Proteomic Analysis of Alternative Protein Tyrosine Phosphorylation in 1,2-Dichlorovinyl-Cysteine-Induced Cytotoxicity in Primary Cultured Rat Renal Proximal Tubular Cells

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ABSTRACT

Toxicant exposure affects the activity of various protein tyrosine kinases. Using phosphotyrosine proteomics, we identified proteins that were differentially phosphorylated before renal cell detachment and apoptosis. Treatment of primary cultured rat proximal tubular epithelial cells with the model nephrotoxicant S-(1,2-dichlorovinyl)-L-cysteine (DCVC) resulted in early reorganization of F-actin stress fibers and formation of lamellipodia, which was followed by cell detachment from the matrix and apoptosis. This was prevented by genistein-mediated inhibition of protein tyrosine kinases and enhanced by inhibition of protein tyrosine phosphatases using vanadate. Phosphotyrosine proteomics revealed that DCVC-induced renal cell apoptosis was preceded by changes in the tyrosine phosphorylation status of a subset of proteins, as identified by matrix-assisted laser desorption ionization/time of flight-mass spectrometry (MS)/MS including actin-related protein 2 (Arp2), cytokeratin 8, f-complex protein 1 (TCP-1), chaperone containing TCP-1, and gelsolin precursor. The major differentially tyrosine-phosphorylated protein was Arp2, whereas phosphorylation of Arp3 was not affected. Arp2 was located in the lamellipodia that were formed before the onset of apoptosis. Because DCVC-induced cell detachment and apoptosis is regulated by tyrosine kinases, we propose that alterations in tyrosine phosphorylation of a subset of proteins, including Arp2, play a role in the regulation of the F-actin reorganization and lamellipodia formation that precede renal cell apoptosis caused by nephrotoxicants.

Renal proximal tubular epithelial (RPTE) cells are an important target for a variety of chemicals, nephrotoxic medicines, and ischemia/reperfusion injury (Lieberthal et al., 1998). (Sub)lethal injury of RPTE cells is associated with loss of cell-extracellular matrix (ECM) and cell-cell interactions (Bates et al., 1994; Frisch and Francis, 1994). Normally, these interactions are maintained by the F-actin cytoskeletal network, but during renal cell injury, the organization of the F-actin cytoskeleton is lost (van de Water et al., 1996). This results in redistribution of integrins to the apical side of the cell in association with a disturbance of focal adhesions and disruption of intercellular adhesions (Goligorsky et al., 1993; Bergin et al., 2000). Moreover, renal cell regeneration is stimulated by collagen IV-induced redistribution of collagen-
binding integrins to the basolateral membrane (Nony and Schnellmann, 2001). In addition, the maintenance of cell adhesions is important for cell survival, whereas loss of RPTE cell adhesion results in the onset of apoptosis (Bates et al., 1994; Frisch and Francis, 1994). In vivo, renal injury is associated with loss of cell adhesion and apoptosis, which both seem important in the pathogenesis of acute renal failure (Thadhani et al., 1996). Little is known about the molecular mechanisms, by which renal cell injury causes cell detachment, and the consequences for cell survival.

Protein tyrosine kinases and phosphatases regulate the phosphorylation of proteins located at both cell-cell and cell-ECM contact sites (Volberg et al., 1992; Roura et al., 1999). Cell stressors, such as nephrotoxicants or ATP depletion, perturb protein tyrosine phosphorylation (pTyr), thereby altering the functional state of these cell contact sites (Volberg et al., 1992; Roura et al., 1999; Wang et al., 2001). This suggests that changes in protein pTyr may determine the outcome of renal cell injury (Schwartz et al., 1999). Indeed, pharmacological inhibition of tyrosine kinases prevents nephrotoxicity and renal cell death. For example, the tyrosine kinase inhibitor AG1714 inhibited cisplatin-induced nephrotoxicity (Novogrodsky et al., 1998). Chemical anoxia in LLC-PK1 cells resulted in increased pTyr, which was blocked by the tyrosine kinase inhibitors genistein, herbimycin A, and tyrphostin, thereby providing protection against chemical-anoxia-induced cell death (Hagar et al., 1997).

Adhesion of cells to the ECM or neighboring cells is mediated through focal adhesions (FA) or adherens junctions (AJ), respectively. The AJs consist of E-cadherin proteins that link two cells in a zipper-like way. E-cadherin is connected to the F-actin cytoskeletal network through catenins (Braga, 2002). The function of both existing and assemble cell-cell adhesions is regulated by tyrosine phosphorylation. ATP depletion of proximal tubular cells resulted in loss of cell-cell interaction, which was associated with hyperphosphorylation of β-catenin and plakoglobin (Schwartz et al., 1999; Wang et al., 2001). This phosphorylation was enhanced by vanadate, a tyrosine phosphatase inhibitor, and inhibited by genistein, a general tyrosine kinase inhibitor (Schwartz et al., 1999).

FA formation is regulated by integrin clustering at sites of contact, which in turn results in pTyr and activation of F-actin-associated proteins like focal adhesion kinase (FAK), Src kinase, and paxillin (Richardson and Parsons, 1996). Previous studies (van de Water et al., 1999, 2001) showed that the levels of tyrosine-phosphorylated FAK and paxillin decreased during S-(1,2-dichlorovinyl)-L-cysteine (DCVC)-induced renal cell injury in both primary cultured RPTE and LLC-PK1 cells, which is associated with reorganization of FAs and actin stress fibers and precedes the onset of apoptosis. Therefore, we hypothesize that nephrotoxins alter the balance in tyrosine kinase and phosphatase activity, resulting in reorganization of FAs and the actin cytoskeleton, which is followed by apoptotic cell death. Likewise, altered protein tyrosine phosphorylation on tyrosine residues of specific subsets of proteins is expected to occur in direct relation to any of the above biological events.

