Changes in Pancreatic Glutathione Peroxidase and Superoxide Dismutase Activities in the Prediabetic Diabetes-Prone BB Rat (43808)

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Abstract. **Pancreatic superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) activities were measured during the development of diabetes in diabetes-prone BB rats (BBdp) prior to insulin dependence. The pancreata from seven to eight BBdp rats of each sex were examined at ages 5, 7, 10, and 18 weeks and compared with age-matched control BB rats (BBc). At Week 18, BBdp rats had moderate to high insulitis but normal levels of blood glucose and insulin. Pancreatic CuZnSOD activity in BBdp rats was two times higher than the activity seen in BBc rats at age 5–10 weeks but then declined to the same level as seen in BBc rats at 18 weeks of age. MnSOD activity increased over time in the BBdp rats but remained very low in BBc rats. These changes in CuZnSOD and MnSOD activity resulted in BBdp rats having twice the pancreatic total SOD activity compared with BBc rats ($P < 0.0001$). Total GSHPx activity was significantly reduced in the pancreata from both male and female BBdp rats compared with their respective controls ($P < 0.01$ and $P < 0.0001$, respectively). The lower total GSHPx activity was due to reduced selenium-dependent GSHPx (SeGSHPx) activity. Erythrocyte and plasma activity of these enzymes was not different between rats with or without insulitis, indicating that differences in enzyme activities were confined to the pancreas. Thus, changes in pancreatic antioxidant enzyme activities occur prior to the development of diabetes symptoms in BBdp rats and may be related to the destruction of the pancreatic B cells and ultimate development of diabetes.** [P.S.E.B.M. 1994, Vol 207]

Superoxide dismutase (SOD, superoxide oxidoreductase, EC 1.15.1.1), along with glutathione peroxidase (GSHPx, glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) and catalase (EC 1.11.1.6), form the primary intracellular defenses against toxic, reactive oxygen metabolites (1). SOD functions to remove superoxide radicals by reducing them to hydrogen peroxide, which is then removed by GSHPx or catalase. SeGSHPx requires selenium (Se) for activity and varies with dietary intakes of Se (2), although there is also peroxidase activity that is independent of Se, referred to as non-Se-dependent GSHPx (non-SeGSHPx). There are two forms of SOD: a mitochondrial enzyme which requires manganese for activity (MnSOD) and another containing copper and zinc (CuZnSOD) which is located in the cytosol. CuZnSOD activity is dependent on copper for activity and blood and tissue activity reflect dietary intakes of copper (3). SOD activity is also found in plasma in the form of extracellular SOD (EC-SOD), enzymatically similar to CuZnSOD, but with a higher molecular weight due to attached carbohydrate (1).

Pancreatic tissue has been reported to contain relatively little activity of the protective enzymes SOD and GSHPx compared with other tissues, and this has been suggested to be a factor in the development of

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insulin-dependent diabetes mellitus (IDDM) (4, 5). The metabolism of the diabetogenic agents streptozotocin (STZ) and alloxan both result in the production of toxic oxygen reduction produces which damage islet B-cells. The reason for the high susceptibility of B-cells to these agents is unknown but has been suggested to be due to the low activity of antioxidant protective enzymes. STZ treatment has been shown to decrease pancreatic CuZnSOD (6) and GSHPx activities (7), and the induction of diabetes has been prevented by exogenous administration of SOD, catalase, or hydroxyl radical scavengers in STZ-induced (8, 9) and alloxan-induced diabetes (10, 11). However, failure of CuZnSOD to protect against STZ-induced diabetes has also been reported (12).

