Cadmium-induced DNA damage triggers G2/M arrest via chk1/2 and cdc2 in p53-deficient kidney proximal tubule cells

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Bork U, Lee WK, Kuchler A, Dittmar T, Thévenod F. Cadmium-induced DNA damage triggers G2/M arrest via chk1/2 and cdc2 in p53-deficient kidney proximal tubule cells. Am J Physiol Renal Physiol 298: F255–F265, 2010. First published November 18, 2009; doi:10.1152/ajprenal.00273.2009.—Carcinogenesis is a multistep process that is frequently associated with p53 inactivation. The class 1 carcinogen cadmium (Cd2+) causes renal cancer and is known to inactivate p53. G2/M transition (M) arrest contributes to stabilization of p53-deficient mutated cells, but its role and regulation in Cd2+-exposed p53-deficient renal cells are unknown. In p53-inactivated kidney proximal tubule (PT) cells, comet assay experiments showed that Cd2+ (50–100 μM) induced DNA damage within 1–6 h. This was associated with peak formation of reactive oxygen species (ROS) at 1–3 h, measured with dihydrorhodamine 123, and G2/M cell cycle arrest at 6 h, which were abolished by the antioxidant α-tocopherol (100 μM). Cd2+-induced G2/M arrest was enhanced approximately twofold on release from cell synchronization (double thymidine block or nocodazole) and resulted in approximately twofold increase of apoptosis, indicating that G2/M arrest mirrors DNA damage and toxicity. The Chk1/2 kinase inhibitor UCN-01 (0.3 μM), which relieves G2/M transition block, abolished Cd2+-induced G2/M arrest and increased apoptosis. This was accompanied by prevention of Cd2+-induced cyclin-dependent kinase cdc2 phosphorylation at tyrosine 15, as shown by immunofluorescence microscopy and immunoblotting. The data indicate that in p53-inactivated PT cells Cd2+-induced ROS formation and DNA damage trigger signaling of checkpoint activating kinases ataxia telangiectasia-mutated kinase (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR) to cause G2/M arrest. This may promote survival of premalignant PT cells and Cd2+ carcinogenesis.

7-hydroxystauosporine; cyclin-dependent kinase; p21WAF1/CIP1; cyclin; DNA strand breaks

Cells respond to DNA damage either by undergoing cell cycle arrest, to facilitate DNA repair, or by developing apoptotic death to eliminate excessively damaged and potentially harmful cells (1, 34, 63). Cell cycle checkpoint control mechanisms delay cell cycle progression, thus giving altered and potentially harmful cells time to repair and/or to initiate apoptosis induction (36). The extent of DNA damage determines whether arrest and DNA repair prevail or whether apoptosis is initiated. If DNA repair occurs, specific enzymes reverse or fix the damage (3).

The tumor suppressor p53 regulates DNA damage-induced cell cycle arrest (53, 54) by directly stimulating the expression of p21WAF1/CIP1, an inhibitor of cyclin-dependent kinases (Cdks). Cdks are key regulators of the cell cycle, working together with their partners, the cyclin proteins, to ensure that, for example, DNA replication in the S phase follows from the G1 resting phase (32). Through its negative effects on various Cdks, p21WAF1/CIP1 inhibits both the G1/S and the G2/M transitions. For instance, according to current dogma (57), p21WAF1/CIP1 induces G2/M phase arrest by inhibiting cyclin B/Cdc2 (Cdk1). While p53 and p21WAF1/CIP1 are not required for initiating G2/M cell cycle arrest, they seem to be critical for sustaining it. Cells lacking p53 and p21WAF1/CIP1 exit prematurely from the G2/M cell cycle arrest and either enter mitosis or reinitiate DNA replication (10), which causes genomic instability and can lead to accumulation of oncogenic mutations.

In p53-deficient cells G2/M arrest is initiated by the protein kinases ataxia telangiectasia-mutated kinase (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR) (37). Both enzymes are phosphatidylinositol 3-kinase (PI3-kinase)-related proteins that phosphorylate multiple substrates in response to DNA damage or replication blocks (26, 42). Both ATM and ATR recognize DNA strand breaks and contain signaling and repair activity (5, 15, 39, 61). The kinases Chk1 and Chk2 are downstream effectors of ATM and ATR, which induce G2/M cell cycle arrest by inactivating cdc25 tyrosine phosphatases through phosphorylation (4). cdc25 is required for removal of inhibitory phosphotyrosines on cyclin B/Cdc2 and cyclin A/Cdk2 kinase complexes that mediate entry into mitosis (9). Interestingly, ATM/ATR are upstream activators of both the p53- and Chk1/2-cdc25 signaling pathways (46).

