

# Poly(ADP-ribose)polymerase activation determines strain sensitivity to streptozotocin-induced $\beta$ cell death in inbred mice

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## ABSTRACT

Streptozotocin (STZ) is believed to induce pancreatic  $\beta$  cell death in mice by depleting the cell of NAD+NADH. The drug is known to cause a greater depletion of  $\beta$  cell NAD+NADH in C57bl/6J mice than in Balb/c mice. To investigate the basis for this strain difference, we compared the effects of streptozotocin on poly(ADP-ribose)-polymerase (PARP) activation – the major site of NAD consumption, and on mitochondrial activity – the major site of NAD production.

A significant strain difference was demonstrated in STZ-induced PARP activation (fmol NAD incorporated/min/ $\mu$ g DNA  $\pm$  s.e.m.: Balb/c control  $2.28 \pm 0.14$ , Balb STZ  $3.11 \pm 0.25$ ; C57bl/6J control  $2.57 \pm 0.29$ , C57bl/6J STZ  $4.17 \pm 0.24$ ). In comparison, no strain difference could be demonstrated in hydrogen-peroxide-induced PARP activation. No strain differences could be detected in the activity of STZ-treated islet mitochondria as measured by determining ATP production (pmol/ $\mu$ g protein/h  $\pm$  s.e.m.: Balb/c control  $0.20 \pm 0.02$ ,

Balb/c STZ  $0.15 \pm 0.02$ ; C57bl/6J control  $0.23 \pm 0.03$ , C57bl/6J STZ  $0.15 \pm 0.02$ ) or by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye reduction (change in optical density/mg protein  $\pm$  s.e.m.: Balb/c control  $10.19 \pm 0.62$ , Balb/c STZ  $6.01 \pm 1.17$ ; C57bl/6J control  $6.15 \pm 0.98$ , C57bl/6J STZ  $5.81 \pm 0.96$ ).

The strain difference in STZ-induced NAD depletion appears to be due to a difference in NAD consumption and not a difference in a mitochondrial process involved in replacing decreasing NAD concentrations. It is unlikely that a strain difference in the enzymic activity of PARP is responsible for strain differences in the effects of STZ, as no strain differences in hydrogen-peroxide-induced PARP activation could be detected. Thus the greater PARP activation, NAD depletion and  $\beta$  cell death observed in C57bl/6J islets may be due to greater levels of DNA damage or differences in the DNA excision repair processes.

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## INTRODUCTION

The drug streptozotocin (STZ) is a glucose analogue (*N*-[methylnitrosocarbamoyl]-D-glucosamine) that specifically damages pancreatic  $\beta$  cells (Schein & Loftus 1968). It is rapidly transported into the  $\beta$  cell via the glucose transporter, Glut2, and is known to be metabolised by the  $\beta$  cell upon entry (Karunanayake *et al.* 1976, Johansson & Tjalve 1989, Kroncke *et al.* 1995). STZ metabolites accumulate inside the cell, presumably because the glucose moiety is lost. STZ then acts on the cellular components and, in particular, the mitochondria and DNA (Schein *et al.* 1973, Kwon *et al.* 1994).

STZ acts on the mitochondria by inhibiting the ability to produce ATP and, in the process, NAD. The major action of STZ on the DNA is believed to be the induction of DNA adducts (Ledoux *et al.* 1988). These adducts are in turn repaired by the nuclear excision repair processes. The strand breaks induced during excision repair activate poly(ADP-ribose)polymerase (PARP), which converts NAD into polymers of ADP-ribose at the site of the DNA strand break (Yamamoto *et al.* 1981). This leads to NAD depletion. If the DNA repair processes are sufficiently activated, PARP activation can cause the depletion of NAD to non-physiological levels. Normally NAD depletion would be replaced by

cellular processes. However, STZ also inhibits the replacement of the NAD by inhibiting mitochondrial ATP generation. Thus, both DNA repair-induced NAD depletion and the inability of the cell to replace decreasing NAD concentrations may combine to cause loss of cell viability.

