Biochemical Pharmacology, Vol 46, No. 8, pp. 1333–1338, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

ASCORBIC ACID UPTAKE BY ISOLATED RAT HEPATOCYTES

STIMULATORY EFFECT OF DIQUAT, A REDOX CYCLING COMPOUND

MARIE CHRISTINE CORNU,*† GREGORY ADAM MOORE,*‡ YOSHIO NAKAGAWA§ and PETER MOLDÉUS*

*Karolinska Institutet, Institutionen för Toxikologi, Box 60400, 104 01 Stockholm and ‡National Chemical Inspectorate, Box 1384, 171 27 Solna, Sweden; and §Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, 3-42-1, Hyakunincho, Shinjuku-ku, Tokyo 169, Japan

(*Received* 30 *June* 1993; *accepted* 12 *July* 1993)

Abstract—The toxicity of redox cycling compounds which generate the formation of active oxygen species is commonly accepted to be associated with a decrease of cellular reductants involved in cellular defence. However, when hepatocytes were incubated with diquat, an established redox cycler, in the presence of ascorbic acid (AA) (1 mM), the intracellular level of AA was increased. The effects of diquat on AA uptake were investigated in isolated rat hepatocytes. Incubation of hepatocytes with diquat plus AA (1 mM) resulted in about a 2-fold increased accumulation which occurred in a time-dependent manner reaching a steady state after 15 min at 37°. The initial AA uptake rate was dependent on the AA concentration added. This process is described by Michaelis–Menten kinetics (apparent K_m = 953 ± 59 μM and V_{max} = 2.68 nmol/min/10^6 cells). Characterization of AA accumulation showed it to be inhibited: by incubation at 4°; with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, an inhibitor of intracellular ATP production; by decreasing the extracellular Na^+ concentration or incubating with ouabain; with pfloretin, a glucose transport inhibitor; and with glucose, a competitive inhibitor of AA transport. Replacement of AA with its oxidized form, dehydroascorbic acid, in the absence of diquat enhanced AA accumulation by 2.5-fold and apparently prevented further accumulation by added diquat. In addition, maintaining AA reduced with dithiothreitol inhibited the diquat effect. Diquat-induced AA accumulation was inhibited (65%) by desferrioxamine, a free-iron chelator, but not by catalase and/or superoxide dismutase or different antioxidants. In contrast, incubation with other active oxygen species generating systems including bipyridilium structural analogues, paraquat and benzyl viologen, had no effect on AA accumulation in hepatocytes. These results suggest that diquat-induced AA accumulation by hepatocytes occurs by a specific mediated transport system rather than as a consequence of cytotoxicity and may involve the presence of free-iron.

During aerobic metabolism, potentially harmful activated oxygen species are formed in cells. The deleterious effects of such species are counterbalanced by a complex antioxidant system consisting of the enzymes catalase, superoxide dismutase and glutathione peroxidase, and low molecular weight antioxidants such as glutathione, ascorbic acid and tocopherols [1].

Ascorbic acid (AA‖) is a major water soluble antioxidant found in the aqueous compartments of cells and extracellular fluids such as blood plasma, aqueous humor and bronchial epithelial lining fluid [2]. AA is effective at scavenging the majority of soluble radicals and is involved in the subsequent regeneration of α-tocopherol by reducing the α-tocopheryl radical at the membrane surface [2]. Consequently, AA plays a prominent role in the protection of biological systems such as the inhibition of oxidative modification of low density lipoproteins in humans [3].