Cadmium (Cd) is a toxic transition metal with no known physiological role in higher eukaryotic organisms. It is widely used in the manufacturing industry, is present in cigarette smoke, and plays an important role as an environmental pollutant in contaminated food and water (2, 44). One of the major targets of Cd2+-induced damage are the kidneys, where it accumulates preferentially in the proximal tubule (PT), which may ultimately result in renal failure (16). At the cellular level, Cd2+-induced nephrotoxicity is reflected by the formation of reactive oxygen species (ROS) (48) and cell death induction (29, 49). Cd2+ also is classified as a class 1 carcinogen and suspected of inducing cancer in many organs, including the kidney (25, 35). Cd2+ carcinogenesis involves multiple mechanisms, including DNA strand breaks as well as inhibition of DNA repair (reviewed in Refs. 7, 55). Cd2+ induces p53-dependent G1/S and/or G2/M cell cycle arrest in various cell lines expressing p53 (8, 12, 27, 60). However, tumorigenesis (as part of a multistep process) frequently involves shedding of p53 function, thus preventing p53-triggered apoptotic responses to genetic damage and providing survival advantages to incipient mutated cancer cells at a number of subsequent steps in tumor progression (23). In fact, Cd2+ has been shown...
to disrupt p53 conformation, resulting in inhibition of its function (33). Therefore it is imperative to understand cell cycle alterations and their impact on death and survival decisions in p53-deficient Cd2⁺-exposed cells, particularly of organs in which Cd2⁺ carcinogenesis occurs. Only one study in cells with mutated p53 has reported Cd2⁺-induced cell cycle alterations, namely, increased mitotic arrest (22).

Our aim was to investigate Cd2⁺-induced cell cycle arrest and cell death in a p53-inactivated kidney PT cell line. Here we demonstrate that 50–100 μM Cd2⁺ induces ROS formation and DNA damage, which results in cell cycle arrest in the G2/M phase and is mediated by cdc2 phosphorylation. The Chk1/2 inhibitor UCN-01 prevents cdc2 phosphorylation and overcomes G2/M arrest, which leads to increased cell death in Cd2⁺-exposed cells. Hence Cd2⁺-induced G2/M phase arrest may contribute to survival of p53-deficient PT cells evolving from premalignant to malignant state and thereby promote renal Cd2⁺ carcinogenesis.

**MATERIALS AND METHODS**

**Materials.** 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazo- lium bromide (MTT), ethidium bromide (EtBr), thymidine, deoxy- cytidine, and α-tocopherol were from Sigma-Aldrich (St. Louis, MO). CdCl₂ was from Merck (Darmstadt, Germany). Nocodazole, hydro- xystaurosporine (HU), and 2’-(4-ethoxypyphenyl)-5-(4-methyl-1-piperazi- nyl)-2,5’-bi-1H-benzimidazole, 3HCl (H-33342) were from Calbio- chem (EMD, La Jolla, CA). Dihydrorhodamine 123 (DHR123) was from Axxora (Lörrach, Germany). SDZ PSC 833 (PSC 833) was a gift from Novartis (Basel, Switzerland). 7-Hydroxyxystaurosporine (UCN- 01) was a gift from the Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health (Bethesda, MD). Other chemicals were obtained at the highest grade available. Inhibitors and drugs were dissolved in water, ethanol, or dimethyl sulfoxide, as appropriate [±0.1% (vol/vol)].

**Antibodies.** Mouse monoclonal antibodies against cdc2 (sc-8395) and MDM2 (sc-965) were from Santa Cruz Biotechnology (Santa Cruz, CA); tyrosine 15-phosphorylated cdc2 (cdc2-p) specific rabbit monoclonal (10A11; no. 4539) and p53-specific rabbit polyclonal (no. 9282) antibodies were from Cell Signaling (Danvers, MA). Alexa Fluor 488 chicken anti-rabbit IgG was from Molecular Probes (cat- log no. A21441). Horseradish peroxidase-conjugated donkey anti- rabbit IgG and sheep anti-mouse IgG were from GE Healthcare (Piscataway, NJ).

**Cell culture.** Immortalized p53-inactivated WKPT-0293 C1.2 rat kidney PT cells were cultured as described previously (49). Cells (passages 100–130) were split two or three times per week before reaching confluence. For experiments, cells were seeded into 24-well plates and grown for 2 days before treatment, unless otherwise indicated.

**Single-cell gel electrophoresis (comet) assay.** The technique followed the protocol of Singh et al. (43) with slight modifications. Cells (1.0–1.5 × 10⁵) were treated with Cd²⁺ in serum-containing medium (SCM), washed once in cold phosphate-buffered saline (PBS), and harvested with a cell scraper. After centrifugation at 4,500 g for 3 min at 4°C, pellets were resuspended in 10 μL of PBS, embedded, and lysed. DNA was allowed to unwind (1 mM EDTA, 300 mM NaOH, pH >13, 20 min) and subjected to electrophoresis in the same solution (18–20 V, 300 mA, 15 min). DNA of individual cells was stained with EtBr (5 μg/ml) for 5 min, rinsed in water, and analyzed by fluorescence microscopy. Cells were visualized using excitation/emission wavelengths (λex/λem) of 518/605 nm for EtBr with a VisiChrome High Speed Monochromator system (Visitron Systems, Puchheim, Germany), which was connected to a Zeiss Axiovert 200M microscope (Carl Zeiss, Jena, Germany) equipped with a ×20 objective. Images were captured, acquired, and processed with MetaMorph software (Universal Imaging, Downingtown, PA). For each experimental time point and condition, ~80 cells in 5 randomly chosen areas were counted according to the absence (no damage) or presence of a fluorescent tail next to the head (damage).

