0026-895X/95/050928-10$3.00/0

MOLECULAR PHARMACOLOGY, 48:928–937 (1995).

Inhibition of Succinate:Ubiquinone Reductase and Decrease of Ubiquinol in Nephrotoxic Cysteine S-Conjugate-Induced Oxidative Cell Injury

BOB VAN DE WATER,[1] J. PAUL ZOETEWEIJ, HANS J. G. M. DE BONT and J. FRED NAGELKERKE

Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, The Netherlands.

Received June 6, 1995; Accepted August 1, 1995

SUMMARY

The role of complex II in the cellular protection against oxidative stress was investigated in freshly isolated rat renal proximal tubular cells (PTC) with the use of the nephrotoxin *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC). DCVC caused oxidative stress in PTC as determined by flow cytometry with dihydrorhodamine-123; this fluorescent probe is readily oxidized by primary hydroperoxides such as those formed during lipid peroxidation. The oxidative stress could be prevented by inhibition of the β-lyase-mediated formation and covalent binding to cellular macromolecules of reactive DCVC metabolites, with amino oxyacetic acid (AOA), or by the antioxidant *N,N'*-diphenyl-*p*-phenylenediamine. Both AOA and DPPD also prevented cell death. The DCVC-induced oxidative stress was associated with a decrease in the succinate:ubiquinone reductase (SQR) activity of complex II, whereas NADH:ubiquinone reductase activity of complex I remained unaffected. AOA prevented the effect on SQR activity, whereas *N,N'*-diphenyl-*p*-phenylenediamine did not. Inhibition of SQR activity with thenoyl trifluoracetone (TTFA) potentiated the DCVC-induced oxidative cell injury, suggesting the involvement of SQR activity in an antioxidant pathway. To investigate this in greater detail, PTC were treated with an inhibitor of cytochrome-*c*-oxidase, KCN, in a buffer containing glycine, which prevents cell death by KCN. Glycine did not affect cell death by DCVC. KCN prevented the DCVC-induced oxidative stress and cell death. KCN cytoprotection could be prevented by inhibition of SQR activity with oxaloacetate or TTFA, whereas inhibition of either complex I or III with rotenone and antimycin, respectively, did not prevent it. The effect of DCVC on complex II was associated with a decrease in the cellular amount of reduced ubiquinone (QH_2); the KCN-mediated cytoprotection was related to a 60% increase of cellular QH_2. Rotenone almost completely inhibited ubiquinone reduction even in the presence of KCN, whereas oxaloacetate in combination with KCN resulted in QH_2 levels comparable to control. This suggests that the SQR activity by complex II rather than the cellular content of reduced ubiquinone (QH_2) is important as a part of the cellular antioxidant machinery in the cytoprotection against oxidative stress.

Oxidative stress is frequently involved in the development of cytotoxicity, either chemically induced or as a result of (patho)physiological conditions (1); in many cases, the mitochondria could be identified as the potential origin of the oxidative stress (2–5). This suggests the failure of cellular antioxidant pathways, especially in the mitochondria. The mitochondrial membranes are protected against oxidative stress by α-tocopherol (vitamin E) and reduced Q (QH_2), both potent antioxidants (6–8), which are present in the inner and outer mitochondrial membranes. Although Q is continuously reduced to ubiquinol at complexes I, II, and III of the MRC, observations in submitochondrial particles suggest that primarily reduction by complex II is important in the antioxidant function of Q (Fig. 1) (9–11). Recent experiments in submitochondrial particles and liposomes indicate that the SQR activity of complex II is involved in the regeneration of α-tocopherol from α-tocopheroxyl radicals formed during lipid peroxidation (12–15). These observations suggest a role of complex II in an antioxidant pathway. However, evidence for a role of complex II in the cellular antioxidant machinery has not yet been documented in intact cells. If this is the case, an inhibition of complex II, caused by either pathological circumstances or xenobiotics, would increase the susceptibility for oxidative cell injury. We decided to study the antioxidant role of complex II with the nephrotoxin DCVC in PTC.

[1] Present address: W. Alton Jones Cell Science Center, 10 Old Barn Road, Lake Placid, New York 12946.

ABBREVIATIONS:: DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; TTFA, thenoyltrifluoroacetone; β-lyase, L-cysteine *S*-conjugate β-lyase (E.C. 4.4.1.13); Q, ubiquinone; QH_2, ubiquinol; PI, propidium iodide; DhRho123, dihydrorhodamine-123; BSA, bovine serum albumin; Δψ, mitochondrial membrane potential; ROS, reactive oxygen species; MRC, mitochondrial respiratory chain; SQR, succinate:ubiquinone reductase; PTC, rat renal proximal tubular cells; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Rho123, rhodamine-123; AOA, aminooxyacetic acid; HPLC, high performance liquid chromatography; DPPD, *N,N'*-diphenyl-*p*-phenylenediamine; Rot, rotenone; Oxa, oxaloacetate; DEF, desferrioxamine; GSH, .

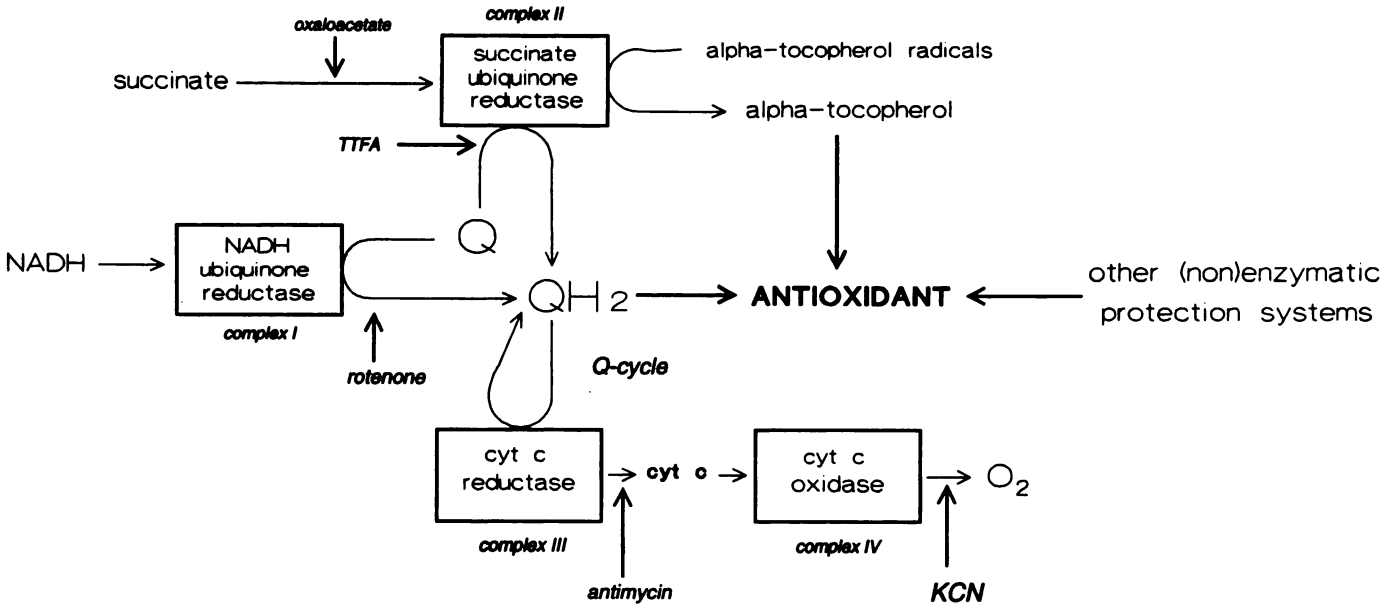

Fig. 1. Simplified scheme of the MRC showing sites of substrate entry, reduction of Q, and potential site for the involvement of complex II in an antioxidant pathway. *cyt*, cytochrome. Other enzymatic protection systems involved in the cellular antioxidant machinery may include vitamin E radical reductase, glutathione-*S*-transferases, glutathione-reductase and -peroxidase, and DT-diaphorase.