Inbred strains of mice vary in their sensitivity to STZ injected as a single dose or as multiple subdiabetogenic doses (Rossini *et al.* 1977). This indicates that genetic susceptibility to the initial  $\beta$ -cell-damaging event seen after a single dose of STZ may contribute to the progression of insulin-dependent diabetes mellitus in the multiple-low-dose STZ mouse model. Previous studies in this laboratory (Cardinal *et al.* 1998) have investigated the basis for differences in STZ sensitivity between the sensitive C57bl/6J and the relatively resistant Balb/c mouse strains. Balb/c mice contained over seven times more pancreatic insulin than C57bl/6J mice after an i.v. dose of 120 mg/kg body weight. The difference in STZ sensitivity was due to the amount of  $\beta$  cell death as confirmed by morphology in *in vivo* studies. STZ sensitivity was found to be at the  $\beta$  cell level, as C57bl/6J islets incubated *in vitro* contained over 14 times more dead cells. After an *in vitro* dose of 2.2 mM STZ for 30 min, islet concentrations of NAD were significantly less in C57bl/6J islets than in Balb/c islets. As no strain differences in STZ/glucose transporter capacity could be detected, these studies concluded that STZ sensitivity is determined at the  $\beta$  cell level and is regulated by events that occur after the drug enters the  $\beta$  cell and before the cell is depleted of NAD. The present study examined whether the strain differences in STZ sensitivity and NAD depletion are due to a greater consumption of NAD by the DNA repair enzyme PARP, or to a difference in the ability to replace the decreasing ATP and NAD levels by the mitochondria.

## MATERIALS AND METHODS

### Mice and reagents

Male Balb/c and C57bl/6J mice were obtained from the Central Animal Breeding House at the University of Queensland. The animals were 8–12 weeks old at the start of each experiment. All animal experimentation was approved by the Queensland University of Technology Research Ethics Committee and was in compliance with the National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes.

### Islet isolation

Islets were isolated using the method of Lake *et al.* (1987). Using this method, 100–200 islets per mouse could be reliably isolated.

### Isolation of islet nuclei and mitochondria

All islets used for isolation of nuclei and mitochondria were prepared using previously described methods (Eizirik *et al.* 1988). After treatment, the islets were washed in Hank's buffered saline solution supplemented with 0.2% bovine serum albumin, 5.5 mM D-glucose and 20 mM Hepes (HBSSH) and resuspended in a solution containing 250 mM sucrose, 2.5 mM EDTA, 2 mM cysteine, 0.02% bovine serum albumin, 10 mM Hepes pH 7.4. The islets were left to lyse partially in this buffer for 5 min on ice and then dispersed by rapidly pipetting the solution in and out of a 1-ml pipette 20 times. The nuclei were pelleted by centrifuging for 3 min at 1000 g. The pellet was then used for PARP activity assays. The supernatant was retained and further spun at 100 000 g for 5 min at 4 °C in a Beckman TL-100 ultracentrifuge. The pellet was used for mitochondrial activity assays.

### Estimation of poly(ADP-ribose)polymerase (PARP) activity

The method used was a modification of that of Uchigata *et al.* (1982). Nuclei from 150 islets (one mouse) were resuspended in 100  $\mu$ l buffer A (50 mM Tris pH 7.5, 30% glycerol, 1 mM EDTA, 0.5 mM EGTA) and 100  $\mu$ l buffer B (100 mM Tris pH 8.0, 10 mM MgCl<sub>2</sub>, 5  $\mu$ Ci/ml [adenine-2,8-<sup>3</sup>H]NAD (NEN), 20 mM  $\beta$  mercaptoethanol). The mixture was vortexed and incubated for 30 min at 37 °C. The DNA was precipitated by adding 1 ml ice-cold stop solution (10% trichloroacetic acid, 2% sodium pyrophosphate decahydrate). The samples were left on ice for 45 min and then spun at 12 000 g in a microfuge for 10 min. The supernatant was removed and the pellet was washed three more times with 1 ml stop solution, followed by one wash in 0.6 M perchloric acid. The pellet was then left to resuspend overnight in 200  $\mu$ l 0.04 M NaOH. A portion (150  $\mu$ l) of the solution was added to 10 ml Instagel scintillant and the amount of radioactivity measured in a  $\beta$  counter. The remainder of the sample was used to measure DNA content as previously described (Kissane & Robins 1958). Results were expressed as fmol NAD incorporated/min/ $\mu$ g DNA.