In Type I diabetes, the destruction of the pancreatic B-cells is associated with an autoimmune process resulting from infiltration of inflammatory cells into the islets, a process termed "insulitis." The spontaneously diabetic BB rat is an excellent animal model of insulin-dependent diabetes mellitus (IDDM) and cell mediated autoimmunity (13). Activated inflammatory cells such as polymorphonucleated neutrophils and macrophages produce large quantities of reactive oxygen species such as $O_2\cdot^-$, $OH\cdot$, $H_2O_2\cdot^-$ and $NO\cdot$ which are normally used to destroy invading pathogens but during autoimmune disease, inflict damage on the host organism (14, 15). In the diabetes-prone BB rat (BBdp), the insulitis is characterized by a widespread lymphocytic infiltration of the pancreatic islets which can be histologically quantitated. This destructive process is initiated prior to overt diabetes symptoms and is in evidence during the asymptomatic period in BBdp rats with normal levels of blood glucose and insulin. Thus with insulitis, the pancreas of the BB rat is subjected to an environment with high levels of free radicals and reactive oxygen species.

The purpose of the present study was to determine if the increased susceptibility of the BB rat to diabetes is related to a deficit in the activity of protective enzymes in the pancreas. The endogenous activities of SOD (both the cytosolic CuZnSOD and the mitochondrial MnSOD) and GSHPx (SeGSHPx and non-SeGSHPx) were determined in the pancreata from diabetes-prone BB rats (BBdp) and compared with that from control BB rats (BBc) derived from the same colony. Enzyme activities were determined at various time intervals prior to insulin-dependence, during the development of insulitis, the earliest symptom of diabetes. The activities of SOD and GSHPx were also examined in erythrocytes and plasma of BBdp rats to determine if the presence or absence of insulitis had any effect on blood enzyme activity, which would distinguish an overall change in antioxidant defenses or trace element status from changes that were confined to the pancreas.

Materials and Methods

Animals. Spontaneously diabetes-prone BB (BBdp) and control BB (BBc) rats were obtained from the colony maintained at the Health Protection Branch, Health Canada (Ottawa, Ontario) and fed a standardized rat diet (NIH-07), containing the American Institute of Nutrition (AIN-76A) recommended amounts of vitamins and minerals. The composition of this diet has been described elsewhere (16) and contained the following levels of minerals: Se, 0.1; Cu, 6; and Mn, 54 mg/kg diet. Seven to eight rats of each sex were sacrificed at 32, 47, 74, and 124 ± 3 days of age, along with an equal number of age-matched control BB rats. Pancreata were removed, and one half was examined histologically for insulitis and the other half used for determination of enzyme activities and protein. In the second study, 41 BBdp animals (22 males and 19 females) were sacrificed at 18 weeks of age, and a blood sample and pancreata were removed. Pancreata were examined for insulitis and blood (erythrocytes and plasma) analyzed for antioxidant enzyme activities.

Pancreas Preparation. The pancreas was removed, dissected free of extraneous fat and lymph tissue and divided into two parts: one for light microscopic examination for insulitis according to methods described by Hoorfar *et al.* (16), and the other half was stored at −70°C until used for enzyme determinations. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless indicated otherwise, and all reagents and samples were maintained at 4°C throughout the preparative steps. On the day of analysis, tissues were thawed at 4°C and homogenized in 10 volumes of cold Triton X100 (0.2%, v/v). A portion of the homogenate was used for the determination of total SOD, SeGSHPx, non-SeGSHPx, total GSHPx activities, and total protein. Another portion of the homogenate was extracted with 0.4 × volumes of a mixture of chloroform/ethanol (15/25, v/v) and used for the determination of CuZnSOD.

Blood Preparation. Rats were fasted overnight and a 75 μl blood sample was removed from the tail vein into a microhematocrit tube containing ammonium heparin (Travenol Lab Inc., McGaw Park, IL). Tubes were centrifuged at 13,700*g* at 4°C, and the plasma and erythrocyte portions were used for the determination of GSHPx and SOD activities. The erythrocyte portion was washed with isotonic saline (0.9%), lysed with 1.0 ml of demineralized water and used for erythrocyte GSHPx and hemoglobin determinations. A portion of the erythrocyte lysate was extracted with 0.4 × volumes of a mixture of chloroform/ethanol (15/25, v/v) and used for the determination of CuZnSOD. The portion of tube containing plasma was washed out with 400 μl of saline and used for determination of plasma enzyme activities and protein.

