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EVENTS THAT PRECEDE AND THAT FOLLOW *S*-(1,2-DICHLOROVINYL)-L-CYSTEINE-INDUCED RELEASE OF MITOCHONDRIAL Ca^{2+} AND THEIR ASSOCIATION WITH CYTOTOXICITY TO RENAL CELLS

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Abstract—Previous studies showed that *S*-(1,2-dichlorovinyl)-L-cysteine perturbs intracellular Ca^{2+} homeostasis [Vamvakas *et al.*, *Mol Pharmacol* **38**: 455–461, 1990]. The objective of the present study was to investigate the cellular events that precede and that follow *S*-(1,2-dichlorovinyl)-L-cysteine-induced mitochondrial Ca^{2+} release. In incubations with isolated kidney mitochondria, *S*-(1,2-dichlorovinyl)-L-cysteine-induced Ca^{2+} efflux is preceded by increased oxidation of mitochondrial pyridine nucleotides and is prevented by ATP, an inhibitor of the hydrolysis of pyridine nucleotides, and by *meta*-iodobenzylguanidine, an acceptor of ADP-ribose moieties. In LLC-PK_1 cells, elevation in the cytosolic Ca^{2+} concentration is followed by a several-fold increase in DNA double-strand breaks which is attributed to the activation of Ca^{2+}- and Mg^{2+}-dependent endonucleases. The formation of DNA double-strand breaks is followed by increased poly(ADP-ribosylation) of nuclear proteins. *S*-(1,2-Dichlorovinyl)-L-cysteine-induced cytotoxicity in LLC-PK_1 cells is blocked by chelation of cytosolic Ca^{2+} with Quin-2, by inhibition of DNA fragmentation with aurintricarboxylic acid and by inhibition of increased poly(ADP-ribosyl)transferase activity by 3-aminobenzamide. These findings indicate that *S*-(1,2-dichlorovinyl)-L-cysteine bioactivation in renal cells may initiate the following cascade of events: increased oxidation and hydrolysis of mitochondrial pyridine nucleotides resulting in the modification of mitochondrial membrane proteins by pyridine nucleotide-derived ADP-ribose moieties, followed by Ca^{2+} release. Elevated Ca^{2+} concentrations may activate Ca^{2+}-dependent endonucleases, which leads to DNA fragmentation followed by increased poly(ADP-ribosylation) of nuclear proteins and, finally, cytotoxicity.

S-(1,2-Dichlorovinyl)-L-cysteine is the key metabolite responsible for the acute and chronic nephrotoxicity and perhaps nephrocarcinogenicity of trichloroethene and dichloroethine [1, 2]. Trichloroethene and dichloroethine are biotransformed to *S*-(1,2-dichlorovinyl)-L-cysteine, which is nephrotoxic *in vivo* and cytotoxic to renal cells [3–6]. *S*-(1,2-Dichlorovinyl)-L-cysteine is cleaved to reactive, sulfur-containing intermediates by cysteine conjugate β-lyase, which is present in the mitochondria and cytosol of renal proximal tubule cells [7].

S-(1,2-Dichlorovinyl)-L-cysteine is a weak inducer of DNA repair in LLC-PK_1 cells and produces DNA single-strand breaks in renal cells *in vivo* and *in vitro* [8, 9]. In addition, *S*-(1,2-dichlorovinyl)-L-cysteine promotes *N*-nitrosodimethylamine-initiated renal tumors in mice [10].

A central event in the cytotoxicity of *S*-(1,2-dichlorovinyl)-L-cysteine is the impairment of the ability of the mitochondria to sequester Ca^{2+} with a resultant increase in cytosolic Ca^{2+} concentrations [11]. The disturbance in intracellular Ca^{2+} homeostasis precedes both the severe perturbations of the mitochondrial membrane potential and cell death. A similar selective depletion of mitochondrial Ca^{2+} has been observed with several compounds that induce increased oxidation and hydrolysis of mitochondrial pyridine nucleotides (for a review, see Ref. 12). The products of this reaction are nicotinamide and ADP-ribose; hence the final step in mitochondrial Ca^{2+} efflux may be the modification of a mitochondrial membrane protein by ADP-ribose moieties.

Increased intracellular Ca^{2+} concentrations may perturb DNA structure and function by activation of Ca^{2+}- and Mg^{2+}-dependent endonucleases, which catalyse the formation of DNA double-strand breaks followed by the induction of increased poly(ADP-ribosylation) of nuclear proteins [13–15]. Since NAD^+ is the substrate for poly (ADP-ribosyl)transferase, an increase in the formation of nuclear poly(ADP-ribosyl)conjugates may deplete the cellular NAD^+ concentration and, consequently, ATP pool. This energy consuming process may be associated with cell death [16]. A similar pathway may be involved in the renal toxicity of the glutathione conjugate of 1,2-dibromo-3-chloropropane [17] and in the toxicity of 2-bromo-3-(*N*-acetylcystein-*S*-yl)hydroquinone to freshly isolated proximal tubule cells (S. Vamvakas, unpublished).

