Title page

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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phosphotyrosine-proteomics of renal cell injury responses

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Abbreviations: DCVC, 1,2-dichloro-vinyl-L-cysteine; RPTE, renal proximal tubular epithelial; FA, focal adhesion; AJ, adherens junctions; PTK, protein tyrosine kinase, MALDI-TOF, matrix assisted laser desorption ionization-time-of-flight.

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Abstract

Toxicant exposure affects the activity of various protein tyrosine kinases. Using phospho-tyrosine proteomics we identified proteins that were differentially phosphorylated prior to 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. Phospho-tyrosine 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 MALDI-TOF-MS/MS including actin-related protein 2 (Arp2), cytokeratin 8, t-complex protein 1, chaperone containing TCP-1 and gelsolin precursor. The major differentially tyrosine phosphorylated protein was Arp2, while phosphorylation of Arp3 was not affected. Arp2 was located in the lamellipodia that were formed prior to the onset of apoptosis. Since 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.
Introduction

Renal proximal tubular epithelial (RPTE) cells are an important target for a variety of chemicals, nephrotoxic medicines as well as 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 (Frisch and Francis, 1994; Bates et al., 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 B. 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 (Frisch and Francis, 1994; Bates et. al., 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 (Roura, et al., 1999; Volberg, et al., 1992; 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 prevent 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 assembling 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 hyper-phosphorylation of β-catenin and plakoglobin (Wang, et al., 2001; Schwartz, et al., 1999). 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 B. et al., 2001; van de Water B. et al., 1999) 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 nephrotoxicants 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 phospho-tyrosine proteomics. This technique is based on high resolution two-dimensional (2D) SDS-PAGE in conjunction with 2D immunoblot analysis with anti-pTyr antibody (Zheng et al., 2002; Soskic et al., 1999). 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 bio-activation is important for DCVC-induced cytotoxicity culminating in apoptosis and necrosis depending on the concentration and cell type used. Apoptosis of RPTE 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 B., 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 B., 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.
Our data show that DCVC-induced actin reorganization, cell detachment and apoptosis are inhibited by general inhibition of tyrosine kinase activity, while 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, which are directly related to the formation of reactive metabolites.
Methods

Materials

Dulbecco’s modified Eagles medium/Ham’s F12, PBS, cholera toxin, insulin and penicillin/streptomycin/amphotericin B (PSA) were from Invitrogen. Fetal bovine serum was from Life Technologies (Grand Island, NY). Collagen (type I, rat tail) and epidermal growth factor (EGF) were from Upstate Biotechnology (Lake Placid, NY). Genistein (Gen) and sodium orthovanadate (Van) were from Sigma (St. Louis, MO). N,N’-Diphenyl-p-phenylenediamine (DPPD) was from Kodak (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) perfusion and separated by density centrifugation using Nycodenz (Sigma) as described previously (Boogaard et al., 1989). Cells were cultured on collagen coated dishes in Dulbecco’s modified Eagles medium/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 and amphotericin B (PSA). RPTE 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 6-9 days after plating. The porcine renal epithelial cell line LLC-PK1 cells were maintained in DMEM supplemented with 10% (v/v) FCS and penicillin/streptomycin at 37°C in a humified 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 DNA of pEGFP-actin (Clontech) using Lipofectamine-Plus reagent according to the manufacturer’s procedures (Life Technologies, Inc). 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 in collagen (MatTek Corp.) coated glass coverslips containing 24-well dishes, 6 wells or 10 cm dishes were washed with PBS once. To ensure a more homogeneous cell population response to DCVC, a requirement for in depth phospho-proteomics analysis, RPTE and LLC-PK1 cells were exposed to 0.25 mM and 1 mM DCVC, respectively, as described previously (van de Water B., et al., 1999; van de Water B., et al., 2001). Briefly, cells were treated with DCVC in phenol red free Hanks’ balanced salt solution (137 mM NaCl, 5 mM KCl, 0.8 mM MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.4 mM Na\textsubscript{2}HPO\textsubscript{4}·2H\textsubscript{2}O, 0.4 mM KH\textsubscript{2}PO\textsubscript{4}, 1.3 mM CaCl\textsubscript{2}, 4 mM NaHCO\textsubscript{3}, 25 mM HEPES, 5 mM D-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 orthovanadate (Van) and Genistein (Gen) were used at a concentration of 25 µM and 100 µM respectively. At these concentrations the inhibitors itself had almost no effect on cell morphology and phosphatase and kinase activity was selectively blocked (van de Water B., et al., 1999).
Cell cycle analysis

