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Antioxidant enzyme activity and mRNA expression in the islets of Langerhans from the BB/S rat model of type 1 diabetes and an insulin-producing cell line

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Abstract It has been proposed that low activities of antioxidant enzymes in pancreatic beta cells may increase their susceptibility to autoimmune attack. We have therefore used the spontaneously diabetic BB/S rat model of type 1 diabetes to compare islet catalase and superoxide dismutase activities in diabetes-prone and diabetes-resistant animals. In parallel studies, we employed the RINm5F beta cell line as a model system (previously validated) to investigate whether regulation of antioxidant enzyme activity by inflammatory mediators (cytokines, nitric oxide) occurs at the gene or protein expression level. Diabetes-prone rat islets had high insulin content at the age used (58–65 days) but showed increased amounts of DNA damage when subjected to cytokine or hydrogen peroxide treatments. There was clear evidence of oxidative damage in freshly isolated rat islets from diabetes-prone animals and significantly lower catalase and superoxide dismutase activities than in islets from age-matched diabetes-resistant BB/S and control Wistar rats. The mRNA expression of antioxidant enzymes in islets

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from diabetes-prone and diabetes-resistant BB/S rats and in RINm5F cells, treated with a combination of cytokines or a nitric oxide donor, DETA-NO, was analysed semi-quantitatively by real time PCR. The mRNA expression of catalase was lower, whereas MnSOD expression was higher, in diabetes-prone compared to diabetes-resistant BB/S rat islets, suggesting regulation at the level of gene expression as well as of the activities of these enzymes in diabetes. The protein expression of catalase, CuZnSOD and MnSOD was assessed by Western blotting and found to be unchanged in DETA-NO treated cells. Protein ex-

pression of MnSOD was increased by cytokines in RINm5F cells whereas the expression of CuZnSOD was slightly decreased and the level of catalase protein was unchanged. We conclude that there are some changes, mostly upregulation, in protein expression but no decreases in the mRNA expression of catalase, CuZnSOD or MnSOD enzymes in beta cells treated with either cytokines or DETA-NO. The lower antioxidant enzyme activities observed in islets from diabetes-prone BB/S rats could be a factor in the development of disease and in susceptibility to DNA damage in vitro and could reflect islet alterations prior to immune attack or inherent differences in the islets of diabetes-prone animals, but are not likely to result from cytokine or nitric oxide exposure in vivo at that stage.

Keywords Nitric oxide · RINm5F cells · Cytokines · Lightcycler PCR · Insulin · Diabetes

Introduction

It has been proposed that production of free radicals in excess of beta cell antioxidant capacity may contribute to the cytokine-induced cytotoxic processes that lead to type 1 diabetes. Cytokines induce free radical production in animal models of autoimmune diabetes [1, 2, 3], see reviews [4, 5, 6, 7]. An increase in radical production under such conditions may be a particular challenge to pancreatic islet cells as levels of the antioxidant enzymes superoxide dismutase (SOD) or catalase have been found to be low in islets relative to other organs, such as kidney or liver [8, 9]. In Wistar rat islets, compared to liver, these activities were: catalase 1%, CuZnSOD 31% and MnSOD 25%, respectively. Gene expression levels have been found to parallel enzyme activities in rat islets, but catalase expression was not detectable with either Northern or Western blotting [10]. Furthermore, in vivo supplementation of antioxidant capacity with a metalloporphyrin-based SOD mimic [11] and decomposition catalysts of the radical peroxynitrite [12] has been shown to reduce autoimmune diabetes.

Previously reported measurements of antioxidant enzyme activities in the Biobreeding (BB) rat model of type 1 diabetes rat are inconclusive. Catalase and SOD activities are lower in whole pancreas from pre-diabetic male BB diabetes-prone (DP) compared to female DP and BB diabetes-resistant (DR) rats [13]. Activities of islet SOD were found to be lower in pre-diabetic BB DP rats compared to Wistar rats [14]. However, another group found higher SOD activities in whole pancreas from BB DP versus BB DR rats in the pre-diabetic period [15]. Catalase activities in islets from BB rats have not, to our knowledge, been reported. There are no reports about mRNA or protein expression of catalase or SOD in islets from BB DP or BB DR rats. The lack of publications on catalase may be understandable in the context of the work by Tiedge et al. [10] on normal rat islets, showing that catalase expression was not detectable by either Northern

or Western blotting. Our aim in this study was to further clarify the potential contribution of antioxidant enzyme levels to disease onset by investigation of the relative levels of gene expression and activities of SOD and catalase in DP and DR BB rat islets. Free radical or cytokine treatments, used to replicate autoimmune attack of beta cells, are known to have many effects [21, 22] including oxidative damage to DNA as indicated by strand breaks [23, 24] and confirmed by enzymatic detection methods [25]. We have therefore also looked for evidence of endogenous oxidative DNA damage in islets from diabetes-prone and diabetes-resistant animals, and investigated if this can be replicated in islets by treatment with cytokines and hydrogen peroxide in vitro and whether DP rat islets are more susceptible.