DCVC is often used to investigate the mechanism of toxicity of nephrotoxic L-cysteine-*S*-conjugates (5, 16–24). It induces proximal tubular necrosis in various mammalian species (25, 26). This has been related to the formation of highly reactive, sulfur-containing metabolites by renal cysteine conjugate β-lyase (E.C. 4.4.1.13) (27–29), whose activity is high in both the cytosol and the mitochondrial matrix of PTC (30, 31). Binding of these reactive metabolites to macromolecules, especially in mitochondria (19), results in the initiation of several events that precede cell death (5, 20–24). Thus, DCVC causes a decrease in the mitochondrial membrane potential ($\Delta\psi$) (5, 23), most likely due to an inhibition of the respiratory chain, since DCVC inhibits mitochondrial site I and II respiration and succinate dehydrogenase activity in isolated mitochondria (18, 19). In addition, DCVC increases both the cytosolic and mitochondrial Ca^{2+} concentrations in PTC and LLC-PK_1 cells (5, 21, 23, 24). The mitochondrial Ca^{2+} increase, in turn, is involved in the initiation of lipid hydroperoxide formation (5, 24); prevention of mitochondrial accumulation of Ca^{2+} by an inhibitor of the mitochondrial Ca^{2+} uniporter, ruthenium red, protects against lipid hydroperoxide generation. Thus, the mitochondria become the site of the initiation of oxidative stress.

In the present study, we investigated the role of complex II in the oxidative cell injury caused by DCVC. The results strongly suggest that SQR activity of complex II is important in the maintenance of a functional cellular antioxidant defense system; interference with this complex may increase the susceptibility for oxidative stress-induced cell damage.

Materials and Methods

Chemicals. DhRho123 was obtained from Molecular Probes (Eugene, OR); collagenase (from *Clostridium histolyticum*), BSA (Fraction V), KCN, rotenone, antimycin, TTFA, oxaloacetic acid, coenzyme Q_9, coenzyme Q_{10}, propidium iodide, Rho123, and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO); and HEPES was obtained from Boehringer Mannheim (Mannheim, FRG). DCVC was kindly provided by Dr. J. M. N. Commandeur (Department of Pharmacochemistry, LACDR, Free University, Amsterdam, The Netherlands). ^{35}S-(1,2-dichlorovinyl)-L-cysteine (specific activity, 21.6 μCi/μmol) was a generous gift from Dr. J.,L. Stevens (W. Alton Jones Cell Science Center, Lake Placid, New York).

Isolation of proximal tubular kidney cells. Male SPF Wistar/Wu rats from the Sylvius Laboratories (Leiden, The Netherlands), weighing 200–250 g, were used throughout all the experiments. Animals were housed in Macrolon cages with hardwood bedding and had free access to food (MRH-B, Hope Farms B.V., Woerden, The Netherlands) and tap water. An alternating 12-hr light/dark cycle was maintained in the animal rooms.

Proximal tubular kidney cells were isolated as described previously (23, 32). With this method, a cell preparation was routinely obtained that stained 95% positive for γ-glutamyltranspeptidase and 90% positive for nonspecific esterase with a viability of 90–95%, as determined by trypan blue exclusion (32). The cells were suspended in Hanks' balanced salt solution, pH 7.4, (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $Na_2HPO_4 \cdot 2H_2O$, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2$, 4 mM $NaHCO_2$, 20 mM HEPES) containing 5 mM glucose, 5 mM glycine, and 2.0% (w/v) BSA (buffer A) gassed for 30 min with 95% O_2/5% CO_2. Glycine was also added to the incubation buffer because in this study we evaluated the effect of various MRC inhibitors that are cytotoxic to PTC (Table 1). The cytotoxicity of these compounds is prevented by the addition of glycine to the incubation buffer (Table 1), as described previously (36, 40). This enabled us to investigate the role of the respiratory chain in DCVC-induced cytotoxicity caused by oxidative stress.

Flow cytometric analysis of oxidative stress, $\Delta\psi$, and cell death in PTC in suspension. Freshly isolated PTC were suspended in buffer A at a density of 0.5–1.0 × 10^6 cells/ml. The suspension routinely contained ~50% single cells, 45% cell clusters consisting of 2–5 cells, and ~5% cell clusters of up to 20 cells, as determined with light microscopy. Cells were incubated in Costar culture flasks at 37° for 30 min before the start of the experiments.

Hydroperoxide formation was determined by flow cytometric analysis using the nonfluorescent probe DhRho123, which can be oxidized by hydroperoxides to the fluorescent Rho123. This probe is specific for hydroperoxides and does not react with superoxide anion (33, 34). PTC were incubated with 10 μM DhRho123 for 15 min at 37°, after which experiments were started. Before flow cytometric

TABLE 1

Effect of glycine on the cytotoxicity of various inhibitors of the respiratory chain

PTC were incubated with 1 mM KCN, 5 μM antimycin A, 20 μM Rot, 250 μM TTFA, or 10 mM Oxa in either the absence or presence of 5 mM glycine. The effect on ΔΨ and viability was determined after 30 min and 60 min, respectively, by flow cytometric analysis using Rho123 and propidium iodide, as described in Materials and Methods. Shown is the ΔΨ as percentage of the control (mean ± standard error of three experiments) and percent cell death (mean ± standard error of three experiments).

Treatment	ΔΨ	Cell death
		%
Control	100	11 ± 1
+ glycine	109 ± 1	9 ± 1
KCN	59 ± 5[a]	42 ± 10[a]
+ glycine	56 ± 4[a]	18 ± 3[a,b]
Antimycin A	65 ± 3[a]	30 ± 8[a]
+ glycine	72 ± 5[a]	11 ± 2[b]
Rot	65 ± 1[a]	29 ± 4[a]
+ glycine	61 ± 3[a]	19 ± 3[a,b]
TTFA	104 ± 2	15 ± 2
+ glycine	113 ± 6	12 ± 1
Oxa	99 ± 2	10 ± 2
+ glycine	112 ± 4	9 ± 1

[a] Significantly different from treatment control (analysis of variance; $p \leq 0.05$).
[b] Significantly different from the same treatment without glycine (analysis of variance; $p \leq 0.05$).

analysis, PI (5 μM final concentration) was added to the cells. The Rho123 and PI fluorescence properties of individual cells and cell clusters were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser, using the Lysis program. Rho123 fluorescence was detected by the Fl_1 detector (emission, 500–550 nm corresponding to green fluorescence), and PI fluorescence was detected by the Fl_3 detector (emission, >600 nm corresponding to red fluorescence). Incubation of PTC with DhRho123 resulted in three distinct populations of cells: population R1 with PI-negative fluorescence properties, indicating viable cells and cell clusters; population R2 with PI-positive fluorescence properties, indicating dead individual cells and cell clusters containing only dead cells; and population R3 with PI-positive fluorescence properties (Fig. 2A). The latter population consisted of cell clusters of both viable and dead cells as indicated by its staining with Rho123 (24). The mean Rho123 fluorescence of PI-negative cells (i.e., the viable cells present in population R1) loaded with DhRho123 was calculated. For each separate experiment, the autofluorescence of PI-negative cells was determined. Oxidative stress was expressed as the percentage of fluorescence at t = 0 and calculated as follows: [(C − A)/(B − A)], where A is the autofluorescence of PI-negative cells, B is the Rho123 fluorescence of PI-negative cells at t = 0, and C is the Rho123 fluorescence of PI-negative cells from the sample.

Δψ and cell death were also determined by flow cytometry, as described previously (5, 23).

Determination of covalent binding of DCVC to macromolecules. PTC were incubated at a density of 1–2 · 10^6 cells/ml in buffer A and incubated with ^{35}S-labeled DCVC (0.27 μCi/μmol) in either the absence or presence of various inhibitors. After a 30-min incubation, PTC were washed three times with PBS, pH 7.4, at 4°; then, cells were resuspended in 500 μl PBS, and 250 μl 20% (w/v) trichloroacetic acid was added. After vigorous vortexing, the cellular precipitate was pelleted by centrifugation for 5 min at 14,000 × *g*. The supernatant was removed for determination of nonprecipitable radioactivity. The pellet was washed two times with 500 μl PBS and 250 μl 20% (w/v) trichloroacetic acid; thereafter, the pellet was dissolved in 250 μl 1 N NaOH. Radioactivity was determined by liquid scintillation counting. Radioactivity was correlated to the amount of cellular protein and expressed as nmol bound/mg cell protein. Protein was measured using the Bradford method with BSA as the standard.

