Inhibition of p38-MAPK Potentiates Cisplatin-Induced Apoptosis via GSH Depletion and Increases Intracellular Drug Accumulation in Growth-Arrested Kidney Tubular Epithelial Cells

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We were interested in analyzing the regulation by mitogen-activated protein kinases (MAPKs) of cisplatin-provoked toxicity in epithelial renal tubule cell lines, when assayed under culture conditions (cell confluence plus serum deprivation), which mimic the characteristics of a nonproliferating epithelium. Under these restrictive growth conditions, cisplatin induced apoptosis with lower efficacy than in exponentially growing cells, and decreased p38-MAPK phosphorylation in NRK-52E and other (LLC-PK1, MDCK, HK2) cell lines. Moreover, cisplatin-provoked apoptosis was potentiated by cotreatment with p38-MAPK-specific inhibitors (SB203580, SB220025) or transfection with a kinase-negative mutant of M KK6, whereas c-Jun NH2-terminal kinase or extracellular signal-regulated kinase/MAPK and ERK Kinase inhibitors were ineffective. By contrast, when applied to exponentially growing cells, cisplatin stimulated p38-MAPK phosphorylation and apoptosis, was attenuated by kinase inhibitors. Treatment of confluent/serum-deprived cells with cisplatin caused mitochondrial transmembrane potential disruption and activated the mitochondrial apoptotic pathway, as indicated by the decrease in Bcl-XL expression, increase in Bax expression and cytochrome c release, and these effects were potentiated by cotreatment with SB203580. Treatment of confluent/serum-deprived cells with cisplatin plus SB203580 decreased the intracellular reduced glutathione (GSH) content, and increased intracellular cisplatin accumulation as well as cisplatin binding to DNA. Cotreatment with the GSH-depleting agent D,L-buthionine-R,S-sulfoximine also potentiated cisplatin-provoked apoptosis. In summary, p38-MAPK inhibition potentiates cisplatin-provoked apoptosis in growth-arrested epithelial renal tubule cells, a result that may be explained at least in part by GSH depletion and drug transport alteration.

Key Words: cisplatin; apoptosis; MAPK kinases; cisplatin uptake; intracellular glutathione; renal tubule cells.

Cisplatin is a DNA alkylation agent with well proved efficacy in the treatment of a wide range of solid tumors and some forms of leukemia (Boulikas and Vougiouka, 2004). It is normally accepted that the cytotoxicity of cisplatin, manifested as the induction of apoptosis and/or necrosis, is mainly due to the generation of DNA lesions—mostly intrastrand cross-links, plus a minor proportion of interstrand cross-links and monofunctional adducts (Fuertes et al., 2003; Jamieson and Lippard, 1999). Nonetheless cisplatin may also bind other cellular targets, including membrane phospholipids, RNA, and proteins, causing cytoskeleton disruption, mitochondrial dysfunction, and intracellular oxidation, which are also important for cell death (Fuertes et al., 2003; Troyano et al., 2001). A common adverse effect of cisplatin treatment is nephrotoxicity, mainly due to the damage and eventual death (apoptotic or necrotic) of renal epithelial tubule cells (Leibbrandt et al., 1995). At the functional level, nephrotoxicity may be manifested as acute renal failure or chronic renal insufficiency and electrolyte wasting (Blachley and Hill, 1981; Safirstein et al., 1987). Apoptotic cell death is a known contributor to nephrotoxic-induced toxicity, and the mechanisms of nephrotoxicity may differ from the actions of the drugs in other cell types (Hortelano et al., 2000; Servais et al., 2008).