**Determination of cellular ROS levels.** DHR123 is a cell-permeant, uncharged, nonfluorescent dye that is oxidized to the highly fluorescent rhodamine 123+. Cells (1.5 × 10⁵) were treated with Cd²⁺ in SCM. When used, α-tocopherol (100 μM) was preincubated for 1 h before addition of Cd²⁺. At each time point, medium was replaced with Hanks’ balanced salt solution (HBSS) + 2.5 μM DHR123 + 1 μM PSC 833 and incubated in the dark for 30 min at 37°C. Cells were then washed once with HBSS and lysed in RIPA buffer for 10 min at 37°C. Cell lysate was measured at λex/λem of 485/535 nm with a Berthold Mithras LB940 fluorescence microplate reader (Berthold Technologies, Bad Wildbad, Germany). Protein content of cell lysate was determined by the method of Lowry et al. (30).

**Cell cycle synchronization.** Cells were synchronized in the S phase with a double thymidine block (24). Into six-well plates 2.5 × 10⁵ and 4.5 × 10⁵ PT cells were plated for control and synchronization experiments, respectively, and remained in exponential growth during synchronization. After 24 h, cells were exposed to 1 mM thymidine in serum-free medium (SFM) for 8 h and then released for 4 h in the presence of 24 μM deoxythymidine in SCM followed by a second thymidine block for a further 8 h. Cells were cultured further in SCM containing 24 μM deoxythymidine. The double thymidine block increased cells in the S phase of cell cycle from 24.9 ± 1.7% to 59.3 ± 12.9% (Table 1). For Cd²⁺ experiments, synchronized cells were incubated for 2.5 h after the second thymidine block before the addition of Cd²⁺ to permit progression into G2/M phase.

Nocodazole is an agent that interferes with the polymerization of microtubules and causes cell cycle arrest in the G2/M phase (24). PT cells (1.5 × 10⁵) were grown for 48 h in 75-cm² culture flasks and reached a density of 20–30%. Cells were synchronized by incubation with a nontoxic concentration of 0.33 μM nocodazole in SCM for 6 h. Nocodazole increased the G2/M fraction in PT cells from 21.9 ±

<table>
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<tr>
<th>Cell Cycle Phase</th>
<th>Control</th>
<th>Thymidine (1 mM 2×)</th>
<th>Control</th>
<th>Nocodazole (0.33 μM)</th>
<th>Control</th>
<th>50 μM, 6 h</th>
<th>100 μM, 6 h</th>
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<tbody>
<tr>
<td>Sub-G₁</td>
<td>3.8 ± 4.0</td>
<td>6.8 ± 4.5</td>
<td>3.7 ± 1.2</td>
<td>8.3 ± 4.2</td>
<td>3.2 ± 1.5</td>
<td>6.5 ± 5.1</td>
<td>7.7 ± 5.0</td>
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<tr>
<td>G₁</td>
<td>48.1 ± 8.7</td>
<td>24.0 ± 8.5</td>
<td>53.0 ± 6.2</td>
<td>15.6 ± 7.3</td>
<td>47.0 ± 2.0</td>
<td>46.7 ± 2.2</td>
<td>40.2 ± 1.5</td>
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<tr>
<td>S</td>
<td>24.9 ± 1.7</td>
<td>59.3 ± 12.9</td>
<td>16.2 ± 5.7</td>
<td>17.4 ± 5.1</td>
<td>26.4 ± 2.8</td>
<td>18.1 ± 2.3</td>
<td>24.3 ± 2.6</td>
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<tr>
<td>G₂/M</td>
<td>21.0 ± 1.2</td>
<td>8.7 ± 3.5</td>
<td>21.9 ± 2.0</td>
<td>51.6 ± 3.7</td>
<td>20.0 ± 2.0</td>
<td>25.4 ± 1.9*</td>
<td>26.6 ± 2.2*</td>
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Values are means ± SD of 3–9 experiments. Flow cytometric analysis of cell cycle distribution of p53-inactivated WKPT-0293 C1.2 rat kidney proximal tubule (PT) cells was performed as described in MATERIALS AND METHODS. Values in bold highlight characteristic alterations of cell cycle phases induced by thymidine and nocodazole synchronization (S and G₂/M phase arrest, respectively) or Cd²⁺ (G₂/M phase arrest). Statistical analysis of control vs. experimental groups was performed with unpaired Student’s t-test or 1-way ANOVA for comparison of multiple groups. *P < 0.01.
2.0% to 51.6 ± 3.7% (Table 1). The increase of cells in the S or G2/M phase was mainly achieved at the expense of cells in the G1 phase. After removal of nocodazole, the synchronized cells were treated with or without Cd2+ in SCM.

Interestingly, after thymidine or nocodazole synchronization was stopped and cells were allowed to grow again, most cells quickly progressed through the cell cycle to the G1 phase within 6 h.