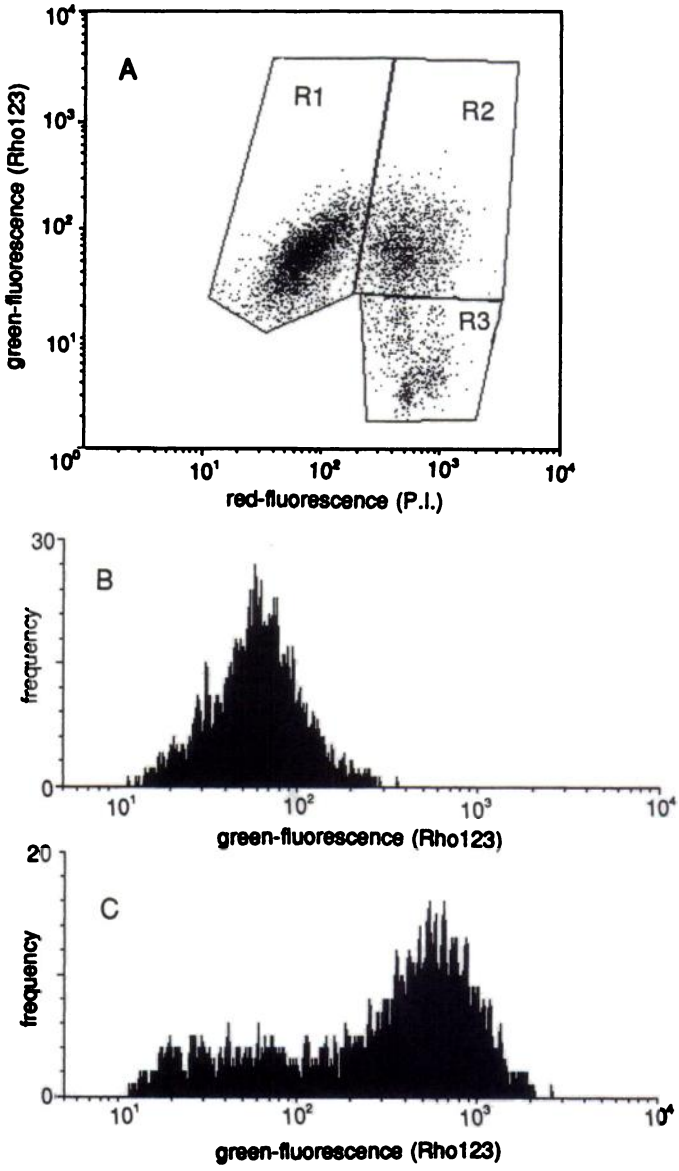

Fig. 2. Flow cytometric analysis of hydroperoxide formation in freshly isolated PTC. PTC loaded with DhRho123 and PI were analyzed as described in Materials and Methods. A, Two-dimensional density plot of green fluorescence (*Rho123*) and red fluorescence (*P.I.*) of untreated. B, Frequency histograms of the green fluorescence of Rho123-positive and PI-negative cells (population R1) of untreated PTC. C, Frequency histograms of the green fluorescence of Rho123-positive and PI-negative cells (population R1) of PTC treated with 400 μM DCVC for 30 min.

Determination of the enzyme activity of complexes I and II. PTC were incubated at a density of 1–2 · 10^6 cells/ml in buffer A with various concentrations of DCVC in absence or presence of inhibitors. After either 15 or 30 min, a 1-ml sample was taken, and PTC were washed two times with PBS, pH 7.4, at 4°, by centrifugation for 2 min at 50 × *g*. Thereafter, cells were resuspended in 1 ml PBS and directly frozen in liquid nitrogen.

Complex I activity was analyzed by determining NADH:Q oxidoreductase activity using a double-wavelength spectrophotometer. Briefly, to 890 μl assay buffer (50 mM KCl, 10 mM Tris·HCl, 1 mM EDTA, 2 mM KCN, pH 7.4, 25°, containing 75 μM NADH), 100 μl cellular homogenate was added. After stabilization of the absorbance (340–380 nm), the reaction was started by the addition of 100 μl Q_0 (100 μM final concentration), and $\Delta A_{340-380}$ was measured. An extinction coefficient of 5.5 mM^{-1} cm^{-1} was used to calculate enzyme activity.

Complex II activity was analyzed by measuring the SQR activity using a double-wavelength spectrophotometer. To 900 μl assay buffer (50 mM $KHPO_4$, 100 μM EDTA, 1 mM KCN, 0.1% BSA [w/v], pH 7.4, at 25°, containing 80 μM Q_0), 100 μl sample was added. After stabilization of the absorbance (270–330 nm), the reaction was started by the addition of 100 μl succinate (10 mM final concentration), and $\Delta A_{270-330}$ was measured. An extinction coefficient of 9.6 mM^{-1} cm^{-1} was used to calculate enzyme activity.

Enzyme activity of complexes I and II was correlated to the amount of cell protein.

Determination of reduced and oxidized Q. Reduced (QH_2) and oxidized (Q) Q_9 and Q_{10} were analyzed as described by others (10). PTC were suspended at a density of 3–6 · 10^6 cells/ml. After a 30-min preincubation, cells were exposed to DCVC (400 μM) with or without inhibitors added just before the addition of DCVC, unless stated otherwise. After 30 min, 2 ml of the cell suspension was added to a tube containing ice-cold 2 ml methanol and 5 ml petroleum ether (fraction 40/60) containing 1 mg pyrogallol/ml to prevent oxidation of ubiquinol during processing. The tube was extensively vortexed and then centrifuged for 10 min at 2000 × *g*. The petroleum ether layer was removed, and 5 ml fresh petroleum ether was added to the tube, and it was again vortexed extensively and centrifuged for 10 min at 2000 × *g*. The latter extraction step was repeated two times. The petroleum ether fractions were pooled, and petroleum ether was evaporated with analytical grade nitrogen gas. Lipids were dissolved in 250 μl methanol just before HPLC analysis. The HPLC system consisted of a Separations pump model 300, an Inertsil 2-C_{18} column (25 × 0.35 cm: particle size, 5 μm), a Separations Spektroflow 759A variable-wavelength detector, and a Shimadzu Chromatopac C-R6A recorder. The mobile phase (40% [v/v] acetonitrile, 40% [v/v] tetrahydrofuran, 15% [v/v] methanol, and 5% [v/v] water) was run at a flow rate of 0.45 ml/min. Reduced and oxidized coenzyme Q_9 eluted at 14 and 18 min and coenzyme Q_{10} eluted at 20 and 23 min, respectively, and were determined at 285 nm. During the entire procedure, >90% of QH_2 remained reduced. The amount of QH_2 and Q was calculated using a standard curve of both reduced and oxidized Q and expressed as the percentage of QH_2 of total Q (QH_2 + Q) present in the cells, calculated as [(QH_2/Q + QH_2) · 100%].

Statistical analysis. Results are expressed as the mean ± standard error of four or five independent experiments, unless stated otherwise. The difference between two groups were analyzed with the use of an unpaired Student's test, and differences between more than two groups were determined with a one-way analysis of variance.

Results

Covalent binding of DCVC to cellular macromolecules. To determine whether there was a correlation between cell death and covalent binding of reactive metabolites of DCVC, cells were incubated with ^{35}S-DCVC. Covalent binding was concentration dependent and increased to 54 nmol/mg cell protein at 400 μM DCVC after a 30-min incubation (Table 2). Binding was prevented by an inhibitor of cysteine conjugate β-lyase, AOA (1 m) (Table 2), indicating that the binding required the formation of reactive metabolites from DCVC. This correlated with complete protection against cell death by AOA. Although the antioxidant DPPD completely prevented the hydroperoxide formation (see later) and cell death, it did not prevent the covalent binding (Table 2).

Relationship between formation of hydroperoxides and cell death caused by DCVC. Hydroperoxide formation was measured with the use of the nonfluorescent compound DhRho123, which is oxidized by hydroperoxides to the fluorescent compound Rho123. To evaluate which class of hydroperoxides (hydrogen peroxide or lipid hydroperoxides) were measured, PTC loaded with DhRho123 were incubated with 1 mM H_2O_2 in the presence or absence of either the iron chelator 1 mM DEF or the chain-breaking lipophilic antioxidant 20 μM DPPD, and after 10 min the fluorescence intensity of PI-negative cells (i.e., living cells) was determined. H_2O_2 increased the green fluorescence intensity of PI-negative cells ~6-fold, indicating the oxidation of DhRho123 to Rho123 (Table 3). To investigate whether this was dependent on H_2O_2 and/or (phospho)lipid hydroperoxides, PTC were incubated with DEF to inhibit the formation of hydroxyl radicals formed through the iron-dependent Haber-Weiss reaction, which in turn can initiate lipid peroxidation. PTC were also incubated with DPPD to inhibit lipid peroxidation. Both DEF and DPPD inhibited the H_2O_2-induced DhRho123 oxidation almost completely (Table 3), although a small increase in oxidation over control was observed. These results indicate that both H_2O_2 and products formed during lipid peroxidation (presumably lipid hydroperoxides) can be detected with the use of DhRho123. In the presence of either DEF or DPPD, the increase in DhRho123 oxidation may mainly reflect H_2O_2, whereas in their absence, both H_2O_2

TABLE 2

Effect of inhibitors on DCVC-induced cytotoxicity and covalent binding to cellular macromolecules

PTC were incubated with various concentrations of [^{35}S]DCVC (specific activity, 0.27 μCi/μmol) in either the presence or absence of inhibitors. After a 30-min incubation, the covalent binding to TCA-precipitable cellular macromolecules was determined as described in Materials and Methods section. Cell death was determined after a 60-min incubation by flow cytometric analysis as described in Materials and Methods. Shown are cell death and the amount of covalent binding given as mean ± standard error of four independent cell isolations.