Biochemical Methods. SOD activity was measured using an automated modification of the xanthine-xanthine oxidase cytochrome c method described previously (17) using an Abbott-VP discrete analyzer (Abbott Laboratories, Mississauga, Ontario). The assay cuvette contained 250 μl of a fresh mixture of: 20 m*M* potassium phosphate buffer (pH 7.8), 0.1 m*M* EDTA, 0.2 μ*M* ferricytochrome c, 50 μ*M* xanthine, and 10 μ*M* fresh potassium cyanide. The reaction was initiated with sufficient xanthine oxidase to give a reaction rate, in the absence of sample, of 0.025 A/min at 30°C using a 550/650 filter. SOD activity was determined using 2.5 μl of sample. Total SOD was determined on the tissue homogenate, CuZnSOD on the chloroform/ethanol extracts, and MnSOD determined by difference. One unit of SOD activity is defined as that amount of enzyme which causes a 50% inhibition of the reaction, under these standardized conditions. SOD activity was expressed per mg protein or per mg hemoglobin.

GSHPx activity was determined by an automated modification of the coupled assay of Paglia and Valentine (18). The assay mixture was prepared fresh daily and contained: 150 m*M* potassium phosphate buffer (pH 7.0), 5 m*M* EDTA (disodium salt), 0.5 m*M* sodium azide, 2 m*M* glutathione (reduced, crystalline free acid), 0.24 m*M* NADPH, and 1 U/ml glutathione reductase. Reactions were carried out at 37°C using an Abbott-VP discrete analyzer with a 340/380 nm filter. The sample cuvette contained 250 μl of assay mixture and 1.25 μl of homogenate. The reaction was initiated by the addition of substrate. SeGSHPx activity was determined using 0.3 m*M* t-butylperoxide as substrate, as blank activity was significantly lower than with hydrogen peroxide. Total GSHPx activity was determined using 1.2 m*M* t-butylperoxide as substrate and non-SeGSHPx determined by difference. One unit of activity catalyzes the oxidation of 1.0 μmol of reduced NADPH per min and was expressed per mg protein or mg hemoglobin.

Hemoglobin was determined on the erythrocyte lysate using an automated cyanomethemoglobin assay (19) and total protein in plasma and pancreas homogenates by a dye binding method (A-Gent kits, Abbott Laboratories, Mississauga, Ontario).

Statistics. All results are reported as means ± SEM. Pancreatic enzyme activities were examined using analysis of variance to determine significant effects due to diabetes, sex and time; and blood activities for insulitis and sex effects. Where no significant differences between males and females were seen, data were pooled for both sexes. Significant differences between means at the same time point were determined by the least significant difference method at the $P < 0.05$ level. All statistical procedures were done using the CSS/3 statistical software package (StatSoft, Tulsa, OK).

Results

On microscopic examination of the pancreata from both male and female BBc and BBdp rats, no evidence of insulitis was seen in the BBc rats or in the BBdp rats during Weeks 5–10. At Week 18, the BBdp animals had a moderate to high degree of insulitis (grade 3–4 on a scale of 4).

The changes in SOD activity in pancreata obtained from both BBdp and age-matched control BBc rats are shown in Figure 1. As there were no significant differences in pancreas SOD activity between male and female rats, data were pooled for both sexes. CuZnSOD activity was much higher in the pancreas of the BBdp rats compared to BBc rats during Weeks 5–10; however, by Week 18, the activity in the BBdp rats had declined to that seen in the BBc rats. MnSOD activity