Diquat, a bipyridyl herbicide, generates active oxygen species by redox cycling and has been shown to be a hepatotoxin *in vivo* and *in vitro* [4]. Toxicity in hepatocytes is greatly enhanced by preincubation with 1,3 bis-(2-chloroethyl)-1-nitrosourea, an inhibitor of glutathione reductase. Because diquat does not participate in alkylation or conjugation reactions, it is therefore a valuable investigative tool for the study of oxidative damage resulting solely as a consequence of active oxygen generation [4]. Recently, we have shown that diquat-induced cytotoxicity is prevented in 1,3 bis-(2-chloroethyl)-1-nitrosourea-pretreated hepatocytes when the medium is supplemented with AA. The cytosolic (cellular) levels of AA were increased although the cellular glutathione level was decreased [5]. In this study, we have characterized intracellular AA accumulation in diquat-treated hepatocytes. It is shown that diquat-induced AA accumulation has several properties characteristic of mediated transport and does not seem to result from cytotoxicity. In addition, diquat-induced AA accumulation does not seem to be dependent on the generation of active

† Corresponding author: Dr M. C. Cornu, ENS.BANA, Laboratoire de Biochimie et Toxicologie Alimentaires, 1 esplanade Erasme, 21000 Dijon, France. Tel. (33) 80 39 66 36; FAX (33) 80 39 66 41.

‖ Abbreviations: AA, ascorbic acid; DHA, dehydroascorbic acid; DTT, dithiothreitol.

oxygen species by other prooxidants but is associated with the presence of diquat and free-iron.

MATERIALS AND METHODS

Chemicals

Diquat dibromide (purity >99%) was a kind gift of Dr L. L. Smith of Imperial Chemical Industries PLC (Millbank, London, U.K.). [1-^{14}C]L-AA (20 mCi/mmol) was purchased from Amersham and L-dehydroascorbic acid (DHA) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Desferrioxamine was obtained from Ciba-Geigy (Basel, Switzerland) and manufactured by Ben Venue labs (Bedford, OH, U.S.A.) as deferoxamine mesylate (Desferal®). Collagenase, superoxide dismutase and catalase were from Boehringer (Mannheim, F.R.G.). L-AA and all other reagents were commercial products of the highest available grade of purity.

Preparation of hepatocytes

Male wistar rats (200 g) were used in all experiments. Hepatocytes were isolated by the method of Moldéus *et al.* [6]. Cell viability was assayed by determining the percentage of the hepatocytes which excluded 0.16% Trypan blue, and approximately >90% of the freshly isolated hepatocytes routinely excluded Trypan blue.

Incubation of hepatocytes

Hepatocytes (10^6 cells/mL) were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM *N*-(2-hydroxyethyl) piperazone-*N'*-(2 ethanesulfonic acid) (HEPES). All incubations were performed in rotating, round-bottomed flasks at 37° under a continuous flow of 95% O_2 and 5% CO_2. Unless otherwise stated, Krebs–Henseleit buffer was supplemented with cold or labelled L-AA (1 mM final concentration) dissolved in Krebs–Henseleit buffer 5 min prior to diquat treatment. Reactions were started by the addition of diquat dissolved in Krebs–Henseleit buffer at a final concentration of 1 mM. All incubations were performed during 15 min except for the time course experiments.

Unless otherwise stated, hepatocytes were incubated in the absence or presence of metabolic inhibitors or other compounds for 10 min before addition of AA. Aliquots of incubation mixture were taken at various times for the analyses of cell viability and AA uptake.

Biochemical assays

Determination of protein content. Proteins was determined using the method of Peterson [7] and using crystallized and lyophilized bovine serum albumin as standard.

AA uptake. Extraction and determination of AA from hepatocytes were based on the methods of Honegger *et al.* [8]. Total AA uptake (reduced + oxidized form) was determined by [1-^{14}C]L-AA analysis. In brief, after extraction, AA radioactivity in isolated cells was determined in 10 mL of scintillation cocktail (Instagel, Packard Instrument Co.) and counted in a LKB Wallac 1216 Spectrometer. Uptake rates were computed based on the specific activity of [1-^{14}C]AA in the media. AA (reduced form) was analysed by HPLC with electrochemical detection; Antec model CU-03 (Antec Instruments, Leiden, Netherlands) in the oxidative mode (at 0.7 V). The analytical column used for separation of AA was 250 mm × 4.6 i.d. containing Supelcosil L-18, 5 μm average particle size (Supelco, Bellefonte, PA, U.S.A.). The mobile phase was composed of 131 mM NaH_2PO_4 buffer and 0.1 mM EDTA with a final pH 2.5 and a flow rate of 0.8 mL/min. The recovery of AA was checked by addition of known amounts of the working standards to hepatocytes and was approximately 95% using this method. Reproducibility of the procedure was checked by analysing representative samples in triplicate which gave a coefficient of variation of 5% for the assay of AA on the same day.

