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Role of mitochondrial dysfunction in cellular responses to S-(1,2-dichlorovinyl)-L-cysteine in primary cultures of human proximal tubular cells

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ABSTRACT

The nephrotoxic metabolite of the environmental contaminant trichloroethylene, S-(1,2-dichlorovinyl)-L-cysteine (DCVC), is known to elicit cytotoxicity in rat and human proximal tubular (rPT and hPT, respectively) cells that involves inhibition of mitochondrial function. DCVC produces a range of cytotoxic and compensatory responses in hPT cells, depending on dose and exposure time, including necrosis, apoptosis, repair, and enhanced cell proliferation. The present study tested the hypothesis that induction of mitochondrial dysfunction is an obligatory step in the cytotoxicity caused by DCVC in primary cultures of hPT cells. DCVC-induced necrosis was primarily a high concentration (≥50 μM) and late (≥24 h) response whereas apoptosis and increased proliferation occurred at relatively low concentrations (<50 μM) and early time points (≤24 h). Decreases in cellular DNA content, indicative of cell loss, were observed at DCVC concentrations as low as 1 μM. Involvement of mitochondrial dysfunction in DCVC-induced cytotoxicity was supported by showing that DCVC caused modest depletion of cellular ATP, inhibition of respiration, and activation of caspase-3/7. Cyclosporin A protected cells against DCVC-induced apoptosis and both cyclosporin A and ruthenium red protected cells against DCVC-induced loss of mitochondrial membrane potential. DCVC caused little or no activation of caspase-8 and did not significantly induce expression of Fas receptor, consistent with apoptosis occurring only by the mitochondrial pathway. These results support the conclusion that mitochondrial dysfunction is an early and obligatory step in DCVC-induced cytotoxicity in hPT cells.

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1. Introduction

Trichloroethylene (TRI; also known as trichloroethene and sometimes abbreviated as TCE) is an environmental contaminant that is considered a known animal carcinogen and a ''probable human carcinogen'' by the International Agency for Research on Cancer [1] and as ''reasonably anticipated to be a human carcinogen'' by the National Toxicology Program [2]. Evidence of the continued interest in the toxicity and human health risk of TRI is highlighted by a series of recent reviews that summarize current state of knowledge and uncertainties [3–6]. Evaluation of the risk for humans potentially exposed to TRI in either the workplace or due to environmental contamination is complicated by the existence of multiple metabolic pathways, multiple target organs, poor or incomplete exposure data for many epidemiology studies, and sex- and species-dependent differences in sensitivity to toxic effects [3,6,7].

The kidneys are one target organ for TRI, and toxicity and carcinogenicity are linked to metabolism of TRI by the

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glutathione (GSH) conjugation pathway [3,5,7,8]. Once the GSH conjugate is formed, it is further metabolized to the cysteine conjugate S-(1,2-dichlorovinyl)-L-cysteine (DCVC), which is the penultimate nephrotoxic species and has been the focus of most of the mechanistic studies of TRI-induced nephrotoxicity. Much of our previous knowledge of the mechanism of DCVC-induced renal injury came from studies in isolated proximal tubular (PT) cells, proximal tubules, renal cell lines, or subcellular fractions from renal cortical homogenates from rats, rabbits, and other non-human mammals. DCVC was demonstrated to be a potent cytotoxicant in freshly isolated rat PT (rPT) cells [9] and in LLC-PK1 cells [10], with production of mitochondrial dysfunction as an early effect. Similar studies in isolated mitochondria from rat kidneys showed DCVC to be a potent inhibitor of respiration and several dehydrogenases [11–17]. Changes in mitochondrial Ca^{2+} ion homeostasis also appear to be a key step in DCVC-induced renal toxicity [13,18–20]. The importance of mitochondria in DCVC-induced renal toxicity is further highlighted by findings that preservation of mitochondrial function is associated with recovery and repair of epithelial cells from rabbit proximal tubules after sublethal injury from DCVC [21–25]. Other studies in primary cultures of rPT cells, which enable assessment of longer term exposures at lower concentrations as compared to studies that can be conducted in suspensions of freshly isolated cells, showed that DCVC primarily caused necrosis at relatively high concentrations but that concentrations as low as 10 μM increased expression of vimentin [26]. Vimentin is a mesenchymal cell marker that is only expressed in dedifferentiated PT cells that are either undergoing neoplastic transformation or are part of a repair and proliferation process [27–29].

Despite these advances in our understanding of mechanisms of action of DCVC in renal injury, extrapolation of these findings to human kidney involves uncertainties because of species differences in metabolism, transport, and overall susceptibility to injury [3–5,7,8]. To solve the problems inherent in interspecies extrapolation, one approach has been to conduct studies in freshly isolated and primary cultures of human PT (hPT) cells. Comparisons with results obtained with rPT cells showed clear species differences in sensitivity and mode of action. For example, freshly isolated hPT cells exhibit significant release of lactate dehydrogenase (LDH) only at DCVC concentrations above 0.5 mM [30], indicating much lower sensitivity to DCVC than rPT cells. Additionally, whereas acute cytotoxicity induced by DCVC in rPT cells could be substantially prevented by inhibition of the cysteine conjugate β-lyase with aminooxyacetic acid [9] and partially prevented by inhibition of the flavin-containing monooxygenase with methimazole [31], acute cytotoxicity of DCVC in hPT cells was not prevented by aminooxyacetic acid but was significantly diminished by methimazole [30]. This suggests that the two bioactivation enzymes for DCVC play quantitatively different roles in rat and human kidney.

Mechanistic studies on the responses of hPT cells to DCVC revealed many of the same effects as observed in rPT cells, including necrosis, apoptosis, repair, proliferation, and mitochondrial dysfunction [32–34]. Although it is clear that, like in rPT cells, mitochondria are early and prominent intracellular targets of DCVC in hPT cells, we do not know whether induction of mitochondrial dysfunction is obligatory for subsequent cytotoxicity or whether other mechanisms are important in the various responses of hPT cells to DCVC. Hence, in the present study we compared effects of DCVC on necrosis, apoptosis, and proliferation with those on mitochondrial function. The ability of mitochondrial inhibitors to alter cellular responses to DCVC was assessed to provide more insight into the relationship between mitochondrial effects and cellular responses. The results suggest that in hPT cells, mitochondrial dysfunction is indeed an obligatory step that precedes many of the diverse responses to DCVC.

2. Materials and methods

2.1. Experimental design

Assessments of time and concentration dependence of DCVC-induced cytotoxicity and proliferation were first conducted to enable placement of the mitochondrial function studies in the proper context with respect to the ultimate cellular response, as the overall goal was to determine the tightness of coupling between cytotoxicity and mitochondrial dysfunction. While our incubations of hPT cells typically use DCVC concentrations of 10 through 500 μM, we also measured time- and concentration-dependent effects of DCVC on DNA and protein synthesis with DCVC concentrations of 1 and 5 μM, to determine if changes in cellular functions could be observable at lower concentrations. The higher concentrations of DCVC that were used (i.e., 50–500 μM) were intended to provide a more complete range of cellular responses.

Although data on potential concentrations of DCVC within PT cells is lacking, there are published data suggesting that the PT cell can be exposed to DCVC under various exposure conditions at concentrations that include at least the lower range of values (i.e., up to 50 μM) that were used in the present study. Humans exposed to relatively low levels of TRI by inhalation (i.e., 100 ppm) transiently exhibit as high as 50 μM concentrations of the GSH conjugate of TRI, S-(1,2-dichlorovinyl)glutathione (DCVG), in the plasma [35] and rats exposed to TRI by oral gavage exhibit renal cortical concentrations of DCVC as high as 50 pmol/g tissue [36]. These data suggest that under typical environmental exposure conditions (<5 ppb), even the lowest concentration of DCVC that was tested (i.e., 1 μM) would be high. Under conditions of exposure to higher concentrations of TRI, such as might occur in occupational exposures and accidental poisonings, intracellular concentrations of DCVC in the PT cell as high as 20–50 μM may, at least transiently, be readily achievable.

Mitochondrial function was assessed by measurement of succinate-dependent State 3 oxygen consumption, membrane potential, and cellular ATP content. To assess whether renal cellular injury can occur without mitochondrial dysfunction, DCVC-induced effects were also measured in the presence of agents that specifically prevent processes within the mitochondria that are associated with toxic events. Thus, cyclosporin A (CsA) was used to block the mitochondrial membrane permeability transition (MPT), ruthenium red (RR) was used to inhibit the calcium uniporter, thereby preventing calcium ion cycling, and PK11195 [1-(2-chlorophenyl)-N-(1-

methylpropyl)-3-isoquinoline-carboxamide] was used as an antagonist of the mitochondrial benzodiazepine receptor (mBzR) or peripheral benzodiazepine receptor (pBzR), which is a component of the permeability transition pore. Whereas protection from mitochondrial dysfunction and cell injury by CsA has been used as a marker for involvement of the MPT for many years [37], CsA is also known to cause renal tubular cell apoptosis [38]. In the present study, as in the original work demonstrating inhibition of the MPT [37], we used a CsA concentration of 0.5 nmol/mg protein. Taking into account the amount of cells and volumes used in the assays with CsA and that 1×10^6 hPT cells has about 1 mg of protein [30,32], the concentration of CsA used here to assess the role of the MPT in DCVC-induced cytotoxicity was approximately 0.8 μM. In contrast, Justo et al. [38] demonstrated renal tubular cell apoptosis at CsA concentrations of 0.8–8 mM, which is three to four orders of magnitude higher than the concentration used here. Hence, CsA-induced cytotoxicity was not a confounding effect in the present studies. Muscarella et al. [39] showed that antagonism of the mBzR with PK11195 leads to loss of mitochondrial membrane potential, release of apoptogenic factors, and exacerbation of cell injury. Hence, the expectation if mitochondrial dysfunction is an obligatory component of DCVC-induced cell injury in hPT cells is that CsA and RR will protect and PK11195 will exacerbate cell injury. Activities of two types of caspases, one whose activation is associated with the mitochondrial pathway of apoptosis (i.e., caspase-3/7) and one whose activation is associated with the non-mitochondrial or extrinsic pathway of apoptosis (i.e., caspase-8) [40], were also measured. Finally, expression of the Fas receptor (FasR), which can be induced in the extrinsic pathway of apoptosis, was also measured by Western blot analysis.

