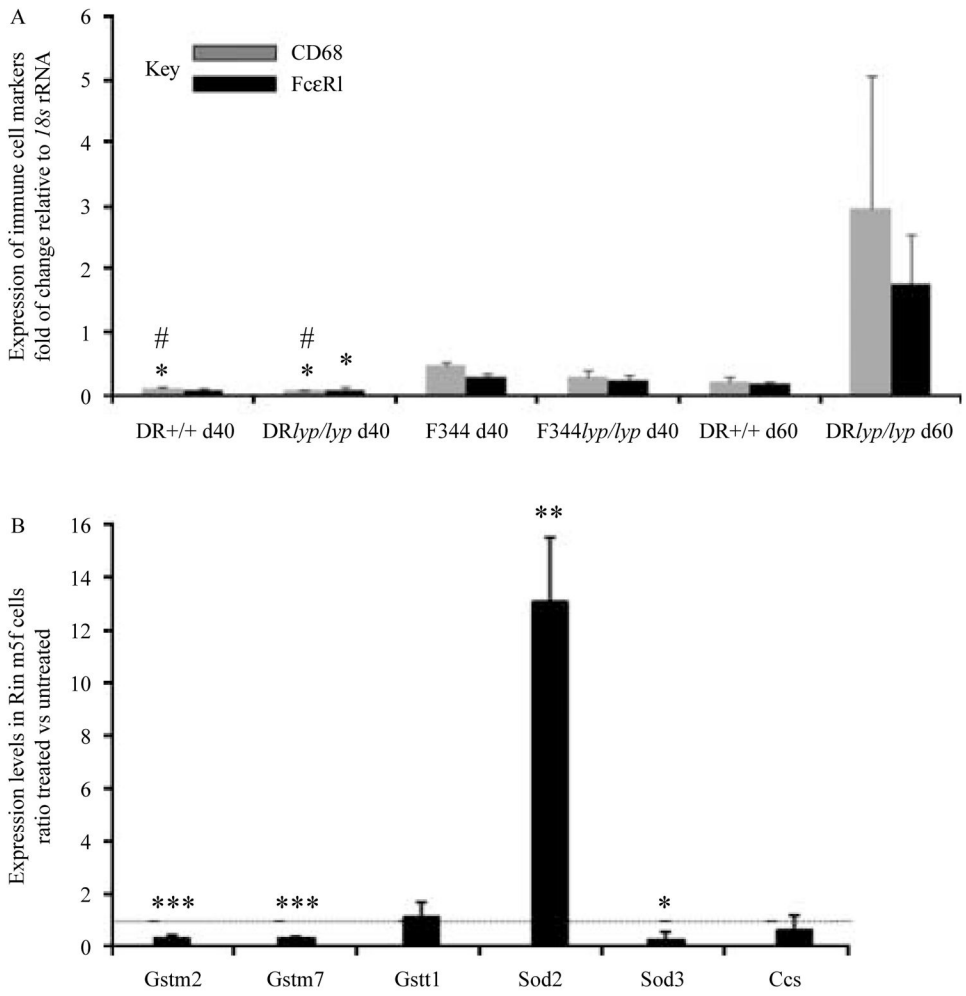

Figure 4.

Influence of inflammatory processes on *Gst* expression. (A) *CD68* and *FcεR1* mRNA expression in BB (DR+/+ and DR*lyp/lyp*) vs Fischer (F344 and F344*lyp/lyp*) rats was examined by quantitative RT-PCR in islets of day 40 rats. A minimum of six animals were examined. Values are expressed as a ratio relative to *18s* rRNA (mean±S.D.), gray=CD68, black=FcεR1. **P*<0·05 vs day 60 DR*lyp/lyp* (positive control), $^{\#}P$<0·05 vs d40 F344 (one-way ANOVA). The variation in CD68 and FcεR1 transcript levels in day 60 DR*lyp/lyp* islets is attributed to varying degrees of insulitis among the individual animals assayed. (B) *Gstm2*, *Gstm7*, *Gstt1*, *Sod2*, *Sod3*, and *Ccs* mRNA expression in RINm5F cells after 24-h treatment with cytokines. The mean values of three independent experiments are expressed as a ratio relative to untreated cultures (mean±S.E.M.), dotted line represents a ratio of 1 (no change), **P*<0·05, ***P*<0·01, ****P*<0·001 relative to untreated (one-way ANOVA).