Apoptosis was determined by cell cycle analysis, which is a reliable marker for DCVC-induced apoptosis (van de Water B., et al., 1999; van de Water B., et al., 2001). Briefly, both floating and trypsinized adherent cells were pooled and subsequently fixed in 90% ethanol (-20 °C). After washing cells twice with PBS-EDTA (1mM), 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 (FACS-Calibur, Becton Dickinson), and the percentage of cells present in sub-G0/G1 was calculated using Cellquest software (Becton Dickinson).

Determination of lactate dehydrogenase (LDH)-release.

Necrotic cell death was monitored by the release of LDH from cells into the culture medium as described previously (van de Water B., et al., 2001). The percentage cell death was calculated from the amount of LDH release caused by treatment with toxicants 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 (10 mM HEPES, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM MgCl₂, and 5 mM EGTA) and frozen in liquid nitrogen, followed by three cycles of freezing and thawing in liquid nitrogen. The supernatant was collected and equal amounts of cell protein were used for measuring caspase activity using Ac-DEVD-AMC as a substrate. The release of AMC was
monitored in time using a fluorescence plate reader (HTS 7000 Bio assay reader; Perkin Elmer). Caspase activity was expressed as pmol/mg of cell protein/minute using free AMC as a standard.

**Western blotting**

Cells were harvested as described previously (de Graauw et al., 2005). Primary antibody incubation was performed overnight at 4 ºC using monoclonal PY99 (0.04 µg/ml, Santa Cruz) antibody. Thereafter blots were incubated with horseradish peroxidase conjugated secondary antibody (GE Healthcare) in TBS-T for 1h at room temperature. Protein signals were detected with ECL-plus method (GE Healthcare) followed by scanning of the blots with a Typhoon 9400 (GE Healthcare).

**Immuno-fluorescence and imaging techniques**

For immuno-fluorescence 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 3 washes with PBS. After cell permeabilization and blocking with TBP (PBS, 0.2% (w/v) Triton X-100, 0.5% (w/v) bovine serum albumin, pH 7.4), cells were stained for β-catenin (0.25 µg/ml, Transduction Lab) or PY99 (0.2 µg/ml, Santa Cruz) overnight at 4 ºC. Cells were washed three times with TPB and subsequently incubated with Alexa-488-labeled goat anti-mouse (1 µg/ml) in combination with rhodamine-phalloidin (0.3 unit/ml, Molecular Probes) to label the F-actin cytoskeletal network, and Hoechst 33258. Cells were mounted on glass slides using Aqua-Poly/Mount (Polysciences Inc.). Cells were analysed using a Bio-Rad Radiance 2100 confocal laser.
scanning system equipped with a Nikon Eclipse TE2000-U inverted microscope and a
60X Plan Apo (NA 1.4; Nikon) oil-emersion objective. Images were processed with Paint
Shop Pro 7.

For live cell imaging, EGFP-actin expressing LLC-PK1 cells were plated on
tissue culture dishes containing a collagen-coated coverslip for 24 h in serum free DMEM
medium. Cells were exposed to DCVC in the presence of DPPD (10 µM) at 37°C in 5 %
CO2 for 5 hours 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 X 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. Movies were
processed with Image-Pro® Plus (Version 5.1; Media Cybernetics).

Preparation of cell extracts for 2D

Cells were lysed in urea lysis buffer (8M urea, 2M thiourea, 4% (w/v) CHAPS, 10 mM
Tris pH 8.0 and 65 mM DTT) 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 NL (GE Healthcare) were rehydrated with the urea samples at 30V for 12 hours. 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 hrs followed by a constant voltage of 8000 V for 60 kVh. After focusing, the IPG strips were equilibrated at RT for 10 min in equilibration buffer (6 M urea, 2% (w/v) SDS, 1% (w/v) DTT, 30 % (v/v) glycerol and 50 mM Tris pH 6.8). The equilibrated IPG strips were transferred onto 20x26 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 (Molecular Probes) or transferred to nitrocellulose membrane (Schleicher and Schuell) overnight at 4 °C. Western blotting was performed as described under ‘Western blotting’.