2.2. Chemicals and reagents

TRI was purchased from Sigma Chemical Co. (St. Louis, MO; Cat. No. T4928, ACS reagent, >99.5% purity). DCVC was synthesized from TRI and L-cysteine in sodium metal and liquid ammonia as described previously [41]. Purity (>95%) was assessed by HPLC and thin layer chromatography and confirmed by GC–MS. Antimycin A (AA), CsA, PK11195, RR, *tert*-butyl hydroperoxide (tBH), cisplatin (CisPt), methyl vinyl ketone (MVK), anisomycin (Anis), staurosporine (Sts), and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. The antibody for actin (Sigma) was a polyclonal rabbit antibody that detects the 42-kDa protein in all mammalian species tested, including humans. All other chemicals for cell isolation and culture and for various assays were purchased from commercial vendors and were of the highest purity available.

2.3. Isolation and primary culture of hPT cells

hPT cells were isolated from fresh human kidneys by a modification of the procedure described by Todd et al. [42] as described previously [30,32–34,43]. Cells were identified as being derived from the proximal tubules by their high activities of brush-border membrane enzymes (i.e., alkaline phosphatase and γ-glutamyltransferase) and low activity of the glycolytic enzyme hexokinase, as described previously [9,26,30–34,43,44]. Human kidneys were purchased from Life Legacy Foundation (Tucson, AZ; formerly International Bioresearch Solutions) or the International Institute for the Advancement of Medicine (Edison, NJ). Donor kidneys were obtained with informed consent and all procedures were approved by the Human Investigation Committee of Wayne State University and judged to qualify for Exemption Category 4 according to the criteria used by the Department of Health and Human Services. Kidney tissue was determined to be normal by a pathologist (i.e., derived from non-cancerous, non-diseased tissue) and was screened for HIV, hepatitis A, B, and C, and CMV. Kidneys were perfused with Wisconsin medium and kept on wet ice until they arrived at the laboratory, which was within 24 h of removal from the donor. Age, gender, and race information, but no other identifying information, was obtained. Because tissue was generally obtained from accident victims or those who have died from cardiovascular incidents and the tissue was rejected for use in transplants (generally because of excessive glomerulosclerosis or arterial plaque), we restricted our donors to adults (age ≥21 years).

All buffers were continuously bubbled with 95% O_2/5% CO_2 and were maintained at 37 °C. Minced cortical pieces from whole kidneys were subjected to collagenase digestion for 60 min, after which the supernatant was filtered through a 70 μm mesh filter to remove tissue fragments, centrifuged at 150 × *g* for 7 min, and the pellet resuspended in Dulbecco's modified Eagle's medium:Ham's F12 Medium (DMEM:F12; 1:1). Approximately (5–7) × 10^6 cells were obtained per 1 g of human kidney cortical tissue. Only cells that excluded >90% trypan blue or exhibited <10% leakage of LDH were used for subsequent primary culture. Basal medium and supplements for hPT primary culture were as described previously [32–34,43]. Basal medium was a DMEM:F-12 (1:1) without serum. Medium was supplemented with 15 mM HEPES, pH 7.4, 20 mM $NaHCO_3$, antibiotics for Day 0 through Day 3 only (192 IU penicillin G/ml + 200 μg streptomycin sulfate/ml) to inhibit bacterial growth, 2.5 μg amphotericin B/ml to inhibit fungal growth, 5 μg bovine insulin/ml (=0.87 μM), 5 μg human transferrin/ml (=66 nM), 30 nM sodium selenite, 100 ng hydrocortisone/ml (=0.28 μM), 100 ng epidermal growth factor/ml (=17 nM), and 7.5 pg 3,3′,5-triiodo-DL-thyronine/ml (=111 nM). Medium was prepared from powder using doubly distilled, deionized water. Before addition of supplements, medium was filtered through 0.22 μm porosity cellular acetate membrane filters for sterilization.

hPT cells were seeded (=Day 0) at densities of 1 × 10^6 cells/ml on Vitrogen-coated, 35-mm dishes, polystyrene culture flasks (T-25 or T-75), or 6- or 24-well plates, depending on the needs of the experiment. Volumes were 1.5, 3, 11, and 1 or 0.5 ml/well for 35-mm dishes, T-25 flasks, T-75 flasks, and 6- or 24-well plates, respectively. Cultures were grown at 37 °C in a humidified incubator under an atmosphere of 95% air/5% CO_2 at pH 7.4. After 24 h (=Day 1), dishes, flasks, or plates were washed with media to remove unattached cells (generally 50–60%, based on protein content in dishes and media [32]) and fresh medium was added. Medium was changed every 2 days thereafter until confluence (generally by Days 4–6). After Day 3, antibiotics were omitted from medium as they inhibit growth. When necessary, cells were harvested from dishes by either

scraping the plates with a Teflon scraper or by brief treatment with Cellstripper (Cellgro, Herndon, VA) (in Ca^{2+}- and Mg^{2+}-free Hanks' buffer). All experiments were performed with confluent cells.

2.4. Measurement of necrosis by LDH release

Cell death by necrosis was determined by measurement of LDH release from cells. LDH activity was determined in media and, after removal of media, washing of cells with PBS, and solubilization of cells with 0.1% (v/v) Triton X-100, in total cells, by addition of pyruvate and NADH and following the decrease in A_{340}. The fraction of LDH release was calculated by the formula: % LDH release = [LDH activity in media/(LDH activity in media + LDH activity in total cells)] × 100%.

2.5. Flow cytometry and cell cycle analysis

Cell cultures were washed twice with sample buffer (PBS plus glucose (1 g/l) filtered through a 0.22 μm filter), dislodged by treatment with Cellstripper, centrifuged at 400 × *g* for 10 min, and resuspended in sample buffer. Cell concentrations were adjusted to (1–3) × 10^6 cells/ml with sample buffer and 1 ml of cell suspension was centrifuged at 400 × *g* for 10 min. All of the supernatant except 0.1 ml/10^6 cells was removed and the remaining cells were mixed on a vortex mixer in the remaining fluid for 10 s. Next, 1 ml of ice-cold ethanol (70%, v/v) was added to the sample drop by drop, with samples being mixed for 10 s between drops. The tubes were capped and fixed in ethanol at 4 °C. After fixation, cells were stained in propidium iodide (50 μg/ml) containing RNase A (100 U/ml). Samples were then mixed, centrifuged at 1000 × *g* for 5 min and all the ethanol except 0.1 ml was removed. Cells were mixed in the residual ethanol and 1 ml of the propidium iodide staining solution was added to each tube. After mixing again, cells were incubated at room temperature for at least 30 min. Samples were analyzed within 4 h by flow cytometry using a Becton Dickinson FACS*Calibur* Flow Cytometer, which is a core facility of the NIEHS Center for Molecular Toxicology with Human Applications at Wayne State University. Analysis was performed with 20,000 events per sample using the ModFit LT v. 2 for Macintosh data acquisition software package (Verity Software House Inc., Topsham, ME; distributed by Becton Dickinson Immunocytometry System BDIS, San Jose, CA). Propidium iodide was detected by the FL-2 photomultiplier tube. Fractions of apoptotic cells were quantified by analysis of the sub-G_1 (sub-diploid) peak with ModFit cell cycle analysis. The percent distribution of cells in the various stages of the cell cycle (G_0/G_1, S, G_2/M) was also calculated. Cell aggregates were discarded in the flow cytometry analysis by post-fixation aggregate discrimination.

2.6. Measurement of cell proliferation by MTT assay

Cell proliferation and cytotoxicity were measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Proliferation Assay kit (ATCC, Manassas, VA) and was expressed in treated samples as the percent of control measurements. Cells were grown on collagen-coated, 24-well plates and incubated with the indicated concentration of DCVC for 4 or 24 h. At the end of the incubation period, media were replaced with fresh media and the MTT reagent was added to each well. Cells were periodically viewed under a phase contrast microscope for the appearance of the intracellular punctate purple precipitate. Once the precipitate was clearly visible, detergent was added to lyse cells and the plate was incubated in the dark for at least 2 h, after which time the absorbance at 570 nm was measured in a SpectraMax 2 plate reader (Molecular Devices, Sunnyvale, CA).

2.7. Confocal microscopy

hPT cells were grown on collagen-coated, 35-mm culture dishes and were viewed with a Zeiss Triple-Laser Scanning Confocal Microscope (LSM 310) with integrated workstation at the Confocal Imaging Core Facility in the School of Medicine at Wayne State University (Detroit, MI). This is a core facility of the NIEHS Center for Molecular Toxicology with Human Applications at Wayne State. Initial magnification was 196×.