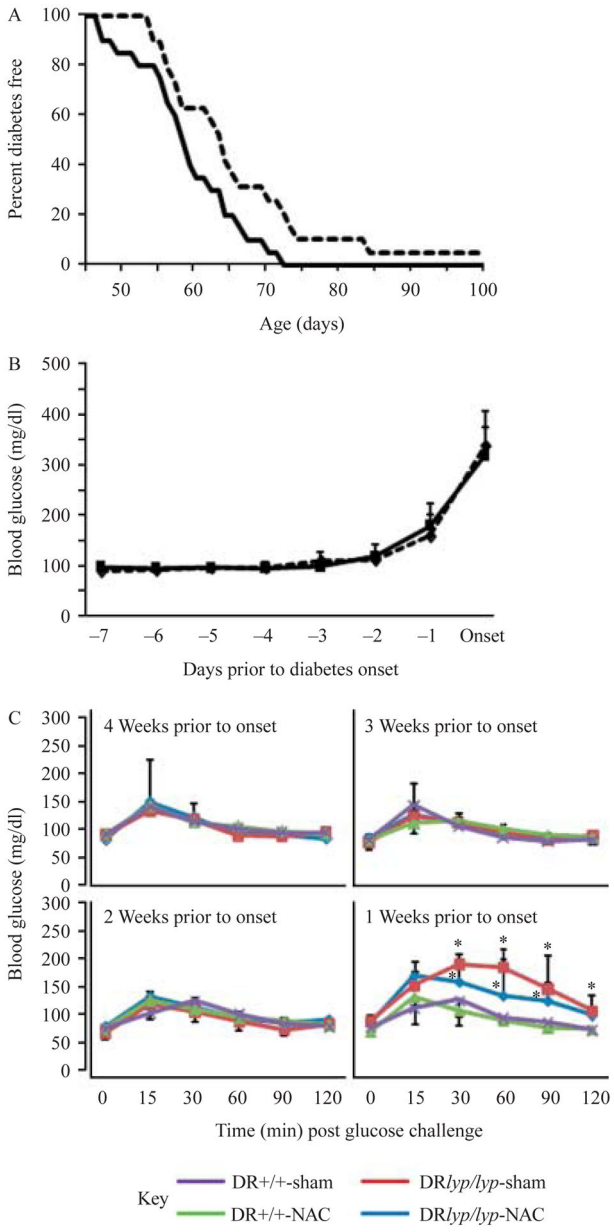

Figure 5.

Treatment of DR*lyp/lyp* rats with NAC. (A) Longitudinal monitoring of DR*lyp/lyp* rats i.p. treated with 200 mg/kg per day NAC (*n*=19, dashed line) or sham-treated controls (*n*=20, solid line). Treatment was initiated at weaning and continued through onset. Dosage was based on previous NAC administration to rats (Tanaka *et al*. 1999) and the fact that higher doses attenuated weight gain in BB rats. NAC-treated rats survived 71±11 days (range 54–230), while sham-treated controls survived 61±6 days (47–72) (*P*=0·033, log-rank test). The sham-treated rats exhibited a similar age of onset compared with completely untreated colony-mates (*n*=202) monitored over the past 5 years (59±6 days, *P*>0·5). At 60 days of age, the percentage of diabetes-free DR*lyp/lyp* rats was 70 and 38% in the NAC- and sham-treated groups respectively and 40 and 8% at 70 days of age. None of the NAC-treated

DR*lyp/lyp* rats developed diabetes before 50 days of age, while 15% of the sham-treated rats did so. (B) Mean daily blood glucose values in NAC-treated (dashed line) and sham-treated (solid line) DR*lyp/lyp* rats during the last week before diabetes onset were not significantly different. (C) Weekly OGTTs were performed on NAC- and sham-treated DR*lyp/lyp* and DR+/+ rats before diabetes onset. Mean values ±S.D. are plotted. Purple line: DR+/+-sham; green line: DR+/+-NAC; red line: DR*lyp/lyp*-sham; blue line: DR*lyp/lyp*-NAC. **P*<0·05 relative to DR+/+ rats (Kruskal–Wallis test).