**MTT cell toxicity assay.** The MTT assay measures cellular viability and can be used to assess, but not distinguish among, cell death, growth, and proliferation. The MTT method was modified as previously described (29). The values were normalized to the control, which was equivalent to 100% cell viability. To account for the cell cycle arrest during synchronization, different cell numbers were plated for nonsynchronized and thymidine-treated cells to obtain a similar cell density before treatment with Cd2+.

**Detection of apoptosis and necrosis with H-33342 and EtBr.** Staining and experiments were conducted essentially as previously described (29). Briefly, control and Cd2+-treated cells were stained with 2 μg/ml H-33342, followed by 5 μg/ml EtBr incubation. Cells were visualized under λex/λem of 350/460 and 518/605 nm for H-33342 and EtBr, respectively, as described above for the comet assay. Cells from five random microscopic fields of view at ×200 magnification were counted per dish, and the average percentages of apoptotic and necrotic cells were calculated.

**Flow cytometric analysis of cell cycle distribution.** The DNA and RNA intercalating fluorescent dye propidium iodide (PI) was used to quantify cellular DNA content and cell cycle distribution (17). After treatment, PT cells were harvested by trypsin digestion and fixed in 20% to 51.6% SFM. Before analysis, cells were incubated with RNase A (20 μg/ml) and stained with PI (50 μg/ml) for 5 min.

Samples were immediately analyzed by flow cytometry with a FACS Calibur flow cytometer (BD Biosciences). A total of 10,000 events were recorded per sample at FL2-H (wavelength 575 ± 26 nm), and the cell fractions in sub-G1, G1, S, and G2/M phases were quantified in histograms with WinMDI software (version 2.9, Joseph Trotter).

**Immunofluorescence staining.** PT cells (3 × 106) were cultured on coverslips (12 mm) pretreated with heat-inactivated fetal calf serum. Cells were fixed, permeabilized, and blocked as described previously (50). Cells were incubated with cdc2-p(Tyr15) primary antibody (1:30) overnight at 4°C and with Alexa Fluor 488-conjugated secondary antibody. cdc2-p(Tyr15) was quantified by densitometry by subtracting background intensity and determining the ratio of cdc2-p to total cdc2 signals.

**Statistical analyses.** All experiments were repeated at least three times. Representative data or means ± SD are shown, unless otherwise indicated. Statistical analysis by unpaired Student’s t-test was performed with Sigma Plot 11.0. For more than two groups, one-way ANOVA was used, assuming equality of variance, with least significant difference (LSD), Tukey, and Bonferroni post hoc tests for pairwise comparison with SPSS 15.0. Results with P < 0.05 were considered statistically significant.

**RESULTS**

A cell line from the S1 segment of rat PT (WKPT-0293 Cl.2) was immortalized by retroviral transfection with SV40 large T-antigen, resulting in p53 inactivation (58). This results in sequestration of p53 by the viral large T-antigen and thereby prevents p53-induced expression of its target gene mdm2 (59). Mdm2 is known to induce p53 ubiquitylation and thereby labels p53 for proteasomal degradation (18). Hence we expected large amounts of p53 and a lack of mdm2 expression. As shown in Fig. 1, rat kidney cortex homogenate, which contains a high percentage of PT, expressed low amounts of p53 (several higher-molecular-mass bands likely represent ubiquitylated p53; data not shown), whereas SV40-transfected cells expressed large amounts of sequestered p53. mdm2, a target gene of p53, was detected in rat kidney cortex homogenate (Fig. 1). In contrast, in WKPT-0293 Cl.2 cells, no mdm2 was found, which confirms the absence of functional p53 in the cell line.

Cd2+ -induced ROS formation causes DNA damage and leads to G2/M phase cell cycle arrest in p53-deficient WKPT-0293 Cl.2 cells. The role of Cd2+ in the induction of DNA damage was examined in p53-inactivated kidney PT cells with the comet assay to detect DNA strand breaks in individual cells. In control PT cells most comets showed no fluorescent tails, indicating nuclear DNA intactness. In contrast, Cd2+ (100 μM) caused an increase of comets with tails of different lengths, indicating nuclear DNA damage.

**Fig. 1.** Expression of p53 and mdm2 in rat kidney cortex homogenate and SV40-immortalized proximal tubule (PT) WKPT-0293 Cl.2 cells. Rat kidney cortex or WKPT-0293 Cl.2 cells were homogenized by sonication. Proteins (50 μg) were separated by SDS-PAGE, transferred overnight, probed for p53 (1:1,000) or mdm2 (1:500), and developed with horseradish peroxidase-conjugated IgG (1:5,000) secondary antibodies. In kidney cortex low levels of p53 expression were observed. In SV40-immortalized PT cells high levels of p53 were detected, possibly representing SV40-sequestered and -inactivated protein. mdm2, a target gene of p53, was expressed in kidney cortex but not in SV40-immortalized WKPT-0293 Cl.2 cells.
fluorescence intensities (Fig. 2A). The percentage of cells with DNA damage was significantly different from controls after exposure to 100 μM Cd\(^{2+}\) within an hour of exposure and remained significantly elevated at 3 and 6 h. Qualitatively similar results were obtained with 50 μM Cd\(^{2+}\) (data not shown).