2.8. Measurement of DNA and protein synthesis

DNA or protein synthesis was quantified by measurement of incorporation of radiolabeled dTTP or L-leucine, respectively, into the acid-insoluble fraction of cell cultures. hPT cells grown on 24-well plates were rinsed with PBS, fresh Krebs–Henseleit buffer (118 mM NaCl, 4.8 mM KCl, 0.96 mM KH_2PO_4, 0.12 mM $MgSO_4 \cdot 7H_2O$, 25 mM HEPES), containing 225 μM dCTP, dATP, and dGTP was added to each flask, and reactions were initiated by addition of 0.5 μCi/ml of [methyl-^{3}H]-dTTP (7 Ci/mmol; ICN Biomedicals Inc., Irvine, CA) for measurement of DNA synthesis. For measurement of protein synthesis, deoxynucleotides were omitted from the buffer and reactions were initiated by addition of 0.5 μCi/ml of [4,5-^{3}H]-L-leucine (120 Ci/mmol; Moravek Biochemicals, Brea, CA). Incubations were performed at 37 °C in a humidified incubator under an atmosphere of 95% air/5% CO_2 for up to 24 h. Buffers were then removed, dishes rinsed three times with ice-cold PBS to remove residual radioactivity, and the cells were scraped into 1-ml PBS. Of the 1-ml mixture, 200 μl was removed for determination of total DNA and protein contents. Trichloroacetic acid (265 μl of 30%, w/v) was added to the remaining 800 μl. Cells were then centrifuged for 2 min at 13,000 × *g* and the resulting acid-insoluble pellet was dissolved in 0.2% (v/v) Triton X-100 in 1 M NaOH, and aliquots were removed for liquid scintillation counting.

2.9. Assays of mitochondrial function

2.9.1. Mitochondrial respiration

Confluent, primary cultures of hPT cells were grown on collagen-coated T-25 flasks. Cells were incubated for various times up to 48 h with either PBS or the indicated concentration of DCVC. At the end of the incubation period, cells were released from the culture surface by brief treatment with Cellstripper and resuspended in PBS at a concentration of 2 × 10^6 cells/ml. Succinate-stimulated O_2 consumption was measured in a Gilson 5/6H Oxygraph as described previously [9,44].

2.9.2. Mitochondrial membrane potential ($\Delta\psi$) measured with JC-1

Mitochondrial membrane potential ($\Delta\psi$) was measured with the fluorescent agent JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzoimidazolylcarbocyanine iodide; Molecular Probes, Eugene, OR) [45,46]. Cells were grown in six-well plates. Following incubation with the appropriate concentration of toxicant, JC-1 was added to each well at a final concentration of 5 μg/ml) and cells were then incubated at 37 °C for 30 min. Cells were then washed and viewed on a confocal microscope using an emission wavelength of 590 nm for the presence of punctate orange–red fluorescence, which indicates polarized mitochondria. In contrast, a diffuse red staining indicates depolarized mitochondria.

2.9.3. Cellular ATP content

ATP content was quantified by the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) and was expressed in treated samples as the percent control. Standard curves with authentic ATP were generated so that luminescence could be equated to ATP concentration. Cells were grown on collagen-coated, six-well plates and incubated with the appropriate toxicant dissolved in media for 4 or 24 h. The cells were washed and scraped into 100 μl of PBS and then 100 μl of Cell Titer-Glo reagent was added. The mixture was incubated for 30 min in the dark and the luminescence was read in a SpectraMax 2 plate reader (Molecular Devices).

2.10. Measurement of caspase activities

Activity of caspases-3/7 and -8 were measured by the Caspase Glo 3/7 kit and Caspase Glo 8 kit, respectively (Promega) and cellular caspase-3/7 or -8 activity in treated cells was expressed as the percent of control. Cells were grown on collagen-coated, six-well plates and incubated with toxicant for 4 or 24 h. Cells were washed and scraped in 100 μl PBS and then 100 μl of Caspase-Glo 3/7 or Caspase-Glo 8 reagent was added. The mixture was then incubated for 2 h in the dark and the multi-well plate was read in the luminescence mode in a SpectraMax 2 plate reader (Molecular Devices).

2.11. Measurement of FasR expression by Western blot analysis

Total cellular protein (150 μg) was loaded in wells of 10% polyacrylamide gels. After electroblotting of protein onto nitrocellulose paper, blots were blocked for 1 h in 5% milk powder solution and incubated overnight with the primary antibody to the FasR. The antibody was a polyclonal antibody made in rabbit that recognizes the human FasR protein (molecular weight 48 kDa) and was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were then washed three times with Tris-buffered saline containing Tween-20 (TTBS) and incubated with a secondary antibody conjugated to alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA) for 1 h. Blots were washed three to six times and then assayed for color development using BCIP/NBT as substrates (Promega, Madison, WI). Alkaline phosphatase staining intensity was determined by scanning laser densitometry with a Fuji Science Imaging System connected to a Macintosh G4 computer.

2.12. Data analysis

Data values for experiments involving measurements of ATP content, caspase activities, respiratory activity, and DNA content were normalized to cellular protein content. Protein was measured using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) and measuring absorbance at 562 nm. Standard curves using bovine serum albumin as the standard were generated to determine sample protein concentrations. Significant differences between mean values of controls and treated samples were first assessed by a one-way or two-way analysis of variance. When significant F values were obtained with the analysis of variance, the Fisher's protected least-significance t-test was performed to determine which means were significantly different from one another, with two-tail probabilities <0.05 considered significant.

3. Results

3.1. Cytotoxicity, proliferation, and morphology

To place mitochondrial dysfunction into the context of cytotoxicity, confluent, primary cultures of hPT cells were incubated for either 4 or 24 h with DCVC, ranging in concentrations from 10 to 300 μM. We first assessed release of LDH as an index of necrosis (Fig. 1). Incubation with 10 μM DCVC had no effect on LDH release at either time point. In contrast, incubation with DCVC concentrations of 50 μM and higher caused both a time- and concentration-dependent

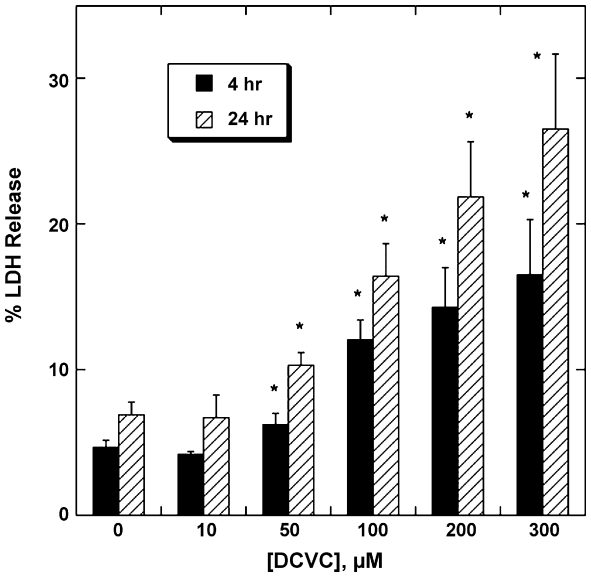

Fig. 1 – DCVC-induced necrosis assessed by LDH release. Confluent primary cultures of hPT cells grown in collagen-coated, six-well plates were incubated for 4 or 24 h with the indicated concentrations of DCVC. LDH release was measured spectrophotometrically and is expressed as percent release. Data are means ± S.E.M. of measurements from four separate cell cultures, each deriving from a different kidney. Significantly different (*$P < 0.05$) from the corresponding control cells.

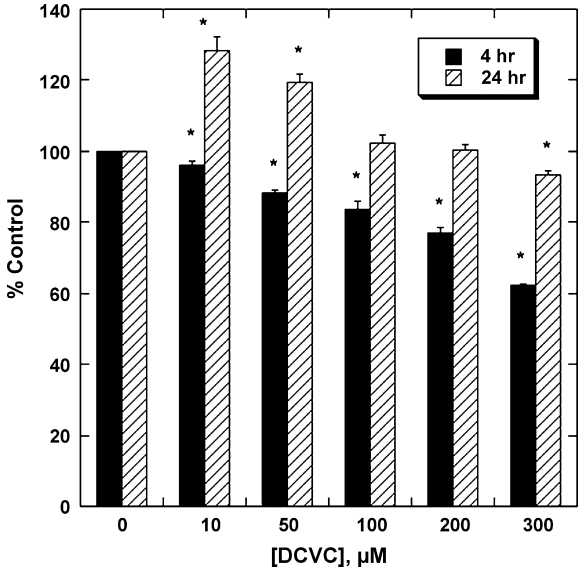

Fig. 2 – DCVC-induced proliferation assessed by MTT reduction. Confluent primary cultures of hPT cells grown in collagen-coated, 24-well plates were incubated for 4 or 24 h with the indicated concentrations of DCVC. MTT reduction was measured spectrophotometrically and is expressed as percent control. Data are means ± S.E.M. of measurements from four separate cell cultures, each deriving from a different kidney. Significantly different (*$P < 0.05$) from the corresponding control cells.

increase in LDH release, with a maximum level of LDH release of approximately 27%.

Assessment of toxicity and proliferation capacity by the MTT assay gave generally similar results as the LDH release assay, except that in this case, 10 μM DCVC did elicit significant effects (Fig. 2). No cell proliferation was observed at the 4-h incubations; rather, a DCVC concentration-dependent decrease in MTT reduction occurred, with the highest concentration tested (300 μM), reducing MTT reduction by approximately 40%. In contrast, DCVC concentrations as low as 10 μM significantly increased MTT reduction at 24 h, suggesting enhanced cell proliferation. As DCVC concentrations were increased, however, the increase in MTT reduction was diminished and was lower than control cells at 300 μM, suggesting cytotoxicity. Because MTT reduction occurs in mitochondria, the decreases with increasing DCVC concentrations are consistent with mitochondrial dysfunction.

Flow cytometry analysis of propidium iodide-stained hPT cells was used to assess the occurrence of apoptosis and the fraction of cells that were in the S phase, indicative of proliferating cells (Fig. 3). DCVC concentrations as low as 10 μM significantly increased apoptosis at both 4- and 24-h exposures; apoptosis was maximal at 50 μM DCVC and was greater at 4 h than at 24-h exposures. The extent of apoptosis decreased at higher concentrations of DCVC and no apoptosis was observed at 300 μM DCVC. As we concluded previously [29–31], the decrease in the amount of apoptosis detected with higher concentrations of DCVC and/or after longer exposure periods is likely due to a greater amount of necrosis in these cell populations. Significant, but modest cell proliferation, as assessed by increases in the proportion of cells in S phase, was only observed at 10 μM DCVC and a 4-h exposure. The proportion of cells in S phase decreased at 24-h exposures at concentrations ≥200 μM DCVC.