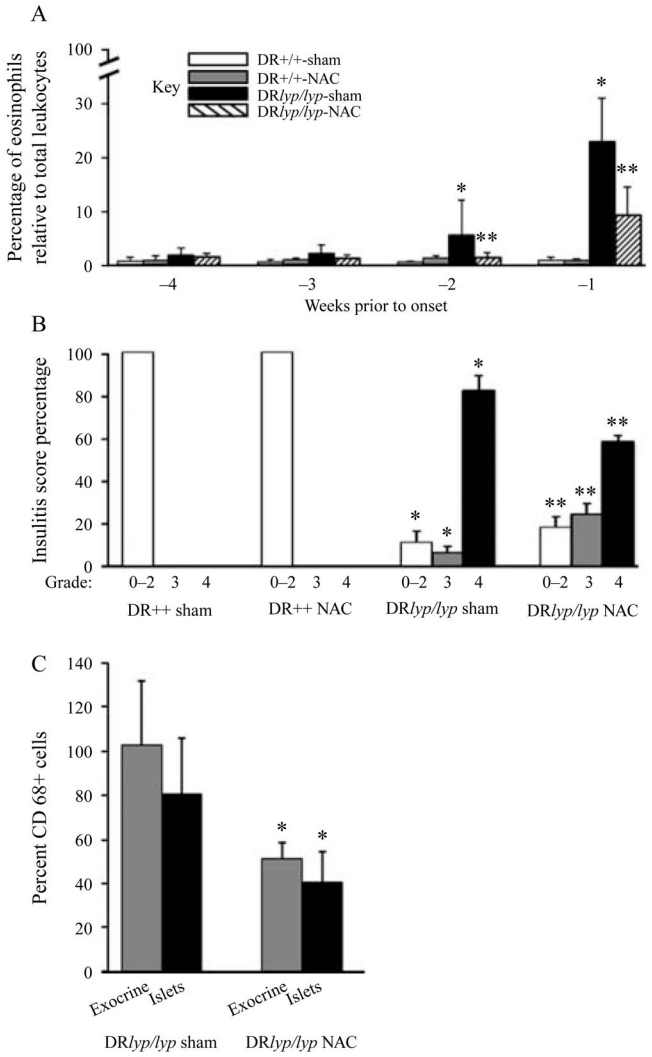

Figure 6.

Effect of NAC on circulating leukocytes and insulitis. (A) Percentage of circulating eosinophils relative to total leukocytes. Results are presented as mean±S.D. of 8–14 per strain/condition. *P<0·05 relative to sham-treated DR+/+ rats, **P<0·05 relative to sham-treated DR*lyp/lyp* rats (Mann–Whitney U test); White column: DR+/+-sham; gray column: DR+/+-NAC; black column: DR*lyp/lyp*-sham; striped column: DR*lyp/lyp*-NAC. (B) Evaluation of mononuclear cell infiltration in and around the pancreatic islets at diabetes onset. Hematoxylin–eosin-stained pancreatic sections of sham- or NAC-treated DR*lyp/lyp* and DR+/+ rats were evaluated at diabetes onset for the severity of the mononuclear cell infiltration (Grades 1–4). Shown are the percentages of islets falling into each category, *P<0·05 relative to age-matched DR+/+ rats, **P<0·05 relative to sham-treated DR*lyp/lyp* rats (Mann–Whitney U test, P<0·05). (C) Evaluation of CD68-positive cell infiltration in exocrine and islet pancreatic tissues of sham- or NAC-treated DR*lyp/lyp* rats at diabetes onset. Shown are the mean (±S.D.) of four to six rats per condition, *P<0·05 relative to sham-treated DR*lyp/lyp* rats (Mann–Whitney U test).