One of the most relevant aspects in the regulation of drug-induced apoptosis is the signaling by mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases which mediate intracellular signal transduction in response to different physiological stimuli and stressing conditions. Three major MAPKs have been identified, namely c-Jun NH2-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK)1/2. It is normally considered that JNK and/or p38 activation is associated to apoptosis induction, and that ERK activation favors proliferation and promotes cell survival (Kim and Chung, 2008; Roux and Blenis, 2004), but this rule is subjected to multiple exceptions. In particular, cisplatin was reported to activate ERK, JNK, and p38 in different cell types, including renal tubule cells (Arany et al., 2004; Ramesh and Reeves, 2005), but the role of these kinases in cell death (i.e., whether they play a proapoptotic or survival effect) depends...
very much on the cell model and experimental conditions (Brozovic and Osmak, 2007). An additional aspect that deserves consideration is that cisplatin uptake by the cells may be regulated by protein kinases (Amran et al., 2005; Basu and Evans, 1994). Moreover, we recently reported that MAPK inhibitors may decrease the intracellular reduced glutathione (GSH) content (Ramos et al., 2006), which is an important factor in the cisplatin detoxification machinery (Zhang et al., 2001). Changes in cisplatin uptake or extrusion would result in alterations in intracellular drug accumulation, and as a consequence in toxicity.

With these considerations in mind, the purpose of this work was to examine the involvement of MAPKs in the regulation of cisplatin-provoked cell death in NRK-52E cells and other renal tubule cell lines. Appropriate cell growth conditions (namely, cell confluence followed by serum deprivation) were selected to mimic the loss of proliferation activity characteristic of tubular epithelial tissue. Under these conditions, cisplatin slightly induced apoptosis, caused a decrease in p38-MAPK phosphorylation and apoptosis was further potentiated by pharmacologic inhibitor- or gene transfer-provoked downregulation of kinase phosphorylation. Cotreatment with p38-MAPK inhibitor decreased intracellular GSH content and increased intracellular cisplatin accumulation and DNA binding, which might explain at least in part the potentiation of drug toxicity.

MATERIALS AND METHODS
Reagents and antibodies. All components for cell culture were obtained from Invitrogen S.A. (Barcelona, Spain). 4,6-Diamino-2-phenylindole (DAPI) was obtained from Serva (Heidelberg, Germany), and rhodamine 123 from Molecular Probes (Eugene, OR). The kinase inhibitors SB203580, SB220025, PD98059, U0126, and SP600125; the caspase-specific substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), and the pan-caspase inhibitor benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), were obtained from Calbiochem (Darmstadt, Germany). Rabbit polyclonal antibodies (pAbs) against human p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p44/42 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), SAPK/JNK, and phospho-SAPK/JNK (Thr183/Tyr185) were from Cell Signalling Technology (Beverly, MA). Rabbit anti-human Bax (N-20), rabbit anti-human pBcl2 (C-20) and goat anti-human Bid (C-20) pAbs, and mouse anti-human Bcl-2 (100) monoclonal antibody (mAb), were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-human Bcl-X_S pAb was from Transduction Laboratories (Lexington, KY). Mouse anti-pigeon cytochrome c mAb (clone 7HB.2C12) was from BD PharnMingen (San Diego, CA). All peroxidase-conjugated immunoglobulin antibodies were from DAKO Diagnostics (Barcelona, Spain). Plasmid encoding enhanced green fluorescence protein (pEGFP) plasmid was obtained from Clontech Laboratories (Palo Alto, CA). MKK6(K82A) and MKK6(Glu) were kindly provided by Prof. R.J. Davis (Massachusetts General Hospital, Boston, MA). Lipofectamine 2000 was obtained from Invitrogen (Invitrogen SA, Barcelona, Spain). OptiMEM was obtained from Gibco-BRL (Carlsbad, CA). All other reagents were from Sigma (Madrid, Spain).