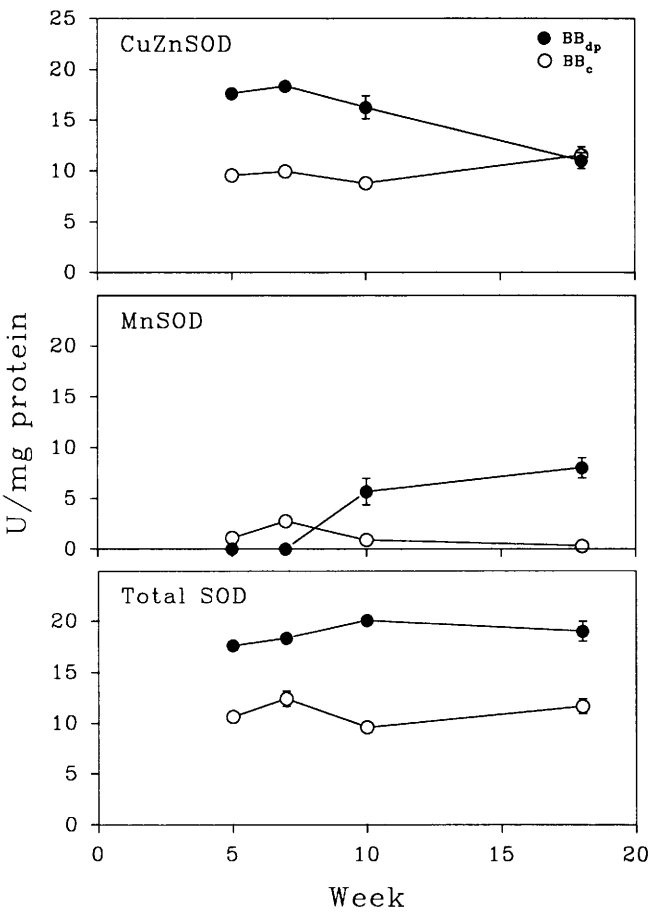

Figure 1. Changes over time in Cu,Zn-superoxide dismutase (CuZnSOD, top), Mn-superoxide dismutase (MnSOD, center), and total superoxide dismutase (total SOD, lower) activities (U/mg protein) in the pancreata of prediabetic diabetes-prone BB rats (BBdp) and age-matched control BB rats (BBc) derived from the same colony. At Week 18, BBdp rats had grade 3–4 insulitis, on a scale of 1–4; no insulitis was evident during Weeks 5–10 or in BBc rats.

was almost negligible in the pancreata of BBc rats throughout the study, while the activity was significantly elevated in the BBdp rats during Weeks 10–18 compared with the BBc rats ($P < 0.01$). Together, these changes were reflected in total SOD that was 150%–210% higher in the pancreata from BBdp rats compared with the BBc rats ($P < 0.0001$). It is noteworthy that in the BBc rats, both forms of SOD activity remained constant throughout the prediabetic period, while in the BBdp rats (with higher total SOD activity), there was a dramatic increase in MnSOD activity (11-fold), as CuZnSOD activity declined.

SeGSHPx activity was significantly lower in the pancreas from BBdp rats compared with BBc rats ($P < 0.00001$) (Fig. 2). This difference was significant in both males and females from Week 7 on, although SeGSHPx activity was higher in the pancreas of female rats compared to male rats ($P < 0.02$) (data not shown). Non-SeGSHPx activity was very low in all groups (<20% of total activity) and was not significantly different between BBc and BBdp rats. Pancreatic total GSHPx activity was also significantly lower in the BBdp rats compared with the BBc rats at each time point during the prediabetic period.