All generated images were exported as .tif 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 Laboratory, Inc.). All phospho-tyrosine (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) with peptide mass fingerprinting.
In-gel digestion

Spots picked from Sypro ruby stained gels were cut in small pieces, washed in 10 µl 50 % acetonitril for 15 min followed by an additional wash in 100 % acetonitril. Spots were dried in a speedvac and incubated for 30 min on ice in 5 µl 5 ng/ul trypsin (Promega) 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. TFA 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) 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 was 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 if 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 (ANOVA; 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 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 with 0.1 and 0.25 mM DCVC for 8 hrs resulted in an increase in apoptosis as well as activation of caspase-3 (Fig. 1A) in the absence and presence of DPPD, an anti-oxidant that blocks the DCVC-induced necrotic pathway. DCVC caused LDH-leakage in the absence of DPPD, which already started at 6 hrs 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, since 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 towards 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 and (van de Water B., et al., 1999)) (Fig. 1B) DCVC-treated cells rounded up, which was associated with formation of stress fibers (Fig. 2A).

Inhibition of protein tyrosine kinase activity by genistein almost completely prevented cell rounding of RPTE at 4 hrs 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. 1A-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 hr (Fig. 1A-B). Although cell rounding was enhanced by addition of vanadate, the percentage apoptosis and caspase-3 activation was not increased, possibly by suppressing pro-apoptotic 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 1A-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 protein tyrosine kinase and phosphatase activity, which is causally linked to renal cell rounding and the onset of apoptosis. Therefore, next we 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 hrs when cell rounding was
just initiated but no apoptosis was yet detected and further increased after 6 and 8 hrs.
Vanadate alone decreased protein tyrosine phosphorylation slightly after 8 hrs of
exposure. Importantly, 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 indicate that the imbalance in protein tyrosine kinase and phosphatase activity
cauased by DCVC is essential for the DCVC-induced morphological changes and
cytotoxicity in RPTE cells. Since, DCVC-induced renal cell injury is directly related to
alterations of the F-actin cytoskeletal network organization prior to the loss of cell
adhesion (van de Water B., 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 were double stained for pTyr and F-actin (Fig.
2A). Four hours exposure to DCVC caused considerable F-actin rearrangement,
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 since a general
inhibitor of caspases, zVADfmk, did not affect this (data not shown). The F-actin
changes were related to alterations in protein pTyr at the sites of cell adhesion: while in untreated cells we observed a specific localization of pTyr staining at the focal adhesions (FAs) and adherens junctions (AJs), exposure of RPTE to DCVC resulted in loss of pTyr staining from the FAs (Fig. 2A, inserts), 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. 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 prior to disruption of AJs (Fig. 2A), while both the increase in protein pTyr at cell-cell junctions and as well as 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). Since 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. Immunofluorescence revealed indeed 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. 2B-C). In cells that still formed cell-cell interactions, we observed broader plaques of β-catenin and E-cadherin at these interactions, compared to control
RPTE. The redistribution of E-cadherin and β-catenin was prevented when blocking kinase activity 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 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. 3A-B, arrows indicate newly formed lamellipodia) at 4 hrs prior to the onset of apoptosis. This lamellipodia formation was dependent on protein tyrosine kinase activity since inhibition of phosphatases by vanadate promoted lamellipodia formation, while genistein prevented this formation either when used alone or in combination with vanadate (Fig. 3A-B, data not shown). Hoechst staining indicated that the nuclei of these (attached) cells still appeared 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 cells and RPTE (Cummings, et al., 2000; van de Water B., et al., 1999). 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 hrs and parallels the time course of actin reorganization observed in RPTE caused by 0.25 mM DCVC (Fig. 3C; and see supplement for movie M1A and M1B). DCVC-induced apoptosis in LLC-PK1 cells occurs after four hours (van de Water B., 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 formation of large F-actin stress fiber clusters as was observed in the RPTE cells (Fig.2A). In addition, DCVC 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 lamellipod (Fig. 3D, zoom right).