Strain differences in BB islet antioxidant enzyme gene expression and activity may be genetically inherent or may arise in response to pathological changes specific to the disease process. Antioxidant enzyme activity is known to be influenced by a number of factors. Insulin treatment of diabetic BB DP rats raised catalase, SOD and glutathione peroxidase activities in whole pancreas compared to BB DR and Wistar rats [16]. Cytokine treatment increases the activity of MnSOD in rat islets [17] and nitric oxide has been shown to inhibit catalase activity in vitro [18, 19]. A potential role for nitric oxide in the pathogenic process in BB rats has been shown by an increase in pancreatic iNOS expression [1] and elevation of urea nitrogen and urinary excretion of nitrate [20] after onset of diabetes. We have shown recently that catalase activity in RIN cells, human islets and rat islets is inhibited by cytokine treatment. Investigation of this effect in RIN cells revealed that the inhibition of catalase activity is nitric oxide-dependent [19]. Here, we have further investigated whether differences in antioxidant capacity arise from effects of cytokines and nitric oxide on levels of gene or protein expression, or enzyme activity.

Materials and methods

Animals

BB/S rats were obtained from the authors' breeding colony maintained at the University of Southampton. The colony consists of two sub-lines: DR animals which have been diabetes-free for more than 17 generations and DP animals which show an 82% incidence of diabetes, with onset at 75–90 days of age [26, 27]. Wistar rats obtained from a commercial breeder (Charles River, Sandwich, UK) were used as strain controls. All animals were housed at a constant temperature (18°C) and humidity (45%) on a 12 h light/dark cycle. They had free access to standard laboratory rat chow and tap water. Diabetes-prone and resistant BB/S animals was previously subjected to timed pancreatic biopsies at days 39, 50, 68, 85, 107; sections of tissues were fixed and immunostained for islet hormones, MHC class 1 and 2 molecules, CD2, CD4, CD8, CD16, Fas, FasL and other antigens [28, 27]. Immune cell infiltration was not usually seen before day 68 and it peaked at day 85.

Rat islets of Langerhans

Islets were isolated by collagenase digestion from age-matched groups of BB/S and Wistar animals at 54–60 days of age [29] and cultured in RPMI 1640 containing 11 mmol/l glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mmol/l L-glutamine and 10% FCS at 37°C in a humidified atmosphere of 95% air/5% CO₂ for 48 h before treatments. Rat islets were treated with IL-1β (140 U/ml), IFN-γ (5 U/ml) and TNFα (53 U/ml) for 24 h. Insulin secretion studies were performed as reported previously using groups of 5 islets [24]. For antioxidant enzyme activity assay, 150–200 islets were transferred with a plastic pipette into Eppendorf tubes (1.5 ml), washed twice in PBS, pelleted at 200 g and phosphate buffer (300 µl, 25 mmol/l) was added. The islet samples were frozen at -70°C, thawed and sonicated for 10 s (probe 3, 50%, Ultrasonic Processor XL, Heat Systems) on ice prior to being assayed for antioxidant enzyme activity.

Cell culture and treatment

RINm5F, radiation-induced rat insulinoma cells, originally from the ATCC (American Type Culture Collection) [30] were grown in RPMI 1640 culture medium containing 11 mmol/l glucose supplemented with 10% FCS, 1% L-glutamine (2 mmol/l), 1% penicillin (100 IU/ml) and 1% streptomycin (100 µg/ml). RINm5F cells were seeded at a density of 4×10⁵ cells/well in 12-well plates. After 24 h, RINm5F cells were cultured in fresh RPMI medium with diethylenetriamine/NO [DETA-NO (100–500 µmol/l)], with a cytokine combination of IL-1β (140 U/ml), IFN-γ (5 U/ml) and TNFα (53 U/ml) or left untreated as control wells. After 24 h, nitrite was determined in the cell media using the modified Griess assay [31] and nitrate was converted to nitrite using nitrate reductase essentially as described elsewhere [32]. For catalase activity assay, cells were trypsinised, washed twice in PBS and spun for 5 min at 4°C, 188 g. PBS was removed and the cell pellet resuspended in 300 µl phosphate buffer (25 mmol/l, pH 7.00). Cell samples were frozen at -70°C, thawed and sonicated as above, on ice, prior to being assayed for catalase activity [33]. Protein content of samples was determined by the Bradford assay [34].

Measurement of enzyme activities

The activity of superoxide dismutase (E.C. 1.15.1.1) was measured by its inhibition of the chemiluminescence of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), which was induced by superoxide anions produced by the action of xanthine oxidase (E.C. 1.1.3.22) on xanthine [35]. By the use of this method interference from other activities in the crude tissue homogenates could be avoided [36]. Portions (25 µl) of tissue homogenates, blanks or appropriate standards were, in duplicate, mixed with 600 µl of a solution consisting of 0.50 mmol/l xanthine, 0.50 mmol/l luminol and 0.1 mmol/l EDTA in a 50 mmol/l carbonate buffer, pH 10.1, in small polystyrene test tubes at room temperature (20°C). The light-emitting reaction was initiated by the addition of 40 µl of a solution of 0.12 g/l xanthine oxidase in carbonate buffer. The chemiluminescence was determined using a LKB-Wallac Luminometer 1250 (LKB-Wallac, Turku, Finland) connected to a potentiometric recorder. The maximum light emission was reached within 1–2 min and remained essentially constant for several minutes. The activity of SOD causing a 50% inhibition of the chemiluminescence was defined as 0.01 unit. This corresponds to 4.2 ng of SOD from bovine erythrocytes. Since the present method is approximately 100 times more sensitive than the original method for SOD determination by McCord and Fridovich [37], this definition would make results obtained by the two methods comparable.