The generation of ROS induced by Cd\(^{2+}\) was next investigated with DHR123. As shown in Fig. 2B, Cd\(^{2+}\) (50 and 100 μM) caused a concentration-dependent increase in ROS as early as 1 h after Cd\(^{2+}\) exposure. ROS reached a peak of 177.2 ± 37.4% above controls (P < 0.025; n = 9) after 1 h with 100 μM Cd\(^{2+}\), whereas with 50 μM Cd\(^{2+}\) peak ROS formation was observed at 3 h (160.5 ± 22.5% above controls; P < 0.05; n = 4). At Cd\(^{2+}\) exposure times of >3 h, ROS levels decreased again but did not reach control levels by 6 h. Incubation with the antioxidant α-tocopherol (100 μM) slightly decreased ROS levels in nontreated cells, but coincubation of α-tocopherol prevented ROS formation induced by Cd\(^{2+}\) (Fig. 2B).

Fig. 2. Effect of Cd\(^{2+}\) on DNA damage, reactive oxygen species (ROS) formation, and G2/M phase cell cycle arrest in p53-inactivated PT cells. A: detection of Cd\(^{2+}\)-induced DNA damage by single-cell gel electrophoresis (comet) assay. PT cells were incubated without or with 100 μM Cd\(^{2+}\) for up to 6 h. Fluorescence microscopic images of PT cells with absence or presence of DNA damage are shown. The % of cells with DNA damage was calculated for each experimental time point and condition tested. Means ± SE of 4 or 5 different experiments are shown. Statistical analysis with unpaired Student’s t-test compares % of cells with DNA damage in controls and cells exposed to 100 μM Cd\(^{2+}\) for 1, 3, or 6 h. B: Cd\(^{2+}\)-induced formation of ROS as measured by oxidation of dihydrorhodamine 123 (DHR123) to fluorescent rhodamine 123+ (Rh123+). PT cells were incubated for up to 6 h with or without 50 and 100 μM Cd\(^{2+}\) and in the presence or absence of the antioxidant α-tocopherol (100 μM) in serum-containing medium. Data are expressed as % of nontreated controls. Means ± SD of 5–7 different experiments are shown. C: effect of Cd\(^{2+}\) and/or α-tocopherol on cell cycle distribution of PT cells. Representative FL-2 histograms display the distribution of cells in the different phases of the cell cycle in unsynchronized PT cells. Arrow indicates the increased cell fraction in the G2/M phase in Cd\(^{2+}\) (100 μM for 6 h)-treated as opposed to untreated cells and α-tocopherol (100 μM) for 6 h)-exposed PT cells. Data are typical of 4 different experiments. D: statistical analysis of Cd\(^{2+}\) (100 μM for 6 h) and/or α-tocopherol (100 μM)-induced changes in the G2/M fraction of unsynchronized PT cells. Statistical analysis with unpaired Student’s t-test compares control cells with Cd\(^{2+}\)-treated cells in the absence or presence of α-tocopherol. Means ± SD of 4 experiments are shown. n.s., No significant difference compared with controls.
Synchronization of PT cells increases proportion of Cd\textsuperscript{2+}-induced G2/M phase cell cycle arrest and apoptosis. To confirm the cell cycle-dependent effects of Cd\textsuperscript{2+}, we synchronized PT cells and monitored Cd\textsuperscript{2+}-induced effects once previously synchronized cells resumed proliferation. We used two synchronization techniques, a double thymidine block and nocodazole, that target the S or G2/M phase (Table 1), respectively. In thymidine-synchronized cells, Cd\textsuperscript{2+} for 6 h showed a clear increase in the fraction of G2/M cells (arrow in Fig. 3A, left). Similar findings were seen with nocodazole-synchronized PT cells (arrow in Fig. 3A, right). Quantitative analysis showed that in nonsynchronized controls 50 \mu M Cd\textsuperscript{2+} for 6 h significantly increased the proportion of PT cells in the G2/M phase by 4.6 \pm 1.8\% compared with 9.9 \pm 3.5\% in nocodazole-synchronized cells (Fig. 3B). Hence, nocodazole synchronization increased the difference between control and Cd\textsuperscript{2+}-treated G2/M-arrested cells by approximately twofold. In contrast, no significant effect of Cd\textsuperscript{2+} on other cell cycle phases was found (data not shown). These data indicate that by synchronizing the cells we increased the number of cells reaching the G2/M phase and hence the proportion of cells arrested in the G2/M phase because of Cd\textsuperscript{2+} damage.