The detection limit of AA was 5 pmol. ^{14}C and HPLC methods used to determine intracellular levels of AA gave similar data (Table 1). Intracellular AA was represented by its reduced form. Therefore, most of our experiments were performed by the HPLC method except when very low amounts of AA, below the HPLC limit of detection, had to be

Table 1. Intracellular levels of AA in control and diquat-treated rat hepatocytes determined by ^{14}C or HPLC as described in Materials and Methods

Treatment	Intracellular AA concentration after 15 min incubation (nmol/10^6 cells) ^{14}C method*	HPLC method†
Control cells	7.50 ± 0.5	7.80 ± 0.8
Diquat-treated cells (1 mM)	14.50 ± 1.2‡	14.00 ± 0.9‡

Krebs–Henseleit buffer was supplemented with 1 mM cold or labelled L-AA (final concentration).

Values are expressed as nmol AA/10^6 cells and represent the means ± SD of six different cell preparations.

* Oxidized and reduced form.

† Reduced form.

‡ Significantly different from control cells, $P < 0.05$.

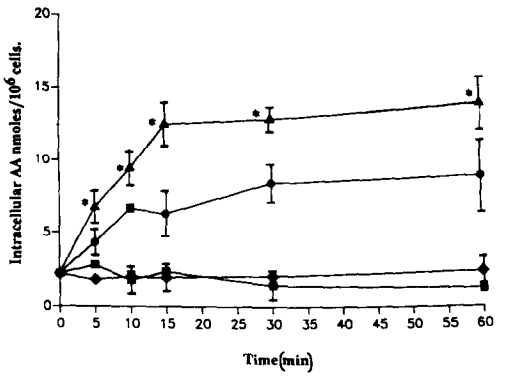

Fig. 1. Time dependency of AA uptake in diquat-treated rat hepatocytes. Hepatocytes were incubated with diquat in the absence and presence of AA (1 mM) as follows: (■) control, (●) AA, (◆) diquat, (▲) AA + diquat. Each symbol represents the mean ± SD of five different cell preparations. *, Significantly different from control value ($P < 0.05$).

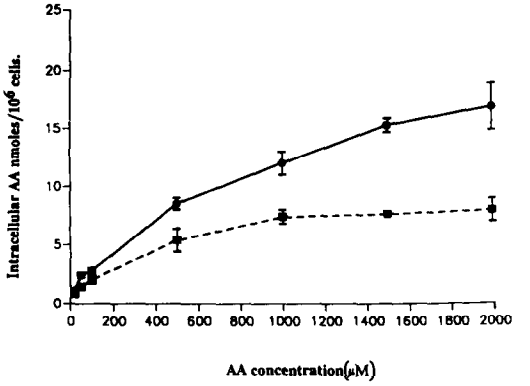

Fig. 2. Concentration dependency of AA uptake in rat hepatocytes in the presence or absence of diquat 1 mM (final concentration). Krebs–Henseleit buffer was supplemented with different concentrations of AA 5 min before adding diquat. AA uptake was determined by the ^{14}C method as described in Materials and Methods. (■) Control, (●) diquat. Each symbol represents the mean ± SD of three different cell preparations. *, Significantly different from control value ($P < 0.05$).

determined. For the concentration dependency experiments, the affinity constant and velocity of AA accumulation were determined by Lineweaver–Burk plots analysed by linear regression.

