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MINIREVIEW

The Mechanism of Alloxan and Streptozotocin Action in B Cells of the Rat Pancreas

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Summary

Alloxan and streptozotocin are widely used to induce experimental diabetes in animals. The mechanism of their action in B cells of the pancreas has been intensively investigated and now is quite well understood. The cytotoxic action of both these diabetogenic agents is mediated by reactive oxygen species, however, the source of their generation is different in the case of alloxan and streptozotocin. Alloxan and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. Thereafter highly reactive hydroxyl radicals are formed by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of B cells. Streptozotocin enters the B cell *via* a glucose transporter (GLUT2) and causes alkylation of DNA. DNA damage induces activation of poly ADP-ribosylation, a process that is more important for the diabetogenicity of streptozotocin than DNA damage itself. Poly ADP-ribosylation leads to depletion of cellular NAD^+ and ATP. Enhanced ATP dephosphorylation after streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of superoxide radicals. Consequently, hydrogen peroxide and hydroxyl radicals are also generated. Furthermore, streptozotocin liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of the streptozotocin action, B cells undergo the destruction by necrosis.

Key words

Alloxan • Streptozotocin • Pancreatic B cells • Mechanism of action • Diabetes

The induction of experimental diabetes in the rat using chemicals which selectively destroy pancreatic B cells is very convenient and simple to use. The most usual substances to induce diabetes in the rat are alloxan and streptozotocin. The understanding of changes in B cells of the pancreas as well as in the whole organism after alloxan or streptozotocin treatment is essential for using these compounds as diabetogenic agents. The metabolic disturbances in alloxan- and streptozotocin-treated rats were described recently by Szkudelski *et al.*

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(1998). This review focuses on the elucidation of the mechanism of cytotoxic action of alloxan and streptozotocin in B cells of the rat pancreas.

1. The mechanism of alloxan action

Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) was first described by Brugnatelli in 1818. Wöhler and Liebig used the name "alloxan" and described its synthesis by uric acid oxidation (for review see Lenzen and Panten 1988). The diabetogenic properties of this drug were reported many years later by Dunn, Sheehan and McLethie (1943), who studied the effect of its administration in rabbits and reported a specific necrosis of pancreatic islets. Since then, alloxan diabetes has been commonly utilized as an animal model of insulin-dependent diabetes mellitus (IDDM).

Alloxan exerts its diabetogenic action when it is administered parenterally: intravenously, intraperitoneally or subcutaneously. The dose of alloxan required for inducing diabetes depends on the animal species, route of administration and nutritional status. Human islets are considerably more resistant to alloxan than those of the rat and mouse (Eizirik *et al.* 1994). The most frequently used intravenous dose of this drug to induce diabetes in rats is 65 mg/kg b.w. (Gruppuso *et al*. 1990, Boylan *et al.* 1992). When alloxan is given intraperitonealy or subcutaneously its effective dose must be 2-3 times higher. The intraperitoneal dose below 150 mg/kg b.w. may be insufficient for inducing diabetes in the rat (Katsumata *et al*. 1992, 1993). Fasted animals are more susceptible to alloxan (Katsumata *et al.* 1992, Szkudelski *et al.* 1998), whereas increased blood glucose provides partial protection (Bansal *et al*. 1980, Szkudelski *et al.* 1998).

The mechanism of alloxan action has been intensively studied, predominantly *in vitro*, and is now characterized quite well. Using isolated islets (Weaver *et al*. 1978b) and perfused rat pancreas (Kliber *et al*. 1996) it was demonstrated that alloxan evokes a sudden rise in insulin secretion in the presence or absence of glucose. This phenomenon appeared just after alloxan treatment and was not observed after repetitive exposure of islets to this diabetogenic agent (Weaver *et al*. 1978b). The sudden rise in blood insulin concentration was also observed *in vivo* just after alloxan injection to rats (Szkudelski *et al*. 1998). Alloxan-induced insulin release is, however, of short duration and is followed by complete suppression of the islet response to glucose, even when high concentrations (16.6 mM) of this sugar were used (Kliber *et al*. 1996).

Alloxan is a hydrophilic and unstable substance. Its half-life at neutral pH and 37 °C is about 1.5 min and is longer at lower temperatures (Lenzen and Munday 1991). On the other hand, when a diabetogenic dose is used, the time of alloxan decomposition is sufficient to allow it to reach the pancreas in amounts that are deleterious.