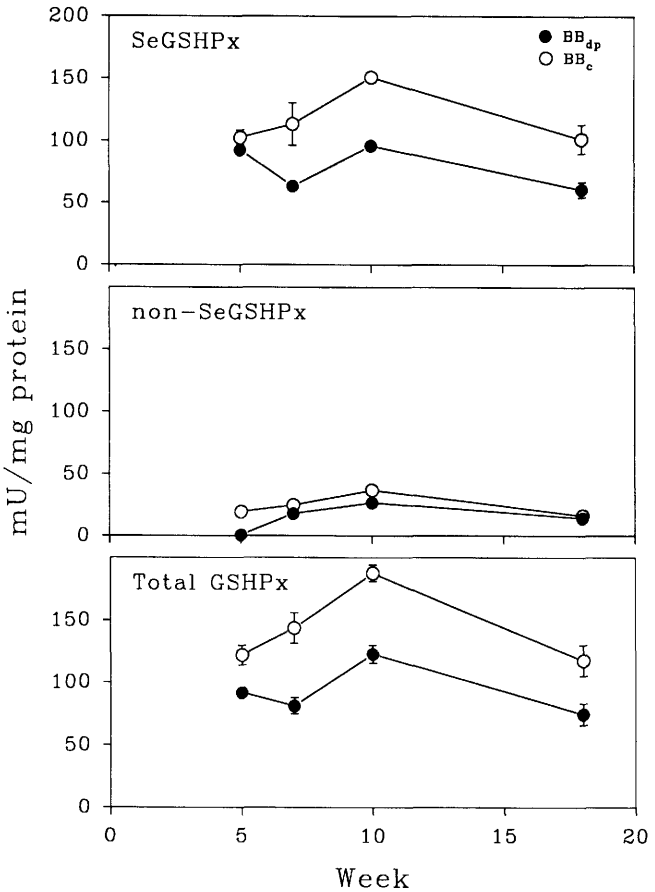

Figure 2. Changes over time in Se-dependent glutathione peroxidase (SeGSHPx, top), non-Se-dependent glutathione peroxidase (non-SeGSHPx, centre) and total glutathione peroxidase (Total GSHPx, lower) activities (mU/mg protein) in the pancreata of prediabetic diabetes-prone BB rats (BBdp) and age-matched control BB rats (BBc) derived from the same colony. At Week 18, BBdp rats had grade 3–4 insulitis, on a scale of 1–4; no insulitis was evident during Weeks 5–10 or in BBc rats.

The presence or absence of insulitis had no effect on SOD or GSHPx activities in either plasma or erythrocytes obtained from BBdp rats (Table I). Erythrocyte and plasma SeGSHPx activities, however, were significantly lower in the female rats compared to male animals ($P < 0.0001$, $P < 0.01$ respectively), while there were no significant sex differences in blood SOD activity (Table I).

Discussion

The present study was undertaken to determine if the increased susceptibility of the BBdp rat to diabetes was related to a deficit in pancreatic antioxidant enzyme activities compared with the nonsusceptible BBc rat. Pancreas has been reported to have relatively little activity of the antioxidant defensive enzymes SOD and GSHPx compared with other tissues (4, 5). Using immunofluorescent staining, however, Gandy *et al.* (20) found that human islet B-cells are rich in CuZnSOD, while the enzyme was not detectable in acinar tissue. In this study, we have also found that pancreas CuZnSOD activity was not abnormally low compared with the activity seen in numerous other tissues from control Wistar rats of the same age that we have examined in our laboratory. CuZnSOD activity was 8–18 U/mg in pancreas compared with 3, 4, 11, and 28 U/mg protein for aorta, spleen, heart, and liver respectively (unpublished data and from Ref. 21). In fact, BBdp rat pancreas was found to be particularly high in MnSOD activity (8 ± 1 U/mg protein at Week 18), next only to that seen in heart (29 U/mg), and to have significantly more activity than numerous other tissues (21). SeGSHPx activity in the BBdp rat pancreas (50–100 mU/mg) was lower than aorta, spleen, kidney, heart, and liver activities, but was still well above the detection limit and much higher than muscle, plasma, and platelet activity. It is also noteworthy that in pancreas, females had more SeGSHPx activity than males, a difference that we have observed previously in liver (21). In a recent study by Cornelius *et al.* (1993) (22), diabetes-prone NOD mice were not deficient in protective enzymes compared with BALB/c mice, although there were significant changes in enzyme activities over time. Thus, like the NOD mouse, the pancreas of the BBdp rat does not appear to have any obvious deficiencies in antioxidant enzyme activity compared with other tissues.

