Pergamon

Biochemical Pharmacology, Vol. 47, No. 7, pp. 1271–1274, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0027-5

Evidence for redox cycling of diquat in rat small intestine

(*Received* 23 *September* 1993; *accepted* 26 *October* 1993)

Abstract—It has previously been established that acute diquat (1,1′-ethylene, 2,2′-bipyridilium) toxicity in the rat is associated with stimulation of net fluid secretion into the gastrointestinal tract. We have examined the possibility that the mechanism of diquat toxicity in the small intestine involves redox cycling of the bipyridyl leading to a disturbance of biochemical function and oxidative stress. Experiments performed *in vitro* showed that diquat (10 μM to 1 mM) produced an increase in activity of the pentose phosphate pathway in rat small intestinal tissue slices, suggesting that there was oxidation of NADPH even at concentrations of diquat which do not cause intestinal fluid secretion in anaesthetized rats. When the effect of diquat on pentose phosphate activity was measured in rats *in situ* at a dose which causes maximal fluid secretion [50 mM diquat dibromide ($DQBr_2$)], production of $^{14}CO_2$ from [1-^{14}C]-glucose increased by 278 ± 28% (N = 4) within 1 hr of exposure to diquat. Under these same conditions, the tissue content of NADPH in the proximal small intestine was significantly depleted, though there was no corresponding increase in $NADP^+$ concentration. Diquat had no effect on tissue concentrations of either the reduced or oxidized forms of NAD. It is likely that NADPH oxidation at low diquat concentrations can be adequately compensated for by mechanisms within the tissue which protect against oxidative stress. However, the data also suggest that diquat-induced fluid secretion in the rat small intestine is associated with redox cycling of bipyridyl leading to depletion of NADPH.

Diquat (1,1′-ethylene, 2,2′-bipyridilium) is a non-selective desiccant herbicide which is moderately toxic to animals [1] and produces renal dysfunction in humans following oral ingestion [2]. The mechanism of toxicity in mammals is unclear but it has been established that renal failure after an oral dose of diquat is secondary to a reduction in plasma volume caused by the abnormally excessive secretion of fluid into the gastrointestinal tract [3, 4]. This intestinal secretory effect, which is also associated with diquat toxicity in man [2], was more recently studied in greater detail in anaesthetized rats [5] where it was found that sublethal doses of diquat produced secretion without obvious histological damage to the intestinal mucosa. This observation has led us to propose that there is a biochemical or pharmacological mechanism involved in the stimulation of gastrointestinal fluid secretion in the rat by diquat.

Diquat is readily converted by one-electron reduction to a cation free radical which reacts rapidly with oxygen to produce a superoxide radical ion (O_2^-) and hydrogen peroxide, with regeneration of the bipyridyl. The reduction of diquat by liver, lung or kidney homogenates [6, 7] is dependent on NADPH [6] which is oxidized during the redox cycling of diquat, and as part of the defence against toxicity associated with generation of oxygen free radicals. Diquat and paraquat, a structurally related bipyridyl, both stimulate the pentose phosphate pathway in rat lung [8] which, under normal circumstances, is a mechanism used by cells to generate NADPH in response to NADPH oxidation [9]. However, despite the increase in glucose metabolism via the pentose phosphate pathway, paraquat (but not diquat) ultimately causes a depletion in lung concentrations of NADPH [10]. Furthermore, both bipyridyls increase glutathione concentrations in lung tissue [11], increasing the likelihood of glutathione conjugation with enzyme proteins and the subsequent perturbation of biochemical events and tissue damage.

It is possible that the intestinal secretion which is observed in rats when diquat is administered orally or instilled into the proximal small bowel is also the result of an altered biochemical balance due to redox cycling of diquat in the intestinal mucosa. Using a concentration of diquat known to stimulate intestinal fluid secretion in the anaesthetized rat, we measured the tissue concentration of pyridine nucleotides to determine if this effective secretory dose of bipyridyl would deplete tissue levels of NADPH. In addition, a series of experiments were performed *in vitro* and *in situ* to determine if diquat stimulates NADPH production through activation of the pentose phosphate pathway.