After demonstrating that Cd\textsuperscript{2+} induces cell cycle arrest in the G2/M phase, we investigated its impact on Cd\textsuperscript{2+}-induced cell death, assuming that G2/M phase arrest reflects DNA damage and hence toxicity. First, we compared Cd\textsuperscript{2+} toxicity in nonsynchronized and thymidine-synchronized cells, using the MTT cell viability assay. In Cd\textsuperscript{2+}-exposed (50 \mu M for 6 h) thymidine-synchronized PT cells, cell viability was significantly decreased by 11.2 \pm 4.2\% in unsynchronized cells and by 27.1 \pm 4.3\% in synchronized cells (i.e., the percentage of dead cells was increased accordingly) (Fig. 3C). This approximately twofold increase in toxicity after synchronization correlates with the approximately twofold increase of cells in the G2/M phase after 50 \mu M Cd\textsuperscript{2+} treatment for 6 h in synchronized cells (see above and Fig. 3B). Similar effects were observed in nocodazole-synchronized cells, in which Cd\textsuperscript{2+}-induced cell viability was significantly decreased from 14.5 \pm 2.9\% in unsynchronized controls to 23.5 \pm 2.8\% (Fig. 3C). To confirm the latter results, we performed H-33342/EtBr nuclear staining of PT cells for detection of apoptosis and necrosis, respectively. Apoptosis by Cd\textsuperscript{2+} (50 \mu M for 6 h) was significantly elevated in nonsynchronized PT cells compared with untreated controls (5.8 \pm 1.5\% vs. 1.2 \pm 0.2\%) (Fig. 3D), which confirms previous observations (29). The values obtained with the MTT test were always higher than those measured by H-33342/EtBr nuclear staining of PT cells. This occurs because the MTT test also measures the number of metabolically active cells and therefore reflects cell cycle arrest in Cd\textsuperscript{2+}-exposed cells, which also results in a decrease of cell number compared with nonarrested cells. Cells synchronized with 0.33 \mu M nocodazole for 6 h displayed 1.7 \pm 0.3\% apoptosis, which is similar to that of unsynchronized cells, confirming that 0.33 \mu M nocodazole is not toxic. Cd\textsuperscript{2+} exposure (50 \mu M for 6 h) significantly increased apoptosis of nocodazole-synchronized cells by approximately twofold (from 5.8 \pm 1.5\% to 10.2 \pm 1.5\%; Fig. 3D). In contrast, necrosis was only increased significantly in synchronized PT cells treated with 50 \mu M Cd\textsuperscript{2+} (2.2 \pm 0.9\% compared with 0.7 \pm 0.2\% in controls).

Cd\textsuperscript{2+}-induced PT cell death is increased and G2/M phase cell cycle arrest abolished by Chk1/2 inhibitor UCN-01. Activation of the G2 checkpoint arrest might serve as a protective mechanism for PT cells to repair DNA damage induced by Cd\textsuperscript{2+} before progressing through mitosis. To test this hypothesis, we used the staurosporine derivative UCN-01 to relieve the G2/M transition block induced by Cd\textsuperscript{2+} (see DISCUSSION) (56). We hypothesized that prevention of cell cycle arrest in the G2/M phase would result in inadequate DNA repair and hence increased cell death. First, we tested the effect of UCN-01 (0.3 \mu M) on the cell cycle of unsynchronized PT cells. As exemplified in a representative histogram, incubation of PT cells with UCN-01 for 6 h increased the percentage of cells in the G1 phase (from 42.1 \pm 2.4\% to 62.4 \pm 4.0\%) at the expense of S and G2/M phases (asterisk in Fig. 4A and Fig. 4B; Table 3) since UCN-01 also arrests cells in the G1 phase in a p53-independent manner (14, 40). Significant increase in the proportion of G2/M phase cells by 50 \mu M Cd\textsuperscript{2+} for 6 h was abolished by coincubation with UCN-01 (arrow in Fig. 4A and Fig. 4B; Table 3), indicating that inhibition of Chk1/2 by UCN-01 overcomes G2/M phase arrest induced by Cd\textsuperscript{2+}.

Similar results were obtained with 100 \mu M Cd\textsuperscript{2+} (data not shown).

We then investigated the effect of UCN-01 on cell viability of nonsynchronized PT cells. After 6-h exposure, cell viability was nonsignificantly reduced by 9.7 \pm 3.7\% with UCN-01 and significantly by 15.8 \pm 6.7\% with 50 \mu M Cd\textsuperscript{2+} (i.e., the percentage of dead cells was increased accordingly) (Fig. 4C). Exposure of PT cells to 100 \mu M Cd\textsuperscript{2+} for 6 h decreased cell viability even more (by 39.0 \pm 5.5\%). Coincubation of 0.3 \mu M UCN-01 with 50 or 100 \mu M Cd\textsuperscript{2+} for 6 h reduced cell viability by 34.2 \pm 7.2\% and 59.3 \pm 11.2\%, respectively (Fig. 4C), which is significantly higher than the effects obtained with Cd\textsuperscript{2+} alone. The increase of cell death induced by UCN-01 was exclusively caused by apoptosis in PT exposed to 50 \mu M Cd\textsuperscript{2+}, whereas with 100 \mu M Cd\textsuperscript{2+} necrosis was also significantly increased (Fig. 4D).