Table 1

Quantitative real-time RT-PCR performance parameters and primer designs

Gene name	UniGene	Slope[a]	R2[b]	E[c]	Primer sequence[d]
Superoxide dismutase copper chaperone (Ccs)	Rn.12311	−3·41	0·999	1·96	5′-GTGATGGGCTCACTATCTGG-3′ 5′-CCCTCTGCAGGAAGATGTT-3′
Superoxide dismutase 2 mitochondrial, mRNA (Sod2)	Rn.10488	−3·45	0·9981	1·95	5′-GGCTTGGCTTCAATAAGG-3′ 5′-GAAGATAGTAAGCGTGCTCC-3′
Superoxide dismutase 3, extracellular (Sod3)	Rn.10358	−3·20	0·9973	2·05	5′-AATGTGCGCAGATAACTCTC-3′ 5′-CAAGGGGAAGACAAACGA-3′
Glutathione S-transferase, mu type 2 (Yb2) (Gstm2)	Rn.625	−3·47	0·9994	1·94	5′-GATTCGTGTGGACGTTTTG-3′ 5′-TCAAAGTCAGGGCTGTAGCA-3′
Glutathione S-transferase mu 7 (Gstm7)	Rn.6036	−3·17	0·9983	2·07	5′-CCCTCGAGCTCATGTGATTG-3′ 5′-CGGTAGGAGCGGGTTACAG-3′
Glutathione S-transferase theta 1 (Gstt1)	Rn.11122	−3·18	0·9979	2·06	5′-CCAGTCTTTGAAGGGCGTCC-3′ 5′-GGGCGCACAGTCGTGTAATG-3′
Cd68 molecule (Cd68)	Rn.12478	−3·56	0·9922	1·91	5′-TGCTTCTCTTGCGCCAGT-3′ 5′-GGGCTGGTAGGTTGATTGTC-3′
Fc fragment of IgE, high affinity I, receptor for; γ polypeptide (FcεR1g)	Rn.201810	−3·38	0·9964	1·97	5′-AGAAACCACCCCAATAGCTT-3′ 5′-GTGGATATGGGGACATTGA-3′

[a] Slope of standard curve.

[b] Linear regression of standard curve.

[c] Reaction efficiency, determined as previously described (Pfaffl 2001).

[d] Top sequence is forward primer, bottom is reverse.

Table 2

Pathway analysis of probe sets differentially regulated by BB vs F344 islets. Genes over-expressed in the BB strains (*n*=2084 probe sets) and those under-expressed in the BB strains (*n*=2545 probe sets) were independently evaluated for biological pathway enrichment (GO molecular function and KEGG) using the DAVID annotation tool. Selected terms that functionally discriminate the islet expression profiles between the BB vs non-BB strains are shown, a complete tabulation is provided in Supplementary Table 2

Annotation category	Term	Count	*P* value
Over-represented in BB (*n*=2084 probe sets)			
GO molecular function	Protein serine/threonine kinase activity	64	$1{\cdot}3\times10^{-8}$
GO molecular function	Transcription regulator activity	125	$6{\cdot}6\times10^{-6}$
GO molecular function	Zinc ion binding	159	$1{\cdot}7\times10^{-5}$
GO molecular function	Transcription repressor activity	41	$2{\cdot}3\times10^{-5}$
KEGG pathway	Wnt signaling	28	$3{\cdot}9\times10^{-5}$
KEGG pathway	MAPK signaling	42	$6{\cdot}1\times10^{-5}$
KEGG pathway	TGFβ signaling	16	$2{\cdot}6\times10^{-3}$
GO molecular function	SMAD binding	11	$2{\cdot}6\times10^{-3}$
GO molecular function	Guanyl-nucleotide exchange factor activity	19	$4{\cdot}5\times10^{-3}$
GO molecular function	GTPase regulator activity	35	$7{\cdot}1\times10^{-3}$
GO molecular function	MAP kinase activity	6	$1{\cdot}9\times10^{-2}$
KEGG pathway	mTOR signaling	9	$5{\cdot}5\times10^{-2}$
Under-represented in BB (*n*=2545 probe sets)			
KEGG pathway	Oxidative phosphorylation	51	$1{\cdot}5\times10^{-10}$
KEGG pathway	Fructose and mannose metabolism	15	$3{\cdot}7\times10^{-4}$
GO molecular function	Intramolecular oxidoreductase activity	15	$4{\cdot}1\times10^{-4}$
KEGG pathway	Glutathione metabolism	17	$1{\cdot}4\times10^{-3}$
KEGG pathway	Proteasome	17	$1{\cdot}4\times10^{-3}$
GO molecular function	NADH dehydrogenase activity	9	$4{\cdot}2\times10^{-3}$
KEGG pathway	Pentose phosphate	10	$9{\cdot}3\times10^{-3}$
GO molecular function	Antioxidant activity	13	$1{\cdot}3\times10^{-2}$
KEGG pathway	Pyruvate metabolism	13	$2{\cdot}2\times10^{-2}$
GO molecular function	Cytochrome *c* oxidase activity	8	$3{\cdot}1\times10^{-2}$
GO molecular function	NADH dehydrogenase (ubiquinone) activity	7	$3{\cdot}2\times10^{-2}$
KEGG pathway	Galactose metabolism	9	$3{\cdot}9\times10^{-2}$

The *P* value defines the significance of the association of a particular biological process with the gene list analyzed.