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Research Article

Alloxan acts as a prooxidant only under reducing conditions: influence of melatonin

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Abstract. Depending on the availability of suitable reducing agents, alloxan can be either a prooxidant or an antioxidant. Alloxan and its reduced derivative, dialuric acid, act as a redox couple, driven by reduced glutathione (GSH) or L-cysteine, generating in vitro in the presence of oxygen, both superoxide radical and hydrogen peroxide. The production of superoxide radicals was shown by the appearance of lucigenin chemiluminescence (CL) as well as by the generation of formazan from nitroblue tetrazolium (NBT). The lucigenin CL as well as the NBT reduction was inhibited by superoxide dismutase and partially by catalase. Melatonin inhibited alloxan-mediated CL. In contrast, in the absence of reducing agents, alloxan is a scavenger of superoxide radicals formed by other reactions. Because of the high content of reducing compounds in the cell (e.g. glutathione), it is suggested that alloxan acts in vivo mainly as a generator of reactive oxygen species.

Key words. Alloxan; free radicals; oxidative stress; diabetes; melatonin; antioxidant; prooxidant; lucigenin; NBT.

Under physiological conditions cells always generate a certain amount of reactive oxygen species (ROS). The endangering potential of a small number of ROS is normally counterbalanced by a well-staggered system of multiple cellular defence mechanisms. But if the formation of radicals temporarily exceeds the capacity for their detoxification, cells suffer from 'oxidative stress' with potentially fatal consequences for a variety of macromolecular compounds and structures inside the cells.

In general, alloxan generates ROS in the presence of reducing compounds. Therefore, ROS formation takes place also when alloxan comes into contact with pancreatic B-cells [1–8]. There are contradictory opinions about the alloxan-mediated formation of ROS in B-cells. It has been reported that either superoxide radicals [1, 2, 4–6], hydrogen peroxide [1, 3–6, 8], hydroxyl radicals [1, 5, 8] or alloxan radicals [4, 9–11] could be responsible for the cytotoxic and diabetogenic effects of alloxan on the B-cells of the islets of Langerhans. Other authors have questioned whether ROS are involved in the diabetogenic action of alloxan and have proposed that alloxan acts as a radical scavenger [9, 10].

Melatonin is successfully able to scavenge ROS [12–15]. Therefore, it is suggested that melatonin may counteract the diabetogenic action of alloxan, provided that this action of alloxan is mediated by ROS.

In the present study we demonstrate that alloxan is able to form primarily superoxide radicals and subsequently hydrogen peroxide in the presence of reducing agents which are sufficiently available in pancreatic B-cells. Thus, the rapid formation of ROS together with a

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simultaneous depletion of the reducing capacity (being part of the cellular defence machinery) gives rise to increasing oxidative stress within pancreatic B-cells, and this might be attenuated by concentrations of melatonin between 0.1 and 1 mM.

Material and methods

Chemicals used were purchased from Sigma Chemical Company, St. Louis, MO, USA [alloxan monohydrate, xanthine oxidase (grade I from buttermilk, 0.68 U/mg protein), nitroblue tetrazolium (NBT), xanthine, bis-*N*-methylacridinium nitrate (lucigenin), L-cysteine, phosphate-buffered saline (PBS), superoxide dismutase (SOD) from bovine erythrocytes, catalase from *Aspergillus niger*, melatonin], from Serva, Heidelberg, Germany [*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethane sulfonic acid (HEPES)], from Calbiochem-Novabiochem, La Jolla, USA [reduced glutathione (GSH)], from ICN Pharmaceuticals, Costa Mesa, CA, USA [Tris(hydroxymethyl)-aminomethan]. All other reagents were obtained either from Merck, Darmstadt, or from Fluka, Deisenhofen, Germany.