To identify differentially tyrosine-phosphorylated proteins during nephrotoxicant-induced renal cell injury, we have used phosphotyrosine proteomics. This technique is based on high-resolution two-dimensional (2D) SDS-PAGE in conjunction with 2D immunoblot analysis with anti-pTyr antibody (Sosick et al., 1999; Zheng et al., 2002). To investigate the effects of altered pTyr in renal cell injury and apoptosis, primary cultured RPTE cells were exposed to the model nephrotoxicant DCVC (Chen et al., 1990; Groves et al., 1993; Cummings et al., 2000). DCVC is metabolized by a β-lyase to a reactive acylating metabolite that covalently modifies cellular macromolecules (Lash et al., 1986; Stevens et al., 1986). This bioactivation is important for DCVC-induced cytotoxicity culminating in apoptosis and necrosis depending on the concentration and cell type used. Apoptosis of RPTE cells caused by DCVC treatment is preceded by disorganization of the F-actin cytoskeletal network and tyrosine dephosphorylation of the FAK and paxillin (van de Water et al., 1999). Moreover, dominant negative-acting FAK deletion mutants promote the onset of apoptosis, indicating an essential role for tyrosine kinase signaling in the control of nephrotoxicant-induced cell death (van de Water et al., 2001). Thus, DCVC is a very useful agent to identify novel alternative protein tyrosine phosphorylation events in the context of cell detachment and apoptosis of RPTE cells.

Our data show that DCVC-induced actin reorganization, cell detachment, and apoptosis are inhibited by general inhibition of tyrosine kinase activity, whereas inhibition of tyrosine phosphatase promotes these events. DCVC causes the altered phosphorylation of a subset of proteins, of which actin-related protein 2 (Arp2) is the most abundant alternative tyrosine-phosphorylated protein. Arp2 localizes at lamellipodia after DCVC treatment, which is associated with increased formation and dynamics of lamellipodia, a process dependent on protein tyrosine kinase activity. Thus, altered tyrosine phosphorylation with combined downstream modulation of actin cytoskeletal organization is important for renal cell toxicity but may also be important for other types of organ toxicities that are directly related to the formation of reactive metabolites.

Materials and Methods

Materials. Dulbecco’s modified Eagles medium/Ham’s F12, PBS, cholera toxin, insulin, and penicillin/streptomycin/ampicillin B were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Invitrogen (Grand Island, NY). Collagen (type I, rat tail) and epidermal growth factor (EGF) were from Upstate Biotechnology (Lake Placid, NY). Gen and sodium orthovanadate (Van) were from Sigma-Aldrich (St. Louis, MO). N,N′,N′′-Diphenyl-p-phenylenediamine (DPPD) was from Eastman Kodak Co. (Rochester, NY). 1,2-dichloro-vinyl-L-cysteine (DCVC) was synthesized as described previously (Hayden and Stevens, 1990).

Isolation and Culture of RPTE and LLC-PK1 Cells. RPTE cells were isolated from male Wistar rats (200–250 g) by collagenase H (Sigma-Aldrich) perfusion and separated by density centrifugation using Nycodenz (Sigma-Aldrich) as described previously (Boogaard et al., 1989). Cells were cultured on collagen-coated dishes in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 containing 1% (v/v) fetal bovine serum, 0.5 mg/ml bovine serum albumin, 10 μg/ml insulin, 10 ng/ml epidermal growth factor, 10 ng/ml cholera toxin, and 1% (v/v) penicillin/streptomycin/ampicillin B. RPTE cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO2 and fed every other day. Cells were used for experiments after they had reached confluence 6 to 9 days after plating. The porcine renal epithelial cell line LLC-PK1 cells were maintained in DMEM supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin at 37°C in a humidified atmosphere of 95% air and 5% carbon
dioxide. For preparation of stable GFP-actin expressing cell lines, LLC-PK1 cells were transfected with 0.8 μg of DNA of pEGFP-actin (Clontech, Mountain View, CA) using Lipofectamine-Plus reagent according to the manufacturer’s procedures (Invitrogen). Stable transfectants were selected using 800 μg/ml G418. Individual clones were picked and maintained in complete medium containing 100 μg/ml G418. Clones were analyzed for expression of GFP-actin using immunofluorescence.

Cell Treatment Conditions. Confluent monolayers of RPTE cells in collagen (MatTek, Ashland, MA)-coated glass coverslips containing 24-well, 6-well, or 10-cm dishes were washed with PBS once. To ensure a more homogenous cell population response to DCVC, a requirement for in-depth proteomics analysis, RPTE and LLC-PK1 cells were exposed to 0.25 and 1 mM DCVC, respectively, as described previously (van de Water et al., 1999). In brief, cells were treated with DCVC in phenol red-free Hanks’ balanced salt solution (137 mM NaCl, 5 mM KCl, 0.8 mM MgSO4, 7.6 mM, 0.4 mM Na2HPO4, 2H2O, 0.4 mM KH2PO4, 1.3 mM CaCl2, 4 mM NaHCO3, 25 mM HEPES, and 5 mM glucose, pH 7.4) for indicated time periods in the presence of the antioxidant DPPD (10 μM), which blocks the lipid peroxidation-dependent necrotic pathway but allows the selective analysis of the onset of apoptosis. Sodium Van and Gen were used at a concentration of 25 and 100 μM, respectively. At these concentrations, the inhibitors itself had almost no effect on cell morphology and phosphatase, and kinase activity was blocked selectively (van de Water et al., 1999).

