Alloxan inhibits ligand binding to adrenoceptors of vascular smooth muscle microsomes

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We have examined the effects of alloxan on the binding of [^3H]prazosin and [^{125}I]monoiodocyanopindolol (ICYP) to plasma-membrane-enriched microsomes isolated from dog aortas and dog mesenteric arteries respectively. Preincubation of the vascular smooth muscle membranes with alloxan reduced the number of binding sites of the α - and β -adrenoceptors in a concentration-dependent manner, whereas the affinity of the radioligands for the adrenoceptors was not affected by alloxan. Streptozotocin, which is also a diabetogenic agent like alloxan, had no effect on the radioligand binding to these adrenoceptors under similar experimental conditions. The inhibitory effects of alloxan on binding to β -adrenoceptors were found to be highly pH-dependent. These results indicate that alloxan exerts adverse effects on cell membrane adrenoceptors in addition to those on the ion-transport function of vascular smooth muscle cell [Kwan (1988) *Biochem. J.* 254, 293–296], and also suggest that the primary site of action of alloxan is the plasma membrane.

INTRODUCTION

Alloxan (2,4,5,6-tetraoxohexahydropyrimidine) is widely used for the chemical induction of diabetes mellitus in experimental animals and is also notorious for its cytotoxic effects on cellular metabolism, including inhibition of a wide spectrum of cellular enzymes (Webb, 1966) and of ion-transport function (Nelson & Boquist, 1982; Frei *et al.*, 1985; Kwan & Beazley, 1987). We have shown that preincubation of alloxan with plasmalemma-enriched microsomes isolated from the smooth muscle of dog aorta and mesenteric arteries of dogs and rats resulted in a dose-dependent inhibition of ATP-driven Ca^{2+} transport (Kwan & Beazley, 1987, 1988; Kwan 1988c). In agreement with the hypothesis put forth by Malaisse (1982), the plasma membrane component seems to be the primary site of action of alloxan (Kwan, 1988b). To further test the hypothesis that alloxan causes diffused damage to the cell membrane components, including not only the ion transport systems but also the membrane receptors, we studied the effects *in vitro* of alloxan on the cell membrane adrenoceptors of the dog vascular smooth muscles. Since α - and β -adrenoceptor densities were found to depend on the size of the vasculature (Kwan *et al.*, 1987, 1988), for α -adrenoceptors we used [^3H]prazosin binding to the dog aortic muscle microsomes, whereas for β -adrenoceptors we studied the [^{125}I]monoiodocyanopindolol (ICYP) binding to dog mesenteric arterial muscle microsomes.

MATERIALS AND METHODS

Preparation of microsomal membranes

Aortas and mesenteric arteries isolated from two or three mongrel dogs were used for each experiment. Dogs were killed by pentobarbital overdose and the blood vessels were removed and placed in ice-cold 250 mM-sucrose solution buffered with 10 mM-Mops buffer at pH 7.0. Aortas were trimmed to remove endothelium and adventitia (Kwan *et al.*, 1984) and mesenteric vasculature was trimmed to remove fat, mesenteries, nerve fibres and veins (Kwan *et al.*, 1983). Isolated arteries were homogenized in sucrose/Mops in a Polytron homogenizer and the microsomal membrane fractions were prepared by differential centrifugation

as previously described in detail (Kwan *et al.*, 1983, 1984). These microsomal fractions have been shown, by marker enzyme distribution and morphological criteria, to be highly enriched in plasma membranes with little contamination by endoplasmic reticulum and mitochondrial membrane fragments (Kwan *et al.*, 1983, 1984, 1987).

Ligand binding to adrenoceptors

We have previously shown that the level of [^3H]prazosin (α_1 -adrenoceptor antagonist) binding to dog mesenteric microsomal membranes was very low compared with that binding to dog aortic muscle microsomes, whereas the reverse was true for the level of [^{125}I]ICYP (β -adrenoceptor antagonist) binding to the microsomal fractions of these two types of arterial muscle (Kwan *et al.*, 1987). Such a heterogeneity in the level of [^3H]prazosin binding sites in dog arteries has also been reported (Bobik, 1982). We therefore used dog aortic muscle microsomes and mesenteric arteries to study the effects of alloxan on α - and β -adrenoceptors respectively.