Cells and treatments. Rat NRK-52E (de Larco and Todaro, 1978), porcine LLC-PK1 (Hull et al., 1976), canine MDCK (Gaus et al., 1966), and human HK-2 (Ryan et al., 1994) renal tubule cells were plated at a density of 5 × 10^3 cells/cm^2 and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamate and antibiotics in a humidified 5% CO2 atmosphere at 37°C. To ensure the acquisition of a nonproliferating state, in most experiments the cells were grown to confluence for 3 days in complete medium, then maintained in confluence for 3 days more with daily change of culture medium, washed with phosphate-buffered saline, and maintained for 16 h in FBS-free culture medium, prior to the initiation of the treatments. The treatments were also carried out in FBS-free medium. In other experiments, the treatments were applied at the time of exponential cell growth (approximately 50% confluence).

At the end of treatments, all cells in the plate (trypsin-detached and nonattached floating cells) were pooled together and collected by centrifugation.

Stock solutions of DAPI (10 μg/ml) and propidium iodide (PI, 1 mg/ml) were prepared in PBS, and stored at 4°C. A stock solution of cisplatin (3.3 mM) was prepared in distilled water, a stock solution of rhodamine 123 in PBS, and stock solutions of SB203580 (20 mM), SB220025 (13.2 mM), PD98059 (20 mM), U10126 (2.63 mM), SP600125 (20 mM), Ac-DEVD-pNA (5 mM), and z-VAD-fmk (25 mM) in dimethyl sulfoxide. These solutions were stored at −20°C. nN-Buthionine- RS-sulfoximine (BSO) was dissolved in distilled water at 50 mM just before application.

Transfection assay. Confusion/serum-deprived cells were transfected with pEGFP plasmid, which codifies the green fluorescent protein; or with a pcDNA3 expression vector containing either MKK6(K82A) cDNA, a kinase-negative mutant of MKK6 obtained by replacement of Lys-82 by Ala, or MKK6(Glu) cDNA, a constitutively activated mutant of MKK6 obtained by replacement of Ser 207 and Thr-211 with Glu (Rainegeaud et al., 1996). For experiments, cells grown in 24-well culture dishes were washed twice with PBS, then fed with 0.5 ml of OptiMEM, and kept at 37°C for 30 min. Plasmid samples (0.75 μg DNA) were mixed with 50 μl of OptiMEM. Aliquots of 2 μl of Lipofectamine 2000 were mixed with 50 μl of OptiMEM, incubated for 5 min at room temperature, then added to the plasmid suspension, and the mixture allowed standing at room temperature for 20 min to form DNA-lipofectamine complexes. The DNA-lipofectamine mixture was added to the cells, which were incubated for 6 h at 37°C. The cells were then washed once with PBS, and allowed to growth for 24 h in 0.5 ml of FBS-containing-DMEM medium and for 16–24 h more in FBS-free medium, after which the treatments were applied under the usual conditions. The efficacy of transfection was estimated in approximately 40–50%.

Determination of cell viability, cell cycle, apoptosis, and caspase activity. Cell viability was estimated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Alley et al., 1988). In short, untreated and drug-treated cells were collected and incubated with 1 mg/ml MTT in fresh culture medium for 4 h at 37°C. The resulting formazan crystals, were dissolved by adding a mixture of isopropanol and 1N HCl (12:1, vol/vol), and the dye absorbance (indicative of cell viability) was measured at 570 nm.