The action of alloxan in the pancreas is preceded by its rapid uptake by the B cells (Weaver *et al*. 1978a, Boquist *et al*. 1983). Rapid uptake by insulin-secreting cells has been proposed to be one of the important features determining alloxan diabetogenicity. Another aspect concerns the formation of reactive oxygen species (Heikkila *et al*. 1976). A similar uptake of alloxan also takes place in the liver. However, the liver and other tissues are more resistant to reactive oxygen species in comparison to pancreatic B cells and this resistance protects them against alloxan toxicity (Malaisse *et al*. 1982, Tiedge *et al*. 1997). The formation of reactive oxygen species is preceded by alloxan reduction. In B cells of the pancreas its reduction occurs in the presence of different reducing agents. Since alloxan exhibits a high affinity to the SH-containing cellular compounds, reduced glutathione (GSH), cysteine and protein-bound sulfhydryl groups (including SH-containing enzymes) are very susceptible to its action (Lenzen and Munday 1991). However, other reducing agents such as ascorbate may also participate in this reduction (Zhang *et al*. 1992). Lenzen *et al*. (1987) proposed that one of the SH-containing compounds essential for proper glucose-induced insulin secretion is glucokinase (EC 2.7.1.2), being very vulnerable to alloxan. Alloxan reacts with two -SH groups in the sugar-binding side of glucokinase resulting in the formation of the disulfide bond and inactivation of the enzyme. Glucose can protect glucokinase against the inactivation hindering the access of alloxan to the -SH groups of the enzyme (Lenzen *et al.* 1987, 1988, Lenzen and Mirzaie-Petri 1991).

Dialuric acid is formed as a result of alloxan reduction. It is then re-oxidized back to alloxan establishing a redox cycle for the generation of superoxide radicals (Munday 1988). The reaction between alloxan and dialuric acid is a process in which intermediate alloxan radicals ($HA^{\bullet}$) and an unidentified "compound 305" (maximum absorption at 305 nm) is formed. The latter appears when alloxan is reduced by GSH (Sakurai and Ogiso 1991). Superoxide radicals are

able to liberate ferric ions from ferritin and reduce them to ferrous ions. Fe^{3+} can also be reduced by alloxan radicals (Sakurai and Ogiso 1995). Moreover, superoxide radicals undergo dismutation to hydrogen peroxide:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2\,H^+ \rightarrow H_2O_2 + O_2$$

This reaction may occur spontaneously or may be catalyzed by superoxide dismutase (EC 1.15.1.1) (Malaisse 1982). In the presence of Fe^{2+} and hydrogen peroxide, highly reactive hydroxyl radicals are then formed according to the Fenton reaction (Fig. 1):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet-}$$

The action of hydroxyl radicals following alloxan treatment was demonstrated *in vitro* (Grankvist 1981, Munday 1988) and *in vivo* (Kurahashi *et al*. 1993).

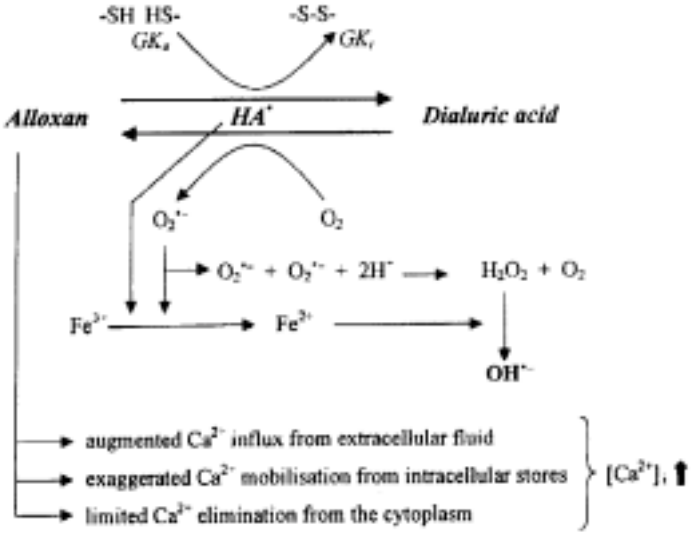

Fig. 1. *The mechanism of alloxan-induced reactive oxygen species generation in B cells of rat pancreas. GKa, GKi – glucokinase active and inactive, respectively; HA$^\bullet$ – alloxan radicals; $[Ca^{2+}]_i$ – intracellular calcium concentration.*