Statistics

Uptake was measured on triplicate samples from at least three different cell preparations for each experiment. Data are expressed as means ± SD. Statistically evaluations were carried out by the Student's *t*-test. Only P values < 0.05 are reported as significant.

RESULTS AND DISCUSSION

AA accumulation in diquat-treated cells

Addition of diquat to isolated hepatocytes supplemented with AA resulted in a time- and AA concentration-dependent increase in intracellular AA content. As shown in Table 1 and Fig. 1, diquat (1 mM) induced about a 2-fold increased accumulation of AA in hepatocytes by 15 min. The accumulated AA was predominantly recovered as the reduced form of AA. Diquat-induced AA accumulation was also dependent on the extracellular AA concentration (Fig. 2). AA accumulation was saturated at about 2 mM and the derived kinetic parameters show an apparent Michaelis constant (K_m) of 953 ± 59 μM and a maximum uptake velocity (V_{max}) of 2.68 nmol/min/10^6 cells (1.91 nmol/min/mg protein).

Because AA accumulation follows saturation kinetics, this indicates that mediated transport is involved. To test this assumption, experiments were performed to characterize further diquat-induced AA accumulation. AA accumulation was temperature dependent: at 4°, AA accumulation was completely inhibited as compared with 37° (results not shown). Preincubation of hepatocytes in the presence of substances that alter cellular energetics and are inhibitors of AA accumulation were investigated. Table 2 shows that carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone, an uncoupler of mitochondrial respiration required for mitochondrial ATP formation and ouabain, an inhibitor of Na^+/K^+ ATPase, inhibited AA uptake in the absence and presence of diquat. Pfloretin, a glucose transport inhibitor, also inhibited AA accumulation in the absence and presence of diquat [9–11]. Because AA is predominantly present as the mono-ionized ascorbate anion (AH^-) at physiological pH, depolarization of the hepatocyte membrane would be expected to interfere with AA accumulation. Hence, the inhibitory effect of ouabain may be due to its ability to increase the intracellular Na^+ concentration. As seen in Table 3, diquat-induced AA accumulation was dependent on the Na^+ concentration. With 5 mM Na^+ or completely replacing Na^+ with choline, diquat-induced AA accumulation was inhibited by 57% or 65%, respectively. Alternatively, increasing the plasma membrane permeability to K^+ by pre-incubation with valinomycin, a K^+ ionophore, inhibited (45%) diquat-induced AA accumulation (Table 2). These data are in agreement with previous studies showing the Na^+ dependency for cellular AA uptake [9–13]. Taken together these results show that diquat-induced AA accumulation exhibits properties characteristic of mediated transport rather than effects due to increased non-specific plasma membrane permeability caused by cytotoxicity.

The characteristics demonstrated here for diquat-induced AA accumulation in hepatocytes are similar to those observed in other cells in the absence of diquat. A direct comparison of the kinetic parameters is difficult because AA transport has generally been conducted under different conditions, e.g. sodium ion concentration. However, several similarities with AA accumulation in other cells are apparent. Firstly, the apparent K_m is characteristic for tissues with

Table 2. Effect of metabolic inhibitors on diquat-induced AA accumulation in rat hepatocytes

Treatment	Concn (μM)	Diquat	(nmol AA/10^6 cells)	% of control	% inhibition of AA accumulation	Viability (%)
Control		−	7.93 ± 0.17	100		96
		+	15.76 ± 0.50	199		94
Ouabain	100	−	5.53 ± 0.15	69	31	90
		+	9.04 ± 0.50*	14	54	91
Valinomycin	1	−	7.86 ± 0.20	99	0	92
		+	12.22 ± 1.40*	154	45	92
FCCP	10	−	5.38 ± 0.15	68	32	92
		+	6.32 ± 0.36*	80	87	91
Pfloretin	200	−	4.01 ± 0.01	51	49	89
		+	5.11 ± 0.17*	64	86	91