Distinctive characteristics of apoptotic cells were the presence of chromatin condensation/fragmentation and the acquisition of sub-G1 DNA content. To analyze changes in chromatin structure, the cells were centrifuged, washed with PBS, resuspended in PBS, and mounted on glass slides. After fixation in 70% (vol/vol) ethanol, the cells were stained for 20 min at room temperature with 1 μg/ml DAPI, and examined by fluorescence microscopy. To analyze cell cycle distribution and to measure sub-G1 DNA content, the cells were incubated for 30 min in PBS containing 0.5 mg/ml RNase A. After the addition of 50 μg/ml PI and permeabilization with 0.1% (wt/vol) Nonidet P-40, the cells were analyzed by flow cytometry using an EPICS XL flow cytometer (Coulter, Hialeah, FL). The fluorescence signal was collected with a 620-nm band pass filter, and the data analyzed using the CXP Analysis software. In some experiments, the relative rate of apoptosis was determined using the APOPercentage kit (Biocolor Ltd, Newtownabbey, Ireland), following the procedure indicated by the manufacturer. This procedure is based on the capacity of cells with translocated phosphorylserine to the outer layer of the plasma membrane (characteristic of apoptosis) to unidirectionally take up the dye, whereas viable and necrotic cells are unable to take or retain the dye, respectively. Dye uptake was measured by spectrometry (absorbance at 550 nm). As a routine, necrosis was also evaluated by measuring trypan blue or PI uptake by nonpermeabilized cells, as an indication of disruption of plasma membrane integrity.
To measure caspase-3 activity, cells were washed twice with ice-cold PBS, resuspended in ice-cold lysis buffer (1 mM dithiothreitol, 0.03% Nonidet P-40 [vol/vol], in 50mM Tris, pH 7.5), kept on ice for 30 min, and finally centrifuged at 14,000 × g for 15 min at 4°C. Samples containing aliquots of the supernatants (corresponding to 50 μg of total protein), 8 μl of Ac-DEVD-pNA, and PBS to complete 200 μl were prepared in triplicate in 96-well microtiter plates and incubated for 1 h at 37°C. The absorption was measured by spectrometry at 405 nm.

**Determination of intracellular GSH content and mitochondrial transmembrane potential.** Nonadherent plus trypsin-detached cells were collected and resuspended in PBS. Intracellular GSH content was measured by monochlorobimane derivatization, and mitochondrial transmembrane potential (∆Ψm) was determined by rhodamine 123–derived fluorescence, following the previously described procedures (Troyano et al., 2001, 2003).

**Immunoblot assays.** To obtain total cellular extracts, the cells were collected by centrifugation, washed with PBS, and lysed by sonication in a buffer containing a protease inhibitor cocktail, 10mM sodium fluoride, and 1mM sodium orthovanadate. To obtain cytosolic extracts, the cells were permeabilized for 5 min in 100 μl of ice-cold PBS containing 80mM KCl, 250mM sucrose, and 250 μg/ml digitonin, then centrifuged at 10,000 × g for 5 min at 4°C, and the supernatants and pellets separated and used as cytosolic and membrane fractions, respectively. For immunoblot, samples containing equal protein amounts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% polyacrylamide minigels and transferred to Immobilon transfer membranes (Millipore Corp., Medford, MA). After blocking with 3% nonfat milk in tween-tris-buffer-saline (TTBS) buffer (0.1% [vol/vol] Tween 20, 25mM Tris, 130mM NaCl, pH 7.5), the membranes were incubated overnight with the primary antibody, then extensively washed with TTBS, and incubated for 1 h with the secondary antibody. After extensive washing with TTBS, the immune complexes were detected by chemiluminescence using the Western blotting kit from Pierce.

**Determination of total intracellular and DNA-bound platinum.** For determination of total intracellular platinum content, cells were extensively washed with cold PBS, resuspended in 400 μl of PBS, and lysed by sonication. Aliquots of 50 μl were then diluted with distilled water to a final volume of 500 μl and digested by addition of nitric acid and hydrogen peroxide (5 μl each), after which samples of 100 μl were analyzed by total reflection X-ray fluorescence (TXRF) using a Seifer Extra-II spectrometer (Seifer, Ahrensburg, Germany), following a previously described procedure (Quiroga et al., 2006). For determination of DNA-bound platinum, cells were collected by centrifugation, washed with cold PBS, and resuspended in 400 μl of PBS. The DNA was extracted using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), as described by the manufacturer. The DNA content in each sample was measured by UV spectrophotometry at 260 nm using a NanoDrop, and the DNA-bound platinum was then measured by TXRF as indicated above.