The objectives of the current study were to explore the biochemical mechanisms mediating the *S*-(1,2-dichlorovinyl)-L-cysteine-induced depletion of mitochondrial Ca^{2+} and to investigate the events that flow from perturbation of the cellular Ca^{2+} homeostasis and their association with cytotoxicity.

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MATERIALS AND METHODS

Chemicals. *S*-(1,2-Dichlorovinyl)-L-cysteine was obtained by synthesis [18] and was 99% pure, as determined by analytical HPLC. [^{3}H]NAD^+ was purchased from Sigma Chemie GmbH (Deisenhofen, F.R.G.). [^{14}C]Thymidine and [^{3}H]thymidine were obtained from Amersham (Braunschweig, F.R.G.). Cell culture reagents were purchased from Gibco BRL (Eggenstein-Leopoldshafen, F.R.G.).

Isolation of pig kidney mitochondria and determination of mitochondrial pyridine nucleotides and Ca^{2+} release. Mitochondria were prepared from the cortex of fresh pig kidneys obtained from a local butcher shop. Immediately after killing the animal, the kidneys were placed in ice-cold 20 mM triethanolamine–HCl buffer (pH 7.4) containing 250 mM sucrose, 1 mM EGTA, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 20 mM KCl and 0.1% bovine serum albumin. The mitochondrial fraction was isolated from renal cortical homogenates, as described previously [19]. Mitochondria (1.5 mg protein/mL) were suspended in 20 mM Tris–HCl buffer (pH 7.6) containing 250 mM sucrose. Before addition of the test compounds, the mitochondria were energized by addition of 2.5 mM succinate and 0.5 μM rotenone. The pyridine nucleotide redox state was monitored fluorimetrically in the absence and presence of 1 mM *S*-(1,2-dichlorovinyl)-L-cysteine at 25° (excitation wavelength 366 nm; emission wavelength 450 nm) [20] in a Kontron SFM 25 fluorimeter (Kontron AG, Zürich). Acetoacetate (5 mM) was used as a positive control [21].

Ca^{2+} release from renal cortical mitochondria was measured with Arsenazo III by dual wavelength spectroscopy at 665 and 685 nm with an Ultraspec II spectrophotometer (LK13 Biochrom, Cambridge, U.K.). For determination of Ca^{2+} release, the mitochondria were preincubated for 3 min with Arsenazo III (30 μM), succinate (2.5 mM), rotenone (0.2 mg/mL), and $CaCl_2$ (50 μM). Ca^{2+} release was determined before and after addition of *S*-(1,2-dichlorovinyl)-L-cysteine at the incubation times indicated in Results. *tert*-Butylhydroperoxide (100 μM), a known depletor of mitochondrial Ca^{2+} [12], was used as a positive control. In some experiments, the mitochondria were preincubated for 3 min with 200 μM ATP, which inhibits pyridine nucleotide hydrolysis [22], or with 10 μM *meta*-iodobenzylguanidine, an ADP-ribose acceptor and inhibitor of the mono(ADP-ribosylation) of mitochondrial proteins [23, 24].

Tissue culture of LLC-PK_1 cells. LLC-PK_1 cells (American Type Culture Collection, passage 196–205) were grown in 150-cm^2 plastic tissue culture flasks in Dulbecco's modified Eagle medium supplemented with 20 mM HEPES, 10% fetal calf serum, 100 U penicillin/mL, 100 mg streptomycin/mL, 1.7 g $NaHCO_3$/L and 3 g glucose/L.

Cells (10^5) were plated in 35-mm wells (six wells per plate). One day later, monolayers in exponential growth were washed twice with phosphate-buffered saline (PBS*) and exposed to *S*-(1,2-dichlorovinyl)-L-cysteine for 48 and 96 hr at the concentrations indicated in Results. In some experiments, the monolayers were treated for 1 hr before addition of *S*-(1,2-dichlorovinyl)-L-cysteine with 3 mM 3-aminobenzamide [an inhibitor of poly(ADP-ribosyl)transferase] [16], with 50 μM aurintricarboxylic acid (an inhibitor of the Ca^{2+}- and Mg^{2+}-dependent endonucleases) [25] or with 5 μM acetoxymethylester of the Ca^{2+} chelator Quin 2 (Quin-2 AM) [26]. After 48 and 96 hr, the morphology of the cells was judged microscopically, and the cell number per well was determined with a Coulter counter after trypsinization. In Results, the cell numbers in the treated samples are calculated as percentage of the control cell number at the corresponding sampling time.