The activity of catalase (E.C. 1.11.1.6) was measured by a sensitive spectrophotometric method [33]. This method utilizes the peroxidatic function of catalase with methanol as the hydrogen donor and the production of formaldehyde is determined with purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) as a chro-

mogen. Samples of tissue homogenates, blanks or formaldehyde standards were incubated in duplicate with 5.9 mol/l methanol and 4.2 mmol/l hydrogen peroxide in a 250 mmol/l phosphate buffer, pH 7.0, for 20 min at room temperature (20°C). After termination of the enzymatic reaction with a 7.8 mol/l potassium hydroxide solution, a second incubation with purpald was performed for 10 min at 20°C. To obtain a coloured compound, the product of the reaction between formaldehyde and purpald was oxidized by potassium periodate. The absorbance was measured at 540 nm.

Western blotting

RINm5F cells were treated for 24 h with a combination of three cytokines (140 U/ml IL-1β, 5 U/ml IFN-γ and 53 U/ml TNFα) or 250 µmol/l DETA-NO and were prepared for electrophoresis and Western blotting as described previously [38]. The protein content was determined [34] and gel lanes were equilibrated. The samples were separated on a 7.5% polyacrylamide gel (SDS-PAGE) and blotted onto a PVDF membrane (pore size 0.45 µm) [38]. The PVDF membrane was blocked with 5% milk protein. Catalase was detected after incubation overnight at 4°C in the primary antibody (1:1000 dilution, polyclonal anti-bovine catalase antibody raised in rabbit; CN Biosciences, Beeston, UK). The incubation with the secondary antibody (1:1000 dilution, goat anti-rabbit IgG-HRP conjugate; Bio-Rad Laboratories, Hemel Hempstead, UK) was for 1 h at room temperature. Primary antibody for CuZnSOD was anti-human erythrocyte, made in sheep (1:1000 dilution) from Calbiochem, Nottingham, UK. Antibody for MnSOD was a generous gift from S. Lortz, Hannover Medical School, Hannover, Germany, and was anti-rat MnSOD made in rabbit by Dr. Kohtaro Asayama (Yamanashi, Japan), 1:3000 dilution. Anti-sheep and anti-rabbit secondary antibodies were used at 1:1000 dilution. Protein standards for each of the antioxidant enzymes were run in respective gels, together with either Rainbow or biotinylated protein molecular weight markers. The proteins were visualised using an enhanced chemiluminescence (ECL) kit from Pierce, Rockford, Ill., USA [38]. The integrated density values (IDV) were determined using 'Alphaease' image analysis software (Alpha Innotech, Cannock, UK).

Comet assay of single cells from islets

Following treatment (cytokine 24 h, or hydrogen peroxide 0.5 mmol/l for 1 h), islets were disaggregated into single cell suspensions using minimal digestion with 1 ml of Accutase solution, incubating for 12–13 min at 37°C. Once a satisfactory separation of islet mass into single cells was obtained, cells were rinsed with RPMI 1640 medium, the cell suspension was centrifuged at 200 g for 5 min and the supernatant was discarded. Dissociation into single cells was carried out gently, in order not to introduce additional DNA strand breakage.

The comet assay was performed by a modified protocol of Singh et al [39] as described previously [25] but using standard microscope slides on which a layer of agarose had been pre-dried. Equal volumes of 1.4% NuSieve (FMC Bioproducts, Rockland, MD, USA) low melting point agarose solution and cell suspension in RPMI 1640 were mixed, 50 µl of the mixture containing approximately 5×10⁴ cells was added on to a coated microscope slide, and a coverslip was added to spread the agarose. Coverslips were removed and the slides were placed in lysis mixture (2.5 mol/l NaCl, 100 mmol/l EDTA-Na₂, 10 mmol/l Tris, 200 mmol/l NaOH, 1% v/v Triton X-100, 10% dimethylsulphoxide, pH 10) at 4°C for at least 1 h. To detect strand breaks (or alkali-labile sites), slides were placed in alkaline buffer (300 mmol/l NaOH, 1 mmol/l EDTA-Na₂, pH >13), incubated for 40 min at 15°C, and subjected to electrophoresis (20 V or 0.8 V/cm, 24 min). To detect EndoIII-sensitive sites, slides were taken from lysis mixture, rinsed three times with enzyme buffer [40 mmol/l HEPES, 100 mmol/l KCl, 0.5 mmol/l EDTA-Na₂, 0.2 mg/ml bovine serum albumin (Fraction V), pH 8], and the surface of the agarose blotted dry. 50 µl of

EndoIII enzyme (gift from Professor Andrew Collins, Rowett Research Institute, Aberdeen, UK), (4 µl/ml of stock extract as supplied), in enzyme buffer was added to the surface of the agarose, a coverslip was applied, and the slide was incubated at 37°C for 30 min. Sites of oxidised pyrimidines are converted to strand breaks by this procedure. The net number of EndoIII-sensitive lesions is seen as the additional strand breaks in a slide treated with EndoIII enzyme over a control slide incubated with buffer and without EndoIII enzyme. The slides were transferred to alkaline buffer, and subjected to immediate electrophoresis (20 V, 24 min), with no unwinding period. Following electrophoresis, the slides were rinsed with Tris-EDTA buffer, pH 7.5, stained with ethidium bromide (20 µg/ml), and scored under a fluorescent microscope with a ×10 objective, using the Casys system (Synoptics, Cambridge, UK). Tail moment was taken as a measure of DNA strand break frequency. At least 50 nuclei on two slides were scored for each treatment.

RNA isolation from islets and cell lines

RNA was isolated from islets and cell lines using modifications of an acid guanidinium thiocyanate-phenol-chloroform extraction method [40]. A minimum of 200 islets were extracted using TriReagent (and the protocol of Sigma-Aldrich). The RNA pellet was resuspended in 15 µl DEPC-treated H₂O at 65°C for 5 min and stored at -70°C. The quality and concentration of the RNA were assessed by the OD 260/280 ratio and only samples with ratios above 1.5 were used in the experiments.