Cell Cycle Analysis. Apoptosis was determined by cell cycle analysis, which is reliable marker for DCVC-induced apoptosis (van de Water et al., 1999, 2001). In brief, both floating and trypsinized adherent cells were pooled and subsequently fixed in 90% ethanol (−20°C). After washing cells twice with PBS-EDTA (1 mM), cells were resuspended in PBS-EDTA containing 7.5 μM propidium iodide and 10 μg/ml RNase A. After 30-min incubation at room temperature, the cell cycle was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ), and the percentage of cells present in sub-G0/G1 was calculated using CellQuest software (BD Biosciences).

Determination of Lactate Dehydrogenase-Release. Necrotic cell death was monitored by the release of lactate dehydrogenase (LDH) from cells into the culture medium as described previously (van de Water et al., 2001). The percentage cell death was calculated from the amount of LDH release caused by treatment with oxidants relative to the amount to that released by 0.1% (w/v) Triton X-100, i.e., 100% release.

Caspase Activity Measurement. Attached cells were harvested and collected by centrifugation together with floating cells. The cell pellet was resuspended in lysis buffer (8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM Tris, pH 8.0, and 65 mM dithiothreitol) and placed on ice for 30 min. The extracts were syringed several times followed by a 15-min centrifugation (10,000 rpm, 10°C). The protein concentration was determined using a Bradford assay with IgG as a standard. For each sample, 150 μg of protein was resuspended in uez a lysis buffer containing 0.5% (v/v) IPG buffer (GE Healthcare). All samples were prepared and run in quadruplicate.

Preparation of Cell Extracts for 2D. Cells were lysed in urea lysis buffer (8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM Tris, pH 8.0, and 65 mM dithiothreitol) and placed on ice for 30 min. The extracts were syringed several times followed by a 15-min centrifugation (10,000 rpm, 10°C). The protein concentration was determined using a Bradford assay with IgG as a standard. For each sample, 150 μg of protein was resuspended in urea lysis buffer containing 0.5% (v/v) IPG buffer (GE Healthcare). All samples were prepared and run in quadruplicate.

Protein Separation by 2D Gel Electrophoresis and 2D Image Analysis. For isoelectric focusing, 24-cm immobilized pH gradient (IPG) strips (pH 3–10 nonlinear) (GE Healthcare) were rehydrated with the urea samples at 30 V for 12 h. Isoelectric focusing was performed at room temperature using the Ettan IPGphor IEF system (GE Healthcare). A gradient of 500 to 8000 V was applied over 2 h followed by a constant voltage of 8000 V for 60 kVh. After focusing, the IPG strips were equilibrated at room temperature for 10 min in equilibration buffer (6 M urea, 2% (v/v) SDS, 1% (w/v) dithiothreitol, 30% (v/v) glycerol, and 50 mM Tris, pH 6.8). The equilibrated IPG strips were transferred onto 20 × 26-cm 9% uniform PAGE gels for separation of proteins based on molecular weight. Gels were run in a Hoeffer DALT 10 gel system (GE Healthcare) overnight at 10°C at a constant amperage of 25 mA per gel. Gels were removed from the plates and either fixed in 30% MeOH/7.5% acetic acid for subsequent Sypro ruby staining (Invitrogen) or transferred to nitrocellulose membrane (Whatman Schleicher and Schuell, Keene, NH) overnight at 4°C. Western blotting was performed as described under “Western Blotting”.

All generated images were exported as tagged imaged file format files for further analysis of protein and phosphorylation profiles. Differences in tyrosine phosphorylation were detected visually by overlaying the images using Adobe Photoshop and quantitatively by PDQuest 2D Gel Analysis Software (Bio-Rad). All phosphotyrosine (PY) profiles were aligned with total protein profiles (Sypro ruby images) to be able to mark proteins undergoing changes in tyrosine phosphorylation. Matched spots from triplicate blots that could be detected on the associated Sypro ruby stained gel were excised from the gel and identified by MALDI-MS/MS (Ultraflex time-of-flight; Bruker Daltonics, Billerica, MA) with peptide mass fingerprinting.

Immunofluorescence and Imaging Techniques. For immunofluorescence studies cells were cultured on collagen-coated glass coverslips in 24-well dishes. After DCVC treatment, cells were fixed with 3.7% formaldehyde for 10 min followed by three washes with PBS. After cell permeabilization and blocking with 0.2% (v/v) Triton X-100, 0.5% (v/v) bovine serum albumin in PBS (TBP), cells were stained for β-catenin (0.25 μg/ml; BD Biosciences Transduction Laboratories, Lexington, KY) or PY99 (0.2 μg/ml; Santa Cruz Biotechnology) overnight at 4°C. Cells were washed three times with TBP and subsequently incubated with Alexa 488-labeled goat anti-mouse (1 μg/ml), in combination with rhodamine phalloidin (0.3 U/ml; Invitrogen) to label the F-actin cytoskeletal network, and Hoechst 33258. Cells were mounted on glass slides using Aqua-Poly/ Mount (Polysciences, Warrington, PA). Cells were analyzed using a Bio-Rad Radiance 2100 confocal laser scanning system (Bio-Rad, Hercules, CA) equipped with a Nikon Eclipse TE2000-U inverted microscope and a 60× Plan Apo (NA 1.4; Nikon, Melville, NY) oil-emersion objective. Images were processed with Paint Shop Pro 7 (Corel Corporation, Ottawa, ON, Canada).