[^{125}I]ICYP binding to vascular β -adrenoceptors. Binding assays were performed in triplicate using 2.2 mCi/nmol of ICYP (New England Nuclear) as previously described (Kwan *et al.*, 1987). The total binding incubation volume was 500 μl and consisted of 100 μl of ICYP diluted to the desired final concentration, 100 μl of microsomal membrane fraction (10–30 μg of protein) and 300 μl of 50 mM-buffer containing final concentrations of 150 mM-NaCl, 1 mM- MgCl_2 and 0.1 mM-ascorbic acid, pH 7.0. Binding experiments were started by addition of membrane fractions and the incubation mixture was maintained at 37 °C with constant shaking for 60 min. The binding was terminated by addition of 2.5 ml of incubation buffer and the mixture was filtered through Millipore HAWP filters on vacuum manifolds followed by four additional rinses, each of 2.5 ml of incubation buffer. Filters were counted for radioactivity for 2 min in a Beckman 5500 gamma counter with a 75% counting efficiency. Non-specific binding to the filters in the absence of added membranes as blanks and 100 μl of each ICYP concentration as standards were counted in each run under similar conditions. Non-specific binding, determined by parallel binding experi-

Fig. 1. Effects of alloxan or [³H]prazosin binding to dog aortic microsomes

(a) Inhibition of prazosin binding to dog aortic microsomes is dependent upon the concentration of alloxan. Microsomal fractions (30–50 μ g) were preincubated with desired concentrations of alloxan at 37 °C for 20 min before assaying for the number of binding sites using 0.3 nM [³H]prazosin. The results are means $\pm$ S.D.; $n = 3$. Binding in the absence of alloxan (89 ± 8 fmol/mg) is taken as 100%. (b) Inhibition of prazosin binding to dog aortic microsomes by alloxan is dependent upon the preincubation time. Experimental conditions were the same as described in (a), except that microsomal fractions were preincubated without (for control, $\circ$) or with 2.5 mg of alloxan/ml ($\bullet$) for the periods indicated (0–30 min) before binding assays. Binding before preincubation (93 ± 11 fmol/mg; $n = 3$) is taken as 100%. (c) Effects of alloxan (5 mg/ml; 20 min preincubation at 37 °C) on the saturation of [³H]prazosin binding to dog aortic microsomes. $\circ$, Control; $\bullet$, alloxan.

ments containing 1 μ M-DL-propranolol in the incubation medium, usually accounted for 20–50% of the total binding, depending upon the ligand concentration used, and increased linearly with both protein and radioligand concentration.

[³H]Prazosin binding to A-adrenoceptors. The binding conditions were basically the same as reported by Shi *et al.* (1989), except that the assay was performed in 50 mM-Tris at pH 7.0. Phentolamine (10 μ M) was used to define the specific binding of [³H]prazosin.

Preincubation of alloxan with membranes. Alloxan solutions at desired concentrations were prepared in 250 mM-sucrose solution buffered with 50 mM-Mops at pH 7.0. Equal volumes of alloxan solution and microsomal membrane suspension were mixed and placed in a temperature-controlled water bath with constant shaking at 37 °C for 20 min (Kwan & Beazley, 1987) or for a specified preincubation period. The control membrane fractions were treated similarly with vehicle solution containing no alloxan. For different experiments, any deviation from the above preincubation conditions is specified in the corresponding Figure legend. When incubation was carried out at different pH values, low-capacity (10 mM) buffer solutions were mixed in equal volume with membranes resuspended in 250 mM-sucrose and brought to the desired pH (sodium acetate for pH 4.8; Tris for pH 7.3; glycine for pH 9.4). At the end of incubation, aliquots were removed for binding assays (see above) in medium containing high buffering capacity (50 mM-Tris) with pH maintained at 7.0.