Neutral filter DNA elution assay. DNA double-trand breaks were quantified with the neutral filter elution method. Monolayers in exponential growth were incubated with [^{14}C]thymidine (0.025 mCi/mL) and [^{3}H]thymidine (0.1 mCi/mL) for 24 hr. After exposure to the radiolabeled thymidine, the monolayers were washed twice with PBS, and the cells were exposed to *S*-(1,2-dichlorovinyl)-L-cysteine for 48 and 96 hr, as described above. DNA double-strand breaks were determined after 48 and 96 hr by a modification of the technique of Bradley and Kohn [27]. Control and treated LLC-PK_1 cells were harvested with a rubber policeman and were washed with ice-cold PBS. Finally the cells were centrifuged at 500 *g* for 5 min, and the pellet was stored at −20°. For the neutral filter elution of the DNA, the cell pellets were allowed to thaw at room temperature and were transferred with 2 mL of ice-cold PBS on polycarbonate filters (Nucleopore 25 mM, 2 μm pore size). The cells were lysed for 1 hr in the dark at room temperature with 1.5 mL of 0.05 M Tris buffer (pH 9.6) containing 0.05 M glycine, 0.025 M Na_2EDTA, 2% (w/v) sodium dodecyl sulfate and 0.5 mg/mL freshly dissolved proteinase K (Boehringer Mannheim, F.R.G.). After lysis, 40 mL of the elution buffer (0.05 M Tris, pH 9.6), containing 0.05 M glycine, 0.025 M Na_2EDTA and 2% w/v SDS) was added to the reservoir above each filter, and the DNA was eluted for 15 hr at 2 mL/hr with an Ismatec IPN (Ismatec, Zürich, Switzerland) 8 pump. Elution samples of each filter were collected separately every 90 min. After addition of 100 μM glacial acetic acid, the radioactivity was determined in a Packard Minaxi Liquid Scintillation counter.

Poly(ADP-ribosyl)transferase activity. LLC-PK_1 monolayers in 75-cm^2 flasks were treated as described above. *N'*-Methyl-*N'*-nitro-*N'*-nitrosoguanidine, an inducer of poly (ADP-ribosyl)transferase activity [28], was employed as a positive control. After 48 and 96 hr, control and treated cells were harvested after trypsinization and were washed once with PBS. A sample was taken for determination of the cell number in the Coulter counter. The washed cells ($2–5 \times 10^6$) were incubated for 10 min at room temperature in suspension with constant shaking in 1 mL of permeabilization buffer (56 mM HEPES, pH 7.4, containing 28 mM KCl and 28 mM $NaCl_2$); 0.01% (w/v) digitonin, 500 μM NAD^+ and 0.5 μCi/mL [^{3}H]NAD^+ were added to the buffer just before use [29]. At the end of the incubation time, the cells were centrifuged for 5 min at 1200 rpm in a Universal/

* Abbreviations: PBS, phosphate-buffered saline; Quin-2 AM, acetoxymethylester of Quin-2.

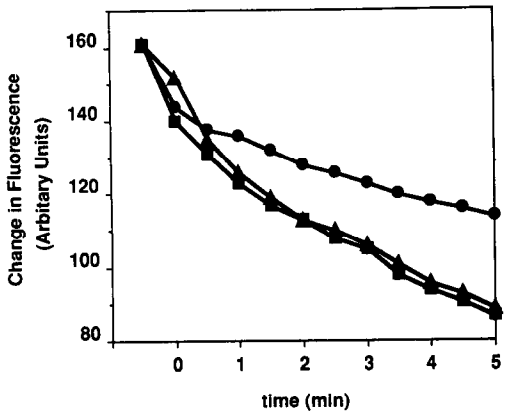

Fig. 1. NAD(P)H oxidation in control mitochondria (●) and in mitochondria treated with 1 mM S-(1,2-dichlorovinyl)-L-cysteine (▲) or 5 mM acetoacetate (■). NAD(P)H concentrations were monitored fluorometrically as described in Materials and Methods. The experiment was repeated three times with similar results (deviations were less than 10%).

k25 centrifuge (Hettich, Tübingen, F.R.G.). After centrifugation a second time, 1 mL of a 1% trypsin solution was added to the pellet, and the cells were stored for 2 hr at −20°. After thawing at room temperature, the cells were solubilized by addition of 1 mL of 2% (w/v) SDS. Two milliliters of 20% trichloroacetic acid were then added to each sample, and the resulting precipitate was collected on Whatmann GF/C glass-fiber filters and washed successively with 10% and 5% trichloroacetic acid and 5% ethanol. Finally the filters were treated with 0.5 mL of tissue solubilizer (Protosol, New England Nuclear, Boston, MA, U.S.A.) for 3 hr at 50°. After addition of 20 μL of glacial acetic acid and toluene scintillator, the radioactivity was determined in a Packard Minaxi Liquid Scintillation counter. In Results the poly(ADP-ribosyl)transferase activity is calculated as percentage of the control rate at the corresponding sampling time.