For live cell imaging, enhanced green fluorescent protein-actin expressing LLC-PK1 cells were plated on tissue culture dishes containing a collagen-coated coverslip for 24 h in serum-free DMEM. Cells were exposed to DCVC in the presence of DPPD (10 μM) at 37°C in 5% CO2 for 5 h in a climate control unit built up on the stage of a Nikon Eclipse TE2000-U inverted microscope. Images were typically taken at 5-min interval using a Bio-Rad Radiance 2100 confocal system with a 60× Plan Apo (NA 1.4; Nikon) objective lens. Image acquisition was controlled using the Laser Sharp software (Bio-Rad) in combination with an in-house-developed macro to maintain auto-focus. Supplementary Videos 1 and 2 were processed with Image-Pro Plus (Version 5.1; Media Cybernetics, Inc., Silver Spring, MD).

Preparation of Cell Extracts for 2D. Cells were lysed in urea lysis buffer (8 M urea, 2 M thiourea, 4% (v/v) CHAPS, 10 mM Tris, pH 8.0, and 65 mM dithiothreitol) and placed on ice for 30 min. The extracts were syringed several times followed by a 15-min centrifugation (10,000 rpm, 10°C). The protein concentration was determined using a Bradford assay with IgG as a standard. For each sample, 150 μg of protein was resuspended in urea lysis buffer containing 0.5% (v/v) IPG buffer (GE Healthcare). All samples were prepared and run in quadruplicate.

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Immunofluorescence and Imaging Techniques. For immunofluorescence studies cells were cultured on collagen-coated glass
In-Gel Digestion. Spots picked from Sypro ruby-stained gels were cut in small pieces, washed in 10 μl of acetonitrile (50%) for 15 min followed by an additional wash in 100% acetonitrile. Spots were dried in a SpeedVac (Thermo Electron, Waltham, MA) and incubated for 30 min on ice in 5 μl of trypsin (5 ng/μl; Promega, Madison, WI) and 50 mM NH₄HCO₃. After addition of 50 mM NH₄HCO₃ so that gel pieces were covered with liquid, tryptic digestion was performed overnight at 37°C. trifluoroacetic acid was added to a final concentration of 0.1% (v/v). A 1-μl aliquot was spotted onto a MALDI target plate using a C18 Zip-Tip (Millipore Corporation, Billerica, MA) for desalting and dihydroxybenzoic acid as a matrix. Analysis of the tryptic peptides was carried out using a MALDI-MS(-MS) (Ultraflex; Bruker Daltonics). Data were analyzed with Flexanalysis 2.0 and Biotools 2.2 (Bruker Daltonics) followed by Mascot search.

Statistical Analysis. Student’s t test was used to determine whether there was a significant difference between two means (p < 0.05). When multiple means were compared, significance was determined by one-way analysis of variance (p < 0.05). Significant differences are marked with an asterisk. In addition, letter designations are used to indicate significant differences. Means with a common letter designation are not different; those with a different letter designation are significantly different from all other means with different letter designations.

Results

DCVC-Induced Cell Rounding and Apoptosis Is Dependent on Increased Protein Tyrosine Kinase Activity. To determine whether differential regulation of protein tyrosine phosphorylation is important in renal cell injury, primary cultured RPTE cells were exposed to the model nephrotoxicant DCVC in combination with genistein, a general tyrosine kinase inhibitor and/or vanadate, a general tyrosine phosphatase inhibitor. Treatment of RPTE cells with 0.1 and 0.25 mM DCVC for 8 h resulted in an increase in apoptosis as well as activation of caspase-3 (Fig. 1A) in the absence and presence of DPPD, an antioxidant that blocks...
the DCVC-induced necrotic pathway. DCVC caused LDH leakage in the absence of DPPD, which already started at 6 h after DCVC treatment (Fig. 1A). To prevent the presence of necrotic cells in our proteomic samples we have used a low amount of DPPD for further studies. In addition, because confluent cultures of primary cells were used, almost all cells were in a G1/G0 phase. DCVC treatment did not cause a shift of cells toward the S or G2/M phase (Supplemental Fig. S1), nor did the inhibitors genistein and vanadate (data not shown). Prior to and independent of apoptosis and caspase activation (data not shown) (van de Water et al., 1999) (Fig. 1B), DCVC-treated cells rounded up, which was associated with the formation of stress fibers (Fig. 2A).

Inhibition of protein tyrosine kinase activity by genistein...
almost completely prevented cell rounding of RPTE cells at 4 h after exposure (Fig. 1A). Although genistein itself induced apoptosis and caspase-3 activation, it inhibited the onset of DCVC-induced apoptosis in the presence as well as the absence of DPPD. This suggests an involvement of altered tyrosine kinase activity in DCVC-induced apoptosis (Fig. 1, A–B). Likewise, inhibition of protein tyrosine phosphatases should exacerbate the DCVC-induced cytotoxicity. Indeed, inhibition of protein tyrosine phosphatase activity by vanadate resulted in enhanced cell rounding at 4 h (Fig. 1, A–B). Although cell rounding was enhanced by the addition of vanadate, the percentage of apoptosis and caspase-3 activation was not increased, possibly by suppressing proapoptotic programs through increased tyrosine phosphorylation of specific apoptosis-regulating proteins. To ensure that the vanadate-induced enhancement of cell adhesion loss was primarily dependent on modulation of protein pTyr levels, we reasoned that inhibition of protein tyrosine kinases with genistein should antagonize the vanadate effect. Indeed, genistein completely ameliorated cell rounding as well as apoptosis of RPTE and caspase-3 activation caused by DCVC/vanadate combination (Fig. 1, A–B).