Chemiluminometric assay. The standard assay system consists of 930 µl of PBS-HEPES buffer (25 mM, pH 8.0, prior equilibration with air), 50 µl of lucigenin (1 mM stock), 10 µl of L-cysteine (100 mM stock). Generation of chemiluminescence (CL) was initiated by addition of 10 µl of alloxan (10 mM stock, dissolved in distilled water, pH 3.0). In some experiments melatonin was present in the reaction mixture at initial concentrations between 0.1 and 1 mM prior to addition of alloxan. SOD, NBT or catalase were added after 4 min of CL registration. To prove the ability of alloxan to scavenge XO-generated superoxide radicals, the following assay system was used: 935 µl of PBS-HEPES buffer (25 mM, pH 8.0), 50 µl of lucigenin (1 mM), 5 µl of XO (3 mU/ml) and 10 µl of xanthine (10 mM dissolved in 10 mM NaOH). After CL registration over 5 min, 10 µl of alloxan (10 mM stock) was added, the sample was rapidly mixed and then CL registration continued for further 3 min. A Lumat LB9501 (EG&G Berthold, Wildbad, Germany) was used for CL determination. The CL measurements were performed at 25 °C.

Spectrophotometric assay. The assay system for performing the wavelength scan consists of 980 µl of Tris-HCl buffer (20 mM, pH 7.2) with or without 10 µl of GSH (100 mM stock). Data were monitored in intervals of 2 min at 25 °C immediately after addition of 10 µl of alloxan (10 mM stock, pH 3.0, dissolved in H_2O) for 18 min using a DU 640-Spectrophotometer (Beckman Instruments, Fullerton, CA, USA).

Spectrophotometric NBT assay. The standard reaction mixture consists of 970 µl of Tris-HCl buffer (20 mM, pH 7.2, well equilibrated with air prior use), 10 µl of NBT (10 mM stock), 10 µl of GSH (100 mM stock). The NBT reduction was started by the addition of 10 µl of alloxan (10 mM stock), and the progress of the reaction was recorded at 570 nm. A part of the experiments was performed in the presence of SOD (4 µg/ml) or catalase (1.5 µg/ml). In other experimental approaches, the Tris-HCl buffer (20 mM, pH 7.2) was equilibrated prior to use either with nitrogen or with oxygen. The measurements were performed at 25 °C. Data are expressed as mean ± SEM of at least three independent measurements.

Results

Alloxan acts as an antioxidant in the absence of reducing agents. In the absence of suitable electron donors, the pH shift of a solution containing alloxan from pH 3.0 to pH 7.2 leads to a rapid disappearance of the alloxan absorption at 245 nm (fig. 1B). Alloxan only acts during this transformation as an antioxidant as demonstrated by its ability to interrupt the lucigenin CL of the xanthine oxidase-triggered superoxide radical formation (fig. 2). After complete disappearance of the peak at 245 nm, the ability to be an antioxidant is lost. Subsequent addition of a reducing agent (e.g. GSH) does not induce further spectral changes (not shown).

Alloxan acts as a prooxidant in the presence of electron donors. On the other hand, addition of GSH prior to

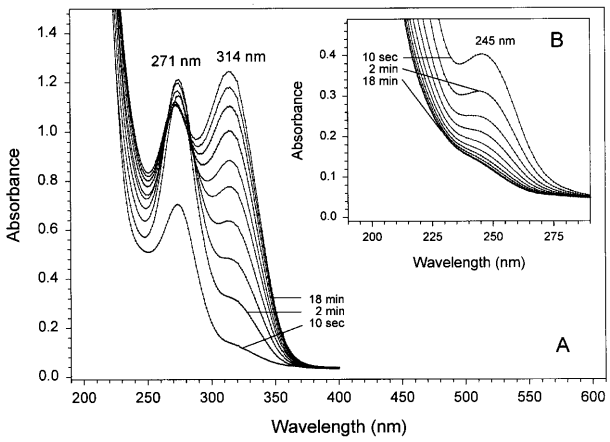

Figure 1. Time-dependent changes in the spectra of alloxan in the presence or absence of GSH at 25 °C and at pH 7.2. Reaction mixture A consists of 980 µl of Tris-HCl buffer (20 mM, pH 7.2), 10 µl of GSH (100 mM stock) and 10 µl of alloxan (10 mM stock, pH 3.0). Reaction mixture B contains 990 µl of Tris-HCl buffer (20 mM, pH 7.2) and 10 µl of alloxan (10 mM stock, pH 3.0). The spectra were recorded at 2-min intervals starting immediately after addition of alloxan to GSH (A) or in the absence of GSH (B).