Cd\textsuperscript{2+} increases phosphorylation of cdc2 at tyrosine 15 in PT cells, which is prevented by UCN-01. To further detail the mechanism of Cd\textsuperscript{2+}-induced cell cycle arrest at the G2 checkpoint, we investigated the role of cdc2 (also known as cdk1 or p34\textsuperscript{cdc1}) as the end point of all cascades leading to G2 arrest. Cdc25 phosphatase dephosphorylates cdc2 at Tyr15 to induce mitosis (see DISCUSSION for details) (19, 21, 45). We used a Tyr15 phosphorylation-specific cdc2 antibody to determine levels of inactive phosphorylated cdc2 protein [cdc2-
Immunofluorescence studies showed positive cdc2-p(Tyr15) staining in control cells, but not when the primary antibody was omitted, confirming specificity of cdc2-p staining and indicating that cdc2-p(Tyr15) is present under basal conditions (Fig. 5A). To further validate the immunofluorescence signals, PT cells were also treated with the positive control 50 μM HU for 24 h to induce cdc2-p(Tyr15) (Fig. 5) (38). The cdc2-p staining was mainly nuclear, but some cytosolic staining was also observed. Exposure of PT cells to 50 μM Cd²⁺ for 4 h increased cdc2-p(Tyr15) staining (Fig. 5A). Similar effects were also observed after 1 and 2 h (data not shown) as well as with 100 μM Cd²⁺ for 4 h (Fig. 5A). Quantitative analysis demonstrated significant increases of cdc2-p(Tyr15) with 50 and 100 μM Cd²⁺ (Fig. 5B). To confirm these observations immunoblots were also performed. Protein loading was controlled by using antibodies against cdc2, which also demonstrated that total cdc2 expression is not affected by the various experimental treatments. In PT cells treated with 50 μM Cd²⁺ for 6 h Cd²⁺-specific increases of cdc2-p(Tyr15) were observed (Fig. 5A and 5B). These data indicate that the G₂ damage checkpoint is activated by Cd²⁺, possibly through prevention of Tyr15 cdc2-p dephosphorylation by cdc25c, which results in cell cycle arrest in the G₂ phase.

Further evidence for this mechanism was also provided with the Chk1/2 inhibitor UCN-01 in immunofluorescence staining experiments with cdc2-p(Tyr15) antibody. A decrease in cdc2-p fluorescence intensity was clearly visible in cells treated with UCN-01 alone (Fig. 5A) and was significant after quan-
Likewise, cdc2-p was attenuated when PT cells were coincubated with 0.3 \( \mu \text{M} \) UCN-01 and 100 \( \mu \text{M} \) Cd\( ^{2+} \) for 4 h (Fig. 5A). A highly significant decrease in cdc2-p(Tyr15) between PT cells treated with Cd\( ^{2+} \) and UCN-01 was observed (Fig. 5B). Immunoblotting showed that treatment with 0.3 \( \mu \text{M} \) UCN-01 alone for 4 h also significantly decreased levels of cdc2-p(Tyr15) compared with untreated controls (Fig. 6, C and D), which confirms the immunofluorescence data (Fig. 5). When PT cells were coincubated with 100 \( \mu \text{M} \) Cd\( ^{2+} \) and 0.3 \( \mu \text{M} \) UCN-01, the increase of cdc2-p(Tyr15) levels induced by 100 \( \mu \text{M} \) Cd\( ^{2+} \) was reduced to the levels obtained with UCN-01 alone, which is in line with the immunofluorescence data (Fig. 6, C and D). This indicates an involvement of the Chk1/2 pathway in Cd\( ^{2+} \)-mediated prevention of Tyr15 cdc2-p dephosphorylation by cdc25c resulting in G2 phase cell cycle arrest.

**DISCUSSION**

The induction of oxidative stress and formation of reactive oxygen and nitrogen species in vivo and in vitro is an attractive hypothesis to account for mutagenic and carcinogenic effects of...
metals in mammalian cells because these radicals are known to cause oxidative damage to lipids, proteins, and DNA (7). Indeed, Cd\textsuperscript{2+}/H\textsubscript{11001} induces rapid and concentration-dependent ROS formation in kidney PT cells (Fig. 2B), which is paralleled by development of DNA damage (Fig. 2A). Although Cd\textsuperscript{2+}/H\textsubscript{11001} is not capable of exerting redox reactions in biological systems, it induces ROS formation in cultured cells of various origins and in vivo, including kidney PT cells, by depletion of endogenous redox scavengers, inhibition of antioxidative enzymes, inhibition of the mitochondrial electron transport chain, and/or displacement of redox-active metals (reviewed in Ref. 47). In the present study we show that ROS mediate Cd\textsuperscript{2+}-induced G\textsubscript{2} checkpoint arrest in p53-deficient kidney PT cells (Fig. 2, C and D), although direct DNA-damaging effects of Cd\textsuperscript{2+} and/or inhibition of DNA mismatch repair may also occur (7, 55). Furthermore, Cd\textsuperscript{2+}-induced p53-independent G\textsubscript{2} checkpoint arrest can be increased in S or G\textsubscript{2}/M phase synchronized cells (Fig. 3).