Renal Cell Injury Caused by DCVC Is Associated with Differential Protein Tyrosine Phosphorylation. The above data are suggestive for a DCVC-induced imbalance in

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**Fig. 3.** DCVC-induced lamellipodia formation is inhibited by genistein and enhanced by vanadate. RPTE cells were exposed to DCVC (0.25 mM) for 4 h in the presence or absence of Gen (100 μM) and/or Van (25 μM). Thereafter, RPTE cells were fixed and stained for paxillin and PY118-paxillin followed by confocal laser-scanning microscopy (A). In subconfluent cultures, the percentage of cells that formed lamellipodia as part of the total amount of cells that could form lamellipodia (cells on the edges of small groups) was calculated (B). LLC-PK1 cells were stably transfected with GFP-actin (pkGFP-actin) and evaluated by immunofluorescence and Western blotting (C). The dynamics of F-actin reorganization and lamellipodia formation was imaged for 4 h in pkGFP-actin cells exposed to DCVC (see Supplemental Videos 1 and 2). Frames were selected to depict cell shape changes at 0, 1, 2, 3, and 4 h (D). Arrows indicate newly formed lamellipodia; zooms of DCVC-induced F-actin fiber reorganization (left) and lamellipodia formation (right) are depicted. Images shown are representative of three independent experiments (n = 3), and the letters indicate statistical differences (p < 0.05).
protein tyrosine kinase and phosphatase activity, which is causally linked to renal cell rounding and the onset of apoptosis. Therefore, we next evaluated DCVC-induced alterations in total protein pTyr status of adhered cells by Western blotting for general tyrosine phosphorylation. DCVC caused a decrease in tyrosine phosphorylation of several proteins (around 70 and 120 kDa) in a time-dependent manner (Fig. 1C). Genistein partly prevented the decrease in protein pTyr caused by DCVC. In contrast, inhibition of phosphatase activity with vanadate resulted in a strong increase in tyrosine phosphorylation when cells were treated with DCVC (Fig. 1C). This increase was already evident at 4 h when cell rounding was just initiated, but no apoptosis was yet detected, and it further increased after 6 and 8 h. Vanadate alone decreased protein tyrosine phosphorylation slightly after 8 h of exposure. It is noteworthy that the vanadate-induced increase in pTyr in DCVC-exposed RPTE cells was completely prevented by genistein, suggesting that DCVC primarily modifies tyrosine kinase activity. Similar observations were made when freshly isolated proximal tubular cells were exposed to DCVC in the presence of vanadate (data not shown), indicating that the DCVC-induced imbalance in tyrosine kinase and phosphatase activity is also relevant for proximal tubular cells with a proteome profile that directly reflects the in vivo condition.

**DCVC-Induced Cell Rounding Is Preceded by Changes in Localization of pTyr Proteins at Cell-Cell and Cell-ECM Contacts.** Our data indicated that the imbalance in protein tyrosine kinase and phosphatase activity caused by DCVC is essential for the DCVC-induced morphological changes and cytotoxicity in RPTE cells. Because DCVC-induced renal cell injury is directly related to alterations of the F-actin cytoskeletal network organization before the loss of cell adhesion (van de Water et al., 1996), we analyzed whether DCVC-induced morphological changes corresponded to F-actin rearrangements that are linked to changes in localization of pTyr proteins. RPTE cells were double-stained for pTyr and F-actin (Fig. 2A). Four hours of exposure to DCVC caused considerable F-actin rearrangements, characterized by loss of thick F-actin bundles and condensation of F-actin into large F-actin clusters. These changes were independent of caspase activity because a general inhibitor of caspases, z-VAD-fmk, did not prevent the DCVC-induced changes in the actin cytoskeletal network (data not shown). The F-actin changes were related to alterations in protein pTyr at the sites of cell adhesion; whereas in untreated cells, we observed a specific localization of pTyr staining at the focal adhesions (FAs) and AJs, exposure of RPTE cells to DCVC resulted in loss of pTyr staining from the FAs (Fig. 2A, insets), possibly due to the reorganization of these structures. Genistein could prevent the increase in stress fiber formation, which was associated with a low amount of pTyr staining at the FA and cell-cell contacts (data not shown). Inhibition of tyrosine phosphatases by vanadate resulted in enhanced DCVC-induced disruption of the FAs.

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**Fig. 4.** 2D protein tyrosine profiling of DCVC-exposed RPTE cells. RPTE cells were treated with DCVC (0.25 mM) in the presence or absence of Van (25 μM) or Van/Gen (100/25 μM) for 4 h and thereafter harvested for 2D-electrophoresis. For both treated and control cell lysates, four 2D gels were run; one preparative Sypro ruby-stained gel (A) and three gels for transfer of proteins to nitrocellulose membranes (B). The 2D Western blots were probed with anti-tyrosine antibody PY99. Phosphotyrosine patterns were visualized using ECL Plus followed by scanning with a Typhoon 9400. All blots were run in triplicate and analyzed using PDQuest software followed by spot picking and MALDI-time of flight-MS identification (also see also Table 1).
This disruption was not completely prevented by genistein, as determined by the redistribution of FAK (data not shown).

In contrast to DCVC-induced loss of pTyr from the FAs, pTyr was increased at AJ and located in broad plaques in cells that still formed cell-cell interactions after DCVC exposure (Fig. 2A). Vanadate further enhanced protein pTyr before disruption of AJs (Fig. 2A), whereas both the increase in protein pTyr at cell-cell junctions and the disruption of F-actin organization were completely ameliorated by genistein, either in DCVC-treated cells or DCVC and vanadate-treated cells (Fig. 2A; data not shown). The formation of stable cell-cell interactions depends on maintaining AJ proteins, such as β-catenin and E-cadherin in a dephosphorylated state, DCVC may cause translocation of these proteins, thereby facilitating the onset of apoptosis. Indeed, immunofluorescence revealed a redistribution of both proteins, normally comprising the AJ, away from the plasma membrane to the cytoplasm upon rounding of RPTE cells after DCVC and DCVC/Van (Fig. 2, B–C). In cells that still formed cell-cell interactions, we observed broader plaques of β-catenin and E-cadherin at these interactions, compared with control RPTE cells. The redistribution of E-cadherin and β-catenin was prevented when kinase activity was blocked with genistein.