Treatment	Cell death	Covalent binding
	%	*nmol/mg cell protein*
Control	14 ± 3	–
[^{35}S]DCVC		
100 μM	22 ± 3	10 ± 1
200 μM	39 ± 4	24 ± 2
400 μM	50 ± 6	54 ± 4
400 μM + AOA[b]	15 ± 3[a]	6 ± 1[a]
400 μM + DPPD[c]	16 ± 2[a]	52 ± 5

[a] Significantly different from DCVC (400 μM) (analysis of variance, $p \leq 0.05$).
[b] PTC were pretreated with AOA (1 mM) for 15 min before the addition of DCVC.
[c] DPPD (20 μM) was added just before the addition of DCVC.

TABLE 3

Effect of DEF and DPPD on oxidation of DhRho123 in PTC caused by H_2O_2

PTC loaded with DhRho123 were treated with 1 mM H_2O_2 in the presence or absence of either DEF (1 mM) or DPPD (20 μM). After 10 min, the amount of oxidation of DhRho123 in PI-negative cells (i.e., living cells) was determined by flow cytometric analysis as described in Materials and Methods. DEF and DPPD were added 15 min before the addition of H_2O_2. Values shown are the relative amount of DhRho123 present in the cells expressed as times over control (mean ± standard error of four experiments).

Treatment	DhRho123 oxidation
Control	1.00[a]
+ H_2O_2	6.41 ± 0.38[b]
+ DPPD	0.79 ± 0.03[b]
+ H_2O_2	0.97 ± 0.06[b,c]
+ DEF	0.82 ± 0.04[b]
+ H_2O_2	1.23 ± 0.20[b,c]

[a] arbitrary fluorescence units.
[b] Significantly different from control (analysis of variance, $p \leq 0.05$).
[c] Significantly different from the same treatment without H_2O_2 (analysis of variance, $p \leq 0.05$).

and, presumably, lipid hydroperoxides are detected with DhRho123. At the concentration used for detection of lipid peroxidation in these experiments, DhRho123 did not cause cell death, nor could it prevent cell death caused by prooxidants such cumene hydroperoxide or *t*-butylhydroperoxide; therefore, there is no pronounced antioxidant effect at this DhRho123 concentration.

Incubation of PTC with DCVC resulted in an increase in the Rho123 fluorescence of the PI-negative (i.e., living) cells (Fig. 2, B and C). The increase was both time and concentration dependent on DCVC (Fig. 3A) and occurred before the onset of cell death (Fig. 3B). With 400 μM DCVC, a maximum in Rho123 fluorescence intensity of PI-negative cells was observed after a 30-min incubation. The antioxidant DPPD completely prevented DhRho123 oxidation (not shown) and cell death (Table 3). Also, the iron chelator DEF (1 mM) prevented DhRho123 oxidation and cell death (not shown). This suggests that the majority of hydroperoxides, measured after exposure of PTC to DCVC, are formed during lipid peroxidation and therefore most likely represent lipid hydroperoxides.

The inhibitor of β-lyase, AOA, also completely prevented hydroperoxide formation (not shown) and cell death (Table 3), suggesting that formation of reactive DCVC metabolites is coupled to oxidative stress.

Miller *et al.* (40) demonstrated that glycine inhibits the cell death of rabbit PTC caused by a low concentration of DCVC (25 μM). To determine whether in our system the presence of glycine influenced the mechanism by which DCVC causes cytotoxicity, PTC were incubated with DCVC in either the absence or presence of glycine. In the presence of glycine, DCVC caused the same extent of hydroperoxide formation (Fig. 4A) and cell death (Fig. 4B) as in its absence. To investigate whether in the absence of glycine lipid peroxidation was also the major cause of DCVC-induced cell death, PTC incubated in the presence or absence of glycine were treated with DCVC plus DPPD. In the absence of glycine, the antioxidant DPPD was still cytoprotective against both hydroperoxide formation and cell death (Fig. 4, A and B). However, at later timepoints, DPPD could not prevent cell death caused by DCVC when glycine was absent.

Effect of DCVC on the enzyme activity of complexes I and II. Both Lash and Anders (18) and Hayden and Stevens (19) demonstrated that treatment of isolated mitochondria with DCVC results in an inhibition of both site 1 and site 2 respiration as well as succinate dehydrogenase activity, suggesting inhibition of both complex I and complex II activity. Because DCVC also causes mitochondrial injury in both freshly isolated PTC (5, 23) and renal proximal tubules from the rabbit (41), the effect of DCVC on the activity of both complex I and II in intact PTC was determined. The activity of complex I, as assessed in the cell homogenate, was decreased by only 10% after a 15-min incubation with 400 μM DCVC and did not decrease any further after a 30-min incubation of PTC. This effect of DCVC was independent on the activity of β-lyase since AOA did not prevent it (Table 4). In contrast, the activity of complex II was already decreased by 36% after a 15-min incubation with DCVC. The β-lyase inhibitor AOA completely protected against this decrease (Table 4), but it was not prevented by the antioxidant DPPD.

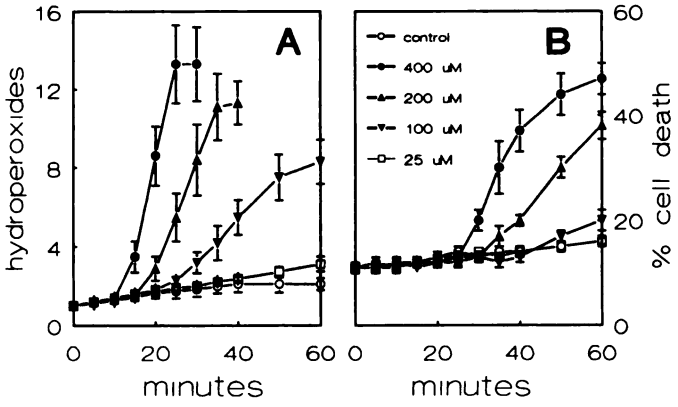

Fig. 3. Effect of DCVC on hydroperoxide formation and cell death. PTC were incubated with either 25, 100, 200, or 400 μM DCVC, and the effects on hydroperoxide formation and cell death were analyzed as described in Materials and Methods. Values are given as mean ± standard error of five independent cell isolations.

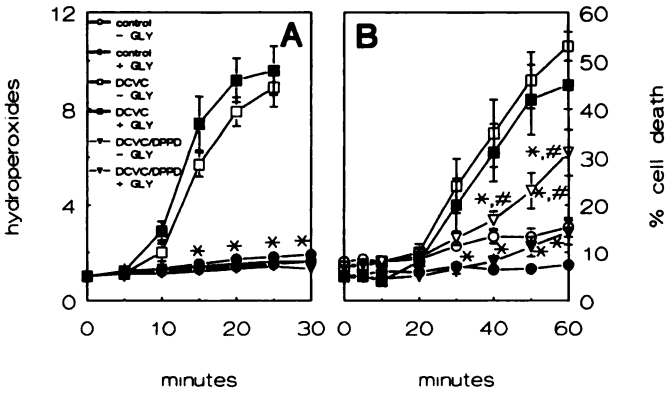

Fig. 4. Effect of glycine on DCVC-induced hydroperoxide formation and cell death. PTC were incubated with DCVC (400 μM) in the presence or absence of DPPD (20 μM) in Hank's balanced salt solution/HEPES buffer in which glycine (5 mM) was either added or omitted. Hydroperoxide formation and cell death were analyzed as described in Fig. 3 legend. Values are given as mean ± standard error of four independent cell isolations. *, Significantly different from DCVC-treated cells alone (analysis of variance, $p \leq 0.05$); #, significantly different from the same treatment with glycine (analysis of variance, $p \leq 0.05$).

TABLE 4

Effect of DCVC on the activity of complexes I and II

PTC were incubated with various concentrations of DCVC for either 15 or 30 min, and the effect on the activities of complexes I and II were determined by measuring NADH:Q oxidoreductase and SQR activity respectively. Activity is expressed as mean ± standard error percentage of untreated control incubations of three or four independent cell isolations. The activities of both complexes I and II were determined for each sample.