**Statistical analysis.** All experiments were repeated at least in triplicate, and the data expressed as mean ± SD. The significance of differences between experimental conditions was examined using the Student’s t-test and when positive represented by asterisks (*p < 0.01; **p < 0.05).

**RESULTS**

**Cell Death**

Firstly we determined the capacity of cisplatin to cause apoptosis in confluent/serum-deprived NRK-52E cells. As demonstrated in Figure 1A, prolonged confluence followed by serum deprivation (see “Materials and Methods” section) resulted in cell accumulation at the G0/G1 phase of the growth cycle, with concomitant decrease in the frequency of cells at S and G2/M phases (left panel), characteristic of a nonproliferating cell population. This contrasts with the heterogeneous distribution (G1–S–G2) in exponentially growing cell cultures (right panel). Treatment of confluent/serum-deprived cell cultures with 50–300μM cisplatin caused a moderate dose-dependent decrease in cell viability, as measured by the MTT reduction assay, and correspondingly increased the frequency of cells with condensed/fragmented chromatin, characteristic of apoptosis (Fig. 1B). At the concentration of 200μM cisplatin, which was selected for further experiments, apoptosis was already detected at 16 h of treatment (Fig. 1C). Additional determinations indicated that cisplatin increased the frequency of cells with reduced (sub-G1) DNA content, as indicated by PI staining and flow cytometry assays (see Fig. 5B); elicited caspase-3 activation, as determined by the increase in DEVDase activity in *in vitro* assays (Fig. 1D), as well as by PKCδ cleavage to generate the apoptosis-associated 41-kDa fragment (Fig. 1E); and cell death was greatly reduced by the pan-caspase inhibitor z-VAD-fmk (Fig. 1C). Staining with trypan blue on the plate surface did not reveal significant increase in dye uptake, indicative of necrosis-derived plasma membrane damage, in cultures treated with 200μM cisplatin, although necrosis was progressively detected at higher concentrations (result not shown). Taken together, these observations indicate that the measured cell death is in fact caspase-dependent apoptosis.

**MAPK Activation and Effect of MAPK Inhibitors**

As indicated above, MAP kinases are essential factors in the signaling of apoptosis (Wada and Penninger, 2004). For this reason, experiments were carried out to determine the capacity of cisplatin to modulate ERK1/2, JNK, and p38-MAPK activation in confluent, serum-deprived NRK-52E cells, as evidenced by changes in their phosphorylation state. The results in Figure 2 indicate that cisplatin decreased p38-MAPK phosphorylation, decreased ERK2 (p42) phosphorylation without apparent effect on ERK1 (p44), and did not affect the phosphorylation state of JNK. The decrease in p38 and ERK2 phosphorylation was already detected at 3–6 h of treatment, thus preceding the timing of apoptosis execution. Cotreatment with z-VAD-fmk did not affect the cisplatin-provoked decrease in p38-MAPK phosphorylation (result not shown). This excludes a possible regulation of p38-MAPK by caspase activities, as was earlier described using other experimental models (Grethe et al., 2004).