RESULTS

Oxidation of mitochondrial pyridine nucleotides and depletion of mitochondrial Ca^{2+}

Oxidation and hydrolysis of mitochondrial pyridine nucleotides is the first step required for Ca^{2+} release from intact respiring mitochondria [12]. Figure 1 shows that S-(1,2-dichlorovinyl)-L-cysteine increased the rate of oxidation of mitochondrial NAD(P)H compared with controls during 5 min of incubation; a similar effect was obtained with acetoacetate, which oxidizes mitochondrial pyridine nucleotides [21].

Incubation of pig kidney mitochondria with S-(1,2-dichlorovinyl)-L-cysteine resulted in a marked release of Ca^{2+} into the incubation medium (Fig. 2a). When compared with the NAD(P)H oxidation, the Ca^{2+} release required longer incubation times. Both control and treated mitochondria retained Ca^{2+} for the first 15 min of the incubation, after which a time-dependent release of Ca^{2+} was observed in the S-(1,2-dichlorovinyl)-L-cysteine-treated samples. The degree of Ca^{2+} efflux was quantitatively comparable to that induced by *tert*-butyl hydroperoxide (Fig. 2a), a known depletor of mitochondrial Ca^{2+} [12]. Inhibition of pyridine nucleotide hydrolysis by ATP [21] and of the mono(ADP-ribosylation) of mitochondrial membrane proteins by *meta*-iodobenzylguanidine [23, 24] abolished S-(1,2-dichlorovinyl)-L-cysteine-induced Ca^{2+} release (Fig. 2b). Aminooxyacetic acid, an inhibitor of the cysteine conjugate β-lyase [30], also prevented the Ca^{2+} release (data not shown).

Cytotoxicity of S-(1,2-dichlorovinyl)-L-cysteine in LLC-PK$_1$ monolayers

Exposure of subconfluent LLC-PK$_1$ monolayers to 100 μM S-(1,2-dichlorovinyl)-L-cysteine did not impair cell proliferation compared with control values (Fig. 3). With 200 μM S-(1,2-dichlorovinyl)-L-cysteine, a modest reduction in cell numbers was observed after 96 hr; this effect was more marked with 400 μM S-(1,2-dichlorovinyl)-L-cysteine, and therefore, this concentration was used for the experiments described below. The ability of S-(1,2-dichlorovinyl)-L-cysteine to increase the cytosolic Ca^{2+} concentration in LLC-PK$_1$ monolayers has been demonstrated previously [11]. In the present study, the participation of Ca^{2+}-dependent mechanisms in S-(1,2-dichlorovinyl)-L-cysteine-induced cell killing was investigated. The membrane-permeable Quin-2 AM [26] protected cells from S-(1,2-dichlorovinyl)-L-cysteine-induced toxicity. Cytotoxicity was also partially blocked by inhibition of the Ca^{2+}- and Mg^{2+}-dependent endonucleases with 50 μM aurintricarboxylic acid [25] or of poly(ADP-ribosyl)transferase with 3 mM 3-aminobenzamide [16] (Fig. 4). At the concentrations used, neither aurintricarboxylic acid nor 3-aminobenzamide interfered with cell replication in the absence of S-(1,2-dichlorovinyl)-L-cysteine (data not shown).

To test our hypothesis further, we investigated the mechanism of S-(1,2-dichlorovinyl)-L-cysteine-induced toxicity in suspensions of freshly isolated proximal tubule cells by the Trypan blue exclusion method. The results obtained in this system were similar to those described above for LLC-PK$_1$ cells: the cytotoxicity of the S-conjugate was blocked when the cells were preincubated with Quin-2 or with the inhibitors of the endonucleases and the poly(ADP-ribosyl)transferase (data not shown).