Materials and Methods

Diquat dibromide ($DQBr_2$) was obtained from Zeneca Agrochemicals (Jealott's Hill Research Station, Bracknell, U.K.). [1-^{14}C]Glucose and [6-^{14}C]glucose were purchased from Amersham International plc. (Amersham, U.K.) and alcohol dehydrogenase in ammonium sulphate solution was obtained from Boehringer Mannheim (East Sussex, U.K.). All other reagents were bought from the Sigma Chemical Co. (Poole, U.K.). Male Sprague–Dawley rats weighing 250–300 g were purchased from Charles River (Margate, U.K.) and housed in stock cages for at least 3 days before use. Rats were fed on standard PCD meal (Special Diet Services Ltd, Witham, U.K.) and water *ad libitum* unless stated otherwise.

Measurement of $^{14}CO_2$ *production.* Rats were killed by an overdose of halothane anaesthetic (Fluothane, ICI Pharmaceuticals, Macclesfield, U.K.) and approximately 15 cm of proximal small intestine was removed from each and placed in ice cold Krebs–Ringer phosphate buffer (NaCl, 136 mM; KCl, 5 mM; $CaCl_2$, 1.9 mM; $MgSO_4$, 1.3 mM; NaH_2PO_4, 7.5 mM; Na_2HPO_4, 13 mM; glucose, 11 mM). The tissue was sliced into 1 mm segments using a tissue chopper (Mickle Laboratory Engineering Co.) and five pieces of tissue were incubated in 3 mL of phosphate buffer containing diquat at concentrations of 0, 0.01, 0.1 or 1.0 mM at 37° under air in a shaking water bath for 30 min. Then, 0.5 μCi of [1-^{14}C]glucose or [6-^{14}C]glucose was added to the medium in the respirometer flasks and for a period of 60 min $^{14}CO_2$ was trapped in 0.2 mL of KOH (20% w/v), placed in the centre well of the flask together with a 2-cm square of hard filter paper (Whatman No. 542) to facilitate absorption. After incubating the tissue, the filter paper and KOH were transferred to plastic scintillation vials containing 1 mL water and 10 mL Optiphase 'MP' (Fisons plc, Loughborough, U.K.) and radioactivity was measured using a Packard Tri-Carb

2000CA liquid scintillation counter. The amount of $^{14}CO_2$ produced was expressed as dpm/10 mg tissue dry weight (tdw).

In a separate experiment, the activity of the pentose phosphate pathway was measured after the tissue had been exposed to 50 mM $DQBr_2$ (26 mM diquat ion) or 150 mM NaBr (control tissue) *in vivo* for 1 hr in anaesthetized rats as described in the methods below. At the end of the 1 hr exposure period, the tissues were removed from the rat, sliced and incubated for 60 min in Krebs–Ringer phosphate buffer containing 0.5 μCi of [1-^{14}C]glucose or [6-^{14}C]glucose according to the procedure described above.

Measurement of intestinal pyridine nucleotide content. The experimental procedure followed that described previously [5]. After an overnight fast, male rats were anaesthetized with sodium pentobarbital (Sagatal, 60 mg/mL, 80 mg/kg) before isolating two 10 cm sacs of proximal small intestine *in situ*, each with their blood supply intact. The proximal sac was filled with 500 μL of a 50 mM $DQBr_2$ solution made isotonic with 75 mM NaBr and the distal sac, which was used as the control, was filled with 500 μL of 150 mM NaBr. After a 1 or 2 hr incubation period, during which anaesthesia was maintained, the tissues were removed from the rat, drained and sliced into 1 mm segments using an automated tissue chopper. To extract the pyridine nucleotides, approximately 100 mg of tissue were placed in either 2 mL of hot (90°) 0.1 M HCl or 0.1 M NaOH and homogenized before being returned to the hot water bath for 1 min. After cooling on ice the extracts were buffered to pH 6 with K_2HPO_4 (acid extract) and to pH 8 with KH_2PO_4 (alkaline extract) and then centrifuged at 10,000 *g* for 10 min. The supernatant was removed and assayed for both the reduced and oxidized forms of NAD and NADP according to the enzymatic method of Nisselbaum and Green [11]. Data were expressed as nmol/100 mg tissue wet weight (tww). In all experiments, data were averaged and expressed as the mean with the SEM. Statistical analysis was performed using two-tailed Student's *t*-test.