RINm5F cells seeded at 8×10⁵ cells/well in 6-well plates were pre-incubated for 24 h at 37°C then left as controls or treated with a combination of three cytokines (140 U/ml IL-1β, 5 U/ml IFN-γ and 53 U/ml TNFα) or 250 µmol/l DETA-NO. Cytokine treatment time was chosen to test for RINm5F cell expression of antioxidant enzymes corresponding to the time at which biological effects were clearly visible, e.g. known cytokine depression of catalase activity [19]. RNAzol B (1 ml) from AMS Biotechnology (Europe), Abingdon Oxon, UK was added to all wells to lyse and detach the cells and RNA extracted according to the protocol. The RNA pellet was resuspended and handled as above for islets.

cDNA synthesis

RNA was treated with RQ1 RNase free DNase from Promega, Southampton, UK to remove any DNA contamination prior to cDNA synthesis. cDNA was synthesized from the RNA samples using the Reverse IT first strand synthesis kit using random decamers (400 ng/µl) as the primers (ABgene, Epsom, UK). MS2 control RNA (50 ng/µl) and MS2 primers supplied in the kit were used as a positive control. A negative "No RT" control was performed by omitting the reverse transcriptase blend in one cDNA sample to check for any DNA contamination in the RNA samples. One microgram of RNA from islets or 2 µg from cells was used. All incubations were performed in a TouchDown thermal cycler (Hybaid, Ashford, UK). The cDNA samples were stored at -70°C.

Semi-quantitative RT-PCR using the LightCycler system

cDNA was constructed from 2 µg RINm5F RNA or from 1 µg islet RNA as described above. PCR amplifications were performed on a Roche LightCycler using the SYBR Green 1 kit; the products were detected by fluorescence and confirmed by melting points. Gene-specific primers (forward and reverse) were designed by TIB Molbiol, Berlin, Germany: Catalase: 5' CTgTgTgAgAACATTgC-CAACCACC, 5' CCAggCTgTgAggTAACATAAgACT; MnSOD: 5' ATTAACgCgCAGATCATgCAG, 5' TTTCAGATAgTCAGgTC-TgACgTT; CuZnSOD: 5' TTCgAgCAGAAggCAAgCggTgAA, 5' AATCCCAATCACACCACAAGCCAA. The results for mRNA expression in RINm5F cells were related to glucose-6-phosphate

dehydrogenase (G6PDH) and the mRNA expression in islets to β-actin as the housekeeping gene.

The quantities of PCR product relative to those of the housekeeping gene were calculated following the manufacturer's recommended method using the cycle threshold (CT) values (CT represents the PCR cycle at which a significant increase in fluorescence above the baseline is first detected). The mean change in CT was calculated from differences between the CT for the gene of interest and the housekeeping gene for control, treated or experimental groups. Relative quantification values are expressed as 2^{-ΔCT}.

Statistical analysis

Sample data for mRNA were tested for normality using the Shapiro-Wilkes test [41]. Statistical significance analyses were performed using Student's *t*-test or analysis of variance in conjunction with Tukey's and Dunnett's tests on Minitab.

Results

Responses of BB/S DP rat islets to glucose, cytokines and hydrogen peroxide: comparison with BB/S DR and Wistar rat islets

Islets isolated from BB/S DP animals were isolated in significant numbers, they contained and secreted insulin but to a lesser extent than islets from diabetes-resistant BB/S DR or Wistar rats (Table 1) and [27]. Use of the comet assay, combined with enzyme detection of specific types of DNA strand breaks, revealed increased oxidised pyrimidines in islets from DP animals and an increased susceptibility to DNA damage when islets were challenged with hydrogen peroxide or cytokine treatments (Table 1).

Catalase and SOD activities in extracts of islets from pre-diabetic DP and DR BB/S or Wistar rats

To assess antioxidant status in islets from pre-diabetic BB/S DP rats, after onset of insulinitis catalase and SOD activities were measured in extracts of freshly isolated islets. There was 70% (*P*<0.01) lower catalase activity in extracts of freshly isolated islets from pre-diabetic BB/S DP rats versus that of age-matched Wistar rats and 60% (*P*<0.01) lower activity versus age-matched BB/S DR rats (Fig. 1a). The SOD activity was 76% (*P*<0.01) lower in pre-diabetic BB/S DP rat islets compared to that of BB/S DR and 89% (*P*<0.01) lower compared to that of Wistar rat islets (Fig. 1b).

mRNA expression of antioxidant enzymes in islets from pre-diabetic DP and DR BB/S rats

RNA from freshly isolated pre-diabetic BB/S DP and age-matched BB/S DR islets was semi-quantified for the expression of catalase, MnSOD and CuZnSOD by relating the enzyme expression to the expression of the house-

Table 1 Islets were isolated from rat strains as shown. The insulin content of islets and the secretory responsiveness to glucose over 1 h were measured. DNA damage was recorded as 'tail moment', a measure of damaged DNA in the tail of the comet, in the comet assay. Islets were freshly isolated and used for hydrogen peroxide

treatment and EndoIII site determination of oxidized pyrimidines, or precultured and then cultured for 24 h in the case of cytokine treatment. The data are expressed as means±SEMs, shown with the number of observations in parentheses