### Assessment of STZ effects on mitochondrial ATP content and ATP production

Groups of 50 islets (one mouse) were incubated with 2.2 mM STZ for 30 min in 1 ml HBSSH. The islets were washed with HBSSH, mitochondria isolated and the pellet resuspended in 200 µl 0.1 M phosphate buffer that had previously been gassed for 5 min with carbogen (5% CO<sub>2</sub> in O<sub>2</sub>). One hundred microlitres of the suspension were added to 100 µl reaction buffer (50 mM NAD, 0.2 mM L-glutamic acid sodium salt, 0.25 mM ADP in 0.1 M phosphate buffer pH 7.4), mixed and incubated at 37 °C. At time points 0, 1, 2 and 3 h, 50 µl was removed, added to 50 µl boiling stop buffer (100 mM Tris pH 7.8, 4 mM EDTA), boiled for 2 min and placed on ice until assayed for ATP. Ten microlitres of this mixture were added to 100 µl luciferase reagent and the luminescence read using a Packard Picolite luminometer. The sample was read for 10 s after a 2 s delay. The ATP standard curve was constructed between 1 and 10 mM. Sample ATP concentrations were read off a log luminescence vs log concentration standard curve. Samples were expressed as pmol ATP/µg protein. Protein was measured using the Bradford method (1976).

### Assessment of STZ effects on mitochondrial MTT dye reduction

Mitochondria from 150 islets (one mouse) were resuspended in 150 µl 0.1 M phosphate buffer pH 7.4. A portion of this suspension (50 µl) was used to measure protein content using the method of Bradford (1976). The mitochondrial suspension (50 µl) was added to 50 µl MTT reaction solution (0.5 mg/ml MTT, 50 mM NAD, 0.2 mM L-glutamic acid sodium salt, 50 mM ADP in 0.1 M phosphate buffer pH 7.4) and incubated at 37 °C. Dye reduction was measured at 30 min and 60 min. The reaction was stopped by adding 500 µl isopropanol. The dye was allowed to dissolve overnight at 4 °C. The contents were then centrifuged at 12 000 *g* in a microcentrifuge for 10 min. The supernatant was read in a Shimadzu UV-1601 spectrophotometer at 540 nm. The rate of change in absorbance from 30 min to 60 min was proportional to the mitochondrial dehydrogenase activity. Activity was expressed as change in optical density and corrected for protein content.

### Statistical analysis methods

Results from groups from each study were first analysed using ANOVA (using the statistical software package 'Statistical Package for Social

Sciences (SPSS) Version 7.5'). If the ANOVA showed a significant difference between groups, Student's *t*-test was used as the *post hoc* test.

## RESULTS

### Poly(ADP-ribose)polymerase activation assay

PARP activation was measured after groups of 150 islets from both strains were treated with 2.2 mM STZ or 200 µM hydrogen peroxide for 30 min. STZ significantly increased islet PARP activation in both strains, with the level of PARP activation being significantly greater in C57bl/6J islets (Fig. 1*a*). There was no significant difference in control PARP activity in islets from the two strains. Hydrogen peroxide also increased islet PARP activation in both strains, however there was no strain difference detected (Fig. 1*b*).

### Mitochondrial ATP content

Mitochondrial ATP content was measured after groups of 50 islets were treated with 2.2 mM STZ for 30 min. There were no significant differences in mitochondrial ATP content between control and STZ-treated islets from both strains. There were also no significant differences between strains in mitochondrial ATP content for both control and STZ-treated islets (Fig. 2).