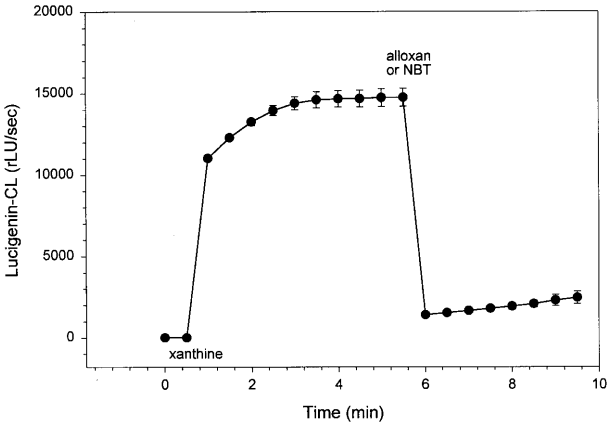

Figure 2. Influence of alloxan on lucigenin CL triggered by the xanthine oxidase reaction. The assay system consists of 935 µl of PBS-HEPES buffer (25 mM, pH 8.0), 50 µl of lucigenin (1 mM) and 5 µl of XO (containing 3 mU/ml). CL formation was initiated by addition of 10 µl of xanthine (10 mM) and stopped by addition of 10 µl of alloxan (10 mM, pH 3.0). The final concentration of alloxan in the assay amounted to 0.1 mM. CL measurements were performed at 25 °C. An analogous experiment with a similar result was performed with NBT instead of alloxan (not shown). In the CL assay, the pH of the sample was not influenced by addition of the reactants.

alloxan induces a rapid increase in absorption at 271 nm (completed within 2 min and lasting over 60 min) which was accompanied by a slower but continuous increase at 314 nm (fig. 1A). The height of this peak at 271 nm, probably corresponding to the amount of dialuric acid formed from alloxan under reducing conditions, depends on the ratio between GSH and alloxan (fig. 3) as well as on the availability of an acceptor of electrons. Under anaerobic conditions, the 271-nm peak is about 15% higher (not shown). This GSH-dependent formation of dialuric acid from alloxan is the decisive precondition for the subsequent ROS generation.

Indications for superoxide radical and hydrogen peroxide formation. Under reducing conditions, lucigenin-derived CL as an indicator for superoxide radicals was immediately generated when alloxan was added to lucigenin in the presence of cysteine (fig. 4). Neither cysteine nor alloxan alone were able to generate a lucigenin CL. Cysteine can be substituted by GSH (not shown). The CL is almost completely blocked after the addition of SOD and partially attenuated by catalase (fig. 4B). Addition of melatonin leads to a concentration-dependent depression of the lucigenin CL (fig. 4A).

NBT is a dye which is thought to react with superoxide radicals by forming blue-coloured formazan [16, 17]. Therefore, we used NBT as an additional tool to prove whether GSH and alloxan at pH 7.2 is a superoxide radical-producing redox couple. Blue-coloured formazan was formed during the interaction between alloxan and GSH (fig. 5). The rate of NBT reduction was influenced by the molar ratio between GSH and alloxan as well as by the pO_2 (partial pressure of oxygen) of the incubation buffer. The rate of superoxide radical formation and as a result the dye generation became faster when the ratio between GSH and alloxan was close to 10 to 1. No formazan was detected at a ratio of GSH to alloxan of 1 to 10 (fig. 5). Formazan generation is partially inhibited by SOD as well as by catalase (fig. 6B). Anaerobic conditions drastically enhanced the NBT transformation, whereas buffer equilibration with oxygen decreased the rate of dye formation (fig. 6A).