Rat strain	BB/S DP	BB/S DR	Wistar
Islet isolation per animal	291±8.4 (4)	440±20.3 (4)	458±46 (5)
Insulin (ng/islet)	30.4±2.8 (20)	38.0±4.9 (4)	40.6±2.2 (8)
Insulin secretion (ng/islet/h)			
2 mmol/l	0.3±0.04 (6)	1.1±0.16 (4)	0.5±0.08 (4)
20 mmol/l	1.2±0.13* (6)	4.0±0.49* (4)	3.4±0.139* (4)
DNA damage untreated	0.18±0.06 (3)	–	0.19±0.09 (8)
DNA damage IL-1β	1.87±0.03 (3)	–	0.41±0.09 (8)
DNA damage 3 cyt	3.03±0.03* (3)	–	1.76±0.06*** (8)
DNA damage control	0.38±0.04 (3)	0.10±0.01** (3)	–
H ₂ O ₂ treated	0.85±0.09* (3)	0.40±0.07*** (3)	–
DNA damage EndoIII sites	0.77±0.07 (3)	0.27±0.08** (3)	–

* $P<0.01$, for treatment versus control effects

** $P<0.01$, for differences from islets from DP animals

Table 2 Nitrite and nitrate in serum in BB/S diabetes-prone, BB/S diabetes-resistant and Wistar rats. Serum was extracted from pre-diabetic BB/S DP (50–60 days old) and age-matched BB/S DR or Wistar rats. Nitrite and nitrate was measured. Values are expressed as mean±SEM, $n=3$. Statistical analysis was performed using

Student's t -test. There was significantly higher nitrite in serum from BB/S DP versus BB/S DR rats, and significantly less nitrite in BB/S DR versus Wistar rat serum. There was more nitrate, and combined nitrate and nitrite, in serum from BB/S DP versus Wistar but not versus BB/S DR rats

Rat strain	Wistar	BB/S DR	BB/S DP
Nitrite ($\mu\text{mol/l}$)	9.0±0.5 (3)	6.5±0.68* (4)	15.2±2.30* (4)
Nitrate ($\mu\text{mol/l}$)	29.8±6.0 (3)	56.1±12.3 (4)	49.1±2.00* (4)
Total nitrite and nitrate ($\mu\text{mol/l}$)	38.8±5.90 (3)	62.6±12.40 (4)	64.3±3.81* (4)

* $P<0.05$

keeping gene β -actin (Fig. 2). There was significantly lower mRNA expression of catalase (74%) ($P<0.05$) and of CuZnSOD (90%) ($P<0.05$) in BB/S DP versus BB/S DR islets, but no significant difference in the mRNA expression of MnSOD between the two groups. There are no reports of β -actin being affected by cytokine or nitric oxide treatment.

Nitrite and nitrate concentration in serum from DP and DR BB/S or Wistar rats

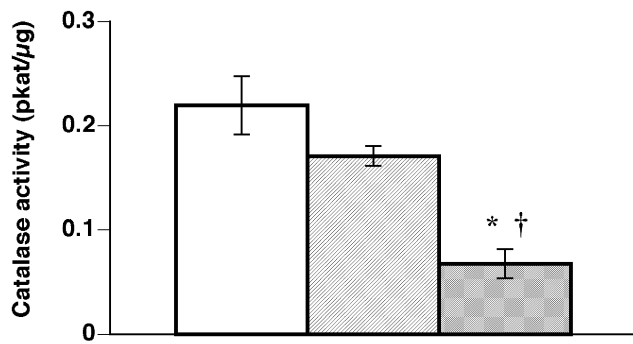
Nitrite and nitrate were measured in serum from pre-diabetic BB/S DP and age-matched BB/S DR and Wistar rats as evidence of possible iNOS activity in the whole animal. There was significantly more ($P<0.05$) nitrite in the serum from BB/S DP rats compared to that of BB/S DR rats and significantly less ($P<0.05$) nitrite in BB/S DR rat serum compared to that of Wistar rats. There was significantly more nitrate ($P<0.05$) and combined nitrate and nitrite ($P<0.05$) in serum from BB/S DP versus Wistar rats but no difference in nitrate or nitrate plus nitrite in serum from BB/S DP versus that of BB/S DR rats (Table 2).

mRNA expression of antioxidant enzymes in RINm5F cells after cytokine or DETA-NO treatment compared to untreated cells

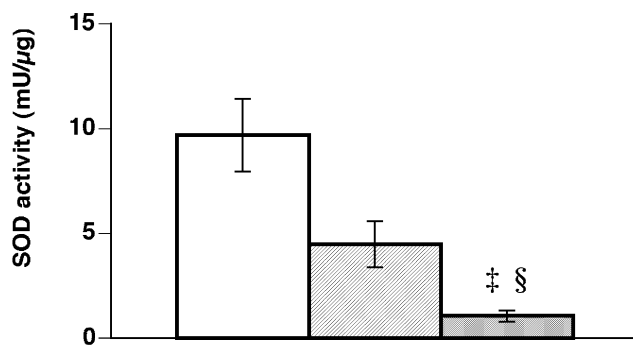
Expression of MnSOD mRNA was significantly increased ($P<0.01$) after treatment of RINm5F cells with a combination of cytokines compared to untreated RINm5F cells, but there were no significant differences in the mRNA expression of catalase or CuZnSOD (Fig. 3a) [though the catalase value is observed to be borderline significantly increased ($P<0.05$) by ANOVA]. Interestingly, there was clearly no difference in the mRNA expression of MnSOD, catalase or CuZnSOD after treatment with DETA-NO (Fig. 4a). There is no difference in G6PDH in islets treated with IL-1β or a combination of IL-1β and IFN-γ for 6 h or 24 h, (personal communication, A.K. Andersson, Uppsala). In our experiments there is no significant difference in G6PDH expression between control 30.9±0.33 and DETA-NO treated cells 31.4±0.5; $n=3$.