One of the targets of the reactive oxygen species is DNA of pancreatic islets. Its fragmentation takes place in B cells exposed to alloxan (Takasu *et al.* 1991a, Sakurai and Ogiso 1995). DNA damage stimulates poly ADP-ribosylation, a process participating in DNA repair. Some inhibitors of poly ADP-ribosylation can partially restrict alloxan toxicity. This effect is, however, suggested to be due to their ability to scavenge free radicals rather than to a restriction of poly ADP-ribosylation initiated by alloxan (Sandler and Swenne 1983, LeDoux *et al.* 1988). Superoxide dismutase, catalase (EC 1.11.1.6) (Grankvist *et al.* 1979, Grankvist 1981, Jörns *et al*. 1999) and non-enzymatic scavengers of hydroxyl radicals (Ebelt *et al.* 2000) were also found to protect against alloxan toxicity. Therefore, chemicals rendering anti-oxidative properties and inhibiting poly ADP-ribosylation can attenuate alloxan toxicity.

It has been argued that glucose counteracts alloxan cytotoxicity *in vitro* and *in vivo*. This ability, however, is not only the result of the protection of glucokinase. The protective effect of glucose against necrotic death of B cells may be due to interaction of the sugar with the glucose transporter GLUT2 resulting in limited alloxan uptake (Jörns *et al*. 1997).

It has been previously proposed that the action of glucose is also related to its metabolism and to the increased generation of reducing equivalents (NADH and NADPH) accelerating the recirculation of glutathione. GSH is known to provide protection against free radicals (Donnini *et al*. 1996). It may thus divert hydrogen peroxide from the pathway leading to the formation of hydroxyl radicals (Malaisse 1982, Malaisse-Lagae *et al*. 1983, Pipeleers and van de Winkel 1986):

$$GSSG + 2\,NADPH \rightarrow 2\,GSH + 2\,NADP^+$$

$$H_2O_2 + 2\,GSH \rightarrow GSSG + 2\,H_2O$$

Moreover, Sakurai and Ogiso (1991) observed that the *in vitro* generation of hydroxyl radicals in the presence of alloxan strongly depends on GSH concentration. GSH in low concentrations potentiated the formation of these radicals, whereas the oxygen consumption, autoxidation of dialuric acid and formation of hydroxyl radicals were significantly inhibited in higher concentrations. GSH at high concentrations can also inhibit HA$^\bullet$ generation and directly neutralize hydroxyl radicals. Thiyl radicals (GS$^\bullet$) formed in this reaction are then converted to GSSG:

$$GSH + OH^{\bullet-} \rightarrow GS^\bullet + H_2O$$

$$GS^\bullet + GS^\bullet \rightarrow GSSG$$

Indeed, in rat islets incubated with alloxan the GSH content and GSH/GSSG ratio were decreased (Malaisse *et al*. 1982), whereas glucose evoked the opposite effect.

In the *in vivo* experiment, glucose given to rats 20 min prior to alloxan partially restricted alloxan-induced increase in the activity of glutathione peroxidase (EC 1.11.1.9) and mitigated the drop of liver nonprotein -SH groups (especially reduced glutathione) (Szkudelski *et al*. 1998). The protective action of this sugar is, however, strongly glucose and alloxan dose-dependent (Harman and Fischer 1982, Gorray *et al*. 1983).

It has been proposed that disturbances in intracellular calcium homeostasis constitute an important step in the diabetogenic action of alloxan. This concept was confirmed by *in vitro* and *in vivo* experiments demonstrating that alloxan elevates cytosolic free Ca^{2+} concentration in pancreatic B cells (Kim *et al*. 1994, Park *et al*. 1995). This effect arises from several events: alloxan-induced calcium influx from extracellular fluid, exaggerated calcium mobilization from intracellular stores and its limited elimination from the cytoplasm. The calcium influx may result from the ability of alloxan to depolarize pancreatic B cells (Dean and Matthews 1972). Depolarization of the cell membrane opens voltage-dependent calcium channels and enhances calcium entry into cells. Alloxan was also found to exert a stimulatory effect on mitochondrial Ca^{2+} efflux with simultaneous inhibitory action on Ca^{2+} uptake by mitochondria (Nelson and Boquist 1982, Lenzen *et al.* 1992). The restriction of calcium removal from the cells due to alloxan-induced inhibition of liver plasma membrane Ca^{2+}-ATPase was also reported (Seckin *et al.* 1993). The effect of alloxan on intracellular calcium concentration seems to be mediated, at least partially, by H_2O_2 since hydrogen peroxide itself exerts a similar effect on calcium concentration in B cells (Park *et al*. 1995).