RESULTS

Fig. 1(a) shows the effects of increasing concentrations of alloxan in the preincubation medium on the binding of [³H]prazosin to α_1 -adrenoceptors of microsomes isolated from dog aortic smooth muscle. A 25–35% inhibition of [³H]prazosin binding was observed at concentrations of alloxan as low as 0.5 mg/ml, and 5 mg of alloxan/ml inhibited between 50 and 70% of binding in different experiments. The membranes were routinely preincubated with alloxan for 20 min, since our previous study showed that a 20 min preincubation period was optimal in causing inhibition of ATP-dependent Ca²⁺ transport (Kwan & Beazley, 1987). Fig. 1(b) shows that this is also true for

Fig. 2. Effects of alloxan on [¹²⁵I]ICYP binding to dog mesenteric artery microsomes

(a) Inhibition of ICYP binding to dog mesenteric artery microsomes is dependent upon the concentration of alloxan. Microsomal fractions (50–80 μ g) were preincubated with desired concentrations of either alloxan ($\bullet$) or streptozotocin ($\circ$) at 37 °C for 20 min before assaying for the number of binding sites using 100 pM [¹²⁵I]ICYP. Results are means $\pm$ S.D.; $n = 3$. (b) Effects of alloxan on the saturation profile (3–100 pM-ICYP) of [¹²⁵I]ICYP binding to dog mesenteric artery microsomes under the described conditions [$\circ$, control; $\bullet$, alloxan (5 mg/ml)]. (c) Effects of alloxan (5 mg/ml; 20 min preincubation at 37 °C) on the binding time (2–90 min at 100 pM-ICYP). Vertical bars represent S.D.; $n = 3$ ($\circ$, control; $\bullet$, alloxan).

[³H]prazosin binding. Without preincubation with membranes, 2.5 mg of alloxan/ml caused less than 20% inhibition of [³H]prazosin binding. Using another potent and commonly employed diabetogenic drug, streptozotocin (at 2.5 mg/ml),

Fig. 3. Effect of preincubation pH on the alloxan inhibition of binding of [^{125} I]ICYP to dog mesenteric artery microsomes

When preincubation with 5 mg of alloxan/ml (●) was carried out at different pH levels, low-capacity (10 mM) buffer solutions were mixed in equal volume with membranes resuspended in 250 mM-sucrose and brought to the desired pH [sodium acetate for pH 4.8 (a); Tris for pH 7.3 (b); glycine for pH 9.4 (c)]. Control samples (○) were treated similarly in the absence of alloxan. At the end of preincubation, aliquots were removed for binding assays in medium containing high buffering capacity (50 mM-Tris) with the pH maintained at 7.0. Each data point represents the mean $\pm$ S.D. from three separate experiments.

under the same experimental conditions, we were unable to detect any notable changes in [^{3} H]prazosin binding (results not shown) Fig. 1(c) shows that the inhibition of [^{3} H]prazosin binding to dog aortic muscle microsomes by alloxan was primarily due to its effect on the receptor density ($B_{\max}$), not on the affinity of the ligand for the receptors (K_d). Scatchard analysis of binding data in two separate experiments gives K_d values of 0.15 and 0.08 nM in control membranes and 0.16 and 0.07 nM in membranes pretreated with 5 mg of alloxan/ml for 20 min. In these two experiments, the $B_{\max}$ values were lowered by alloxan to 57% and 34% of the control value.

Fig. 2(a) shows that binding of ICYP to the β -adrenoceptors of smooth muscle microsomes from dog mesenteric arteries was inhibited by alloxan in a concentration-dependent manner (20 min preincubation). Such an inhibition, however, was not observed when the membranes were preincubated with streptozotocin. The results from both the equilibrium and the kinetic binding experiments (Fig. 2b) suggest that the primary change caused by alloxan was a decrease of the number of binding sites for ICYP and not the affinity of the ligand for the membrane receptors. Since our earlier study (Kwan & Beazley, 1987) indicated that the inhibitory effect of alloxan on ATP-dependent Ca^{2+} transport depends on the pH of the preincubation medium, we also examined the effect of pH on the inhibition of ICYP binding by alloxan. Fig. 3 shows that the inhibitory effect of alloxan was highly pH-dependent, being particularly potent at acidic pH. At pH 4.8, more than 90% of ICYP binding was inhibited by 5 mg of alloxan/ml. This is not a non-specific effect of acidic pH on membranes, since ICYP binding to the control membranes treated similarly but in the absence of alloxan was practically unaffected by the preincubation pH.