Treatment	Complex I	Complex II	
	30 min	15 min	30 min
Control	100	100[a]	100[b]
DCVC			
100 μM	ND[c]	101 ± 6	75 ± 7[d]
200 μM	ND[c]	77 ± 13[d]	64 ± 8[d]
400 μM	88 ± 4	64 ± 9[d]	55 ± 8[d]
400 μM + AOA	86 ± 7	106 ± 10[•]	95 ± 7[•]
400 μM + DPPD	79 ± 5[d]	71 ± 11[d]	52 ± 12[d]

[a] Mean activity of NADH:Q oxidoreductase activity of untreated PTC was 2.1 ± 0.4 μmol/mg cell protein/min (mean ± standard error of four different cell isolations).

[b] Mean activity of SQR activity of untreated PTC was 6.6 ± 1.1 μmol/mg cell protein/min (mean ± standard error of four different cell isolations).

[c] Not determined.

[d] Significantly different from control (analysis of variance, $p < 0.05$).

[•] Significantly different from DCVC (analysis of variance, $p \leq 0.05$).

Effect of inhibition of complex II on the DCVC-induced oxidative cell injury. To investigate whether inhibition of complex II affects the antioxidant properties of PTC, cells were incubated with TTFA, an inhibitor of SQR (35). Exposure of PTC to DCVC (400 μM) together with TTFA (250 μM) potentiated hydroperoxide formation and cell death caused by DCVC (Fig. 5, C and F). TTFA also potentiated hydroperoxide formation and cell death caused by 100 μM (Fig. 5, A and D) and 200 μM DCVC (Fig. 5, B and E). TTFA (250 μM) did not cause cytotoxicity during the time period of the experiment (Table 1).

Effect of inhibition of cytochrome *c* oxidase on the DCVC-induced oxidative stress and cell death. To investigate the role of SQR activity as part of a cellular antioxidant pathway, we treated cells with an inhibitor of cytochrome *c* oxidase, KCN, which increases the succinate-mediated prevention of oxidative stress in submitochondrial particles (9). KCN could be used because glycine was present in the buffer, which protects against KCN-induced toxicity to proximal tubules (Table 1; Ref. 36). KCN (1 mM) clearly prevented the formation of hydroperoxides and cell death caused by DCVC (Fig. 6, A and B). To investigate whether the cytoprotection by KCN was related to either NADH:Q reductase activity or SQR activity, PTC were treated with KCN in combination with an inhibitor of complex I, Rot, or an inhibitor of complex II, Oxa, respectively. Inhibition of complex I by Rot (20 μM) (Fig. 6) and complex III by antimycin (5 μM) (results not shown) did not prevent the KCN-mediated cytoprotection. In contrast, Oxa (10 mM) almost completely overruled the KCN cytoprotection against the DCVC-induced oxidative stress and cell death (Fig. 6). This effect was even more pronounced when Oxa was used in combination with Rot (Fig. 6). A similar inhibition of the KCN-mediated protection against DCVC-induced oxidative stress was observed with another inhibitor of complex II, TTFA (250 μM) (not shown).

Effect of DCVC on the redox state of cellular Q. The antioxidant properties resulting from the SQR activity of complex II in isolated submitochondrial particles are related to the redox state of Q; formation of QH_2 resulted in protection against oxidative stress (9–11). We hypothesized that inhibition of complex II caused by DCVC might affect the redox state of Q and thereby reduce the cellular protection against oxidative stress. To investigate this, we determined whether the DCVC-induced cytotoxicity was associated with a decrease in the redox state of Q (expressed as $QH_2/Q + QH_2$). In untreated PTC, QH_2 was 41% of total Q. After a 30-min incubation with DCVC (400 μM), QH_2 had diminished to only 10% of total Q, clearly demonstrating a decrease in the amount of reduced Q (Fig. 7). The total amount of Q after exposure to DCVC was unchanged, indicating that DCVC affected only the redox state of Q and left Q intact. The decrease of QH_2 was largely prevented by AOA (Table 5). In contrast, the antioxidant DPPD, although equal in preventing DCVC-induced cell death, did not prevent the DCVC-induced decrease of QH_2 (Table 5), indicating that this decrease is not caused by oxidative stress.

Effect of KCN on the redox state of Q. To investigate whether KCN-mediated cytoprotection against the DCVC-induced oxidative cellular injury was related to changes in the redox state of Q, we also defined the effect of KCN on $(QH_2/Q + QH_2)$. Treatment of PTC with KCN (1 mM) for 30 min resulted in a 60% increase in the percentage of QH_2 (Fig. 7). In the presence of KCN, the decrease in $(QH_2/Q + QH_2)$

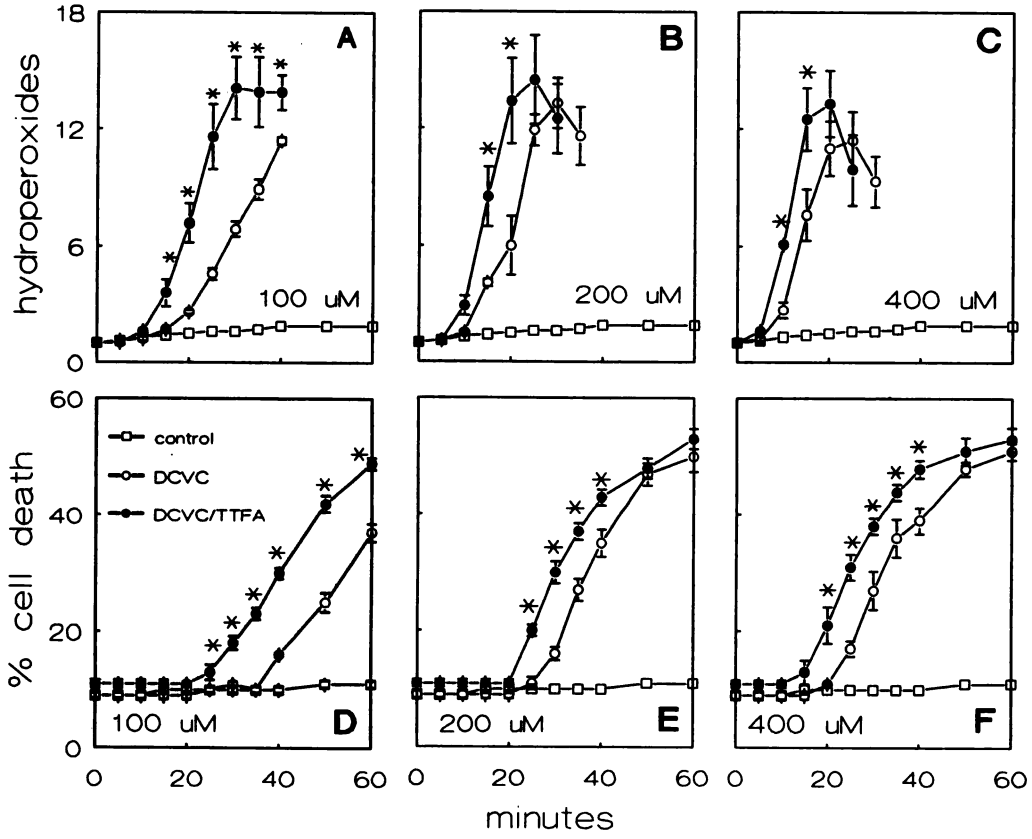

Fig. 5. Effect of TTFA on the DCVC-induced hydroperoxide formation and cell death. PTC were treated with either 100 (A and D), 200 (B and E), or 400 (C and F) μM DCVC in the absence or presence of TTFA, and the effects on hydroperoxide formation (A through C) and cell death (D through F) were analyzed as described in Fig. 3 legend. TTFA (250 μM) was added just before the start of the experiment. Values are given as mean ± standard error of four independent cell isolations. *, Significantly different from DCVC-treated cells alone (Student's *t* test; $p \leq 0.05$).