Thus, the previously mentioned sudden rise in insulin release from B cells treated with alloxan (Weaver *et al*. 1978b, Kliber *et al.* 1996) may be one of the effects of alloxan-induced augmentation in cytosolic Ca^{2+} concentration (Weaver *et al*. 1978b, Kim *et al*. 1994). The exaggerated concentration of this ion contributes to supraphysiological insulin release and, together with reactive oxygen species, causes damage of pancreatic B cells.

The results of experiments with calcium channel antagonists have confirmed the important role of cytosolic calcium in the cytotoxic action of alloxan. Pretreatment of rats with verapamil prevented the alloxan-induced increase in B cell Ca^{2+} concentration and abolished the stimulatory effect of alloxan on insulin release (Kim *et al*. 1994). The calcium channel antagonists (verapamil and diltiazem) also suppressed hyperglycemia and the onset of alloxan diabetes in rats (Katsumata *et al*. 1992, Kim *et al*. 1994).

Summing up, the toxic action of alloxan on pancreatic B cells, described many years ago by Dunn *et al.* (1943), are the sum of several processes such as oxidation of essential -SH groups, inhibition of glucokinase, generation of free radicals and disturbances in intracellular calcium homeostasis.

Many investigators suggested that the selectivity of alloxan action is not quite satisfactory. Recent experiments confirmed this objection. The diabetogenic dose of alloxan was found to decrease -SH groups accompanied by a simultaneous rise in glutathione peroxidase activity in the rat liver two minutes after its administration (Szkudelski *et al*. 1998). At the same time, the blood insulin concentration rose dramatically. This exaggerated insulinemia did not evoke, however, any significant reduction of blood glucose suggesting impaired peripheral insulin sensitivity in the short time after alloxan treatment (Szkudelski *et al.* 1998). It was also observed that alloxan intensified basal and epinephrine-induced lipolysis in isolated rat adipocytes and insulin failed to restrict this effect (Kandulska *et al*. 1999).

Thus, using alloxan to evoke diabetes, animals should be examined after proper period of time to minimize side effects of alloxan action. It should also be emphasized that the range of the diabetogenic dose of alloxan is quite narrow and even light overdosing may be generally toxic causing the loss of many animals. This loss is most likely due to kidney tubular cell necrotic toxicity, in particular when too high doses of alloxan are administered (Lenzen *et al.* 1996).

2. The mechanism of streptozotocin action

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is synthesized by *Streptomycetes achromogenes* and is used to induce both insulin-dependent and non-insulin-dependent diabetes mellitus (IDDM and NIDDM, respectively).

The range of the STZ dose is not as narrow as in the case of alloxan. The frequently used single intravenous dose in adult rats to induce IDDM is between 40 and 60

mg/kg b.w. (Ganda *et al*. 1976), but higher doses are also used. STZ is also efficacious after intraperitoneal administration of a similar or higher dose, but single dose below 40 mg/kg b.w. may be ineffective (Katsumata *et al*. 1992). For instance, when 50 mg/kg b.w. STZ are injected intravenously to fed rats, blood glucose (determined 2 weeks after treatment) can reach about 15 mM (Szkudelski, unpublished observations).

STZ may also be given in multiple low doses. Such treatment is used predominantly in the mouse and the induction of IDDM is mediated by the activation of immune mechanisms. However, Ziegler *et al.* (1984) and Wright and Lacy (1988) demonstrated that the non-specific activation of the immune system *via* complete Freund's adjuvant prior to STZ injections allows to reduce its diabetogenic dose even in the rat.