Results and Discussion

Diquat has been shown to stimulate the production of $^{14}CO_2$ from [1-^{14}C]glucose by slices of rat lung *in vitro* at concentrations as low as 10 μM [8] so the question which we wished to address in the present study was whether diquat was equally reactive in intestinal tissue under similar circumstances. Diquat, at concentrations ranging from 10 μM to 1 mM, did not increase $^{14}CO_2$ production from [6-^{14}C]glucose in slices of rat intestine *in vitro* (Fig. 1), demonstrating that glycolysis was not stimulated beyond background activity in the intestinal tissue. In contrast, incubating slices of small intestine with [1-^{14}C]glucose in the presence of 10 μM diquat ion resulted in a significant ($P < 0.01$) elevation in $^{14}CO_2$ production, providing evidence for increased activation of the pentose phosphate pathway. Similarly, at a concentration of 1 mM diquat ion, the average production of $^{14}CO_2$ increased 207% when compared to control values ($P < 0.001$). This response was comparable to that obtained with the lower diquat concentration of 10 μM which would suggest that the pentose phosphate pathway may be maximally stimulated even by very low concentrations of bipyridyl. The pentose phosphate pathway is stimulated in response to NADPH oxidation [9] so it is apparent from these data that diquat has the ability to initiate redox cycling and oxidation of NADPH within the intestinal cells. However, since diquat at a concentration of 1 mM does not inhibit net fluid absorption [5], but does activate the pentose phosphate pathway, it seems likely that the rate of NADPH oxidation associated with this level of redox activity can be adequately compensated for by mechanisms within the tissue whose role it is to protect cells from damage by oxidative stress.

It has previously been established that near maximal intestinal fluid secretion is obtained after exposure *in situ* to 50 mM $DQBr_2$ in the anaesthetized rat [5]. Although fluid secretion was not measured in the present series of *in situ* experiments, it was evident from the yellow-green mucous secretion which could be drained from each diquat treated tissue that 50 mM $DQBr_2$ was indeed stimulating intestinal fluid secretion. The proximal small intestine from these diquat treated rats was removed in order to measure activity of the pentose phosphate pathway and to determine the pyridine nucleotide content of the small intestine. After exposure *in situ* to 50 mM $DQBr_2$ for 1 hr followed by incubation *in vitro* with [1-^{14}C]glucose, there was an average increase ($P < 0.05$) of 278 ± 28% ($N = 4$ rats) in tissue $^{14}CO_2$ production when data from control and diquat treated intestine were compared (Table 1). In contrast, $^{14}CO_2$ production from [6-^{14}C]glucose, which was converted to pyruvate by glycolysis, was unaffected by the presence