When superoxide radical generation via the xanthine oxidase system was chemiluminometrically measured, addition of NBT led to an immediate breakdown of lucigenin CL (fig. 2). During this interaction formazan was also generated, demonstrating the ability of superoxide radicals to reduce NBT. Neither alloxan nor NBT inhibit xanthine oxidase (not shown).

Discussion

The schematic diagram (fig. 7) summarises our understanding which was deduced from data taken from literature as well as from our own results concerning the ambivalence of alloxan properties determined mainly from the availability of appropriate donors and acceptors of electrons.

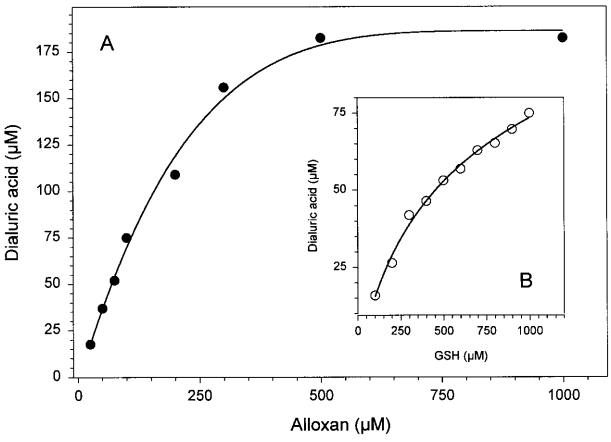

Figure 3. Effect of alloxan and constant GSH concentration on formation of dialuric acid at 25 °C. Measurements were performed at 271 nm in Tris-HCl buffer (20 mM, pH 7.2) at either constant GSH concentration (1 mM, fig. 3A), varying alloxan concentrations, or at constant alloxan concentration (0.1 mM, fig. 3B), varying GSH concentrations, in the cuvette. From the resulting maximal difference in the absorption between blank and sample, the corresponding concentration of dialuric acid was calculated using the molar extinction coefficient given by Munday et al. [22].

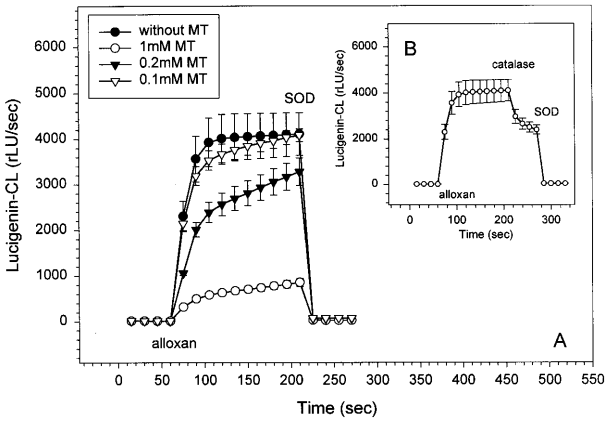

Figure 4. Detection of superoxide radical formation by lucigenin CL triggered by the interaction between L-cysteine and alloxan and inhibited either by melatonin, SOD or catalase. Reaction mixture A consists of 935 µl of PBS-HEPES buffer (25 mM, pH 8.0), 50 µl of lucigenin (1 mM stock), melatonin (MT, final concentration between 0 and 1 mM) and 5 µl of XO (3 mU/ml). CL formation was started by addition of 10 µl of xanthine (10 mM stock). The reaction was stopped by addition of SOD (4 µg/ml). Reaction mixture B consists of 935 µl of PBS-HEPES buffer (25 mM, pH 8.0), 50 µl of lucigenin (1 mM stock), 5 µl of XO (3 mU/ml) and 10 µl of xanthine (10 mM stock). Addition of catalase (1.5 µg/ml) or SOD (4 µg/ml) was used to stop CL.