As an example of effects of DCVC on cellular morphology, time-dependent effects of 50 μM DCVC were assessed by phase contrast microscopy (Fig. 4). We chose 50 μM as the concentration of DCVC because this was the concentration that produced maximal amounts of apoptosis but only small increases in LDH release. Little change in cellular morphology

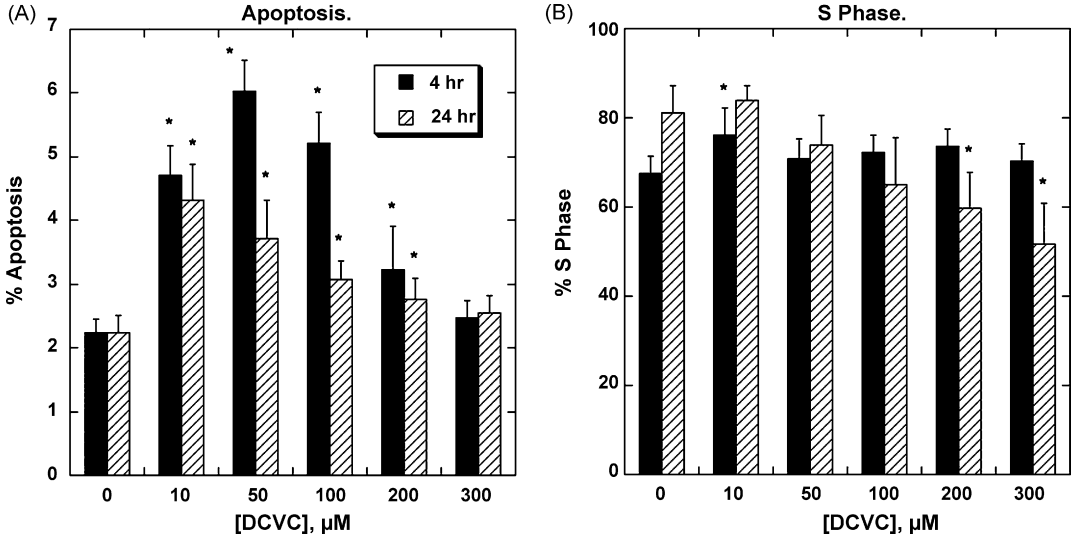

Fig. 3 – DCVC-induced apoptosis and cell proliferation assessed by flow cytometry and FACS analysis. hPT cells grown in collagen-coated, six-well plates were incubated for 4 or 24 h with the indicated concentrations of DCVC. Cells were stained with propidium iodide and cell cycle status was analyzed by flow cytometry and FACS analysis. Results are the percentage of cells undergoing apoptosis (sub-diploid) (A) and the percentage of cell in S phase (B). Values are means ± S.E.M. of measurements from four separate cell cultures, each deriving from a different kidney. Significantly different (*$P < 0.05$) from the corresponding control cells.

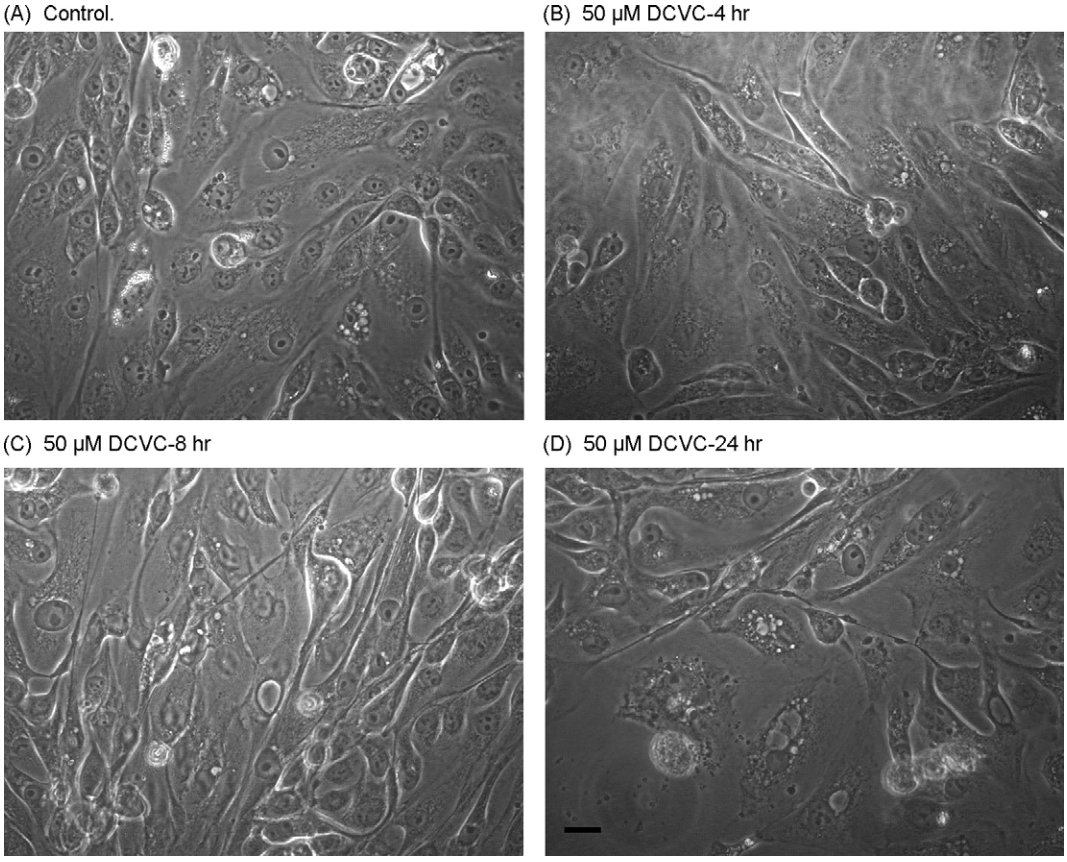

Fig. 4 – Time-dependent changes in morphology induced by DCVC. hPT cells were cultured for 5 days in supplemented, serum-free, hormonally defined DMEM:F12 medium on collagen-coated, 35-mm polystyrene dishes until they were confluent. Cells were then incubated for the indicated times up to 24 h with either medium (=Control) or 50 μM DCVC. At the indicated times, photomicrographs were taken at 196× magnification on a Carl-Zeiss Confocal Laser Microscope. Bar = 5 μm.

was observed after a 4-h incubation with 50 μM DCVC. With increasing incubation time, however, progressive alterations in cellular morphology were observed, as indicated by increases in formation of intracellular vesicles, loss of cuboidal epithelial shape, and increasing amounts of debris.

As a potentially more sensitive indicator of cell function, we measured amounts of DNA and protein in hPT cells incubated for up to 24 h with between 1 and 50 μM DCVC, to derive rates of DNA and protein synthesis (Fig. 5). Rates of DNA synthesis increased approximately five- to sixfold over the time course of culture, reaching maximal levels at 4 h and decreasing slightly at 6 and 24 h. Based on DNA synthesis rates, there was no evidence of net proliferation at DCVC concentrations up to 50 μM. Although rates of protein synthesis also tended to increase with time in culture, the increases were more gradual (i.e., maximal at 24 h) and modest (approximately twofold). Exposure to 25 μM DCVC significantly increased rates of protein synthesis at 4, 6, and 24 h, suggesting some promotion of proliferation.

3.2. *DCVC-induced mitochondrial dysfunction*

As a basic assessment of mitochondrial function, succinate-dependent oxygen consumption was measured in hPT cells incubated for up to 48 h with media (=Control), DCVC (10–500 μM), or Sts (Fig. 6). Succinate-dependent respiration was maintained over 48 h of culture in control cells but decreased in a time- and concentration-dependent manner in cells incubated with 10–500 μM DCVC. Besides increasing with increasing DCVC concentration, the extent of inhibition of respiration markedly increased with time; for example, whereas 100 μM DCVC only produced about 25% inhibition at 1 h, it produced 38% inhibition at 4 h, 50% inhibition at 8 h, 65% inhibition at 24 h, and 80% inhibition at 48 h. For comparison, the effect of 1 μM Sts, as a control for an agent that produces apoptosis, was determined. Sts produced no effect on respiration at 1 and 2 h, only 25% inhibition at 4 h, 50% inhibition at 8 and 24 h, and about 65% inhibition at 48 h. This delayed time course suggests that Sts-induced apoptosis occurs primarily at a time when Sts does not significantly affect mitochondrial function (i.e., $\leq$4 h).