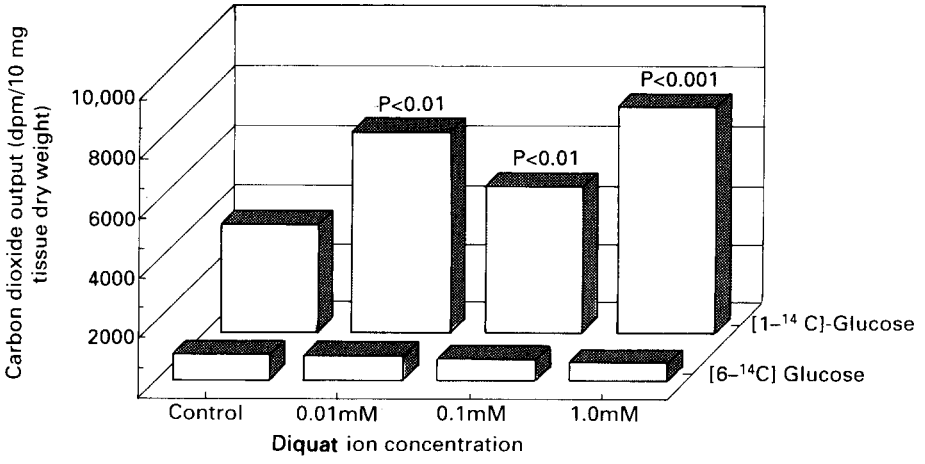

Fig. 1. The effect of diquat on the activity of the pentose phosphate pathway in slices of rat small intestine incubated *in vitro* with [1-^{14}C]glucose or [6-^{14}C]glucose. Activity was determined by the production of $^{14}CO_2$ expressed as dpm/10 mg dry weight tissue (tdw). Each measurement represents the mean of four determinations.

Table 1. The production of $^{14}CO_2$ from tissue incubated with [1-^{14}C]glucose or [6-^{14}C]glucose after exposure to 150 mM NaBr (control) or 50 mM $DQBr_2$ (diquat) *in vivo* in anaesthetized rats

	Control (dpm/10 mg tdw)	Diquat (dpm/10 mg tdw)
[1-^{14}C]Glucose	3573 ± 1023	9128 ± 1610*
[6-^{14}C]Glucose	1948 ± 408	1903 ± 323

The data are expressed as the mean ± SEM for four rats and standardized for tissue dry weight (tdw). *$P < 0.05$.

of diquat, signifying that the increased $^{14}CO_2$ production from [1-^{14}C]glucose was due to activity of the pentose phosphate pathway. This increase in the pentose phosphate pathway activity was similar to that obtained *in vitro* and confirms the view that there is limited stimulation of the pentose phosphate pathway in response to diquat induced NADPH oxidation.

The average control value of NADPH measured in segments of intestine after 1 hr of incubation *in situ* with 50 mM $DQBr_2$ was 1.38 ± 0.17 nmol/100 mg tww (N = 3), a level which did not change after 2 hr (1.40 ± 0.11 nmol/100 mg tww; N = 3) and which was similar to the concentration (0.90 ± 0.20 nmol/100 mg tww; N = 4 determinations) measured in tissue taken from a freshly killed rat, demonstrating that the surgical procedure did not alter tissue levels of NADPH. Similarly, the amount of $NADP^+$ measured in this same freshly obtained tissue was not significantly different from the concentration measured after 1 or 2 hr incubation *in situ* with 150 mM NaBr (0.72 ± 0.22 nmol/100 mg tww; mean ± SD for combined data, N = 7). The appearance of fluid within diquat treated sacs was associated with depletion in the tissue content of NADPH (Fig. 2) which was significant when compared to control values both at 1 hr ($P < 0.05$) and at 2 hr ($P < 0.002$). In contrast, there was no change in the tissue levels of $NADP^+$ (Fig. 2) and the ratio of NADPH to $NADP^+$ was reduced ($P < 0.05$) from a control value of 1.45 ± 0.50 (N = 3) to 0.31 ± 0.13 (N = 3) in the presence of diquat after 1 hr and from 2.46 ± 0.68 (N = 3) to 0.60 ± 0.07 (N = 3) after 2 hr exposure. This fall in the ratio of reduced to oxidized pyridine nucleotide appears to be due not simply to oxidation of NADPH to $NADP^+$ but to depletion of reduced pyridine nucleotide through some mechanism which has yet to be identified but which results in its irreversible loss or destruction. In liver microsomes prepared from Sprague–Dawley rats, rapid reduction of diquat is catalysed by microsomal NADPH-cytochrome P450 reductase [12], indicating that there is involvement of cytochrome-containing redox systems in the oxidation and possible depletion of NADPH. Although cytochrome P450 microsomal enzyme activity has been measured in rat intestine [13] there are quantitative and qualitative differences between liver and intestinal tissues, with the greater activity and variability observed in hepatic tissue. Whether cytochrome P450 enzymes are involved in the oxidation and depletion of NADPH in intestinal tissue has not been determined.