Protein tyrosine phosphorylation profiling of DCVC-exposed RPTE cells

So far, our results demonstrate that DCVC-induced cell rounding and apoptosis, which is preceded by F-actin rearrangement and lamellipodia formation, is 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 phospho-tyrosine proteomic analysis (two-dimensional SDS-PAGE (2D) 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 phospho-tyrosine 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 for 4 hrs; a time point before the onset of cell detachment and apoptosis. Protein samples were separated according to MW and pI, transferred to membranes followed by pTyr detection using PY99 antibody (Fig. 4A-B). Matching of spots from triplicate blots in PDQuest software resulted in detection of 50-57 pTyr spots in total, of which 25 spots 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-TOF-MS analysis we identified both proteins with a changed as well as with an 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, like actin-related protein 2 (Arp2) and 3 (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 and supplemental table S1 for detailed information). The major differentially phosphorylated proteins were Arp2, G6PDH and a yet unidentified protein.
Treatment of RPTE 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 proteins in DCVC-induced renal cell-injury. Instead of an increase in pTyr, the phospho-proteins Arp2, pyruvate kinase, ribonucleoprotein, cytokeratin 8 and HSC 70 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. Since DCVC causes a drastic dynamic restructuring of the actin cytoskeleton in both RPTE and LLC-PK1 cells, we further evaluated the Arp2 protein. The phospho-tyrosine 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 co-localized 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 co-localized 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 as well as pkGFP-actin cells. In both cell lines Arp2 is localized in the lamellipodia that were formed early after DCVC exposure (Fig. 5D and E) at the time point at which the phospho-tyrosine 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 appeared 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 phospho-specific 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 prior to renal cell injury. We were able to 1) show that DCVC-induced cell detachment and apoptosis was 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 phospho-tyrosine proteomics. These differentially phosphorylated proteins included 1) cytoskeleton-related proteins, like actin-related protein 2 (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 glucose-6-phosphate dehydrogenase (G6PDH). The identification of Arp2, a component of the Arp2/3 complex, as a major alternatively tyrosine phosphorylated proteins 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. While Arp2 was phosphorylated in DCVC exposed RPTE, 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-WASP through protein tyrosine kinase pathways (Millard et al., 2004; Machesky and Gould, 1999). 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 was 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). Since Arp2/3 complex is 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 prior to 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 as well as 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 as well as PDGF stimulated tyrosine phosphorylation of Arp2/3 to a similar extent in human mesenchymal stem cells. Whether DCVC-induced Arp2 phosphorylation is also dependent on EGFR activation is yet unclear. However, both 2,3,5-Tris-(glutathion-S-yl)hydroquinone (TGHQ) (Dong et al., 2004) as well as DCVC cause activation of ERK1/2 in LLC-PK1 cells (our own observation and (Vaidya et al., 2003)).

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. Y28, 130, 136, 201, 221) 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 phospho-specific 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 TCP1, 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 prior to DCVC-induced apoptosis, including cofilin, Hsp27 and alpha-B-crystallin. The turn-over of F-actin, which is also needed for
formation of lamellipodia, is regulated by a close collaboration between cofilin, gelsolin and the Arp2/3 complex (Ressad et al., 1999; DesMarais et al., 2004). Together this suggests that the observed F-actin reorganization prior to 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. Phospho-tyrosine 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, phospho-proteomics 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.

Acknowledgements

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References


Footnotes

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Legends for figures

Figure 1: DCVC-induced cell rounding and apoptosis is regulated by tyrosine kinases.
RPTE were treated with DCVC (0.1 and 0.25 mM) in Hank’s HEPES in the presence (bottom row) or absence (upper row) of DPPD (10 µM) and in the presence or absence of vanadate (Van, 25 µM) and/or genistein (Gen, 100 µM). After 8 hrs of incubation, apoptosis was determined using flow cytometry analysis as well as caspase-3 activation and LDH-leakage (A). After 4 hrs, phase-contrast images were taken of control and treated cells (0.25 mM DCVC) (B). These images are also representative for 0.1 mM DCVC treatment. In addition, samples were taken for 1D western blotting and analyzed for tyrosine phosphorylation using anti-tyrosine antibody (PY99). Changes in pTyr after DCVC (0.25 mM) exposure were studied in time in either the absence or the presence of Gen or Van (C). Data shown represent the mean ± S.E.M. for three independent experiments (n=3) and the letters indicate statistical differences (p<0.05). Western blots are representative of three independent experiments (n=3).