In normal cells DNA damage induced by oxidative stress leads to cell cycle arrest at the G\textsubscript{1} and G\textsubscript{2}/M cell cycle checkpoints (6). Although p53 mediates cell cycle arrest in the G\textsubscript{1} phase and G\textsubscript{2}/M phases, the G\textsubscript{2}/M checkpoint is especially important for understanding cell cycle alterations and death and survival decisions linked to growth and differentiation in p53-deficient cells, such as premalignant or malignant cells. Cells deficient in p53 rely on ATM/ATR-mediated checkpoint signaling for survival (28, 37). The critical regulatory step in the activation of cdc2 during progression from the G\textsubscript{2} phase into mitosis is dephosphorylation of cdc2 at Tyr15, which is carried out by cdc25c (19, 21, 45). Hence activation of ATM/ATR by DNA damage initiates a signaling cascade, which inactivates cdc25c and thereby prevents dephosphorylation of cdc2 at Tyr15 to induce mitosis. Here we further demonstrate that Cd\textsuperscript{2+}-induced G\textsubscript{2}/M cell cycle arrest correlates with an increase of cdc2 phosphorylation at Tyr15 (Figs. 5 and 6), indicating that ROS-induced DNA damage activates the ATM/ATR signaling pathway, and thus Chk1/2, to induce G\textsubscript{2} checkpoint arrest. A previous study in the p53-proficient rat PT cell line NRK-52E demonstrated G\textsubscript{2}/M phase arrest mediated by Cd\textsuperscript{2+} (60). The authors observed upregulation of p53 and p21\textsuperscript{WAF1/CIP1} that was held responsible for mediating inhibition of Cdk1 and Cdk2 and, as expected, resulted in G\textsubscript{2}/M arrest. Surprisingly, p53 upregulation induced by Cd\textsuperscript{2+} was not associated with G\textsubscript{1} phase arrest. Multiple overlapping upstream signals may have triggered p53 activation (57), including ATM/ATR, but this was not investigated further.

UCN-01 overcomes cell cycle arrest in the G\textsubscript{2} phase by targeting Chk1/2 (11, 20, 62). In the presence of UCN-01 Cd\textsuperscript{2+}-induced cdc2-p(Tyr15) phosphorylation was abolished and even decreased compared with controls (Fig. 5, Fig. 6, C and D), which is in line with the presumed mode of action of UCN-01 and suggests that under these conditions cdc2 was more active to promote mitosis. Accordingly, cell cycle arrest
induced by $\text{Cd}^{2+}$ was also abolished (Fig. 4, A and B). Interestingly, by overcoming G2 checkpoint arrest with UCN-01, $\text{Cd}^{2+}$-induced cell death was increased (Fig. 4, C and D). This indicates that the G2/M cell cycle arrest induced by $\text{Cd}^{2+}$ occurs in a population of damaged PT cells that can potentially die unless this DNA damage is partially or completely fixed. Active cdc2 is also a key player in the regulatory processes linking cell cycle arrest to apoptosis via the dissociation of the survivin/caspase 9 complex and by inhibiting B cell lymphoma 2 (Bcl-2), an antiapoptotic protein (13). Prevention of G2 checkpoint arrest by UCN-01 gives $\text{Cd}^{2+}$-damaged cells no opportunity to repair damaged DNA and in turn precipitates entry of these p53-inactivated PT cells into mitosis (10) concluding with apoptosis (Fig. 4D) (possibly via caspase 9 activation and Bcl-2 inhibition). Alternatively, cells can undergo mitotic catastrophe (37, 52).

It is well established that p53 inactivation provides an advantage to premalignant cells (23). At first sight, G2/M phase arrest of p53-inactivated cells provides an opportunity for repair of DNA damage. However, this mechanism turns out to be a double-edged sword because repair mechanisms have a limited fidelity, which allow some nonlethal mutations to slip through when p53-inactivated cells exit prematurely from the G2/M cell cycle arrest and enter mitosis (10). The worst-case scenario occurs when mutations are generated, which promote cell survival and/or proliferation. They are passed on to subsequent generations of cells, which may eventually evolve into malignant cells. UCN-01 represents a therapeutic strategy that takes these considerations into account, and in fact it has been shown to increase the cytotoxicity of chemotherapy and radiation (31, 41, 51). Consequently, UCN-01 is currently used in clinical trials.

In summary, $\text{Cd}^{2+}$ induces cellular stress in p53-inactivated kidney PT cells, leading to ROS formation and DNA damage. This triggers activation of the ATM/ATR pathway, which abrogates dephosphorylation of cdc2/cyclin B complex at cdc2-p(Tyr15). This results in G2 checkpoint cell cycle arrest to provide an opportunity for DNA repair and delays entry of p53-inactivated PT cells into mitosis. The inhibitor of the ATM/ATR pathway UCN-01, which targets Chk1/2 upstream kinases and thereby enhances cdc25c-mediated dephosphorylation of cdc2, overcomes G2 checkpoint arrest. Hence $\text{Cd}^{2+}$-damaged cells enter mitosis and die. The data suggest that in DNA-damaged p53-inactivated kidney PT cells induced by $\text{Cd}^{2+}$, G2 checkpoint arrest may allow premalignant cells to survive and subsequently develop into malignant cells.

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DISCLOSURES
No conflicts of interest are declared by the authors.

REFERENCES