Several groups have investigated the pancreatic activity of antioxidant enzymes in BB rats and its relationship with diabetes. In the 6- to 7-month-old insulin-dependent BB rat (on insulin therapy), pancreas

Table I. Effects of Insulitis on Blood Enzyme Activities in Diabetes-prone BB Rats

Enzyme	Sex	Insulitis	
		Present	Absent
Erythrocyte			
SeGSHPx (mU/mg Hb)	M	260 ± 7 (*n* = 13)	284 ± 8 (*n* = 9)
	F	242 ± 8 (*n* = 5)	231 ± 4 (*n* = 14)
non-SeGSHPx (mU/mg Hb)	M + F	148 ± 9	150 ± 7
Total GSHPx (mU/mg Hb)	M	414 ± 11	433 ± 20
	F	374 ± 20	381 ± 6
CuZnSOD (U/mg Hb)	M + F	119 ± 2	113 ± 3
Plasma			
SeGSHPx (mU/mg prot)	M	47 ± 4 (*n* = 13)	56 ± 3 (*n* = 9)
	F	41 ± 4 (*n* = 4)	42 ± 3 (*n* = 14)
non-SeGSHPx (mU/mg prot)	M + F	16 ± 2	16 ± 1
Total GSHPx (mU/mg prot)	M	65 ± 5	74 ± 4
	F	54 ± 6	56 ± 4
EC-SOD (U/mg prot)	M + F	6.3 ± 0.5	5.5 ± 0.2

Note. Mean age 126 ± 3 days. Values are Mean ± SEM (*n*) and are given for males and females separately where there was a significant ($P < 0.05$) sex difference by two-way ANOVA. SeGSHPx = Selenium-dependent glutathione peroxidase; non-SeGSHPx = non-Selenium-dependent glutathione peroxidase; CuZnSOD = Copper, Zinc-dependent superoxide dismutase; MnSOD = Manganese-dependent superoxide dismutase; and EC-SOD = Extracellular superoxide dismutase.

CuZnSOD, GSHPx, and glutathione reductase (GSHR) activities were increased compared with nondiabetic littermates (23). In addition, the severity of antioxidant alterations paralleled the degree of hypoinsulinemia in these animals. These animals, however, were already diabetic and receiving insulin therapy. Thus, the alterations observed in their study may have been related to suboptimal insulin therapy, and no information was given concerning changes occurring during the prediabetic period. In another study, prediabetic BBdp rats were found to have lower islet total SOD activity compared with control Wistar rats (24). However, BBdp rats were compared with commercial Wistar rats rather than BBc rats, so these differences cannot be attributed directly to the development of diabetes in the BB rat. In addition, no attempt was made to distinguish between the two forms of SOD, which, as can be seen from the present study, do not respond in a similar manner. Thus, from previous studies it is not clear which changes in the BB rat pancreas are associated with the development of diabetes, with the diabetic state, or with suboptimal insulin therapy during diabetes.

In the present study, we observed significantly higher CuZnSOD and total SOD activity in BBdp rats compared with BBc animals, long before the appearance of insulitis or clinical diabetic symptoms. CuZnSOD activity was almost twice as high in the BBdp rats compared with the BBc rats early in the prediabetic period (Weeks 5, 7, 10), while from Week 10, CuZnSOD activity began to decline, so that at 18 weeks, we found no difference in CuZnSOD activity between BBdp and BBc rats, although total SOD activity remained significantly higher in the BBdp rats. The marked reduction in pancreatic CuZnSOD activity in the BBdp rat coincided with the development of insulitis seen at Week 18. A similar decline in CuZnSOD activity (as a % of total activity) was also noted in the diabetes-prone NOD mouse, although the absolute amounts of the two forms of SOD were not indicated (22).

There are several possible explanations for the fall in pancreatic CuZnSOD activity seen in the BBdp rats compared with the BBc rats in this study. The reduction may be due to the lower GSHPx activity in the pancreas of BBdp rats; or due to a response to autoimmune inflammation; or due to glucosylation of CuZnSOD, all of which have been shown to reduce SOD activity (13, 14, 25, 27, 28). Inhibition of islet SOD activity has been suggested as the mechanism whereby diabetogenic chemicals damage B-cells (25). Enzymatic CuZnSOD activity, but not immunoreactive SOD levels or MnSOD activity, was decreased by 50% in alloxan exposed islets, and CuZnSOD, but not MnSOD injection prevented or reduced STZ-induced diabetes. SOD activity is also inhibited by high levels of its product, H_2O_2 (26). In the present study, BBdp rats had less pancreatic GSHPx activity than age-matched BBc rats, throughout the prediabetic period (from Week 7 on). This suggests that with lowered GSHPx activity there may be elevated H_2O_2 levels, which could be inhibiting CuZnSOD activity, as CuZnSOD activity rapidly dropped from Week 7 on, the time at which SeGSHPx activity was also significantly reduced. The decrease in CuZnSOD activity may also be related to inflammation, as turpentine (a potent inflammatory agent), reduced liver CuZnSOD activity (27). A final possible mechanism may be due

to autooxidative glucosylation of proteins, as increased glucosylation of CuZnSOD has been seen in diabetics and shown to reduce enzyme activity (28).