DNA fragmentation

The protection against S-(1,2-dichlorovinyl)-L-cysteine-induced cytotoxicity afforded by aurintricarboxylic acid indicated that DNA double-strand breaks caused by the activation of Ca^{2+}- and Mg^{2+}-dependent endonucleases may be involved in the toxic effects of the S-conjugate. Hence, DNA double-strand breaks were quantified with the neutral filter elution assay. No difference in the amount of DNA double-strand breaks was found when LLC-PK$_1$ monolayers were treated with 20 or 50 μM S-(1,2-dichlorovinyl)-L-cysteine for 48 and 96 hr (data not shown). With the same experimental protocol, 100 μM of the S-conjugate failed to induce

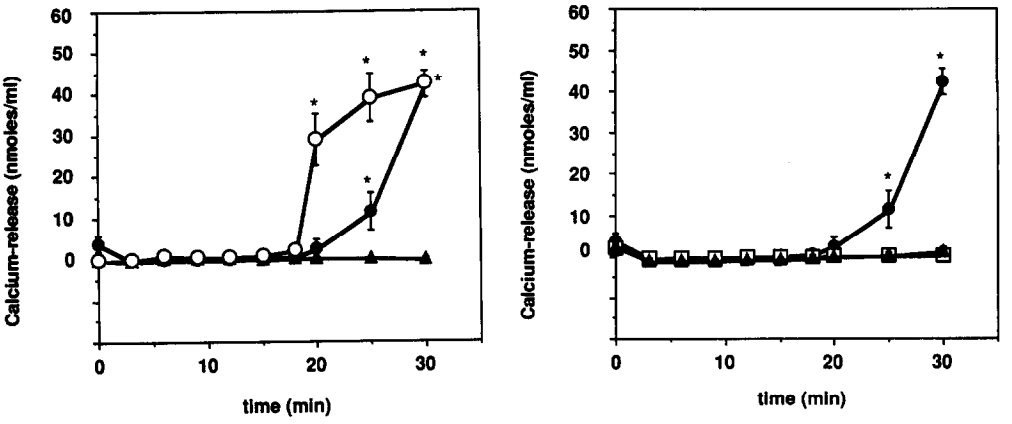

Fig. 2. *S*-(1,2-Dichlorovinyl)-L-cysteine-induced Ca^{2+} release from pig kidney mitochondria (1.5 mg/mL protein). At the times indicated, Ca^{2+} release was determined from control mitochondria (▲) and from mitochondria treated with 1 mM *S*-(1,2-dichlorovinyl)-L-cysteine (●) or with 100 μM *tert*-butylhydroperoxide (○). Results are means ± SD from six samples prepared from three pig kidneys. The asterisk indicates significant difference ($P < 0.01$) to controls as determined by Student's *t*-test. (b) Inhibition of *S*-(1,2-dichlorovinyl)-L-cysteine-induced Ca^{2+} release from pig kidney mitochondria (1.5 mg/mL protein) by ATP and *meta*-iodobenzylguanidine. At the times indicated, Ca^{2+} release was determined from mitochondria treated with 1 mM *S*-(1,2-dichlorovinyl)-L-cysteine in the absence (●) or presence of 200 μM ATP (▲) or 10 μM *meta*-iodobenzylguanidine (□). Results are means ± SD from six samples prepared from three pig kidneys. The asterisk indicates significant difference ($P < 0.01$) to controls as determined by Student's *t*-test.

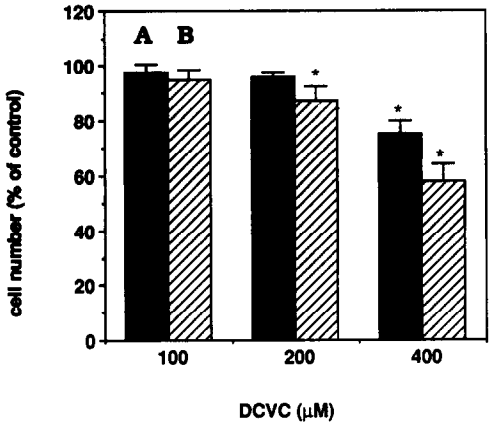

Fig. 3. Effect of *S*-(1,2-dichlorovinyl)-L-cysteine on cell proliferation in LLC-PK_1 cells. Cells were exposed to 100, 200 or 400 μM *S*-(1,2-dichlorovinyl)-L-cysteine for 48 (A) or 96 (B) hr. Cell number per plate was determined as described in Materials and Methods. Data are shown as means ± SD for nine cultures from three separate experiments. The asterisk indicates significant difference ($P < 0.01$) to controls as determined by Student's *t*-test.

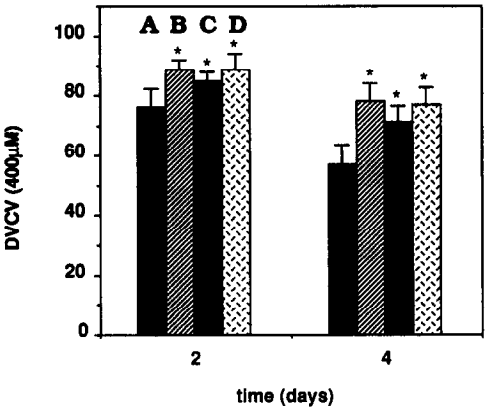

Fig. 4. Effect of *S*-(1,2-dichlorovinyl)-L-cysteine on cell proliferation in LLC-PK_1 cells. Cells were exposed to 400 μM *S*-(1,2-dichlorovinyl)-L-cysteine for 48 hr in the absence (A) or presence of 5 μM Quin-2 AM (B), 50 μM aurintricarboxylic acid (C) or 3 mM 3-aminobenzamide (D). Cell number per plate was determined as described in Materials and Methods. Data are shown as means ± SD for nine cultures from three separate experiments. The asterisk indicates significant protection as determined by Student's *t*-test ($P < 0.01$) compared to the monolayers treated with *S*-(1,2-dichlorovinyl)-L-cysteine only.