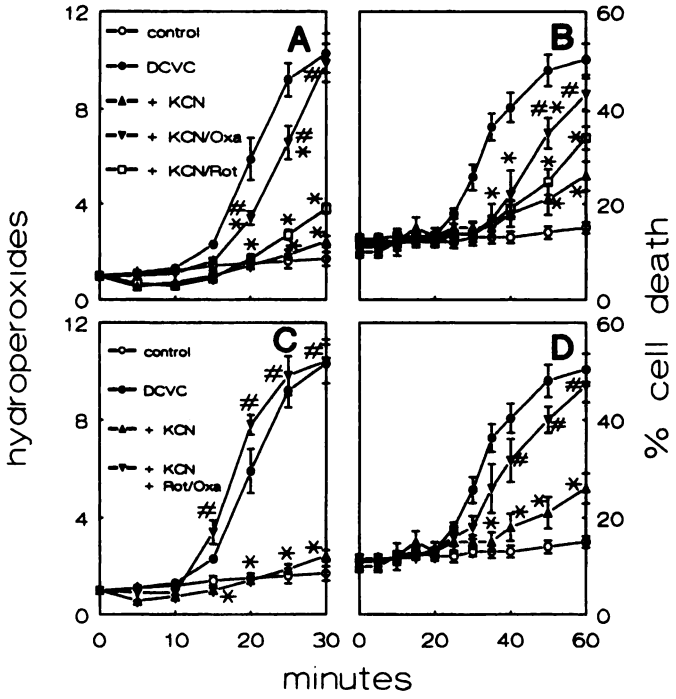

Fig. 6. Effect of KCN in combination with either Rot and/or Oxa on the DCVC-induced hydroperoxide formation and cell death. PTC were treated with 400 μM DCVC in the absence or presence of inhibitors. KCN (1 mM) and Rot (20 μM) were added just before the start of the experiment, whereas Oxa (10 mM) was added 15 min before the addition of DCVC. The effect on hydroperoxide formation and cell death were analyzed as described in Fig. 3 legend. Values are given as mean ± standard error of four independent cell isolations. *, Significantly different from DCVC-treated cells alone (analysis of variance, $p \leq 0.05$); #, significantly different from DCVC/KCN-treated cells (analysis of variance, $p \leq 0.05$).

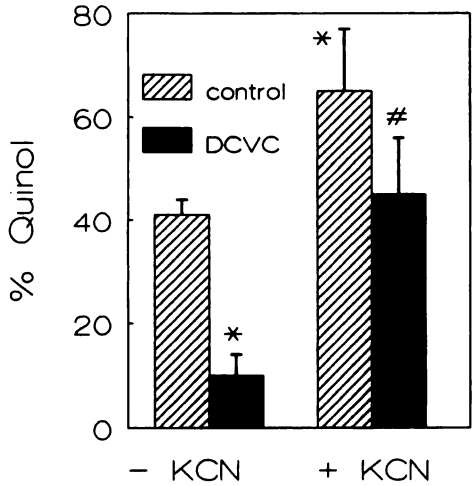

Fig. 7. Effect of KCN on the DCVC-induced decrease of the redox state of Q. PTC were treated with DCVC (400 μM) in the presence or absence of KCN (1 mM). KCN was added just before the start of the experiment. After a 30-min incubation, Q was extracted from the cells, and reduced and oxidized Q were analyzed by HPLC analysis as described in Materials and Methods. Values are expressed as mean ± standard error percentage quinol content in the cells versus total Q present in the cells of four to seven independent cell isolations. *, Significantly different from untreated PTC (analysis of variance, $p \leq 0.05$).

TABLE 5

Effect of DPPD and AOA on the DCVC-induced decrease of (QH_2/Q + QH_2)

PTC were incubated with DCVC (400 μM) in either the presence or absence of AOA (1 mM) or DPPD (20 μM). AOA was added 15 min before the start of the experiments. After a 30-min incubation, Q was extracted and analyzed by HPLC as described in Materials and Methods. Numbers in parentheses indicate percent of control.

Treatment	(QH_2/Q + QH_2)
	% *control*
Control	41 ± 3 (100)
DCVC	10 ± 4[a] (25)
+ AOA	33 ± 4[b] (80)
+ DPPD	12 ± 2 (29)

[a] Significantly different from control (analysis of variance, $p < 0.05$).
[b] Significantly different from DCVC (analysis of variance, $p \leq 0.05$).

caused by DCVC was less; in this case, the percentage of QH_2 was equal to the control level without DCVC (Fig. 7). When PTC were treated with Rot (20 μM), which itself decreased QH_2 by 74%, KCN could not increase QH_2 (Table 6). However, when PTC were incubated with an inhibitor of complex II, Oxa (10 mM), KCN could still increase the QH_2 in the cells, although the absolute amount of QH_2 present was less than with KCN alone (Table 6). Inhibition of complex II with either Oxa or TTFA alone also decreased QH_2 in the PTC (Table 6).

Discussion

To study the role of complex II in protection against xenobiotic-induced oxidative stress, we used the nephrotoxic L-cysteine *S*-conjugate DCVC. This conjugates causes oxidative stress in renal tubular epithelial cells (Fig. 3; Ref. 5), which is recognized as a major event in the initiation of cell death by this compound (5, 20, 22, 24). Observations in submitochondrial particles suggested that complex II of the MRC plays an important role in the protection of cells against oxidative stress (9–12, 15). Because nephrotoxic cysteine conjugates cause mitochondrial injury (5, 18, 19, 23), including inhibition of succinate dehydrogenase activity in isolated mitochondria from renal cortex (18), we hypothesized that the oxidative stress caused by the cysteine conjugates was related to decreased activity of complex II. DCVC treatment of

TABLE 6

Effect of various inhibitors of the MRC on the KCN-mediated increase of (QH_2/Q + QH_2)

PTC were incubated either with KCN (1 mM), Rot (20 μM), Oxa (10 mM), or TTFA (250 μM). In cotreatments with KCN, other inhibitors were added 15 min before the addition of KCN. Oxa was always added 15 min before the start of the experiment. After a 30-min incubation, Q was extracted and analyzed by HPLC as described in Materials and Methods. Values are given as mean ± standard error of three to five experiments.

Treatment	(QH_2/Q + QH_2)	% of control
Control	39 ± 5	100
KCN	64 ± 11[a]	164
+ Rot	12 ± 4[a,b]	31
+ Oxa	37 ± 14[b]	95
+ Rot/Oxa	9 ± 4[a,b]	23
Rot	10 ± 2[a]	26
Oxa	8 ± 2[a]	21
Rot/Oxa	8 ± 3[a]	21
TTFA	15 ± 6[a]	38

[a] Significantly different from control (analysis of variance, $p < 0.05$).
[b] Significantly different from KCN (analysis of variance, $p \leq 0.05$).

PTC decreased SQR activity after a 15-min incubation (Table 3), before the increase in the formation of hydroperoxides occurred. The NADH:Q reductase activity of complex I remained unaffected. This suggests that complex II is a target of the respiratory chain in DCVC-induced PTC injury. The latter hypothesis is supported by observations that DCVC inhibits succinate-dehydrogenase activity and succinate-dependent respiration in isolated rat renal mitochondria (18, 19). The DCVC-induced decrease in the activity of complex II is dependent on cysteine-conjugate β-lyase metabolism (Tables 2 and 3). This suggests that covalent binding of reactive metabolites of DCVC to specific proteins of the MRC, possibly also to components of complex II, may cause the decrease in SQR activity and thereby increase the susceptibility to oxidative stress. Although several mitochondrial matrix proteins, e.g., HSP60, mortalin, 2-oxoglutarate, malate dehydrogenase, and methylmalonate semialdehyde dehydrogenase, have been identified as targets for reactive metabolites of the nephrotoxic cysteine conjugates (37, 38), this has not been investigated for the complex II proteins. It would be interesting to investigate whether covalent binding occurs to these proteins to further delineate the molecular pathways involved in failure of the antioxidant machinery and/or the initiation of the oxidative stress after exposure to nephrotoxic cysteine conjugates.

If complex II is involved in the protection against oxidative stress, then an inhibition of complex II (preventing SQR activity) should potentiate the hydroperoxide formation and cell death caused by DCVC. Therefore, we treated cells with TTFA, which interacts with the iron-sulfur center S-3 of complex II, resulting in prevention of Q reduction (35, 39; Table 4). TTFA clearly potentiated the DCVC-induced oxidative stress and cell death, even at the very low DCVC concentration of 100 μM; this indicated that inactivation of complex II increases the susceptibility to oxidative cell injury.

Under normal conditions, complex II activity is linked to reduction of cytochrome *c*, so that only a portion of the reducing equivalents entering complex II may be used for its antioxidant function (Fig. 1). Therefore, blockade of the respiratory chain at a site distal from complex II would prevent the cytochrome *c* reductase-related activity of complex II and therefore might increase the availability of reducing equivalents for the antioxidant-related SQR activity of complex II (Fig. 1). To investigate this, we incubated PTC with an inhibitor of cytochrome *c* oxidase, KCN, which almost completely prevented the oxidative cell injury by DCVC. The KCN-mediated cytoprotection was not related to activity of complex I or III, since neither Rot nor antimycin could prevent it. In contrast, a competitive inhibitor of succinate dehydrogenase, Oxa, overruled the KCN-mediated protection against oxidative stress. Together, these data strongly suggest that complex II is part of a cellular antioxidant pathway.