We next determined cellular ATP concentrations in hPT cells treated for 24 h with either DCVC (10–300 μM) or several positive and negative controls (Table 1). DCVC caused a progressive decrease in ATP contents: At concentrations at which significant apoptosis occurs ($\leq$200 μM), no more than 40% depletion of ATP was observed; at a higher concentration of DCVC that is primarily necrotic rather than apoptotic, ATP

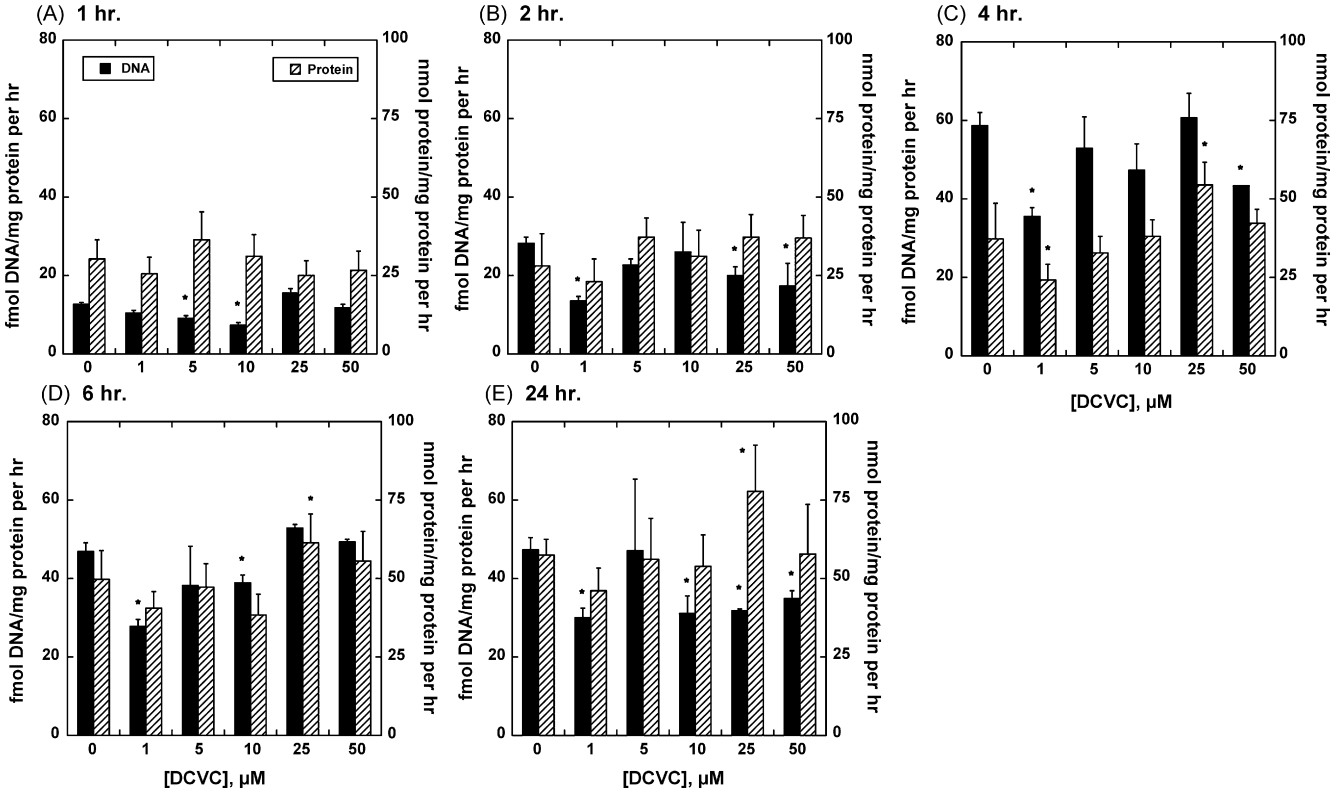

Fig. 5 – DCVC-induced changes in DNA and protein synthesis. Confluent hPT cells grown on collagen-coated, 24-well plates were incubated with the indicated concentrations of DCVC for up to 24 h. At the specified times, dishes were washed and then incubated for 1 h with precursors for DNA, including [methyl-^{3}H]-dTTP, for measurement of rates of DNA synthesis, or with [4,5-^{3}H]-L-leucine for measurement or rates of protein synthesis. Samples were processed for determination of radioactivity in the acid-insoluble fraction and results were normalized to protein content. Results are the means ± S.E.M. of measurements from four separate cell cultures, each deriving from a different kidney. Significantly different ($^*P < 0.05$) from corresponding control values.

depletion was >50%. By contrast, incubation with 1 mM KCN + 0.1 mM iodoacetate (KCN/IAA) or 50 μM tBH produced approximately 80% ATP depletion, whereas 1 μM AA or 1 μM Sts only produced approximately 20% ATP depletion.

The influence of DCVC on caspase activities was assessed as another indicator of the involvement of mitochondria in DCVC-induced toxicity (Table 2). Activation of caspase-3/7 is indicative of apoptosis that occurs through the mitochondrial pathway whereas activation of caspase-8 is indicative of apoptosis that occurs through the extrinsic or extra-mitochondrial pathway. AA had no effect on either caspase-3/7 at 4 or 24 h or caspase-8 at 4 h, but significantly increased activity of casapase-8 at 24 h. Sts significantly increased both types of caspases at both time points; effects on caspase-3/7 were greater than those on caspase-8, suggesting that the mitochondrial pathway is more important in Sts-induced apoptosis. At the 4-h incubation, DCVC produced a significant increase in caspase-3/7 activity at 10 and 50 μM and in caspase-8 only at 10 μM; the increase in caspase-3/7 activity was larger than that in caspase-8 activity, suggesting a greater role for mitochondrial-mediated vs. extra-mitochondrial-mediated apoptosis. At the 24-h time point, however, DCVC only increased caspase-8 activity at ≤100 μM; caspase-3/7 activity decreased in a DCVC concentration-dependent manner, suggesting primarily necrosis at later time points and higher concentrations of DCVC.

3.3. *Influence of modulation of mitochondrial function on DCVC-induced toxicity*

hPT cells were incubated for 2, 6, or 24 h with 0, 10, 50, or 100 μM DCVC in the absence or presence of the MPT inhibitor CsA (0.5 nmol/mg protein = approximately 0.8 μM), to examine the influence of CsA on DCVC-induced apoptosis (Fig. 7). In the absence of CsA, DCVC induced modest amounts of apoptosis as compared to untreated control cells at 2 h (2.7–4.6-fold increase vs. control cells), larger amounts at 6 h (2.7–16.0-fold increase vs. control cells), and modest amounts at 24 h (2.4–9.2-fold increase vs. control cells). CsA significantly decreased DCVC-induced apoptosis at relatively low DCVC concentrations at the 6-h incubation and at 10 μM DCVC at 24 h. Thus, at the 2-h incubation time, DCVC-treated cells increased the number of apoptotic cells only 1.3–2.2-fold in the presence of CsA. In contrast, CsA increased apoptosis with 100 μM DCVC at 24 h (44-fold increase over control with CsA as compared to 16.0-fold increase over control without CsA), which is a time and DCVC concentration at which the primary cellular response is necrosis. Regardless of the complexity of

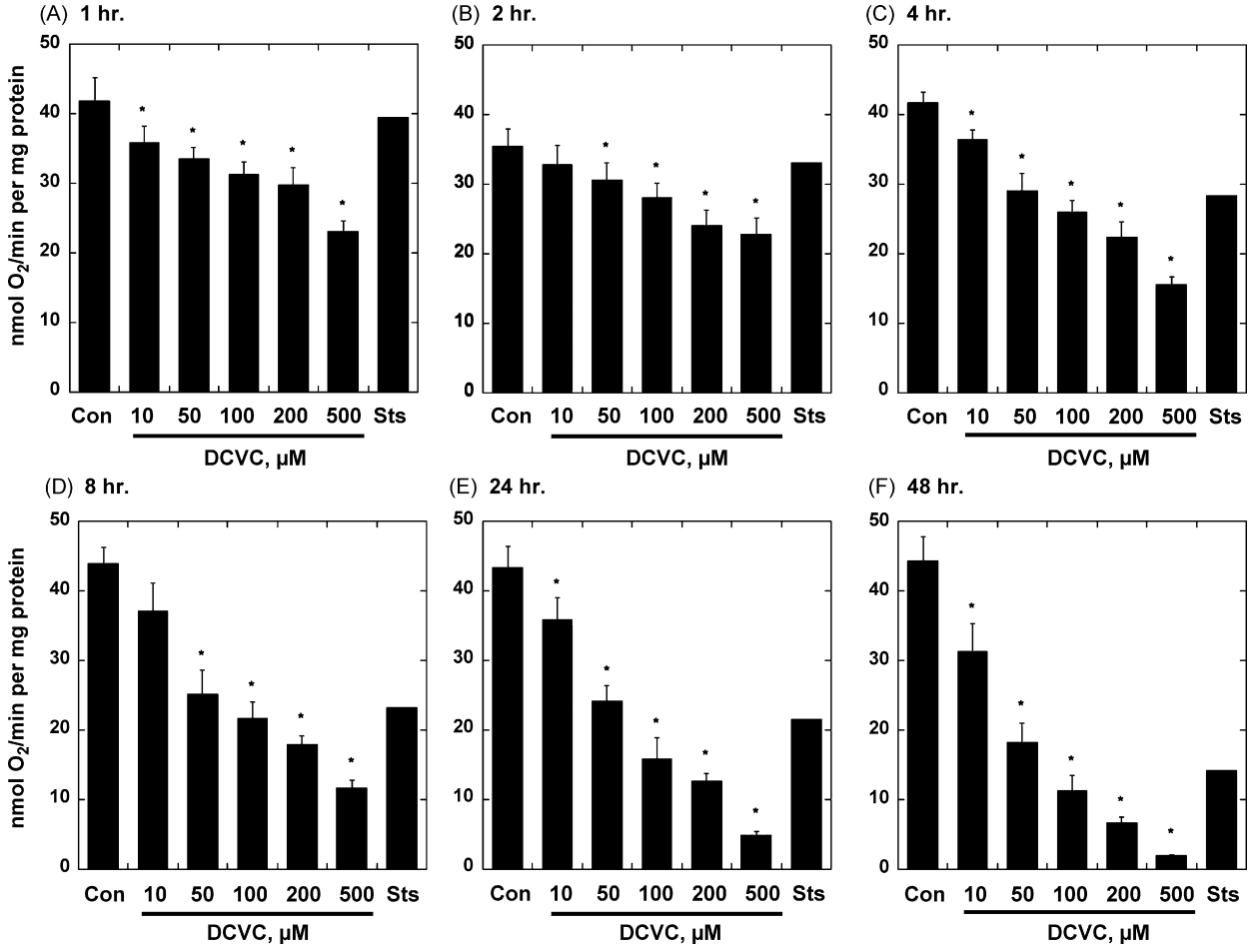

Fig. 6 – Time- and concentration-dependent inhibition of cellular respiration by DCVC. Confluent hPT cells grown on collagen-coated, T-25 flasks were incubated up to 48 h with PBS (=Control, Con), the indicated concentrations of DCVC, or 1 μM staurosporine (Sts). At the indicated times, cells were released from flasks by brief treatment with trypsin/EDTA, resuspended in PBS at a cell concentration of 2×10^6 ml^{-1}, and were added to the Oxygraph electrode chamber for measurement of succinate-stimulated O_2 consumption. Results are means ± S.E.M. of measurements from four separate cell cultures, each deriving from a different kidney, except for incubation with Sts, which were averages of measurements from two separate cell cultures. Significantly different (*$P < 0.05$) from corresponding control values.

the influence of CsA, the results clearly demonstrate a prominent role for mitochondrial dysfunction and the MPT in the hPT cell response to DCVC.