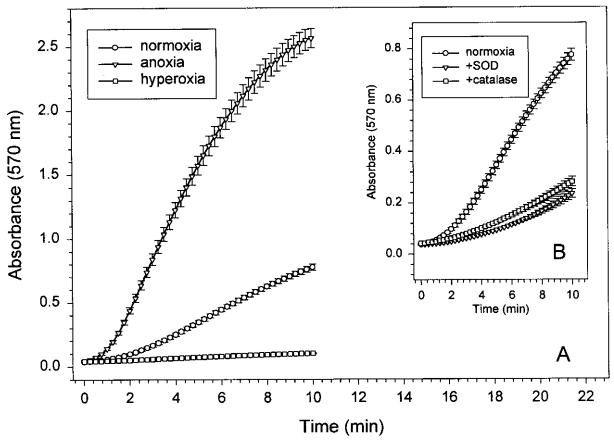

Figure 6. Effect of pO_2, SOD and catalase on the formazan generation from NBT in the presence of alloxan and GSH. The formation of formazan by alloxan and GSH was recorded at 570 nm (the absorption maximum of formazan), 25 °C and different pO_2 (fig. 6A) or under normoxic conditions in the presence of either SOD or catalase (fig. 6B). Reaction mixture A consisted of 970 µl of Tris-HCl buffer (20 mM, equilibrated either with air, nitrogen or oxygen for about 30 min, pH 7.2), 10 µl of NBT (10 mM stock), 10 µl of GSH (100 mM stock) and 10 µl of alloxan (10 mM stock). Reaction mixture B consists of 960 µl of air-saturated Tris-HCl buffer (20 mM, pH 7.2), 10 µl of NBT (10 mM stock), 10 µl of GSH (100 µM stock), 10 µl of catalase (1.5 µg/ml) or 10 µl of SOD (4 µg/ml) and 10 µl of alloxan (10 mM stock, pH 3.0).

Without reducing agents alloxan can act as a scavenger for superoxide radicals [9], as was also shown in figure 2. On the other hand, alloxan acts as a ROS generator in the presence of reducing equivalents via the redox couple alloxan and dialuric acid. This demonstrates that the property of alloxan is either an antioxidant or a prooxidant depending mainly on the availability of reducing agents. Because of the high GSH content in mammalian cells [18], alloxan probably acts in vivo mainly as a prooxidant.

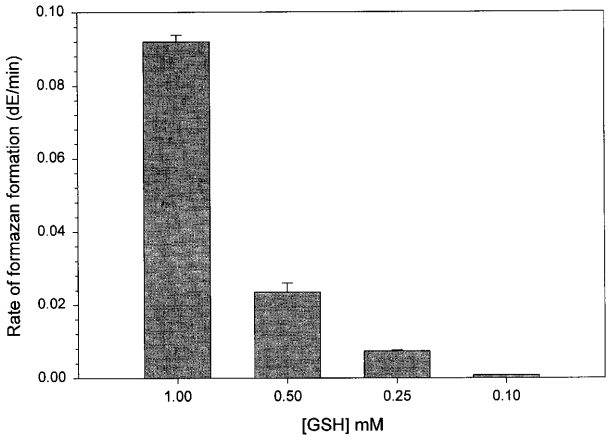

Figure 5. Influence of GSH and alloxan on the rate of formazan formation from NBT. The reaction mixture consists of 970 µl of Tris-HCl buffer (20 mM, pH 7.2), 10 µl of NBT (10 mM stock) and 1–10 µl of GSH (100 mM stock). Formazan formation at 25 °C was started after addition of 10 µl of alloxan (10 mM stock, pH 3.0). The increase in absorbance at 570 nm was used for calculation of the rate of formazan formation.

The decrease of light absorption at 245 nm reflects the irreversible decomposition of alloxan into alloxanic acid (fig. 1B). This observation corresponds well with data from literature [19]. From data presented in figure 1B, a half-life of alloxan at 25 °C and pH 7.2 can be calculated as 2–3 min. This value is in the same range as other data from literature [7, 19–21]. On the other hand, the increase of light absorption at 271 nm is probably connected with the formation of dialuric acid (fig. 1A), whereas the nature of the peak at 314 nm (not identical with alloxantin) remains unknown [22]. As for the peak at 271 nm, the rate of peak formation as well as the maximum height of the peak at 314 nm is increased at 25 °C under anaerobic conditions (data not shown).