In submitochondrial particles, the antioxidant properties of complex II have been related to the SQR activity, which results in the reduction of Q to QH_2, a potential antioxidant that prevents oxidative stress in various biological systems (6–8). The DCVC-induced decrease of SQR activity might therefore decrease the reduction of Q, resulting in a decrease in (QH_2/Q + QH_2), which would increase the susceptibility for oxidative cell injury. DCVC decreased the amount of cellular QH_2 without affecting the total cellular content of Q. The antioxidant DPPD did not prevent the DCVC-induced decrease of QH_2, indicating that the decrease in QH_2 is not the consequence of the oxidative stress. Other inhibitors of complex II, Oxa and TTFA, were also capable of decreasing QH_2 in PTC, indicating a direct relationship between inhibition of complex II and the redox state of Q. The KCN-mediated cytoprotection against the DCVC-induced oxidative stress (as discussed) was clearly associated with an increased level of cellular QH_2, confirming an inverse relationship between total cellular QH_2 and oxidative stress.

However, the role of QH_2 was not equally straightforward in all of our experiments. Thus, the increase of QH_2 caused by KCN was dependent on the activity of complex I, since Rot inhibited the increase of QH_2 completely. However, surprisingly, Rot did not affect the cytoprotection by KCN. In contrast, inhibition of complex II with Oxa only partially prevented the KCN-mediated increase of QH_2, leaving QH_2 values around control levels. However, Oxa overruled the cytoprotection against oxidative stress provided by KCN. These data suggest that the KCN-mediated cytoprotection may depend on the reduction of Q by complex II rather than on the total amount of reduced Q present in the cells. Although the cumulative evidence indicates that SQR activity of complex II is necessary for proper cellular protection against oxidative stress, it thus is not clear whether this activity is related to the formation of QH_2 or to the recycling of the even more potent antioxidant, α-tocopherol (12–15). Because Oxa did prevent the KCN-mediated cytoprotection (Fig. 6) at QH_2 levels around control values (Table 4), we believe that the antioxidant function related to the SQR activity of complex II is coupled to another system with antioxidant properties, which may be the recycling of α-tocopherol. Although this has been demonstrated in isolated mitochondria (13, 15), further experiments are needed to verify this in intact cells.

Inhibition of complex II with TTFA did not cause oxidative stress, suggesting that there are other antioxidant systems acting in parallel to the SQR-related antioxidant pathway that may be inhibited by DCVC. Vitamin E radical reductase, DT-diaphorase, GSH-*S*-transferases, and GSH reductase and GSH peroxidase (i.e., GSH redox cycle) have been implicated in the protection against lipid peroxidation in various systems (42–44). Although at present we do not know the effect of DCVC on all of these enzyme systems, Lock and Schnellmann recently found that DCVC inhibits GSH reductase (45). We also found that DCVC inhibits the enzymes of the GSH redox cycle, GSH reductase and GSH peroxidase, in PTC (46). This inhibition occurred after 10 min, before the increase in hydroperoxide generation. Furthermore, the thiol reductant dithiothreitol prevented DCVC-induced hydroperoxide formation and cell death. Both depletion of mitochondrial glutathione, using 3-hydroxy-4-pentenoic acid, or oxidation of thiols, using diamide, potentiated DCVC-induced hydroperoxide formation and cell death (46). These data strongly support the idea that in addition to inhibition of complex II, the DCVC-induced perturbation of the GSH redox cycle is involved in the oxidative cell injury caused by DCVC. Depletion of mitochondrial GSH, using 3-hydroxy-4-pentenoic acid, did not cause oxidative cell injury; however, the addition of TTFA, which also does not cause oxidative stress, rapidly increased hydroperoxide formation, followed by cell death. Together, these data suggest that the antioxidant function of the complex II acts in parallel with another

antioxidant function, presumably GSH redox cycle, although the role of other antioxidant systems cannot be excluded.

We propose the following mechanism for the DCVC-induced oxidative cellular injury: DCVC is metabolized by renal cysteine conjugate β-lyase to reactive metabolites, which bind covalently to macromolecules, especially in the mitochondria (19). This covalent binding affects the function of these proteins, presumably including components of complex II (as discussed) and enzymes of the glutathione redox cycle (46). The decrease in activity of these enzymes causes a failure of the cellular antioxidant system, thereby increasing the susceptibility to oxidative cell injury. Oxidative stress likely arises from the mitochondria because (i) prevention of the uptake of mitochondrial Ca^{2+} inhibited the oxidative stress (5) and (ii) various inhibitors of the MRC affected the DCVC-induced hydroperoxide formation (results from present study). The oxidative stress aggravates mitochondrial injury already caused by the binding of reactive metabolites of DCVC (5, 18, 19). Both the oxidative stress and mitochondrial injury lead to irreversible cellular injury.

In conclusion, the present data demonstrate that complex II of the MRC plays a role in a cellular antioxidant pathway and that the inactivation of this complex by xenobiotics may increase the susceptibility to oxidative cell injury.

Acknowledgments

We would like to acknowledge Prof. Dr. Gerard J. Mulder for critical reading of the manuscript and Jaap van Hellemond for helpful discussions and enthusiastic support with the enzymatic assays.