**DCVC-Induced Lamellipodia Formation Is Tyrosine Kinase-Dependent.** In addition to the DCVC-induced changes in F-actin fibers and cell adhesion sites, we observed the formation of membrane ruffles or so-called lamellipodia in RPTE cells that were treated with DCVC. To analyze for lamellipodia formation, RPTE cells were stained for paxillin and tyrosine-phosphorylated paxillin, a protein that is localized at newly formed focal complexes at the lamellipodia (Blagoev et al., 2004a). DCVC caused formation of lamellipodia (Fig. 3, A–B, arrows indicate newly formed lamellipodia) at 4 h before the onset of apoptosis. This lamellipodia formation was dependent on protein tyrosine kinase activity, because inhibition of phosphatases by vanadate promoted lamellipodia formation, whereas genistein prevented this formation either when used alone or in combination with vanadate (Fig. 3, A–B; data not shown). Hoechst staining indicated that the nuclei of these (attached) cells still seemed normal (data not shown).

The formation of lamellipodia is driven by active F-actin reorganization. To obtain more detailed information of the dynamics the lamellipodia in relation to DCVC-induced cytoskeletal changes, we performed live cell imaging using the well-characterized renal epithelial cell line LLC-PK1. The mechanism of DCVC-induced cytotoxicity is similar in LLC-PK1 and RPTE cells (van de Water et al., 1999; Cummings et al., 2000). The LLC-PK1 cells allow the expression of GFP-tagged proteins. To study the dynamics of DCVC-induced lamellipodia formation and F-actin reorganization, GFP-actin was stably expressed in LLC-PK1 cells (pkGFP-actin) (Fig. 3C). The pkGFP-actin cells were exposed to DCVC (1 mM), which allowed us to follow the actin dynamics during a relatively short period of 4 h and paralleled the time course of actin reorganization observed in RPTE cells caused by 0.25 mM DCVC (Fig. 3C; see Supplemental Videos 1 and 2). DCVC-induced apoptosis in LLC-PK1 cells occurred after 4 h (van de Water et al., 2001). Control pkGFP-actin cells showed little rearrangement in their F-actin stress fiber network, whereas random formation of small lamellipodia was observed at the cell border. In contrast, DCVC treatment resulted in a strong reorganization of the F-actin cytoskeletal network within the pkGFP-actin cells (Fig. 3D, zoom left), with the formation of large F-actin stress fiber clusters as observed in the RPTE cells (Fig. 2A). In addition, DCVC

### TABLE 1

<table>
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<th>Spot No.</th>
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<th>DCVC</th>
<th>DCVC/Van</th>
<th>DCVC/Van/Gen</th>
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<th>Protein Function</th>
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a Average spot intensity of control blots (n = 3).

b Average fold differences, obtained by dividing averaged spot intensities of DCVC, DCVC/Van, or DCVC/Van/Gen-exposed cells by averaged spot intensities of control cells.
caused a rapid formation of lamellipodia with GFP-actin localization in newly formed lamellipodia. F-actin fibers were formed and reorganized as the lamellipodia extended into a broad lamellipodia (Fig. 3D, zoom right).

**Protein Tyrosine Phosphorylation Profiling of DCVC-Exposed RPTE Cells.** Thus far, our results demonstrated that DCVC-induced cell rounding and apoptosis, which is preceded by F-actin rearrangement and lamellipodia formation, are regulated by tyrosine kinase activity. Thus, it would be essential to identify tyrosine-phosphorylated proteins that are linked to the effects mediated by DCVC. To screen for differentially phosphorylated proteins after DCVC treatment, we used phosphotyrosine proteomic analysis (2D SDS-PAGE in conjunction with 2D immunoblot analysis with anti-pTyr antibody detection) to identify the proteins with a change in tyrosine phosphorylation before the onset of renal cell injury.

For 2D, pTyr protein profiling four groups were used: control, DCVC, DCVC/Van, and DCVC/Van/Gen. In this way, we were able to identify phosphotyrosine proteins affected by DCVC alone but also obtain detailed information on changes in tyrosine phosphorylation during DCVC-induced renal cell injury in the absence of either tyrosine phosphatase or tyrosine kinase activity. All RPTE cells were exposed to DCVC in the presence or absence of genistein and vanadate for 4 h and stained for F-actin, tyrosine-phosphorylated proteins (PY99) (E). Images shown are representative of three independent experiments (n = 3). Statistical analysis shows *, p < 0.05 and **, p < 0.01 with n = 3.
could be matched to a corresponding protein spot on the preparative Sypro ruby gel (Fig. 4). For the remaining spots, the Sypro ruby intensity (i.e., protein amount) was too low. Using MALDI-time of flight-MS analysis, we identified both proteins with a changed and unchanged pTyr status. Knowledge of both groups could increase our understanding in the role of protein tyrosine phosphorylation in either normal or injured renal cells. Only three spots could not be identified, possibly due to their low protein concentration, even though the tyrosine phosphorylation signals were strong. The identified proteins included: 1) cytoskeleton-related proteins, such as actin-related proteins 2 and 3 (Arp2 and Arp3), cytokeratin 8, t-complex protein 1, chaperone containing TCP-1, and gelsolin precursor; 2) stress-response proteins, such as HSC70; 3) transcription and translation control proteins, including nuclear ribonucleoprotein A2/B1; and 4) proteins involved in metabolism, including pyruvate kinase and glucose-6-phosphate dehydrogenase (G6PDH) (Table 1; Supplemental Table S1 for detailed information). The major differentially phosphorylated proteins were Arp2, G6PDH, and a yet unidentified protein, spot 3. Treatment of RPTE cells with DCVC and vanadate did not change the overall 2D pTyr profile but rather showed differences in signal intensities. In particular, phospho-spots 3, 8, 9, 11, 12, and 13 showed an enhanced tyrosine phosphorylation when blocking tyrosine phosphatases. G6PDH (spot 9) was already phosphorylated in DCVC-treated cells, whereas programmed cell death-6-interacting protein (spot 8) could now be recognized as differentially phosphorylated protein in DCVC-induced renal cell injury. Instead of an increase in pTyr, the phosphoproteins Arp2, pyruvate kinase, ribonucleoprotein, cytokeratin 8, and HSC70 showed a decrease in pTyr, possibly due to changes in the dynamics of activation of signal transduction pathways when blocking phosphatase activity. Finally, genistein decreased the pTyr status of a subset of proteins, including pyruvate kinase, G6PDH, Arp2, and programmed cell death-6-interacting protein.