DNA double-strand breaks after 48 hr (Fig. 5A), whereas an increase in DNA double-strand breaks was observed after 96 hr (Fig. 5B). Both 200 and 400 μM *S*-(1,2-dichlorovinyl)-L-cysteine increased the percentage of fragmented DNA, and the effect was more marked after 96 hr incubation time than after 48 hr. Preincubation of the cells with 10 μM of *meta*-iodobenzylguanidine, an inhibitor of the mitochondrial mono(ADP-ribosylation) [23, 24], reduced the DNA double-strand breaks induced by 200 μM *S*-(1,2-dichlorovinyl)-L-cysteine by approximately 50%.

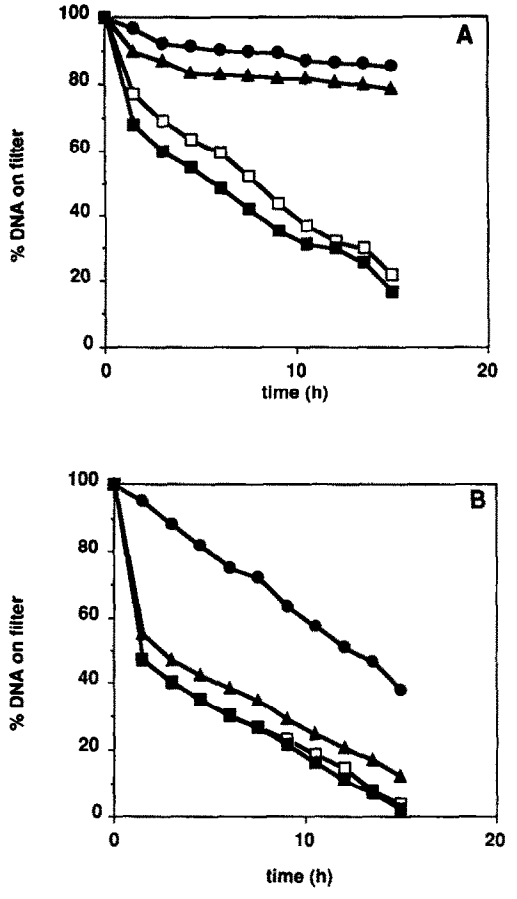

Fig. 5. DNA neutral filter elution profile from untreated (●) LLC-PK$_1$ monolayers and from cells treated for 48 (A) or 96 (B) hr with (▲) 100, (□) 200 or (■) 400 μM S-(1,2-dichlorovinyl)-L-cysteine. Results are from one experiment representative of three; values determined in different experiments deviated by less than 10%.

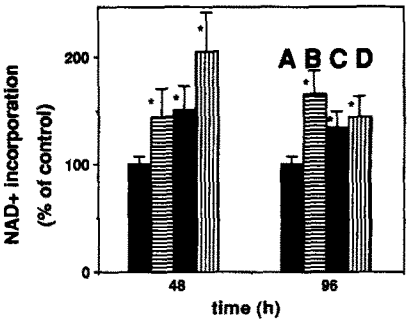

Fig. 6. Effects of S-(1,2-dichlorovinyl)-L-cysteine on poly(ADP-ribosyl)transferase activity in LLC-PK$_1$ cells. Monolayers were treated for 48 and 96 hr with several concentrations of S-(1,2-dichlorovinyl)-L-cysteine: (A) control, (B) 100, (C) 200 and (D) 400 μM. Data are shown as means ± SD of nine cultures from three separate experiments. The asterisk indicates significant difference ($P < 0.01$) to controls as determined by Student's *t*-test.