The effect of diquat on intestinal NADH and NAD^+ content was also determined (Fig. 2). Diquat had no significant effect on the tissue concentrations of either the reduced or oxidized form of this pyridine nucleotide, which is consistent with the finding that NADH is not oxidized by NADH-cytochrome b_5 reductase in microsomes from rat hepatocytes exposed to diquat [12]. Cytochrome b_5 is present in both microsomes and microvilli derived from rat intestine [14], but the failure of diquat to alter tissue NADH concentrations in rat small intestine would suggest that there is little if no involvement of NADH-cytochrome b_5 reductase in diquat redox cycling within this tissue. It is interesting to note, however, that the structurally related bipyridyl paraquat inhibits not only NADH concentrations in *Escherichia coli* bacteria but also the *de novo* synthesis of NAD [15].

The information derived from these experiments would suggest that diquat undergoes redox cycling within the small intestine. At a concentration which can induce fluid secretion, diquat depletes tissue levels of NADPH and this

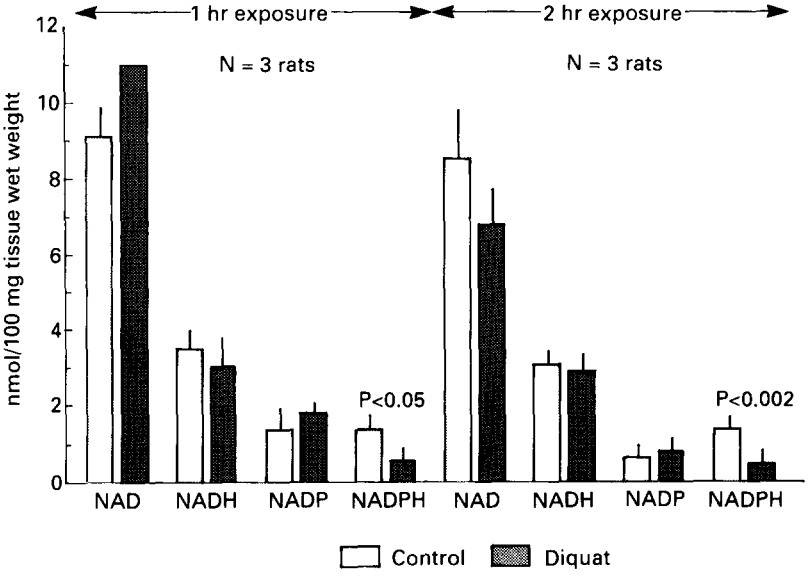

Fig. 2. Effect of 50 mM $DQBr_2$ on the oxidized and reduced forms of pyridine nucleotide content of rat small intestine after 1 and 2 hr incubation *in vivo*. Data are expressed as the means and SEM.

could ultimately lead to oxidative stress and disruption of biochemical events within the intestinal mucosa. Whether the biochemical disturbances themselves would directly result in a shift in the equilibrium for ion transport across epithelial membranes from an absorptive to a secretory status remains unclear, since it is not known if fluid transport in response to diquat is actively or passively driven. However, diquat does increase cytosolic free Ca^{2+} in rat liver [16] and since release of Ca^{2+} into the cytosol from cellular stores and the subsequent activation of Ca^{2+}-dependent ATPases is a prerequisite for fluid secretion by a number of tissues [17], there is reason to suggest that diquat-induced fluid secretion in the small intestine may indeed involve changes in active ion transport across epithelial membranes.