### Mitochondrial activity

Islet mitochondrial activity was measured after islets were treated with 2.2 mM STZ for 30 min. Mitochondrial activity, as measured by ATP production, decreased significantly in C57bl/6J islets ( $P < 0.05$ ), but not in Balb/c islets, after treatment with STZ (Fig. 3). However, no strain difference in islet mitochondrial ATP production was detected between the two strains after STZ treatment.

Using the MTT dye reduction assay, mitochondrial activity from control Balb/c islets was significantly greater than that from C57bl/6J islets ( $P < 0.01$ ). After streptozotocin treatment, there was no significant difference between strains (Fig. 4).

## DISCUSSION

This study demonstrated a significantly greater activation of PARP in the islets of C57bl/6J mice than in those of Balb/c mice in response to STZ treatment. There was no significant difference in mitochondrial ATP content or activity in STZ-treated islets between the two strains. Thus it is

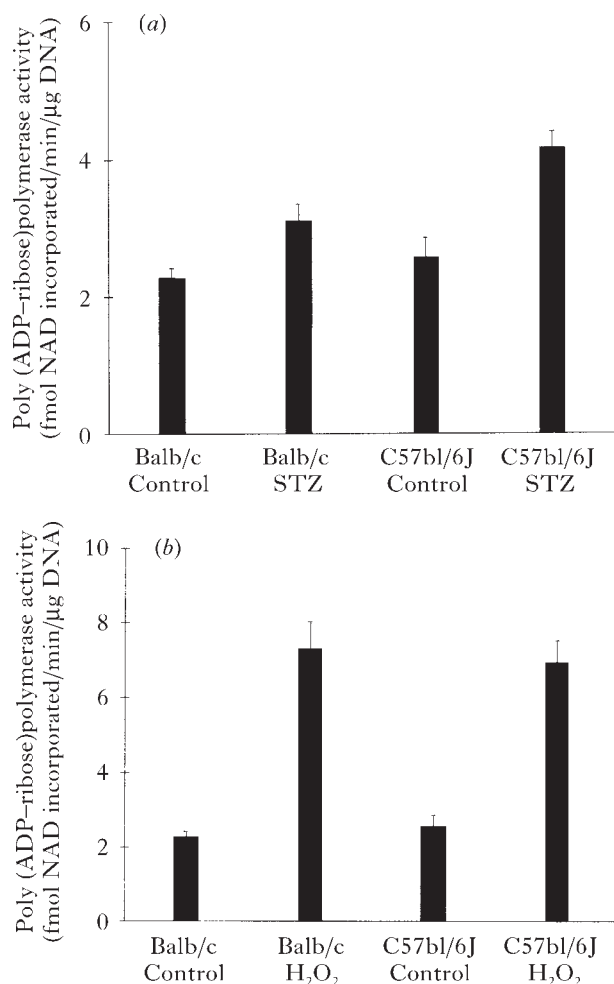


FIGURE 1. STZ and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) activation of PARP activity. (a) PARP activity was significantly increased in both Balb/c and C57bl/6J islets treated with 2.2 mM STZ compared with control groups (vehicle only) ( $P < 0.05$ ,  $P < 0.005$  respectively). STZ-treated PARP activity was significantly greater in C57bl/6J islets than in Balb/c islets ( $P < 0.05$ ). Each bar is the mean  $\pm$  S.E.M. of four separate experiments. The total number of mice and replicates used for each group was seven to eight. (b) There were no significant differences in PARP activation between strains in control or hydrogen-peroxide-treated islets. There was a significant difference between control and hydrogen-peroxide-treated islets in both strains ( $P < 0.001$ ). Each bar is the mean  $\pm$  S.E.M. of four separate experiments. The total number of mice and replicates used for each group was six to eight.

proposed that the previously observed differences in islet NAD between the strains (Cardinal *et al.* 1998) result from differences in the rate of NAD consumption and not in the differences in NAD generation. This is likely to be the basis for the