NIDDM can easily be induced in rats by intravenous or intraperitoneal treatment with 100 mg/kg b.w. STZ on the day of birth. This method of NIDDM induction was described for the first time by Portha *et al.* (1974). At 8-10 weeks of age and thereafter, rats neonatally treated with STZ manifest mild basal hyperglycemia, an impaired response to the glucose tolerance test (Portha *et al.* 1979) and a loss of B cell sensitivity to glucose (Giroix *et al*. 1983).

Streptozotocin action in B cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. Two hours after injection, the hyperglycemia is observed with a concomitant drop in blood insulin. About six hours later, hypoglycemia occurs with high levels of blood insulin. Finally, hyperglycemia develops and blood insulin levels decrease (West *et al.* 1996). These changes in blood glucose and insulin concentrations reflect abnormalities in B cell function. STZ impairs glucose oxidation (Bedoya *et al*. 1996) and decreases insulin biosynthesis and secretion (Bolaffi *et al.* 1987, Nukatsuka *et al*. 1990b). It was observed that STZ at first abolished the B cell response to glucose. Temporary return of responsiveness then appears which is followed by its permanent loss and cells are damaged (West *et al*. 1996).

STZ is taken up by pancreatic B cells *via* glucose transporter GLUT2. A reduced expression of GLUT2 has been found to prevent the diabetogenic action of STZ (Schnedl *et al*. 1994, Thulesen *et al*. 1997). Wang and Gleichmann (1995, 1998) observed that STZ itself restricts GLUT2 expression *in vivo* and *in vitro* when administered in multiple doses.

Intracellular action of STZ results in changes of DNA in pancreatic B cells comprising its fragmentation (Yamamoto *et al*. 1981, Morgan *et al*. 1994). Recent experiments have proved that the main reason for the STZ-induced B cell death is alkylation of DNA (Delaney *et al.* 1995, Elsner *et al.* 2000). The alkylating activity of STZ is related to its nitrosourea moiety, especially at the O^6 position of guanine. After STZ injection to rats, different methylated purines were found in tissues of these animals (Bennett and Pegg 1981).

Since STZ is a nitric oxide (NO) donor and NO was found to bring about the destruction of pancreatic islet cells, it was proposed that this molecule contributes to STZ-induced DNA damage (Kröncke *et al*. 1995, Morgan *et al*. 1994). The participation of NO in the cytotoxic effect of STZ was confirmed in several experiments (Turk *et al*. 1993, Kröncke *et al*. 1995). Pancreatic B cells exposed to STZ manifested changes characteristic for NO action, i.e. increased activity of guanylyl cyclase and enhanced formation of cGMP (Turk *et al*. 1993). STZ is, however, not a spontaneous nitric oxide donor (Kröncke *et al*. 1995). This molecule is liberated when STZ is metabolized inside cells, but NO synthase is not required for this effect (Kröncke *et al*. 1995). On the other hand, the lowering of NO concentration in pancreatic islet cells by inhibition of the inducible form of nitric oxide synthase partially counteracted DNA cleavage induced by STZ (Bedoya *et al*. 1996). A similar effect can be attained by NO scavengers (Kröncke *et al*. 1995). However, the results of several experiments provide the evidence that NO is not the only molecule responsible for the cytotoxic effect of STZ. STZ was found to generate reactive oxygen species, which also contribute to DNA fragmentation and evoke other deleterious changes in the cells (Takasu *et al*. 1991b, Bedoya *et al*. 1996). The formation of superoxide anions results from both STZ action on mitochondria and increased activity of xanthine oxidase (EC 1.1.3.22). It was demonstrated that STZ inhibits the Krebs cycle (Turk *et al*. 1993) and substantially decreases oxygen consumption by mitochondria (Nukatsuka *et al*. 1990b). These effects strongly limit mitochondrial ATP production and cause depletion of this nucleotide in B cells (Nukatsuka *et al*. 1990b, Sofue *et al*. 1991). Restriction of mitochondrial ATP generation is partially mediated by NO. This molecule was found to bind to the iron-containing aconitase inhibiting enzyme activity (Welsh and Sandler 1994).