**Tyrosine Phosphorylation of Arp2 Precedes DCVC-Induced Apoptosis.** Arp2 was identified as the major differentially tyrosine-phosphorylated protein during DCVC-induced RPTE cell injury. Arp2 regulates the assembly and maintenance of many actin-based structures in the cell as part of the Arp2/3 complex. Because DCVC causes a drastic dynamic restructuring of the actin cytoskeleton in both RPTE and LLC-PK1 cells, we further evaluated the Arp2 protein. The phosphotyrosine content of Arp2 increased 17 times after DCVC treatment and was inhibited by genistein/vanadate treatment (Fig. 5A). To confirm the tyrosine phosphorylation of Arp2 in RPTE cells, 2D blots were reprobed for Arp2 (Fig. 5B). Two Arp2 spots were visualized, of which the right spot colocalized with tyrosine spot number 4, which was identified as Arp2 by MS analysis. Interestingly, spot number 3, which could not be identified by MS analysis, also colocalized with Arp2. The pTyr of this spot increased after DCVC-vanadate combination treatment, whereas the pTyr content of spot 4 decreased under these conditions. This suggests that Arp2 is phosphorylated on an additional Tyr site after DCVC/Van treatment, thereby generating a more negative protein charge causing the spot to shift to the acidic site of the gel (Fig. 5B). In addition, DCVC increased the tyrosine phosphorylation status of Arp2 in the pkGFP-actin cells (Fig. 5C), which were used for live-cell imaging (Fig. 3C). Although we also identified Arp3 as a phosphotyrosine protein, no significant changes were observed in its tyrosine phosphorylation status.

The Arp2/3 complex components localize at lamellipodia (Machesky and Gould, 1999) and are required for actin filament branching and lamellipodia extension. As described above, DCVC caused lamellipodia formation in both RPTE and pkGFP-actin cells. In both cell lines, Arp2 was localized in the lamellipodia that were formed early after DCVC exposure (Fig. 5, D and E) at the time point at which the phosphotyrosine content of Arp2 was increased (Fig. 5C). The ruffles that were formed in the RPTE cells contained F-actin as well as tyrosine-phosphorylated proteins. Hoechst staining indicated that the nuclei of these (attached) cells still seemed normal (data not shown). Expression of GFP-Arp2 was cytotoxic for the LLC-PK1 cells, making it impossible to study the wild-type protein or one of its phosphospecific mutants in live cells in the context of mechanisms of DCVC-induced cytotoxicity. Together our data show that the nephrotoxicant DCVC caused tyrosine kinase-dependent lamellipodia formation, which is linked to increased tyrosine phosphorylation of the actin regulatory protein Arp2. These events precede actin-regulated cell detachment and apoptosis, which in turn are also dependent on increased tyrosine kinase activity.