DISCUSSION

The cytotoxic effects of alloxan have long been recognized (Webb, 1966). Considering the pivotal role of intracellular Ca^{2+} in cellular injury and cell death, one probable mechanism of the cytotoxic actions of alloxan may be related to its adverse action on intracellular Ca^{2+} homeostasis. Since the intracellular Ca^{2+} concentration is under multiple membrane regulatory systems, and alloxan has been reported to interfere with mitochondrial and microsomal membrane Ca^{2+} -transport systems (Nelson & Boquist, 1982; Frei *et al.*, 1985; Kwan & Beazley, 1987), the subcellular site of action of alloxan on membrane functions has

remained obscure. More recently, we have provided evidence suggesting that the plasmalemmal ATP-dependent Ca^{2+} transport is the primary site of action of alloxan (Kwan, 1988a). Since adrenoceptors in the microsomal membrane fractions isolated from these canine vascular muscles are of plasmalemmal origin (Kwan *et al.*, 1987, 1988; Shi *et al.*, 1989), our novel finding in the present study of the inhibitory action of alloxan on cell membrane receptors provides additional support to our contention that the plasma membrane is indeed the primary site of action of alloxan. Furthermore, together with our previous findings (Kwan & Beazley, 1987, 1988; Kwan, 1988a,b), these results collectively support the notion that alloxan can cause a diffused change in cell membranes *in vitro*, including effects on both ion-transport function and ligand-receptor interactions.

Although this study was not intended to examine the molecular mechanism by which alloxan exerts its adverse effects on membrane receptors, we have previously presented evidence (Kwan & Beazley, 1988) that the inhibitory effect of alloxan on the ATP-driven Ca^{2+} transport of the vascular muscle membranes can be largely attributed to the action of hydrogen peroxide as well as the resulting hydroxyl free radicals generated during the oxidation-reduction cycles between alloxan and its reduced form, dialuric acid (Deamer *et al.*, 1971). This property is not shared with streptozotocin, which is another commonly used diabetogenic drug. Since the effects of alloxan on adrenoceptor binding shared common features with those on Ca^{2+} transport, including the requirement of preincubation of the membranes with alloxan, insensitivity to streptozotocin and alloxan concentration dependence, it is likely that alloxan may act via a similar mechanism in these two cases. In this regard, lipid peroxidation resulting from the attack of cell membranes by oxygen-derived free radicals has been shown to decrease the β -adrenoceptor density (Heikkilä, 1983). Some of these cell membrane changes *in vitro* (Kwan, 1988c) may provide a plausible interpretation for the transient and localized vascular effects of alloxan near the site of drug administration (MacQueen, 1952; Aviado & Schmidt, 1957). However, we are not aware of any long-term cytotoxic effect of alloxan *in vivo* on the vascular smooth muscle. Neither have we come across any study of the effect of alloxan on the receptor function of vascular muscle strips *in vitro*. In the light of the rapid degradation of alloxan in the circulation (Webb, 1966), the possible protection by the endothelial cell lining (Kwan, 1988c) and the high concentration of alloxan, as well as the non-physiological conditions (such as acidic pH) required for its

optimal effect *in vitro*, these biochemical results may have limited physiological significance. Nevertheless, our present findings using vascular smooth muscle membranes are of cytotoxicological significance if they can be extrapolated to pancreatic β -cells which are known to be selectively more susceptible to the cytotoxic effects associated with alloxan (Malaisse, 1982). It would also be of biochemical interest to further exploit the chemical nature of the β -adrenoceptor which is characteristically sensitive to insult by alloxan at acidic pH.

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