To determine the effect of DCVC and mitochondrial inhibitors on mitochondrial $\Delta\psi$, we first assessed effects of several mitochondrial inhibitors to provide a basis for comparison and to validate the assay in the primary cultures of hPT cells. Control hPT cells exhibited red punctate fluorescence, indicative of maintenance of a high $\Delta\psi$ (Fig. 8A). In contrast, incubation of cells with AA resulted in a complete loss of $\Delta\psi$, as would be expected of this inhibitor (Fig. 8B). Incubation with the MPT inhibitor CsA had a minimal effect on $\Delta\psi$ (Fig. 8C), suggesting that mitochondrial function is maintained. Similarly, the Ca^{2+}-uniporter inhibitor RR also resulted in maintenance of $\Delta\psi$, which again is expected based on the ability of this chemical to prevent futile cycling of Ca^{2+} ions, which would lead to a dissipation of $\Delta\psi$ (Fig. 8D). Co-incubation of hPT cells with AA and CsA resulted in some restoration of $\Delta\psi$ (Fig. 8E), again consistent with inhibition of the MPT leading to preservation of mitochondrial function. Co-incubation of hPT cells with AA, the peripheral benzodiazepine receptor inhibitor PK11195, and CsA also resulted in some restoration of $\Delta\psi$ (Fig. 8F), consistent with inhibition of the MPT and Ca^{2+} cycling leading to preservation of mitochondrial function, although the preservation appeared to be somewhat less than that with AA and CsA alone. In contrast, hPT cells co-incubated with RR and PK11195 exhibited virtually no JC-1 fluorescence (Fig. 8G), indicating that simultaneous inhibition of Ca^{2+} uptake and the pBzR results in inhibition of mitochondrial function.

In comparison with control hPT cells (Fig. 9A), cells incubated for 4 h with 50 μM DCVC exhibited modestly decreased intensity of JC-1 fluorescence (Fig. 9B), suggesting a modest decrease in $\Delta\psi$, which is consistent with the modest ATP depletion (cf. Table 1). Co-incubation of hPT cells for 4 h with 50 μM DCVC and CsA resulted in some maintenance of

Table 1 – Effect of DCVC and mitochondrial inhibitors on ATP concentrations of hPT cells

Incubation	ATP (% Control)
Control	100 ± 5.1
1 mM KCN + 0.1 mM iodoacetate (KCN/IAA) (0.5 h)	22.6 ± 7.3
1 μM antimycin A (AA)	78.7 ± 3.3
1 μM staurosporine (Sts)	78.7 ± 8.4
50 μM *tert*-butyl hydroperoxide (tBH)	18.2 ± 2.1
DCVC	
10 μM	80.0 ± 2.1
50 μM	71.7 ± 3.9
100 μM	60.7 ± 13.4
200 μM	61.0 ± 8.0
300 μM	48.3 ± 8.4

Primary cultures of hPT cells were grown to ~80% confluence and then incubated with the indicated additions for 24 h, except for KCN/IAA as noted. Cellular contents of ATP were determined by a luminescence assay and are expressed as the percent of control. Control = 8.63 nmol ATP/mg protein. Results are means ± S.E.M. of measurements from three separate cell cultures, each derived from a different kidney. All values were significant different ($P < 0.05$) from those for Control cells.

JC-1 fluorescence (Fig. 9C), consistent with modest protection. In contrast, co-incubation of hPT cells for 4 h with 50 μM DCVC and PK11195 resulted in an almost complete loss of JC-1 fluorescence (Fig. 9D), suggesting that inhibition of the pBzR exacerbates DCVC-induced mitochondrial dysfunction. As with CsA, co-incubation of hPT cells for 4 h with 50 μM DCVC and RR resulted in maintenance of JC-1 fluorescence (Fig. 9E), consistent with changes in mitochondrial Ca^{2+} ion homeostasis playing a role in DCVC-induced mitochondrial dysfunction. Finally, co-incubation of hPT cells for 4 h with 50 μM DCVC and CsA and PK11195 resulted in an almost complete loss of JC-1 fluorescence (Fig. 9F), suggesting that inhibition of the pBzR abrogates or prevents the protective effect of CsA.

3.4. *FasR expression*

To further investigate whether DCVC-induced apoptosis in hPT cells can also occur through a non-mitochondrial mechanism, we determined expression of FasR after 4-h incubations with either model chemicals or DCVC or its parent molecule, TRI (Fig. 10). While 50 μM tBH had no effect, 100 μM CisPt decreased and 100 μM MVK increased FasR expression (Fig. 10A). Because we have shown that tBH is a potent mitochondrial toxicant in renal PT cells [47–49], the absence of FasR induction is consistent with it producing either necrosis or apoptosis through the mitochondrial pathway. MVK, however, did modestly increase FasR expression, consistent with it acting through both a mitochondrial and extra-mitochondrial mechanism [50]. Anis and Sts were also tested as controls: Anis is a non-selective mitogen-activated protein kinase inhibitor [51] and Sts is a protein kinase inhibitor that is frequently used as a positive control for inducing apoptosis and produces apoptosis in primary cultures of hPT cells [32]. Anis slightly decreased whereas Sts had no apparent effect on FasR expression.

Neither DCVC nor its parent molecule TRI significantly altered FasR expression (Fig. 10B), suggesting little involvement of the extrinsic pathway in DCVC-induced apoptosis. hPT cells were also exposed to 1 μg/ml LPS as a positive control for induction of FasR. FasR expression was only slightly higher in LPS-treated cells at 4 h but was lower at 24 h, suggesting that this pathway does not function well in these hPT cell cultures (data not shown). The decrease in FasR expression at 24 h with LPS treatment is likely due to LPS-induced cytotoxicity.

4. Discussion

The objective of the present work was to assess the importance of mitochondrial dysfunction in the mechanisms by which hPT cells respond to DCVC exposure. The hypothesis that was tested is that mitochondrial dysfunction is a critical

Table 2 – Effect of DCVC and mitochondrial inhibitors on caspase activities of hPT cells

Incubation	Caspase-3/7		Caspase-8	
	4 h	24 h	4 h	24 h
Control	100 ± 6.4	100 ± 4.1	100 ± 5.5	100 ± 4.2
1 μM antimycin A (AA)	90.5 ± 6.5	115 ± 10	74.6 ± 4.1*	155 ± 7*
1 μM staurosporine (Sts)	212 ± 11*	262 ± 15*	175 ± 8*	137 ± 6*
DCVC				
10 μM	115 ± 5*	92.5 ± 4.3	117 ± 6*	108 ± 4
50 μM	141 ± 8*	94.1 ± 5.5	107 ± 6	110 ± 4*
100 μM	105 ± 6	82.3 ± 4.4*	94.2 ± 4.1	124 ± 10*
200 μM	85.5 ± 3.5*	67.7 ± 4.1*	82.2 ± 4.5*	97.6 ± 4.4
300 μM	74.7 ± 3.5*	52.1 ± 3.2*	72.5 ± 2.7*	86.4 ± 4.2*

Primary cultures of hPT cells were grown to ~80% confluence and then incubated with the indicated additions for 4 or 24 h. At the end of the incubation time, cells were washed, scraped into 100 μl of PBS in 96-well plates, and then 100 μl of either the Caspase-Glo 3/7 or Caspase-Glo 8 reagent was added. After 2-h incubation in the dark, plates were read in luminescence mode in a SpectraMax 2 plate reader (Molecular Devices). Results are expressed as relative luminescence normalized to protein relative to controls (set at 100 for each experiment) and are means ± S.E.M. of measurements from three separate cell cultures, each derived from a different kidney. Significantly different (*$P < 0.05$) from the corresponding control value.