These observations are comparable to those of other authors who found at pH 7.4 peak absorption of dialuric acid at 273 nm and an additional peak in the presence of GSH at 305 nm [7, 19].

Formation of dialuric acid is an essential prerequisite for any radical formation from alloxan under reducing conditions [3, 5, 8, 22]. This was demonstrated by us using chemiluminometric as well as colourimetric methods (see figs 4 and 6). Both methods are established systems for detecting superoxide radicals [16, 17, 23–25].

In CL experiments, addition of SOD almost completely stops light formation by alloxan and cysteine. This, as well as the ability of NBT to completely block superoxide radical-induced CL in the xanthine oxidase system, suggests a predominant generation of superoxide radicals by the interaction of alloxan and cysteine. The partial inhibition of redox couple-driven CL by catalase does not exclude a simultaneous formation of hydrogen peroxide. These results confirm earlier observations on SOD inhibition of oxygen uptake maintained by alloxan and GSH or cysteine by others [7, 26].

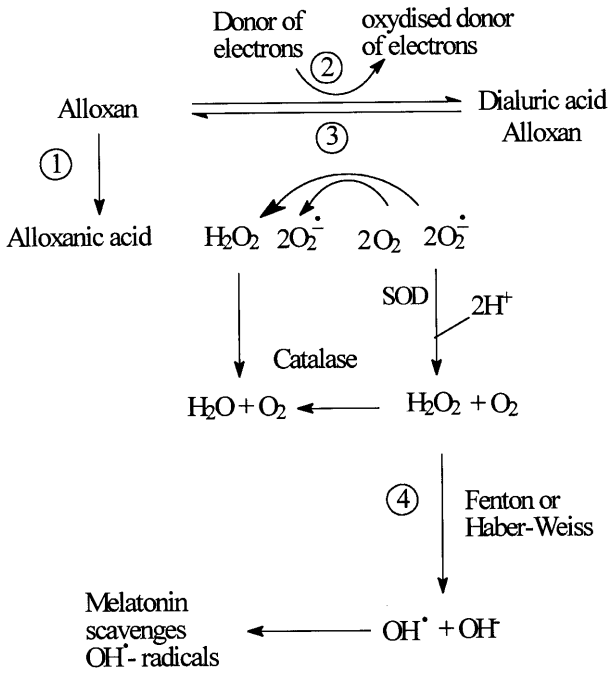

Figure 7. Schematic diagram: Alloxan is either able to act as a ROS generator or as a scavenger of superoxide anions depending on the presence or absence of a suitable donor of electrons. At neutral pH and in the absence of reducing agents, alloxan is rapidly oxidised to alloxanic acid (1), and during this decomposition alloxan can act as a scavenger of superoxide radicals. Once formed, alloxanic acid is not able to act as a generator of ROS. In the presence of an electron donor, alloxan can be reduced to dialuric acid (2). For this purpose, GSH appears to be the most important reducing agent within the cell, and the rapid generation of dialuric acid depends on the ratio between GSH and alloxan. Thus, a redox couple consisting of dialuric acid and alloxan is established, consuming GSH and oxygen and producing superoxide anions and hydrogen peroxide (3). The activity of this redox couple is limited only by the availability of alloxan, reducing agents (e.g. GSH) and suitable electron acceptors (e.g. oxygen). From hydrogen peroxide, the extremely aggressive hydroxyl radical can either be generated by the Haber-Weiss reaction, which additionally consumes superoxide radicals or, if transition metals are available, by the Fenton reaction (4). Melatonin is an effective scavenger of hydroxyl radicals and, at high concentrations, of superoxide anion radicals. Therefore, to a certain degree melatonin can counteract the diabetogenic effect of alloxan.