Poly(ADP-ribosylation)

Among the different types of DNA damage, DNA double-strand breaks are the most effective inducers of poly(ADP-ribosylation) of nuclear proteins [31]. The DNA fragmentation demonstrated with the neutral filter elution assay together with the protective effect of the poly(ADP-ribosyl)transferase inhibitor 3-aminobenzamide against S-(1,2-dichlorovinyl)-L-cysteine-induced toxicity indicated a role for increased poly(ADP-ribosyl)transferase activity in S-conjugate-induced cytotoxicity. Poly(ADP-ribosyl)transferase activity was quantified by measuring the incorporation of [^{3}H]NAD$^+$ in permeabilized control and treated LLC-PK$_1$ cells (Fig. 6). S-(1,2-Dichlorovinyl)-L-cysteine increased poly(ADP-ribosyl)transferase activity in LLC-PK$_1$ cells up to 2-fold compared with control levels after 48 hr. A greater effect was obtained with N'-methyl-N'-nitro-N'-nitrosoquanidine (30 μM), a known inducer of the poly (ADP-ribosyl)transferase [28], which increased [^{3}H]NAD$^+$ incorporation approximately 6-fold after the same incubation time (data not shown). With 100 μM S-(1,2-dichlorovinyl)-L-cysteine, a sustained increase in poly(ADP-ribosyl)transferase activity was observed between the 48 and the 96 hr sampling times; in contrast, with 200 and 400 μM S-conjugate, [^{3}H]NAD$^+$ incorporation after 96 hr was greater than control rates, but was lower than the 48-hr values (Fig. 6).

DISCUSSION

In the present study we investigated the mechanisms responsible for the S-(1,2-dichlorovinyl)-L-cysteine-induced depletion of mitochondrial Ca^{2+} from isolated pig kidney mitochondria, and we also explored the role of Ca^{2+}-dependent events in S-(1,2-dichlorovinyl)-L-cysteine-induced cytotoxicity in renal cells. The hypothesis that Ca^{2+}-dependent degradative pathways may be involved in the renal effects of S-(1,2-dichlorovinyl)-L-cysteine originated from a previous fluorescence digital imaging microscopy study. These experiments demonstrated that an early, central event in the cytotoxicity of S-(1,2-dichlorovinyl)-L-cysteine is the impairment of the ability of the mitochondria to sequester Ca^{2+} with a resultant increase in cytosolic Ca^{2+} concentrations in the submicromolar range [11].

The striking S-(1,2-dichlorovinyl)-L-cysteine-induced efflux of Ca^{2+} from freshly isolated pig kidney mitochondria observed in the present study provides evidence for the importance of Ca^{2+}-dependent mechanisms in S-conjugate-induced cytotoxicity. The efflux was quantitatively similar to the Ca^{2+} release induced by *tert*-butyl hydroperoxide, a model depletor of mitochondrial Ca^{2+} [12], and was preceded by increased oxidation of mitochondrial pyridine nucleotides. Moreover, inhibition of the hydrolysis of oxidized pyridine nucleotides and of the mono(ADP-ribosylation) of mitochondrial membrane proteins blocked S-(1,2-dichlorovinyl)-L-cysteine-induced Ca^{2+} release. These results indicate

that S-(1,2-dichlorovinyl)-L-cysteine may cause a selective depletion of mitochondrial Ca^{2+} by modification of mitochondrial membrane proteins by the ADP-ribose moieties formed by the hydrolysis of the oxidized pyridine nucleotides. This mechanism operates with several compounds that induce a prooxidant state in mitochondria [12]. The ability of haloalkenyl cysteine S-conjugates to induce selective mitochondrial toxicity and Ca^{2+} efflux from isolated kidney mitochondria has been demonstrated previously [32–35]. Other studies demonstrated the role of perturbation of the cellular redox state in the renal toxicity of S-(1,2-dichlorovinyl)-L-cysteine [36, 37], and Chen and Stevens [38] recently showed that there are common elements in the toxic effects of alkylating agents and organic peroxidants in LLC-PK_1 cells. The results presented here provide the first mechanistic and temporal link between changes in the mitochondrial redox state and Ca^{2+} homeostasis in S-conjugate-induced cytotoxicity.