**Discussion**

In the present study, we examined the role of altered protein tyrosine phosphorylation in toxicant-induced renal cell injury and identified proteins with a change in tyrosine phosphorylation status before renal cell injury. We were able to 1) show that DCVC-induced cell detachment and apoptosis were blocked by genistein, a general tyrosine kinase inhibitor, and enhanced by vanadate, a general tyrosine phosphatase inhibitor; 2) demonstrate that the onset of DCVC-induced apoptosis was preceded by increased tyrosine phosphorylation of a subset of proteins when inhibiting phosphatase activity, which was blocked by genistein; and 3) identify proteins with a change in tyrosine phosphorylation status during renal cell injury using phosphotyrosine proteomics. These differentially phosphorylated proteins included 1) cytoskeleton-related proteins, such as Arp2, cytokeratin 8, t-complex protein 1, chaperone containing TCP-1, and gelsolin precursor; 2) stress response proteins, such as HSC70; 3) transcription and translation control proteins, including nuclear ribonucleoprotein A2/B1; and 4) proteins involved in metabolism, including pyruvate kinase and G6PDH. The identification of Arp2, a member of the Arp2/3 complex, as a major alternatively tyrosine-phosphorylated protein seems directly associated with the DCVC-induced formation of lamellipodia early after treatment. The tyrosine phosphorylation content of Arp2, the major differentially phosphorylated protein in this study, was approximately 17 times higher after DCVC exposure (Fig. 5A). Arp2 is a member of the Arp2/3 complex. Although Arp2 was phosphorylated in DCVC-exposed RPTE cells, the phosphorylation of Arp3 was not affected. The Arp2/3 complex is important for the assembly and maintenance of actin-based structures in the cell and localizes in lamellipodia during cell shape changes. The Arp2/3 complex is activated by cortactin or N-Wiskott-Aldrich syndrome protein through protein ty-
tyrosine kinases pathways (Machesky and Gould, 1999; Millard et al., 2004). In addition, the Arp2/3 complex is able to recruit actin-related proteins, such as vinculin and E-cadherin (Kovacs et al., 2002). Apoptosis is generally preceded by profound changes of the actin cytoskeleton. DCVC-induced cell detachment and apoptosis were preceded by dynamic rearrangements in the F-actin cytoskeleton and formation of lamellipodia. Inhibition of phosphatase activity by vanadate resulted in enhanced lamellipodia formation during DCVC exposure, which was completely inhibited by genistein, a general tyrosine kinase inhibitor (Fig. 5C). Because Arp2/3 complex was able to localize in lamellipodia and its tyrosine phosphorylation was affected by DCVC, we propose an essential function for (tyrosine-phosphorylated) Arp2 in toxicant-induced cytoskeletal rearrangement preceding renal cell apoptosis. This fits with a genistein-mediated inhibition of DCVC-induced Arp2 phosphorylation, lamellipodia formation, and apoptosis. Previously, we showed that cisplatin also caused stress fiber formation in proximal tubule cells (Imamdi et al., 2004). However, this occurred in the absence of drastic changes in protein tyrosine phosphorylation, and no lamellipodia were formed (unpublished data). This suggests that phosphorylated Arp2 might not play a role in cisplatin-induced apoptosis. So far, no data exist in the current literature implicating a role for Arp2 phosphorylation in the cytoskeletal changes that occur before apoptotic cell death induced by cytotoxic agents.

We observed a DCVC-induced increase in tyrosine phosphorylation of Arp2 upon rearrangement of the F-actin cytoskeletal network, which was decreased by inhibition of tyrosine kinase activity. DCVC and vanadate combination treatment caused a shift of phosphorylated Arp2 to the acidic side of the gel, suggesting that Arp2 is phosphorylated on an additional Tyr site after DCVC/Van treatment; Arp2 and Arp3 have been identified as differentially tyrosine phosphorylated proteins in relation to growth factor-induced F-actin rearrangement (Blagoev et al., 2004b; Kratchmarova et al., 2005). Both EGF and platelet-derived growth factor stimulated tyrosine phosphorylation of Arp2/3 to a similar extent in human mesenchymal stem cells. Whether DCVC-induced Arp2 phosphorylation is also dependent on EGF receptor activation is yet unclear. However, both 2,3,5-Tris-(glutathion-S-yl)hydroquinone (Dong et al., 2004) and DCVC cause activation of extracellular signal-regulated kinase 1/2 in human mesenchymal stem cells. Whether DCVC-induced Arp2 phosphorylation, lamellipodia formation, and apoptosis. Previously, we showed that cisplatin also caused stress fiber formation in proximal tubule cells (Imamdi et al., 2004). However, this occurred in the absence of drastic changes in protein tyrosine phosphorylation, and no lamellipodia were formed (unpublished data). This suggests that phosphorylated Arp2 might not play a role in cisplatin-induced apoptosis. So far, no data exist in the current literature implicating a role for Arp2 phosphorylation in the cytoskeletal changes that occur before apoptotic cell death induced by cytotoxic agents.

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Although tyrosine phosphorylation of Arp2/3 was recognized in several cell systems, the tyrosine phosphorylation sites in Arp2/3 remain unknown. Using NetPhos 2.0, a sequence-based prediction database for protein phosphorylation sites, we could identify five tyrosine residues (e.g., Tyr28, Tyr130, Tyr136, Tyr201, and Tyr221) as potential tyrosine phosphorylation sites in Arp2. The cytotoxicity of GFP-Arp2 for our LLC-PK1 cells makes it at this moment difficult to study the role of phosphospecific mutants of Arp2 in relation to renal cell injury.

In addition to Arp2, we have also identified several other proteins that were differentially phosphorylated and important in the control of the actin cytoskeletal network, including chaperone containing TCP-1, 1-t complex protein, and gelsolin. In a previous study (de Graauw et al., 2005), we identified several F-actin regulatory proteins as differentially expressed proteins before DCVC-induced apoptosis, including coflin, heat shock protein 27 (Hsp27), and α-B-crystallin. The turnover of F-actin, which is also needed for formation of lamellipodia, was regulated by a close collaboration between coflin, gelsolin, and the Arp2/3 complex (Ressad et al., 1999; DesMarais et al., 2004). Together this suggests that the observed F-actin reorganization before DCVC-induced cell detachment and apoptosis is regulated by a coordinated (in)activation of these actin cytoskeletal proteins.

In summary, our data show that tyrosine phosphorylation plays an important role in DCVC-induced renal cell detachment and apoptosis. Phosphotyrosine proteomic analysis is a valuable tool to identify proteins involved in different cellular processes (e.g., apoptosis, cell migration, and tumor metastasis), thereby providing information on signal transduction pathways involved in these processes. In addition, phosphoproteomics may be used in drug toxicity to shed light on the protein networks that are affected during toxicant exposure, thereby enabling prediction of toxicity (Liebler and Guengerich, 2005). Some of the proteins identified in this study might be crucial in the mechanism of both loss of cell adhesion and apoptosis during nephrotoxicity. Assessing the role of proteins, such as Arp2, enables a better understanding of the mechanisms of renal cell injury in renal diseases and may also shed light on reactive metabolite-induced toxicities in other target organs.

Acknowledgments

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References


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