Inhibitors were added 10 min prior to AA (and diquat) except for ouabain which was pre-incubated for 90 min.
Values represent the mean ± SD of three separate hepatocyte preparations. The percentage inhibition was calculated for hepatocytes incubated in the absence or presence of diquat, respectively.
* Significantly different from the control cells, $P < 0.05$.
FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Table 3. Effect of external sodium concentration in Krebs–Henseleit buffer on diquat-induced AA accumulation in rat hepatocytes

Treatment	Na^+ (mM)	Diquat	Intracellular AA levels (% of control)	% inhibition of AA accumulation
Control	150	−	100 ± 7.5	
		+	184 ± 6*	
[K^+] 150 mM	5	−	54 ± 2*	46
		+	81 ± 6*	57
Choline chloride 150 mM	0	−	69 ± 5*	31
		+	88 ± 8*	65

Each value represents the mean ± SD of three separate cell preparations. Control value (100%) = 7.50 ± 0.2 nmol AA/10^6 cells.
* Significantly different from control value (in the absence of diquat), $P < 0.05$.

high metabolic requirements for AA such as the adrenal medulla and pituitary gland [10]. Secondly, a sodium dependency is more often associated with an anion transport mechanism [9, 12, 13]. Finally, intracellular recovery predominantly of reduced AA seems to be a common feature of AA transport [14–16]. However, concerning this last characteristic it has been demonstrated that both AA and DHA can be transported by cells [13].

Mechanism of diquat-induced AA uptake

Previous studies with other cells (see Rose for review [13]) have shown that AA accumulation can occur *via* two different transport systems: by the transport of reduced AA (AH^-) or oxidized AA (DHA). Oxidized AA has been shown to be the preferred form of AA transported by leucocytes, erythrocytes and the crystalline lens of the eye [14, 17–19]. Transported DHA is then reduced to AH^- by glutathione either non-enzymatically or enzymatically catalysed by cytosolic DHA reductase [15, 20]. Hence, although AH^- is the predominantly recovered form in hepatocytes, it cannot be concluded which transport system(s) is responsible for diquat-induced AA accumulation. To resolve which redox form(s) of AA is transported, hepatocytes were incubated with AA or DHA in the absence and presence of diquat. Table 4 shows that AA accumulation was 2.5-fold greater with DHA than AA. However, diquat failed to enhance AA accumulation in the presence of DHA. Maintaining AA reduced with dithiothreitol (DTT) totally inhibited AA accumulation enhanced by the presence of diquat but not in its absence. Furthermore, incubation of hepatocytes with glucose, a potent inhibitor of DHA transport, inhibited diquat-induced AA accumulation in the presence of DHA or AA [17, 19]. These data indicate that the oxidized form of AA, DHA, is the redox form of AA which is transported by hepatocytes in the presence of diquat but in its absence AA is also transported. The mechanism by which diquat oxidizes AA is not understood. One possible mechanism by which diquat may oxidize AA is by active oxygen species $O_2^{\cdot}$, $OH^{\cdot}$, H_2O_2 generated by redox cycling of diquat. Indeed the duration of AA accumulation in diquat-treated hepatocytes is similar to the duration of redox cycling of diquat that occurs in hepatocytes with 1 mM diquat [21].

Table 4. Effect of DTT and glucose on diquat-induced AA accumulation in rat hepatocytes incubated with AA and DHA

Treatment	Concn (mM)	Diquat	Intracellular AA accumulation [% of control (non-treated cells)] Krebs–Henseleit with 1 mM AA	Krebs–Henseleit with 1 mM DHA	P[1]
Untreated		−	100 ± 3	243 ± 12.5	<0.01
		+	181 ± 14	262 ± 23	<0.01
		P[2]	<0.01	NS	
DTT	3	−	100 ± 5	88 ± 6	NS
		+	90 ± 3	92 ± 12	NS
		P[2]	NS	NS	
Glucose	7.5	−	50 ± 5	110 ± 5	<0.01
		+	98 ± 6	111 ± 2	<0.05
		P[2]	<0.01	NS	

Each value represents the mean ± SD of three separate cell preparations.