References

1. H. Sies, ed. *Oxidative Stress*. Academic Press, New York/London (1985).
2. Ambrosio, G., J. L. Zweier, C. Duilio, P. Kuppusamy, G. Santoro, P. P. Elia, I. Tritto, P. Cirillo, M. Condorelli, M. Chiariello, M., and J. T. Flaherty. Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *J. Biol. Chem.* **268:**18532–18541 (1993).
3. Schulze-Osthof, K., A. C. Bakker, B. Vanhaesebroeck, R. Beyaert, W. A. Jacob, and W. Fiers. Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions: evidence for the involvement of mitochondrial radical generation. *J. Biol. Chem.* **267:**5317–5323 (1992).
4. Dawson, T. L., G. J. Gores, A.-L. Nieminen, B. Herman, and J. J. Lemasters. Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes. *Am. J. Physiol.* **264:**C962–C967 (1993).
5. Van de Water, B., J. P. Zoeteweij, H. J. G. M. de Bont, G. J. Mulder, and J. F. Nagelkerke. The role of mitochondrial Ca^{2+} in the oxidative stress-induced dissipation of the mitochondrial membrane potential: studies in isolated proximal tubular cells using the nephrotoxin 1,2-dichlorovinylcysteine. *J. Biol. Chem.* **269:**14546–14552 (1994).
6. Kagan, V. E., E. A. Serbinova, G. M. Koynova, S. A. Kitanova, V. A. Tyurin, T. S. Stoytchev, P. J. Quinn, and L. Packer. Antioxidant action of ubiquinol homologues with different isoprenoid chain length in biomembranes. *Free Rad. Biol. Med.* **9:**117–126 (1990).
7. Frei, B., M. C. Kim, and B. N. Ames. Ubiquinol is an effective lipid-soluble antioxidant at physiological concentrations. *Proc. Natl. Acad. Sci. USA* **87:**4879–4883 (1990).
8. Forsmark, P., F. Aberg, B. Norling, K. Nordenbrand, G. Dallner, and L. Ernster. Inhibition of lipid peroxidation by ubiquinol in submitochondrial particles in the absence of vitamin E. *FEBS Lett.* **285:**39–43 (1991).
9. Takayanagi, R., K. Takesige, and S. Minakami. NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochem. J.* **192:**853–860 (1980).
10. Hasegawa, E., K. Takeshige, T. Oishi, Y. Murai, and S. Minakami. 1-Methyl-4-phenylpyridinium (MPP+) induces NADH-dependent super-oxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochem. Biophys. Res. Commun.* **170:**1049–1055 (1990).
11. Eto, Y., D. Kang, E. Hasegawa, K. Takeshige, and S. Minakami. Succinate-dependent lipid peroxidation and its prevention by reduced ubiquinone in beef heart submitochondrial particles. *Biochim. Biophys. Acta* **295:**101–106 (1992).
12. Maguire, J. J., D. S. Wilson, and L. Packer. Mitochondrial electron transport-linked tocopheroxyl radical reduction. *J. Biol. Chem.* **264:**21462–21465 (1989).
13. Kagan, V., E. Serbinova, and L. Packer. Antioxidant effects of ubiquinones in microsomes and mitochondria are mediated by tocopherol recycling. *Biochem. Biophys. Res. Commun.* **169:**851–857 (1990).
14. Hiramatsu, M., R. D. Valesco, D. S. Wilson, and L. Packer. Ubiquinone protects against loss of tocopherol in rat liver microsomes and mitochondrial membranes. *Res. Commun. Chem. Pathol. Pharmacol.* **72:**231–241 (1991).
15. Maguire, J. J., V. Kagan, B. A. C. Ackrell, E. Serbinova, and L. Packer. Succinate-ubiquinone reductase linked recycling of α-tocopherol in reconstituted systems and mitochondria: requirement for reduced ubiquinone. *Arch. Biochem. Biophys.* **292:**47–53 (1992).
16. Parker, V. H. A biochemical study of S-dichlorovinyl-L-cysteine. *Food Cosmet. Toxicol.* **3:**75 (1965).
17. Stevens, J., P. Hayden, and G. Taylor. The role of glutathione conjugate metabolism and cysteine conjugate β-lyase in the mechanism of S-cysteine conjugate toxicity in LLC-PK_1 cells. *J. Biol. Chem.* **261:**3325–3332 (1986).
18. Lash, L. H., and M. W. Anders. Mechanism of S-(1,2-dichlorovinyl)-L-cysteine- and S-(1,2-dichlorovinyl)-L-homocysteine-induced renal mitochondrial toxicity. *Mol. Pharmacol.* **32:**549–556 (1987).
19. Hayden, P. J., and J. L. Stevens. Cysteine conjugate toxicity, metabolism, and binding to macromolecules in isolated rat kidney mitochondria. *Mol. Pharmacol.* **37:**468–476 (1990).
20. Chen, Q., T. W. Jones, P. C. Brown, and J. L. Stevens. The mechanism of cysteine conjugate cytotoxicity in renal epithelial cells: covalent binding leads to thiol depletion and lipid peroxidation. *J. Biol. Chem.* **265:**21603–21611 (1990).
21. Vamvakas, S., V. K. Sharma, S. Sheu, and M. W. Anders. Perturbations of intracellular calcium distribution in kidney cells by nephrotoxic haloalkenyl cysteine *S*-conjugates. *Mol. Pharmacol.* **38:**455–461 (1990).
22. Groves, C. E., E. A. Lock, and R. G. Schnellmann. Role of lipid peroxidation in renal proximal tubule cell death induced by haloalkenyl cysteine conjugates. *Toxicol. Appl. Pharmacol.* **107:**54–62 (1991).
23. Van de Water, B., J. P. Zoeteweij, H. G. J. M. de Bont, G. J. Mulder, and J. F. Nagelkerke. The relationship between intracellular Ca^{2+} and the mitochondrial membrane potential in isolated proximal tubular cells from rat kidney exposed to the nephrotoxin 1,2-dichlorovinyl-cysteine. *Biochem. Pharmacol.* **45:**2259–2267 (1993).
24. Chen, Q., T. W. Jones, and J. L. Stevens. Early cellular events couple covalent binding of reactive metabolites to cell killing by nephrotoxic cysteine conjugates. *J. Cell. Physiol.* **161:** 293–303 (1996).
25. Silber, P. M., A. J. Gandolfi, and K. Brendel. Early biological indicators of S-(1,2-dichlorovinyl)-L-cysteine nephrotoxicity in the rabbit. *Drug Chem. Toxicol.* **9:**285–303 (1986).
26. Koechel, D. A., M. E. Krejci, and R. E. Ridgewell. The acute effects of S-(1,2-dichlorovinyl)-L-cysteine and related chemicals on renal function and ultrastructure in the pentobarbital-anesthetized dog: structure-activity relationships, biotransformation, and unique site-specific nephrotoxicity. *Fundam. Appl. Toxicol.* **17:**17–33 (1991).
27. Dekant, W., L. H. Lash, and M. W. Anders. Bioactivation mechanism of the cytotoxic and nephrotoxic S-conjugate S-(2-dichloro-1,1,2-trifluoroethyl)-L-cysteine. *Proc. Natl. Acad. Sci. USA* **84:**7443–7447 (1987).
28. Dekant W., Vamvakas S., and Anders, M. W. Bioactivation of nephrotoxic haloalkenes by glutathione conjugation: formation of toxic and mutagenic intermediates by cysteine conjugate β-lyase. *Drug. Metab. Rev.* **20:**43–83 (1989).
29. Commandeur, J. M. N., and N. P. E. Vermeulen. Identification of N-acetyl-S-(2,2-dichlorovinyl)- and N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine as two regioisomeric mercapturic acids of trichloroethylene in the rat. *Chem. Res. Toxicol.* **3:**212–218 (1990).
30. Lash, L. H., A. A. Elfarra, and M. W. Anders. Renal cysteine conjugate β-lyase: bioactivation of nephrotoxic cysteine S-conjugates in mitochondrial outer membrane. *J. Biol. Chem.* **261:**5930–5935 (1986).
31. Stevens, J. L., J. D. Robbins, and R. A. Byrd. A purified cysteine conjugate β-lyase from rat renal cytosol: requirements for an α-keto acid or an amino acid oxidase for activity and identity with soluble glutamine transaminase K. *J. Biol. Chem.* **261:**15529–15537 (1986).
32. Boogaard, P. J., G. J. Mulder, and J. F. Nagelkerke. Isolated proximal tubular cells as an *in vitro* model for studies on nephrotoxicity. I. An improved method for preparation of a homogeneous suspension of proximal tubular cells and characterization of α-methylglucose uptake. *Toxicol. Appl. Pharmacol.* **101:**135–143 (1989).
33. Henderson, L. M., and J. B. Chapell. Dihydrorhodamin 123: a fluorescent probe for superoxide generation? *Eur. J. Biochem.* **217:**973–980 (1993).
34. Royall, J. A., and H. Ischiropoulus. Evaluation of 2′7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. *Arch. Biochem. Biophys.* **302:**348–355 (1993).
35. Bagenski, M. L., and Y. Hatefi. Reconstitution of succinate-coenzyme Q reductase (complex II) and succinate oxidase activities by highly purified, reactivated succinate dehydrogenase. *J. Biol. Chem.* **244:**5313–5319 (1969).
36. Weinberg, J. M., and M. A. Venkatachalam. Relationship between oxidant and non-oxidant mechanisms in the pathogenesis of acute renal failure. *Miner. Electrolyte Metab.* **18:**123–132 (1992).

37. Bruschi, S. A., J. W. Crabb, and J. L. Stevens. Mitochondrial HSP60 (Pa protein) and a HSP70-like protein (mortalin) are major targets for modification during *S*-(1,1,2,2-tetrafluoroethyl)-*L*-cysteine-induced nephrotoxicity. *J. Biol. Chem.* **268**:23157–23161 (1993).
38. Bruschi, S. A., J. W. Crabb, and J. L. Stevens. The E_3 subunit of 2-oxoglutarate, branched chain α-keto acid, and malate dehydrogenase are adducted during nephrotoxic cysteine-conjugate injury. *Toxicologist* **14**:428 (1994).
39. Ingledew, W. J., and T. Ohnishi. The probable site of action of thenoyltrifluoroacetone on the respiratory chain. *Biochem. J.* **164**:617–620 (1977).
40. Miller, G. W., E. A. Lock, and R. G. Schnellmann. Strychnine and glycine protect renal proximal tubules from various nephrotoxicants and act in the late phase of necrotic cell injury. *Toxicol. Appl. Pharmacol.* **125**:192–197 (1994).
41. Groves, C. E., P. J. Hayden, E. A. Lock, and R. G. Schnellmann. Differential cellular effects in the toxicity of haloalkene and haloalkene cysteine conjugates to rabbit renal proximal tubules. *J. Biochem. Toxicol.* **8**:49–56 (1993).
42. Mosialou, E., G. Ekström, A. E. P. Adang, and R. Mogenstern. Evidence that rat liver microsomal glutathione transferase is responsible for glutathione-dependent protection against lipid peroxidation. *Biochem. Pharmacol.* **45**:1645–1651 (1993).
43. Beyer, R. E. The participation of coenzyme Q in free radical production and antioxidation. *Free Rad. Biol. Med.* **8**:545–565 (1990).
44. Reed, D. J. Regulation of reductive processes by glutathione. *Biochem. Pharmacol.* **35**:7–13 (1985).
45. Lock, E. A., and Schnellmann, R. G. The effect of haloalkene cysteine conjugates on rat renal glutathione reductase and lipoyl dehydrogenase activities. *Toxicol. Appl. Pharmacol.* **104**:180–190 (1990).
46. van de Water, B. Molecular mechanisms of nephrotoxin-induced necrotic and apoptotic cell death. Ph.D. Thesis, Leiden University, The Netherlands, 1995.

Send reprint requests to: Dr. J. Fred Nagelkerke, Division of Toxicology, Sylvius Laboratory, P.O. Box 9503, 2300 RA Leiden, The Netherlands.