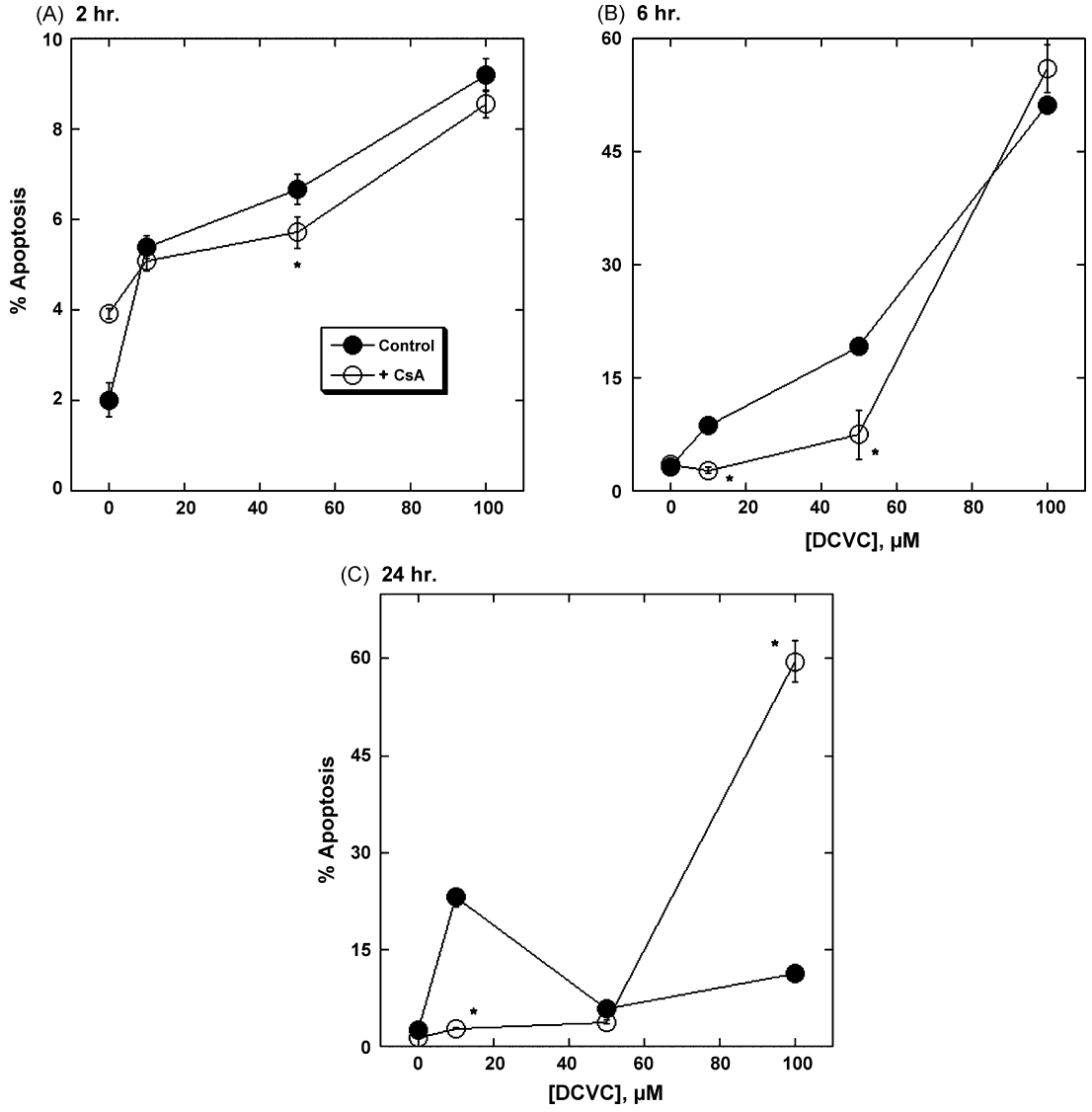

Fig. 7 – Effect of CsA on DCVC-induced apoptosis. hPT cells grown in collagen-coated, six-well plates were incubated for 4 or 24 h with the indicated concentrations of DCVC in the absence or presence of 0.5 nmol CsA/mg protein. Cells were stained with propidium iodide and cell cycle status was analyzed by flow cytometry and FACS analysis. Results are the percentage of cells undergoing apoptosis (sub-diploid) and are means ± S.E.M. of measurements from four separate cell cultures, each deriving from a different kidney. Where not visible, error bars were within the bounds of the data point. Significantly different (*$P < 0.05$) from the corresponding control cells.

and necessary step in DCVC-induced cytotoxicity. Indeed, results of the present study confirm that exposure of hPT cells to DCVC elicits necrosis, apoptosis, and a modest degree of proliferation, that the extent of these responses varies depending on DCVC concentration and exposure time, and that mitochondrial dysfunction occurs at an early time after the initial exposure. Moreover, inhibition of key mitochondrial processes significantly modulated the cytotoxic responses to DCVC, supporting the hypothesis that mitochondrial dysfunction is an obligatory step.

In our earlier studies on DCVC-induced necrosis, apoptosis, and proliferation in hPT cells [32,34], we found that, depending on exposure time and the process being measured, the lowest DCVC concentration at which we could detect significant changes in hPT cells was 10 μM for some processes but was 50 μM or higher for others. For LDH release (cf. Fig. 1), 50 μM DCVC was the lowest concentration that had a significant effect, although the effect was quite modest. For MTT fluorescence and cell cycle analysis (cf. Figs. 2 and 3), significant changes were detected at 10 μM DCVC. Decreases in MTT fluorescence are often taken to imply cytotoxicity, but are also influenced by loss of mitochondrial energization, which can occur without or prior to cell death. Using rates of DNA and protein synthesis as a potentially more sensitive indicator of cytotoxicity or proliferation, however, small and statistically significant changes were observed with 1 and 5 μM DCVC. Importantly, evidence of DCVC-induced proliferation, although modest in scope, was only observed at the

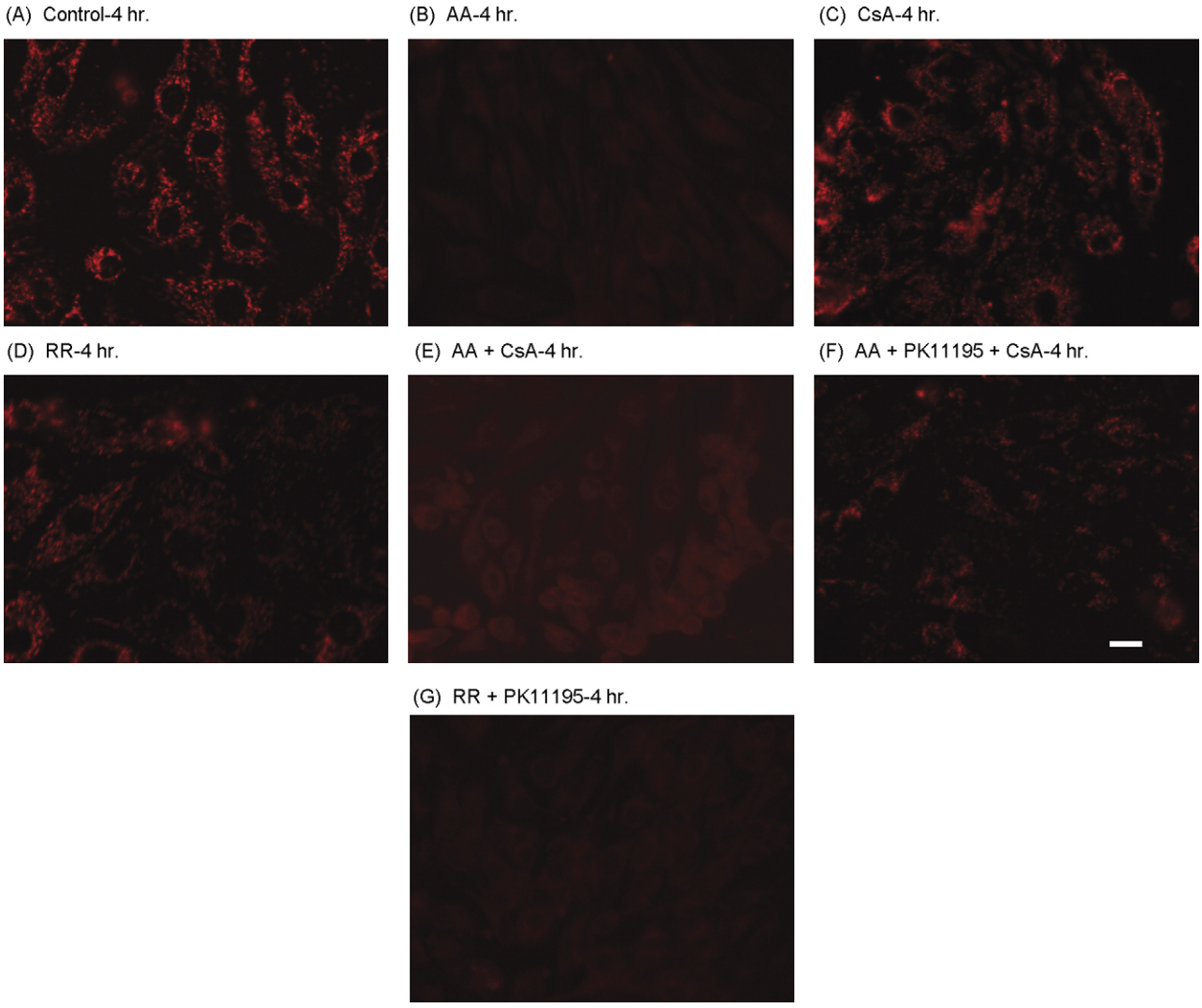

Fig. 8 – Effect of mitochondrial inhibitors on mitochondrial membrane potential. Confluent, primary cultures of hPT cells were grown on collagen-coated, glass cover slips in 35-mm tissue culture dishes and were incubated for 4 h with media (=Control) or mitochondrial inhibitors, added singly or together, as indicated, at the following concentrations: 1 μM AA, 0.5 nmol CsA/mg protein, 30 μM RR, and 120 μM PK11195. JC-1 fluorescence was measured by confocal microscopy assessing the emission of punctate red (~590 nm) in polarized mitochondria using 488-nm excitation. Red fluorescence is shown. Polarized mitochondria are indicated by yellow–red punctate staining whereas depolarized mitochondria exhibit diffuse or no staining. White bars = 5 μm.

lowest concentrations tested. Cytotoxicity becomes more prominent at higher concentrations (cf. Fig. 1), making the cells incompetent to undergo repair and proliferation.

The prominence and potency of the mitochondria as intracellular targets and sites of action are evident from examination of the time- and concentration-dependent effects of DCVC on succinate-dependent respiration (cf. Fig. 6). As found previously in hPT cells [32–34], rPT cells [9,12–15,17–20,26,31], and rabbit proximal tubules [21–25], hPT cells in the current study exhibited decreases in respiration. Modest effects were observed at 10 μM DCVC and none were detected at 1 or 5 μM DCVC. Sts, a broad-specificity protein kinase inhibitor that we have used previously as a positive control for inducing apoptosis [32], was a modest inhibitor of respiration, consistent with the need for some cellular energy for undergoing apoptosis. Similar findings were observed with measurements of cellular ATP contents. Both Sts and DCVC concentrations that produce primarily apoptosis in hPT cells, produced only modest depletion of ATP, in contrast with tBH and IAA/KCN, which caused >75% depletion of ATP. This contrasts with the much larger decreases observed in respiration rates, suggesting that the hPT cell responds to the initial toxic insult by down-regulating ATP consumption to preserve ATP stores.