Augmented ATP dephosphorylation increases the supply of substrate for xanthine oxidase (B cells possess high activity of this enzyme) and enhances the production of uric acid – the final product of ATP

degradation (Nukatsuka *et al*. 1990a). Then, xanthine oxidase catalyses reaction in which the superoxide anion is formed (Nukatsuka *et al*. 1988). As a result of superoxide anion generation hydrogen peroxide and hydroxyl radicals are formed (Nukatsuka *et al*. 1990a, Takasu *et al*. 1991b). The inhibition of xanthine oxidase by allopurinol restricts the cytotoxic effect of STZ *in vitro*. Pretreatment of B cells with this inhibitor prevented the STZ-induced decrease of insulin secretion (Nakatsuka *et al*. 1990a).

It can be stated that potent alkylating properties of STZ are the main reason of its toxicity. However, the synergistic action of both NO and reactive oxygen species may also contribute to DNA fragmentation and other deleterious changes caused by STZ. NO and reactive oxygen species can act separately or form the highly toxic peroxynitrate (ONOO; Fig. 2). Therefore, intracellular antioxidants or NO scavengers substantially attenuate STZ toxicity.

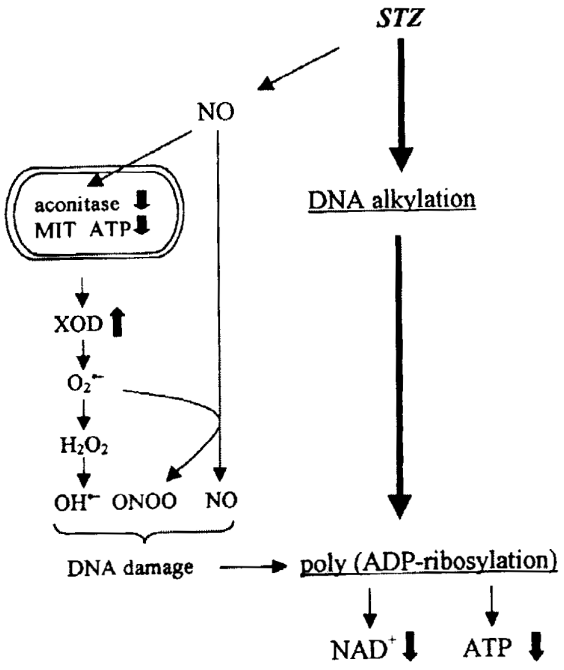

Fig. 2. *The mechanism of streptozotocin (STZ)-induced toxic events in B cells of rat pancreas. MIT – mitochondria; XOD – xanthine oxidase*

STZ-induced DNA damage activates poly ADP-ribosylation (Sandler and Swenne 1983). This process leads to depletion of cellular NAD^+, further reduction of the ATP content (Heller *et al.* 1994) and subsequent inhibition of insulin synthesis and secretion (Nukatsuka *et al*. 1990b). The concept of unfavorable consequences of augmented poly ADP-ribosylation as a result of STZ action was confirmed by experiments revealing that the inhibition of this process prevents the toxicity of this diabetogenic agent. It was found that 3-aminobenzamide, a strong inhibitor of poly(ADP-ribose) synthase, protected against the action of STZ in rats, even when this substance was administered 45-60 min after STZ (Masiello *et al*. 1985, 1990). Another inhibitor of poly(ADP-ribose) synthase, nicotinamide, which is also scavenging oxygen free radicals, exerted best protection when it was administered shortly after STZ (Masiello *et al*. 1990). The failure of protective action of nicotinamide administered after STZ is probably due to a potent reduction of the cellular ATP content by STZ since nicotinamide uptake is ATP-dependent (Sofue *et al*. 1991). The protective effect of 3-aminobenzamide and nicotinamide was also confirmed *in vitro* (Masiello *et al*. 1990).

It has been suggested that some inhibitors of poly ADP-ribosylation may also exert a protective effect due to their hydroxyl radical scavenging properties (LeDoux *et al*. 1988). However, in the case of STZ, recent investigations in poly(ADP-ribose) polymerase-deficient mice demonstrated that the inhibition of poly ADP-ribosylation itself prevents STZ-induced B cell damage and hyperglycemia (Pieper *et al*. 1999). Thus, it can be stated that the activation of poly ADP-ribosylation is of greater importance for the diabetogenicity of STZ than generation of free radicals and DNA damage *per se*.

Calcium, which may also induce necrosis, does not seem to play a significant role in the necrosis evoked by STZ since calcium channel antagonists do not protect B cells against streptozotocin, as they do in the case of alloxan (Katsumata *et al*. 1992).

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