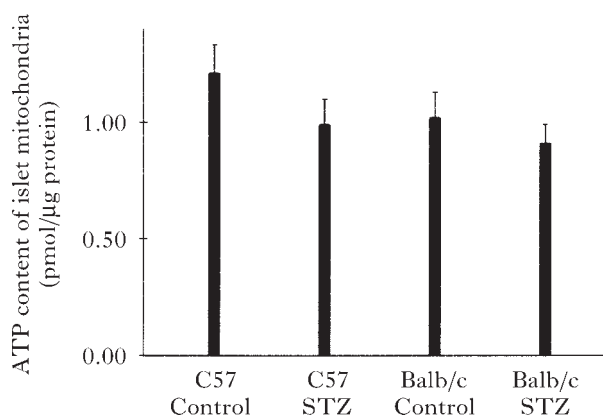


FIGURE 2. Mitochondrial ATP content of control and 2.2 mM STZ-treated islets. No significant differences were found between control and treated groups within strains or between strains. Each bar is the mean  $\pm$  S.E.M. of four separate experiments. The total number of mice and replicates used for each group was nine to eleven.

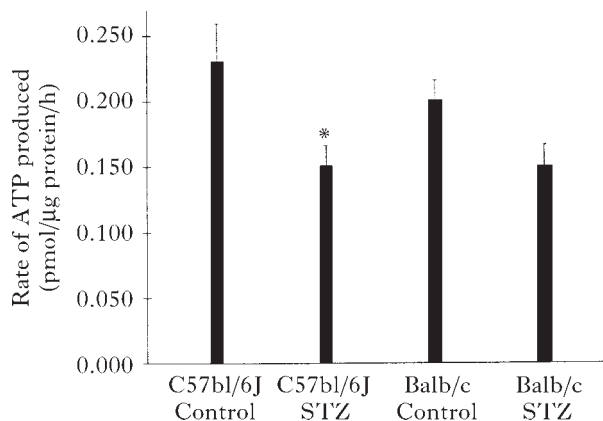


FIGURE 3. Mitochondrial activity (rate of ATP produced) of control and 2.2 mM STZ-treated islets from C57bl/6J and Balb/c mice. There was a significant difference between control and STZ-treated groups in C57bl/6J islets (\*  $P < 0.05$ ). There were no significant differences in control and STZ-treated groups between strains. Each bar is the mean  $\pm$  S.E.M. of four separate experiments. The total number of mice and replicates used for each group was nine to eleven.

different sensitivity to STZ-induced diabetes between the two strains.

The greater PARP activation seen in C57bl/6J islets compared with Balb/c islets is in keeping with our previous observation of greater NAD depletion in this strain (Cardinal *et al.* 1998). This observation is consistent with the work of Okamoto (1982), who proposed that the primary cytotoxic effects of STZ were via the induction of DNA strand breaks and subsequent PARP activation. The results of the present study suggest that strain

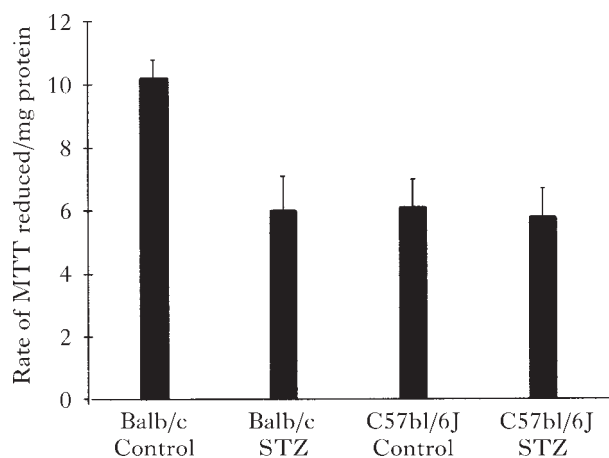


FIGURE 4. The mitochondrial activities of control and 2.2 mM STZ-treated islets as measured by the MTT dye method. There were no significant differences between C57bl/6J control and treated islets. Control Balb/c islets were significantly different from STZ-treated Balb/c islets ( $P < 0.05$ ) and control C57bl/6J islets ( $P < 0.01$ ). There were no significant differences between strains in STZ-treated groups. Each bar is the mean  $\pm$  S.E.M. from four separate experiments. The total number of mice and replicates used for each group was six to eight.

differences in STZ sensitivity and NAD depletion are determined by interactions at the DNA level.