Examination of effects on two types of caspase activities, caspases-3/7 and -8, whose activation are associated with the mitochondrial and extrinsic pathways of apoptosis, respectively, provided additional evidence consistent with the former and not the latter pathway being the predominant one involved in DCVC-induced apoptosis. DCVC concentra-

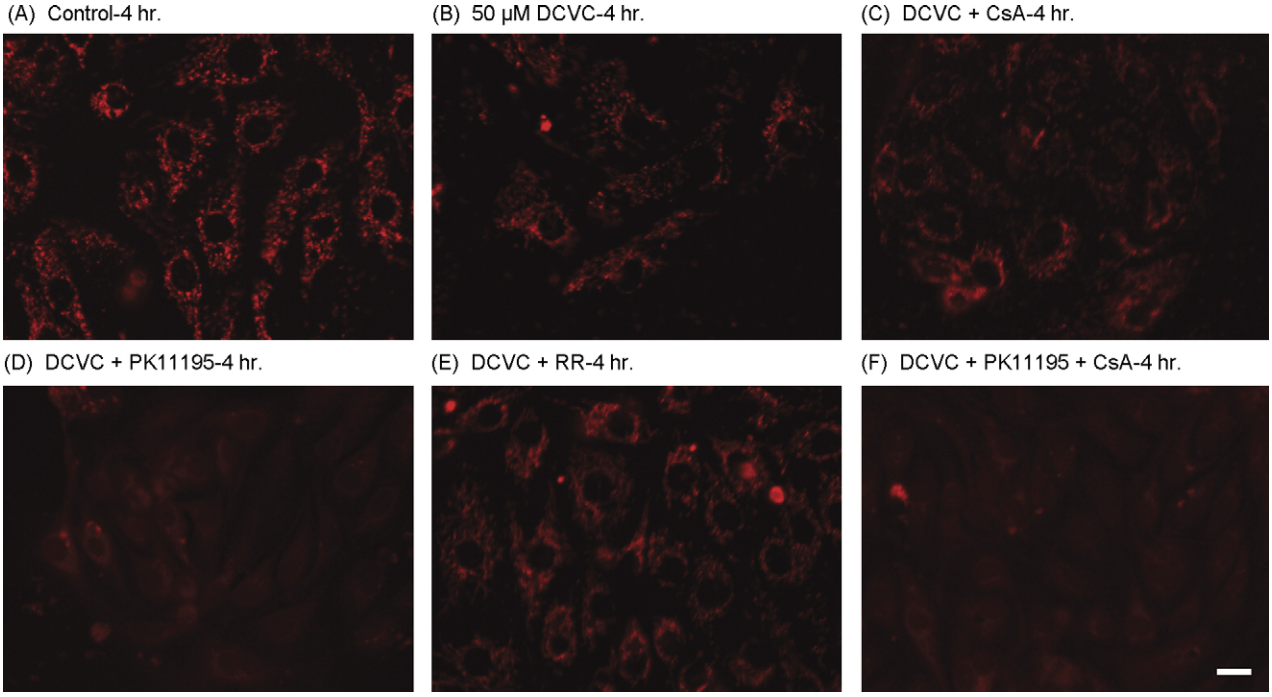

Fig. 9 – Effect of DCVC and mitochondrial inhibitors on mitochondrial membrane potential. Confluent, primary cultures of hPT cells were grown on collagen-coated, glass cover slips in 35-mm tissue culture dishes and were incubated for 4 h with media (=Control) or 50 μM DCVC alone or with the indicated mitochondrial inhibitors as in Fig. 8. JC-1 fluorescence was measured by confocal microscopy as described in the legend to Fig. 8. White bars = 5 μm.

tions ≥100 μM, particularly at the 24-h time point, were associated with significant inhibition of caspase activity, presumably due to the increasing extent of cell death. The relatively modest amounts of activation and differences between responses of the two types of caspases preclude a clear conclusion, based on these data alone, about the role of the two pathways in DCVC-induced apoptosis.

To assess further whether mitochondrial involvement in the mechanism of action of DCVC is obligatory, selected parameters of cytotoxicity and mitochondrial function were measured in the presence and absence of inhibitors of specific mitochondrial processes. As expected, CsA and RR, which block the MPT and Ca^{2+} ion cycling, respectively, were both protective, as evidenced by retention of mitochondrial $\Delta\psi$ in DCVC-treated hPT cells. In contrast, the mBzR antagonist PK11195 exacerbated loss of mitochondrial $\Delta\psi$ due to DCVC, consistent with its ability to facilitate opening of the MPT [39]. Effects of CsA on DCVC-induced apoptosis (cf. Fig. 7) provided additional insight into the key role of mitochondrial dysfunction in DCVC-induced cytotoxicity. At relatively low DCVC concentrations and/or early exposure times, CsA significantly decreased the extent of apoptosis in hPT cells. At higher DCVC concentrations and/or later exposure times (e.g., 100 μM DCVC at 24 h; Fig. 7C), however, hPT cells pretreated with CsA exhibited higher levels of apoptosis than those incubated with DCVC alone. We interpret this finding by considering the range of cellular responses to represent a continuum, as shown in the scheme in Fig. 11. Thus, at the higher dose and later time, CsA protection would be suggested to involve a shift in cellular response from necrosis to apoptosis. At the lower concentra-

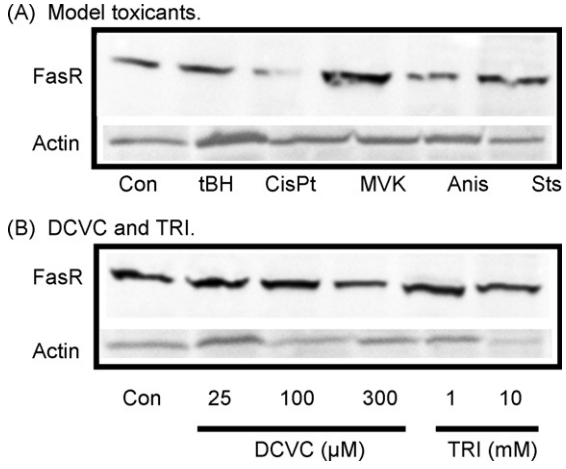

Fig. 10 – Effects of DCVC and model chemicals on FasR expression. Primary cultures of hPT cells were grown on collagen-coated T-75 flasks to ~90–100% confluence and were treated for 4 h with either (A) media (=Control, Con) or model chemicals, including 50 μM tBH, 100 μM CisPt, 100 μM MVK, 10 μg/ml anisomycin (Anis), or 1 μM Sts, or (B) media (=Control, Con), DCVC (25, 100, or 300 μM), or TRI (1 or 10 mM). Western blot analysis was conducted using a rabbit polyclonal antibody to the FasR protein (MW = 48 kDa) that recognizes the human FasR protein. Parallel samples were also run using a polyclonal antibody that detects human actin.

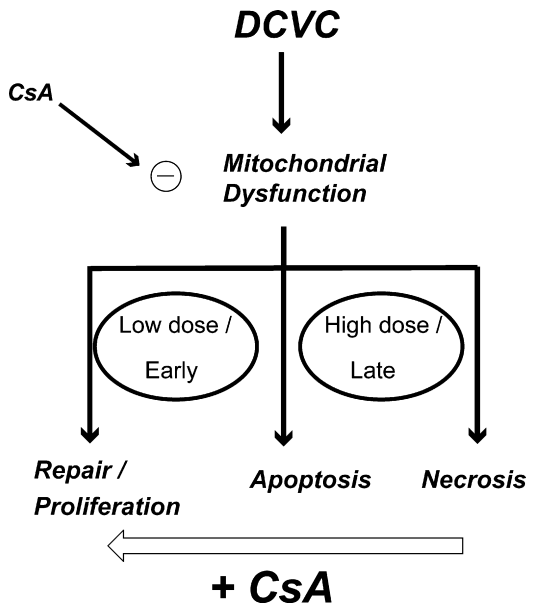

Fig. 11 – Simplified scheme illustrating the influence of CsA on DCVC-induced mitochondrial dysfunction and cytotoxicity in hPT cells.

tions of DCVC and/or earlier exposure times, we suggest that CsA shifts the cellular response from apoptosis to repair and proliferation.

Although our preliminary conclusion is that the extrinsic pathway of apoptosis induction plays little role for DCVC in hPT cells, based on the smaller increase in activity of caspase-8 vs. caspase-3/7 and the FasR expression measurements, additional work is needed to confirm this and to assess if there are any exposure conditions that could result in a stronger enhancement of the extra-mitochondrial pathway. Additionally, the lack of a robust response in the FasR measurements with LPS suggests that this pathway may not have optimal function in the primary cell cultures, making assessment of its role in toxicity difficult. Thus, although this pathway may have more of a role than indicated here, the available data can be predominantly explained by the mitochondrial pathway.

In conclusion, the initial studies presented here were designed to assess time and concentration dependence of cellular responses to DCVC in primary cultures of hPT cells so that mitochondrial dysfunction could be placed in the proper context. The cellular responses involved a range, from repair and proliferation to apoptosis to necrosis. We then tested the hypothesis that mitochondrial dysfunction is an integral and obligatory component of DCVC-induced cytotoxicity. The results support the hypothesis, suggesting that alterations in mitochondrial function are an early event subsequent to DCVC exposure and that cytotoxic responses ensue from that point. Hence, mitochondria can be viewed as the gateway that regulates the cellular response to environmental stresses and pathological states. Strategies to intervene after exposure to DCVC or similar chemicals, designed to prevent irreversible tissue injury, should focus on early steps such as mitochondrial function, to have the broadest applicability.

Acknowledgments

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