Protein expression of catalase, CuZnSOD and MnSOD in RINm5F cells after treatment with cytokines or DETA-NO

The expression of all three proteins was measured by Western blotting in RINm5F cells treated with either a combination of IL-1β, IFN-γ and TNFα or DETA-NO.



a)	Wistar	BB DR	BB DP
Age (days):	54	60	56
n:	9	4	6



b)	Wistar	BB DR	BB DP
Age (days):	85	61	52
n:	4	7	8

Fig. 1 Catalase and superoxide dismutase activity in islet extracts of DP and DR BB/S or Wistar rat islets. Freshly isolated islets were assayed for catalase and for superoxide dismutase activity. Values are expressed in units of activity per microgram of islet protein (mean±SEM). Statistical analysis used one-way ANOVA. There is significantly lower catalase activity in BB/S DP versus BB/S DR islets, * $P<0.01$; and versus Wistar islets, † $P<0.01$. There is significantly lower superoxide dismutase activity in BB/S DP versus BB/S DR islets, ‡ $P<0.01$ and versus Wistar islets, § $P<0.01$.

There were no significant differences in the protein expression of catalase in RINm5F cells after 24 h treatment with a combination of cytokines. IDVs for the protein bands $\times 10^{-3}$ are as follows: cytokine treated 12.9 ± 1.6 versus control 12.8 ± 1.2 (Fig. 3b) or after treatment with 250 $\mu\text{mol/l}$ DETA-NO 16.1 ± 2.5 , $n=3$ (Fig. 4b). Expression of MnSOD was increased by cytokines (184 ± 4 versus 163 ± 3 IDV $\times 10^{-3}$) (Fig. 3b), and this increase was nitric oxide independent (control 290 ± 65 , DETA-NO 285 ± 66 IDV $\times 10^{-3}$) $n=4$ (Fig. 4b). Expression of CuZnSOD was slightly decreased following cytokine treatment (224 ± 25 versus control 280 ± 36 IDV $\times 10^{-3}$,

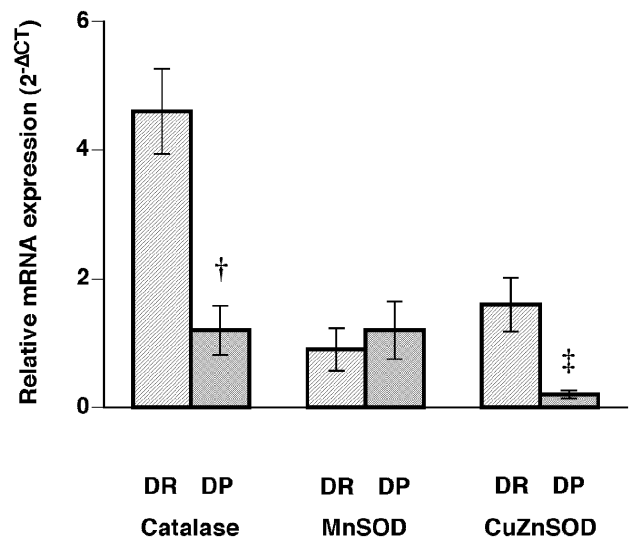
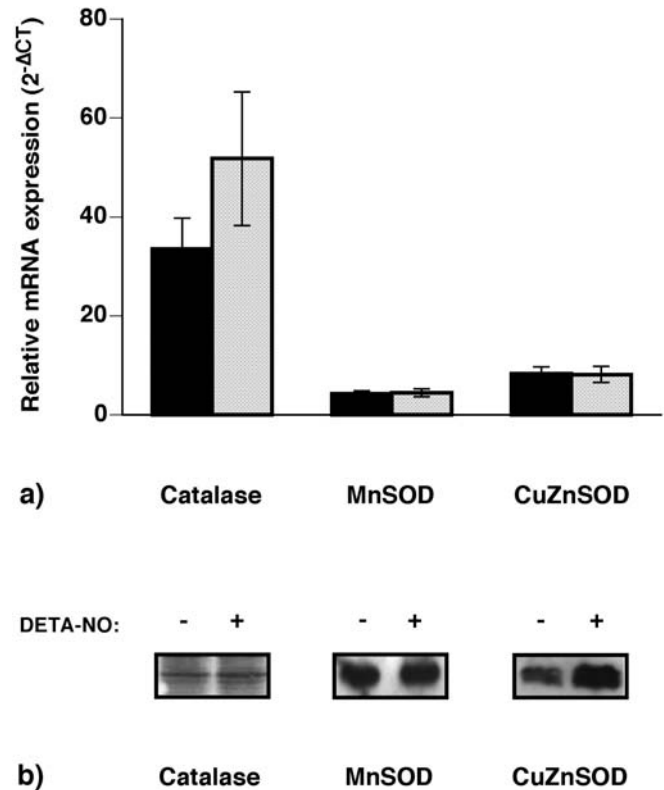
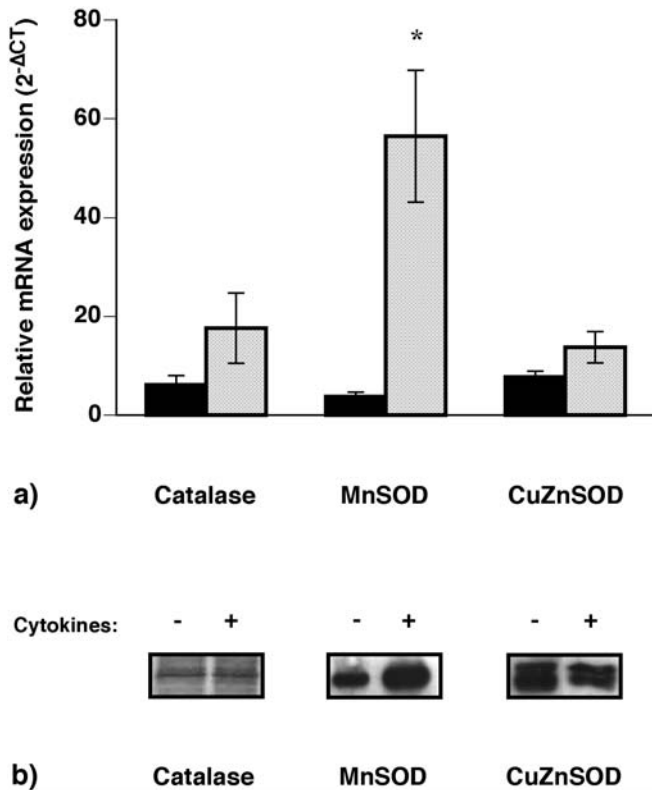