PARP activation is determined by the number of DNA strand breaks present and the activity/amount of PARP enzyme (Shall 1984). It follows, then, that the observed strain variation in PARP activity must be due to the number of DNA strand breaks or the amount/activity of PARP enzyme. To exclude the latter possibility, PARP activation was measured after hydrogen peroxide treatment. STZ and other alkylating agents induce DNA strand breaks indirectly by modifying DNA bases. DNA strand breaks are caused by endonucleases during the process of DNA repair (Saffhill *et al.* 1985, Ledoux *et al.* 1986, Wilson *et al.* 1988). Hydrogen peroxide was used because it directly induces DNA strand breaks, without the involvement of excision repair processes (Imlay & Linn 1988).  $\beta$  cells have been reported to contain low levels of catalase and peroxidase (Eizirik 1996, Lenzen *et al.* 1996). Assuming this to be so, the hydrogen peroxide dose given in this study will generate approximately the same number of DNA strand breaks in both strains. Thus, with the same number of DNA strand breaks, PARP activity is proportional to the amount of enzyme present. The lack of strain difference seen in PARP activation after hydrogen peroxide treatment suggests that there is no difference in the

amount/activity of PARP present in the sensitive and non-sensitive strains.

If there are no strain differences in the amount/activity of PARP, the differences seen in STZ-induced PARP activation would be due to the number of strand breaks present. It is unknown whether, at the dose given, STZ would induce DNA strand breaks directly. However, it is known that STZ induces the formation of the DNA adduct, *N*-7-methylguanine, and, to a lesser extent, *O*-6-methylguanine and 3-methyladenine (Saffhill *et al.* 1985, Ledoux *et al.* 1986, Wilson *et al.* 1988). In the process of excision repair, the damaged DNA is cut enzymically at the site of the adducts and repaired. The number of STZ-induced DNA strand breaks is determined first by the amount of DNA adducts and, secondly, by the activity of nuclear excision repair enzymes.

The present study provides evidence for the STZ-induced mitochondrial inhibition of ATP generation and, thus, inhibition of NAD production in the C57bl/6J islets. Inhibition of mitochondrial MTT dye reduction was also demonstrated, but only in Balb/c islets. Basal levels of MTT dye reduction were lower in the C57bl/6J strain, possibly indicating lower basal glutamate dehydrogenase activity. It would follow then that inhibition of glutamate dehydrogenase would be difficult to demonstrate in this strain. There were no strain differences detected in ATP production or electron transport chain function (as measured by MTT dye reduction) in STZ-treated islet mitochondria, indicating that the differences in NAD depletion are not due to differences in mitochondrial ATP production.

It is concluded from this study that the previously reported differences (Cardinal *et al.* 1998) in STZ sensitivity and NAD depletion and between C57bl/6J and Balb/c mice are determined at the level of the DNA, and not the mitochondria. The greater PARP activation in C57bl/6J islets is likely to be due to greater initial DNA damage, faster excision repair mechanisms, or both. Previous studies (Cardinal *et al.* 1998) have shown that C57bl/6J islets accumulate more STZ metabolites than do Balb/c islets. The presence of a greater level of STZ metabolite accumulation may lead to a greater level of DNA damage. Alternatively, C57bl/6J DNA adduct repair rates have previously been shown to be greater than those of Balb/c mice (Boerrigter *et al.* 1993), and an increased rate of DNA excision repair would cause greater levels of DNA strand breaks (Coquerelle *et al.* 1995). Further studies are warranted to determine the amount of DNA damage induced by STZ.

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