Increases in cytosolic Ca^{2+} concentrations may activate Ca^{2+}-dependent endonucleases that produce DNA double-strand breaks followed by increased poly(ADP-ribosylation) of nuclear proteins [13, 16, 39]. The substrate for poly(ADP-ribosyl)transferase is NAD^+. Hence, a marked increase in the poly(ADP-ribosylation) of nuclear proteins may deplete cellular NAD^+ and consequently ATP pools, and this perturbation of the cellular energy homeostasis may be associated with cell death. Chelation of intracellular Ca^{2+} with Quin-2 and inhibition of the Ca^{2+}-dependent endonucleases by aurintricarboxylic acid and of the poly(ADP-ribosyl)transferase by 3-aminobenzamide protected renal cells from S-(1,2-dichlorovinyl)-L-cysteine-induced cytotoxicity, indicating a role for this pathway in cell damage. The ability of S-(1,2-dichlorovinyl)-L-cysteine to induce DNA double-strand breaks and to increase poly(ADP-ribosylation) of nuclear proteins was, therefore, investigated. S-(1,2-Dichlorovinyl)-L-cysteine induced DNA double-strand breaks in LLC-PK_1 cells at concentrations as low as 100 μM, which do not impair the proliferative capability of the cells; this provides evidence that the cells can survive an increase in DNA double-strand breaks up to a certain level and that the strand breaks are not an epiphenomenon associated with cell death. The data also agree with the previously published fluorescence digital microscopy observations that demonstrated that S-(1,2-dichlorovinyl)-L-cysteine induces an early increase in the cytosolic Ca^{2+} concentrations in viable cells [11]. The reduction of the S-(1,2-dichlorovinyl)-L-cysteine-induced DNA double-strand breaks afforded with *meta*-iodobenzylguanidine, which also blocked Ca^{2+} release, provides further evidence for the key role of the perturbation of the mitochondrial Ca^{2+} homeostasis in DNA fragmentation and, consequently, in cytotoxicity. The use of *meta*-iodobenzylquanidine, a high affinity substrate for mono(ADP-ribosyl)transferase, to study the role of mitochondrial mono(ADP-ribosylation) reactions in intact cells has been demonstrated [40]. Finally, determination of rates of $[^3H]NAD^+$ incorporation in treated and control monolayers showed that S-(1,2-dichlorovinyl)-L-cysteine increased the rate of poly(ADP-ribosylation) of nuclear proteins. It is noteworthy that with 200 and 400 μM of the S-conjugate $[^3H]NAD^+$ incorporation was not higher after 96 hr compared with the 48-hr sampling time, although there was a marked increase both in the impairment of cell proliferation and in the amount of DNA double-strand breaks between 48 and 96 hr. The failure to observe a sustained, time-dependent increase in the formation of poly(ADP-ribosyl)conjugates with the higher S-conjugate concentrations may be due to additional toxic effects of S-(1,2-dichlorovinyl)-L-cysteine, but this has not been established. One may speculate that reactive intermediates derived from cleavage of the S-conjugate may react with the poly(ADP-ribosyl)transferase itself, thus impairing its function. As indicated by the protective role of the poly(ADP-ribosyl)transferase inhibitor 3-aminobenzamide, this pathway may be involved in the S-(1,2-dichlorovinyl)-L-cysteine-induced impairment of cell growth.

The consequences of increased poly(ADP-ribosylation) depend upon the extent of NAD^+ utilization. When cells are subjected to high levels of DNA breakage, extensive polymer synthesis causes a rapid and irreversible decrease in cellular NAD^+ and ATP contents, which has been associated with cell death [29, 41]. Alternatively, during a moderate, temporary induction of DNA double-strand breaks, the poly(ADP-ribosyl)conjugates may promote repair of DNA double-strand breaks by facilitating important steps in excision repair, such as DNA polymerization and ligation of breaks. A non-lethal increase in the amount of nuclear poly(ADP-ribosyl)conjugates may also alter gene expression by changing the structure and function of nuclear proteins; hence, poly(ADP-ribosylation) represents a unique link between DNA damage, cell death and modulation of gene expression, as has been shown with several oxidants [42, 43]. Oxidants may induce modulation of genes, and both the increase in Ca^{2+} concentration and the induction of poly(ADP-ribosylation) appear to be crucial for this effect. The finding that S-(1,2-dichlorovinyl)-L-cysteine and several oxidants share the selective mitochondrial Ca^{2+} release and the increase in poly(ADP-ribosylation) is of particular interest for the S-(1,2-dichlorovinyl)-L-cysteine-induced chronic nephrotoxicity and its possible contribution to the formation of renal adenocarcinomas observed with trichloroethene and dichloroethine, especially as increases in DNA double-strand breaks and poly(ADP-ribosylation) were observed with concentrations that did not impair cell proliferation. The present study demonstrated the role of this pathway in the acute S-(1,2-dichlorovinyl)-L-cysteine toxicity to renal cells. The possible contribution to the chronic nephrotoxicity of S-(1,2-dichlorovinyl)-L-cysteine and the nephrocarcinogenicity of its precursors warrant further investigation.

In summary, the results indicate that S-(1,2-dichlorovinyl)-L-cysteine bioactivation in renal cells may initiate the following cascade of events: increased oxidation and hydrolysis of mitochondrial pyridine nucleotides resulting in the modification of mitochondrial membrane proteins by pyridine

nucleotide-derived ADP-ribose moieties, followed by Ca^{2+} release into the cytosol. Elevated cellular Ca^{2+} concentrations may activate Ca^{2+}-dependent endonucleases, which leads to DNA fragmentation followed by increased poly(ADP-ribosylation) of nuclear proteins and, finally, cytotoxicity.

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