Fig. 2 mRNA expression of antioxidant enzymes related to β -actin in islets from pre-diabetic DP versus DR BB/S rats. Semi-quantitative mRNA expression of the antioxidant enzymes catalase, MnSOD and CuZnSOD was related to the housekeeping gene β -actin in islets from BB/S DR and BB/S DP rats. RNA was extracted and cDNA was synthesised from 1 μg RNA. The cDNA was amplified and quantified using the LightCycler System. The mean change in cycle threshold (CT) was calculated from differences between the CT for gene of interest and housekeeping gene. Data are expressed as mean±SEM. The mRNA expression of catalase related to β -actin was significantly lower in islets from BB/S DP ($n=4$) versus BB/S DR rats ($n=3$), † $P<0.05$. The mRNA expression of CuZnSOD was also significantly lower in islets from BB/S DP ($n=4$) compared to BB/S DR ($n=3$) rats, ‡ $P<0.05$. There were no significant differences in the mRNA expression of MnSOD between BB/S DP and BB/S DR islets

$P<0.05$ by ANOVA, $n=4$) (Fig. 3b) but not DETA-NO treatment (control 347 ± 10.8 , DETA-NO 368 ± 12.7 IDV $\times 10^{-3}$, $n=4$) (Fig. 4b).

Discussion

Here we have shown that freshly isolated islets from pre-diabetic BB/S DP rats have significantly less catalase and SOD activities than those of BB/S DR and Wistar rats. Our results are consistent with previous studies showing that SOD activity was lower in islets from BB DP versus Wistar rats [14] and that catalase activity was lower in whole pancreas from male BB DP versus BB DR rats [13]. Slight contamination of islet preparations with acinar tissue is possible, but may not affect the results since the activities of antioxidant enzymes in pancreatic tissue have been reported to be representative of the activity in islets in NOD mice [42]. The erythrocyte content of islet homogenates has been found to be negligible [33]. We do not have any information on catalase and SOD enzyme activity in lymphocytes or macrophages and the latter may be lost from the BB/S DP islet periphery upon isolation [27]. We have observed that islets from diabetic BB/S rats which spontaneously



recovered to normoglycaemia after insulin therapy contained higher catalase activity compared to Wistar rats (unpublished observations, Sigfrid, Cunningham, Bone and Green). This supports a role for antioxidant enzymes in the ability of beta cells to recover from an autoimmune attack.

Lower antioxidant enzyme activities seen in islets from BB/S DP rats compared to BB/S DR and Wistar rat islets could be linked to insufficient insulin as was reported in one BB study [16]. BB DP rats have been shown to lose pancreatic insulin content both at the onset of insulinitis [43, 44] and after development of diabetes [27, 45]. There are several reports of an impaired insulin secretory response to glucose in islets [46] and perfused pancreas

[47, 48] from BB DP rats. This contrasts with one report which shows an increase in insulin secretion after glucose stimulation of perfused islets from BB DP compared to BB control and Wistar rats [45]. In related studies, the smaller islets from pre-diabetic BB DP rats secreted significantly less insulin per islet in response to both 2 mmol/l and 20 mmol/l glucose [49] and had significantly lower insulin content per islet compared to islets from Wistar and BB DR rats; though, importantly, when values for secretion or insulin content were related to islet protein the differences between Wistar and BB/S DP were removed; granulation was the same [46]. We therefore think it is unlikely that the lower antioxidant enzyme activities seen in islets from BB/S DP rats compared to BB/S DR and Wistar rat islets are linked to insufficient insulin at this pre-diabetic stage.

The mRNA expression of catalase and CuZnSOD was also lower in freshly isolated islets from pre-diabetic

BB/S DP islets compared to islets from age-matched BB/S DR rats. There were no differences in the mRNA expression of MnSOD between BB/S DP and BB/S DR islets. We wanted to investigate the causes underlying the lower activity and expression of some of the antioxidant enzymes in DP animals, and whether in particular this difference was due to pro-inflammatory events within the islets during insulinitis.