Zeneca Central Toxicology Laboratory
Alderley Park
Macclesfield
Cheshire SK10 4TJ
U.K.

JOHN M. RAWLINGS*
IAN WYATT
JON R. HEYLINGS

REFERENCES

1. Clark DG and Hurst WE, The toxicity of diquat. *Br J Ind Med* **23**: 51–55, 1970.
2. McCarthy LG and Speth CL, Diquat intoxication. *Ann Emerg Med* **12**: 394–396, 1983.
3. Crabtree HC, Lock EA and Rose MS, Effects of diquat on the gastrointestinal tract. *Toxicol Appl Pharmacol* **41**: 585–595, 1977.
4. Lock EA, The effect of paraquat and diquat on renal function in the rat. *Toxicol Appl Pharmacol* **48**: 327–336, 1979.
5. Rawlings JM, Foster JR and Heylings JR, Diquat-induced intestinal secretion in the anaesthetised rat. *Hum Exp Toxicol* **11**: 524–529, 1992.
6. Gage JC, The action of paraquat and diquat on the respiration of liver cell fractions. *Biochem J* **109**: 757–761, 1968.
7. Baldwin RC, Pasi A, MacGregor JT and Hine CH, The rates of radical formation from the dipyridylium herbicides paraquat, diquat, and morfamquat in homogenates of rat lung, kidney, and liver: an inhibitory effect of carbon monoxide. *Toxicol Appl Pharmacol* **32**: 297–304, 1975.
8. Rose MS, Smith LL and Wyatt I, The relevance of pentose phosphate pathway stimulation in rat lung to the mechanism of paraquat toxicity. *Biochem Pharmacol* **25**: 1763–1767, 1976.
9. Holzer H, Carbohydrate metabolism. *Annu Rev Biochem* **28**: 171–222, 1959.
10. Keeling PL and Smith LL, Relevance of NADPH depletion and mixed disulphide formation in rat lung to the mechanism of cell damage following paraquat administration. *Biochem Pharmacol* **31**: 3243–3249, 1982.
11. Nisselbaum JS and Green S, A simple ultramicro method for determination of pyridine nucleotides in tissues. *Anal Biochem* **27**: 212–217, 1969.
12. Tsokus-Kuhn JO, Lethal injury by diquat redox cycling in an isolated hepatocyte model. *Arch Biochem Biophys* **265**: 415–424, 1988.
13. Shirkey RS, Chakraborty J and Bridges JW, Comparison of the drug metabolising ability of rat intestinal mucosal microsomes with that of liver. *Biochem Pharmacol* **28**: 2835–2839, 1979.
14. Bruder G, Bretscher A, Franke WW and Jarasch ED, Plasma membranes from intestinal microvilli and erythrocytes contain cytochromes b_5 and P-420. *Biochim Biophys Acta* **600**: 729–755, 1980.
15. Heitkamp M and Brown OR, Inhibition of NAD biosynthesis by paraquat in *Escherichia coli*. *Biochim Biophys Acta* **676**: 345–349, 1981.
16. Tsokus-Kuhn JO, Evidence *in vivo* for elevation of intracellular free Ca^{2+} in the liver after diquat, acetominophen, and CCl_4. *Biochem Pharmacol* **38**: 3061–3065, 1989.
17. Case RM, The role of Ca^{2+} stores in secretion. In: *Secretion: Mechanism and Control. Sydney, Australia, August 1983* (Eds. Case RM, Lingard JM and Young JA), pp. 149–167. Manchester University Press, Manchester, U.K., 1984.

* Corresponding author at present address: Waltham Centre for Pet Nutrition, Waltham-on-the Wolds, Melton Mowbray, Leicestershire LE14 4RT, U.K. Tel. 0664 415380