MnSOD activity increased over time (Week 10–18) in the pancreas of BBdp rats, while the activity remained almost negligible in the pancreas of BBc rats. This rise in MnSOD activity in the BBdp rats during the development of insulitis may be a compensatory mechanism for the declining CuZnSOD activity seen during the same time period. These results are also consistent with *in vitro* studies which have shown that CuZnSOD activity, but not MnSOD activity, is inhibited by H_2O_2 (26). Alternatively, MnSOD activity may be rising in response to increased production of γ-interferon by the insulitis lesion (22, 29). Regardless of the possible mechanism, the pancreas of the BBdp rat had twice the total SOD activity compared with the pancreas from BBc rats, with a dramatic shift in the relative amounts of the CuZnSOD and MnSOD forms. A similar shift has also been observed in the diabetes-prone NOD mouse (22).

There are no obvious explanations for the reduced SeGSHPx and total GSHPx activities observed in the pancreas of BBdp rats compared with BBc animals; however, this reduction was significant very early in the prediabetic period (by Week 7), long before any clinical or histological symptoms. STZ treatment was shown to decrease pancreatic GSHPx activity in one study (7), while another group of researchers found elevated SeGSHPx activity in diabetic rat pancreas (23, 30). In another study, no differences in SeGSHPx activity were seen in the pancreas of the diabetes-prone NOD mouse compared with the BALB/c strain (22). Thus, there does not appear to be any consistent changes in SeGSHPx activity in other diabetes studies; although in the present study, SeGSHPx activity was consistently 35%–45% lower in the pancreas of the BBdp rat compared with BBc rats from Week 7 on.

No differences in plasma or erythrocyte antioxidant enzyme activities were seen in BBdp rats with or without insulitis, indicating that it is unlikely that there were any changes in the copper or selenium status of the BBdp rat with the development of insulitis. Failla and Gardell (31) found that pancreatic Cu content was not altered in the diabetic BB rat compared with non-diabetic or control animals, although they observed decreased plasma Cu in diabetic animals which was increased with insulin treatment. These discrepancies in copper status are probably a consequence of the diabetic state and not related to the development of the disease, as the hypocupremia of diabetes could be reversed by insulin therapy (31), further suggesting that the low blood Cu seen in their study was related to insulin deficiency rather than related to the development of diabetes. Thus, since blood antioxidant enzyme activities did not vary with insulitis in the present study, yet pancreatic activities did, these results suggest that the alterations in antioxidant enzyme activities are specific to the pancreas during the development of diabetes rather than being indicative of overall changes in antioxidant status or a trace element deficiency.

In conclusion, the data from the present experiments indicate that the activities of pancreatic antioxidant enzymes change very early during the development of diabetes in the spontaneously diabetes-prone BB rat and that these changes are demonstrable long before any pancreatic insulitis can be seen. The initial hypothesis that low antioxidant enzyme activity might be associated with the development of diabetes in the BBdp rat appears to be an oversimplification. The relatively low SeGSHPx activity and declining CuZnSOD activity are consistent with this hypothesis, however total SOD activity remained high in the BBdp rat. In addition, there appears to be dramatic shifts in enzyme activities as well as a possible imbalance between GSHPx and SOD activities. These alterations in protective enzyme activities may play a role in the pathogenesis of insulin-dependent diabetes and may affect the susceptibility of the pancreas of the BBdp rat to oxidative stress. The role these changes in enzyme systems play in the protection against viral, chemical, or autoimmune oxidative assaults needs to be further examined.

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