Figure 2: DCVC exposure results in F-actin reorganization and translocation of tyrosine phosphorylated proteins. RPTE were exposed to DCVC (0.25 mM) for 4 hrs in the presence or absence of vanadate (Van, 25 µM) and/or genistein (Gen, 100 µM). Thereafter RPTE were fixed and double-stained for tyrosine phosphorylation (PY99) and F-actin (rhodamin/phalloidin) (A), β-catenin (B) or E-cadherin (C) followed by confocal laser scanning microscopy. Inserts represent enlarged focal adhesions. Images shown are representative of three independent experiments (n=3).
**Figure 3:** DCVC-induced lamellipodia formation is inhibited by genistein and enhanced by vanadate. RPTE were exposed to DCVC (0.25 mM) for 4 hrs in the presence or absence of genistein (Gen, 100 µM) and/or vanadate (Van, 25 µM). Thereafter RPTE were fixed and stained for paxillin and PY118-paxillin followed by confocal laser scanning microscopy (A). In sub-confluent 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 hrs in pkGFP-actin cells exposed to DCVC (see movies M1A and M1B in supplemental data). Frames were selected to depict cell shape changes at 0, 1, 2, 3 and 4 hrs (D). Arrows indicate newly formed lamellipodia, zooms are depicted of DCVC-induced F-actin fiber reorganization (left) and lamellipodia formation (right). Images shown are representative of three independent experiments (n=3) and the letters indicate statistical differences (p<0.05).

**Figure 4:** 2-Dimensional protein tyrosine profiling of DCVC-exposed RPTE. RPTE were treated with DCVC (0.25 mM) in the presence or absence of vanadate (Van, 25 µM) or vanadate/genistein (Van/Gen, 100/25 µM) for 4 hrs 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 3 gels for transfer of proteins to nitrocellulose membranes (B). The 2D Western blots were probed with anti-tyrosine antibody (PY99).
Phospho-tyrosine 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-TOF-MS identification (See also Table 1).

**Figure 5: Verification of DCVC-induced Arp2 tyrosine phosphorylation.** Tyrosine spot intensities of Arp2 (spot #3 and 4) were calculated using PDQuest analysis (A). 2D anti-tyrosine Western blots of RPTE cells were reprobed for Arp2 (B). LLC-PK1 cells were exposed to DCVC (1mM) for 4 hrs. After IP of pTyr proteins using PY99-agarose beads and SDS-PAGE, blots were probed with anti-Arp2 antibody (C). To determine the localization of Arp2 in LLC-PK1 cells were exposed to DCVC for 4 hrs, fixated and stained for Arp2 followed by confocal laser scanning microscopy (D). RPTE cells were exposed to DCVC in the presence or absence of genistein and vanadate for 4 hrs and stained for arp2, F-actin and 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.
### Table 1: Data analysis of all phospho-protein spots subjected for MALDI-TOF-MS identification

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a) Average spot intensity of control blots (n=3)

b) Average fold differences, obtained by dividing averaged spot intensities of DCVC⁴, DCVC/Van² or DCVC/Van/Gen³ exposed cells by averaged spot intensities of control cells
Figure 1

(A) Bar graphs showing the effect of different concentrations of DCVC and DCVC/DPPD on % apoptosis and pmoles/min/mg protein. Asterisks indicate significant differences.

(B) Microscopic images showing cell morphology under various conditions: Control, Gen, Van, and Van/Gen after treatment with DCVC/DPPD.

(C) Western blot analysis showing protein expression over time (2, 4*, 6, 8 h) for Control, DCVC, and DCVC/Gen.