Just prior to and at diabetes onset macrophages, activated T-cells and NK cells have been detected in the endocrine and exocrine pancreas of BB/OK [50, 51], BB/E [52] and BB/S rats [27]. Macrophages secrete cytokines such as IL-1 β and TNF α [4, 53, 54, 55, 56]. There is evidence of increased cytokine concentrations in the BB DP rat, e.g. an increased expression of IFN- γ and IL-10 in the pancreas [57]. Increased expression of IL-1 β [58], IFN- γ and IL-12 [59] was found in islets as well as increased expression of IFN- γ and IL-2 in islet-infiltrating mononuclear leukocytes of BB DP rats [60]. Islet graft survival may also depend on cytokine and free radical activities. Transplanted islet protein expression [61] and graft function [62, 63] have been studied; improvement in graft survival may be achieved by some [63] but not all antioxidant enzyme treatment [64].

Cytokines, such as IL-1 β and IFN- γ are known to induce iNOS [65, 66] and cause increased production of nitric oxide [21] and other reactive oxygen species [4] in insulin-producing cells. There are reports of increased iNOS mRNA [1, 57] and increased staining with iNOS antibodies [1] in the pancreas of BB DP (Mollegard, Ottawa, Edinburgh) rats after onset of insulinitis, but not in BB DR or Wistar rat pancreas. There is also evidence of increased nitric oxide production in vivo at or after onset of diabetes in BB rats [20, 67]. Urea nitrogen was found to be doubled at onset of diabetes [67] and urinary excretion of nitrate was enhanced by 150–200% after onset of diabetes [20]. These published results are corroborated here by the findings that serum from pre-diabetic BB/S DP rats had a higher nitrite concentration compared to serum from age-matched Wistar and BB/S DR rats. BB/S DP rats also had a higher nitrate concentration compared to Wistar rats but not compared to BB/S DR rats. Serum nitrite and nitrate are not expected to precisely reflect nitric oxide production in the vicinity of the pancreatic islet. Nitrite has a short half-life in blood after extraction and is rapidly converted to nitrate [68]. All the rat blood samples were treated in the same way during and after extraction and the assumption was made that the conversion rate to nitrate would be the same in the different sera. Nitrite is reported to be a better measurement than nitrate of nitric oxide produced in vivo since nitrate is affected by many factors such as inhaled nitric oxide, rate of nitrate consumption and excretion, pH and the biochemical microenvironment [68].

Inhibition of catalase activity [19] can be added to the list of processes affected by increased nitric oxide production [69], see reviews [70, 71]. Nitric oxide has been shown to inhibit catalase activity in vitro [18, 72] as well as in cell lines [73] and catalase has been found to

break down nitric oxide to oxygen in the presence of hydrogen peroxide in vitro [18]. Increases in cytokines and nitric oxide in the pancreas of BB/S DP rats could inhibit catalase activity in BB/S DP islets as was seen in RINm5F cells, Wistar rat and human islets [19]. However, we found that RINm5F cells treated with cytokines or the specific nitric oxide-donor DETA-NO for 24 h showed no significant changes in mRNA expression of CuZnSOD and a marginal increase in expression of catalase. The latter is interesting in the context of proteomics data showing de novo synthesis of catalase to be upregulated following IL-1 β treatment of islets from BB DP rats [61]. If extrapolated to the BB/S DP rat islet experiments this would suggest that cytokines or cytokine-induced NO are unlikely to have brought about decreases in expression seen in BB/S DP islets. The low catalase mRNA expression seen in BB/S DP islets compared to BB/S DR islets might therefore be an inherent difference. Another possibility is that the mRNA expression is inhibited by prolonged exposure to reactive oxygen species among other factors [74, 75]. CuZnSOD has been shown to be inhibited by prolonged exposure to hydrogen peroxide and catalase by exposure to superoxide [76]. There is evidence of increased formation of reactive oxygen species in type 1 diabetes mellitus [3, 55] and cytokine treatment of islet cells in vitro has been shown to result in generation of both reactive oxygen species [77] and nitric oxide [21].

IL-1 β has been shown to increase MnSOD but not CuZnSOD activity in islets, which may be through a direct action mediated by an increase in gene expression [17, 78]. IL-1 β has been reported to increase the activity and the mRNA expression of MnSOD in RINm5F cells [17, 79] and in primary rat beta cells [80]. The action of IL-1 β in increasing MnSOD protein levels in rat beta cells was largely nitric oxide independent, and consistent with this, a low dose of a nitric oxide donor had no effect on MnSOD levels [81]. Our observation that cytokines, but not nitric oxide, treatment increased expression of MnSOD in RINm5F cells suggests that the higher MnSOD mRNA expression seen in BB/S DP compared to BB/S DR islets may be mediated by a cytokine-induced production of reactive oxygen species or a direct effect of inflammatory cytokines in the pre-diabetic state.

In conclusion, cytokines induced MnSOD mRNA expression in RINm5F cells, an effect likely to be mediated by a direct action of the cytokines or by cytokine derived reactive oxygen species, but not by nitric oxide since treatment with the NO donor DETA-NO failed to induce the same effect. There were no differences in mRNA expression of catalase or CuZnSOD after cytokine or nitric oxide treatment of RINm5F cells. Islets from pre-diabetic BB/S DP rats showed a lower catalase and CuZnSOD mRNA expression compared to islets from age-matched BB/S DR rats, which is not likely to be mediated by nitric oxide but might be an inherent difference between the two groups. The low expression of the antioxidant enzymes in BB/S DP rat islets compared to BB/S DR islets might make BB/S DP islets more

susceptible to an autoimmune attack since the islets were more susceptible to cytokine or hydrogen peroxide induced DNA damage.

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