Colourimetric experiments also revealed only a partial inhibition of dye formation from NBT by SOD. This might be explained by competition between oxygen and NBT for the reducing equivalents formed by the redox couple. In the presence of both electron acceptors (oxygen and NBT), only a certain amount of reducing equivalents can be used for superoxide radical generation. In addition to the direct transfer of electrons to NBT, the formed superoxide radicals can subsequently also reduce NBT, and thus further enhance dye formation. The high affinity of NBT to superoxide radicals was demonstrated by the immediate and complete inhibition of CL in the presence of NBT in a mixture generating superoxide radicals via xanthine oxidase (see fig. 2). In this reaction formazan formation also takes place. Therefore, the addition of SOD stops the formation of formazan which was maintained by superoxide radicals. This assumption is supported by the fact that in the absence of oxygen the rate of dye formation increases. In contrast, any increase of the pO_2 in the buffer directly decreases NBT reduction, since oxygen seems to be the more efficient acceptor of electrons in comparison to NBT.

The inhibition of formazan generation from NBT in the presence of catalase demonstrates that part of the redox couple-driven superoxide radical formation could be transformed by GSH oxidation into hydrogen peroxide. Hydrogen peroxide per se does not influence dye formation. But after addition of catalase, the oxygen content of the buffer is partially regenerated, and so oxygen again competes successfully with NBT for the electrons. The GSH concentration in animal cells is between 1 and 10 mM [18]. The diabetogenic intravenous (i.v.) dosage of alloxan for rats amounts to 25–60 mg of alloxan/kg body weight [20]. The blood volume of a Wistar rat with a body weight of 250 g is $\sim$ 10 ml [27]. Thus, the initial concentration of alloxan in the blood is in the range of 3–10 mM. Alloxan is accumulated preferably in B-cells of the islets of Langerhans [28, 29]. Thus, our in vitro conditions for ROS generation by alloxan and GSH could approximately reflect the situation in B-cells within the first minutes after alloxan uptake. This means that, at the moment of the influx of alloxan into the B-cell, the precondition for the creation of a temporary active redox couple between alloxan and dialuric acid is fulfilled, as long as a sufficient amount of suitable reducing agents (e.g. GSH) is available in the cell. Redox cycling consumes GSH and oxygen. GSH is

oxidised to GSSG, and oxygen will be reduced to superoxide anion radicals and possibly further to hydrogen peroxide. On the other hand, GSH can also be a scavenger for the formed superoxide radicals. This indicates that besides an ongoing generation of superoxide radicals by the redox couple, an important antioxidative defence system (GSH) is simultaneously increasingly used up. As a consequence, certain SH groups in proteins could be more accessible to alloxan, which could lead to subsequent redox cycling and therefore to an increased probability of their oxidation [4, 5, 8, 20]. The superoxide radicals formed are transformed by SOD into hydrogen peroxide. Glutathione peroxidase and catalase, normally responsible for detoxification of H_2O_2 in the cytosolic compartment, are expressed at very low levels in B-cells [29, 30]. Together with the decrease in the GSH content of the B-cell in the presence of alloxan, shown by [29], the low activity of glutathione peroxidase as well as of catalase could cause a situation where the steady-state concentration of H_2O_2 in the B-cell is transiently increased, and thereby conditions for hydroxyl radical formation are favoured.

This explains why a pineal extract scavenges ROS formed by alloxan in rats [31]. Using a highly selective new spin trap for oxygen-centred radicals (DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide), we were able to confirm earlier observations of the remarkable scavenging properties of melatonin especially for hydroxyl radicals [12–15, 32, 33]. This scavenging effect was dependent on the concentration of melatonin in a range of 0.1 to 1.0 mM. Furthermore, it was shown that the presence of melatonin counteracts the alloxan-induced leakage of insulin from pancreatic B-cells and inhibits hydroxyl radical-mediated lipid peroxidation in liposomes [33]. It could also be demonstrated that melatonin reduces morphological damage of B-cells after application of alloxan [32, 33].

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