P[1], significance of AA accumulation in hepatocytes incubated in the presence of DHA compared with AA; P[2], significance of AA accumulation in hepatocytes incubated with diquat compared with its absence. NS, non-significant.

Untreated cells (AA 1 mM without diquat) = 6.52 ± 0.2 nmol/10^6 cells.

Table 5. Effect of active oxygen species on AA accumulation in rat hepatocytes

Treatment	Final concentration (mM* or IU/mL†)	% of control	Viability (%)
Control	—	100 ± 2	94
Diquat	1*	185‡ ± 2	95
Paraquat	1*	98 ± 5	93
Benzyl viologen	1*	108 ± 5	95
H_2O_2	1*	95 ± 17	92
TBuOOH	1*	97 ± 14	93
Diamide	0.25*	80‡ ± 2	91
	0.75*	46‡ ± 3	80
DMNQ	0.20*	52‡ ± 9	75
Menadione	0.05*	101 ± 5	93
Duroquinone	0.25*	100 ± 3	88
GOD	0.05†	69‡ ± 9	91
XOD	xanthine: 0.05* xanthine oxidase: 0.005†	84‡ ± 6	92

Each value represents the mean ± SD of three separate hepatocyte preparations.

Control value (100%) = 6.52 ± 0.5 nmol/10^6 cells.

tBuOOH, *tert*-butylhydroperoxide; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; GOD, glucose oxidase; XOD, xanthine/xanthine oxidase.

‡ Significantly different from control value, $P < 0.05$.

Role of oxidative stress

The effect of several different prooxidants which generate active oxygen species intra- and extracellularly on AA accumulation was investigated. Table 5 shows that intracellular AA accumulation was not increased. In some instances, the level of intracellular AA was decreased as with 2,3-dimethoxy-1,4-naphthoquinone. However, this was probably associated with increased cytotoxicity. The structurally similar bipyridilium compounds of diquat, paraquat and benzyl viologen, also failed to induce AA accumulation in rat hepatocytes. This may be correlated with their relative toxicities and associated capacity to generate oxidative species (diquat > benzyl viologen > paraquat) [22]. That the prooxidants tested here failed to increase AA accumulation in rat hepatocytes indicates that the diquat effect does not solely depend on the generation of active oxygen species. Because active oxygen species are formed during diquat metabolism it is conceivable that these are involved in diquat-induced AA accumulation. This assumption was tested by adding different scavengers of oxygen metabolites and antioxidants. No effect on the diquat-induced AA accumulation occurred in the presence of

the membrane impermeable enzymes, superoxide dismutase (733 U/mL) and/or catalase (650, or 1300 U/mL alone), or in the presence of antioxidants *N,N'*-diphenyl-*p*-phenylenediamine (10 μM), promethazine (50 μM) and 3-*tert*-butyl-4-hydroxyanisole (10 μM). By contrast, desferrioxamine (100 μM), a free-iron chelating agent significantly ($P < 0.05$) inhibited diquat-induced AA accumulation by 65%.

In conclusion, our findings demonstrate that diquat-induced AA uptake occurs by a specific transport mechanism rather than as a consequence of cytotoxicity. Induced AA accumulation apparently occurs independently of prooxidant-generated active oxygen species but is associated with the presence of diquat and free-iron. It appears therefore that a redox interaction between diquat and free-iron oxidizes AA to DHA which is subsequently transported into hepatocytes.

Acknowledgements—The authors thank Mrs Monica Kimland for excellent technical assistance in parts of this work and Mrs Florence Ferré for typing this manuscript. This work was supported by MFR-INSERM (MCC), Swedish Medical Research Council and National Chemical Inspectorate.

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