The observed downregulation of p38-MAPK and ERK2 might indicate that these kinases play a protective, anti-apoptotic action in cisplatin-treated growth-arrested NRK-52E cells. If this is the case, we could expect that potentiating kinase de-phosphorylation will increase cisplatin toxicity. To analyze this possibility, experiments were carried out using kinase pharmacologic inhibitors, namely the p38-MAPK inhibitors SB203580 (15μM) and SB220025 (2μM), the MEK/ERK inhibitors PD98059 (40μM) and
U0126 (10μM), and the JNK inhibitor SP600125 (20μM). These concentrations were adopted from our previous studies, which indicated that they effectively prevented drug-induced kinase activation (Fernandez et al., 2004; Ramos et al., 2006). It was observed that SB203580 and SB229025, which were innocuous when used alone, potentiated apoptosis induction by cisplatin, as demonstrated by chromatin condensation/fragmentation (Fig. 3A), and increased caspase-3 activation (Fig. 3B). On the other hand, no potentiation was observed using the MEK/ERK and JNK inhibitors (Figs. 3A,B, and results not-shown). Control determinations indicated that the p38-MAPK inhibitors slightly accelerated the decrease in kinase phosphorylation caused by cisplatin (see Fig. 3C).
The importance of p38-MAPK as a regulator of cisplatin-provoked apoptosis in NRK-52E cells was further corroborated by transient transfection assays using the kinase-negative MKK6(K82A) and constitutively activated MKK6(Glu). In spite of the low transfection efficacy in confluent/serum-deprived cells (40–50%), a significant increase or decrease in cisplatin-provoked apoptosis could be detected in MKK6(K82A)- or MKK6(Glu)-transfected cells, respectively, in relation to cells transfected with an irrelevant control plasmid (pEGFP) (Fig. 3C). Control determinations indicated that MKK6(K82A) and MKK6(Glu) slightly augmented or reduced, respectively, the cisplatin-provoked decrease in p38-MAPK phosphorylation, when compared with pEGFP-transfected cells (Fig. 3C).

The possible role of p38-MAPK as a regulator of cisplatin-provoked apoptosis was examined in epithelial tubule kidney cell lines other than NRK-52E, namely LLC-PK1 (porcine), HK-2 (human), and MDCK (canine). The concentrations of 100–300 μM cisplatin for LLC-PK1 cells, and 50–100 μM for HK-2 and MDCK cells, were selected from preliminary dose-response studies (results not shown). The results in Figure 4 indicate that treatment of confluent/serum-deprived cell cultures with cisplatin downregulated p38-MAPK phosphorylation and caused apoptosis in the three assayed cell lines, and apoptosis was exacerbated by cotreatment with the p38-MAPK inhibitor SB203580, as in the case of NRK-52E cells.

**FIG. 2.** MAPK phosphorylation/activation in growth-arrested NRK-52E cells. Cell extracts obtained from confluent/serum-deprived untreated (Cont) cells and cells treated for the indicated time periods with 200 μM cisplatin were assayed by immunoblot using antibodies that recognize total (tot) and phosphorylated (P) p38, ERK1/2, and JNK.

**FIG. 3.** Effect of MAPK inhibition on cisplatin-provoked apoptosis in growth-arrested NRK-52E cells. (A) Frequency of apoptotic cells, as determined by chromatin fragmentation, in confluent/serum-deprived cultures treated for 24 h with the p38 inhibitors SB203580 (SB1, 15 μM) and SB220025 (SB2, 2 μM), the MEK/ERK inhibitors PD98059 (PD, 40 μM) and U0126 (U, 10 μM) and the JNK inhibitor SP600125 (SP, 20 μM), as well as with 200 μM cisplatin, in the absence (-) or the presence of the kinase inhibitors. (B) Caspase-3 activity in untreated cells and cells subjected to the same treatments as in (A). (C) Apoptosis induction in cell cultures transfected with kinase-negative MKK6(K82A) [K82A], constitutively activated MKK6(Glu) [Glu], or an irrelevant plasmid (pEGFP) [GFP], either untreated or treated for 24 h with the indicated concentrations of cisplatin; and in nontransfected cells treated for 24 h with the indicated concentrations of cisplatin, alone (-) and with SB203580. Cells were processed using the APOPercentage kit system, and dye intensity, indicative of apoptosis, was determined by spectrometry (see "Materials and Methods") and expressed in relation to untreated cells, which received the arbitrary value of one. The lower blot represents expression of total and phosphorylated p38-MAPK at 40-h post-transfection with GFP, MKK6(K82A) or MKK6(Glu), upon treatment with 200 μM cisplatin, and in nontransfected cells untreated (-) and treated (+) for 24 h with 200 μM cisplatin, alone (-) and in the presence (+) of SB203580. The results in panels (A), (B), and (C) represent the mean ± SD of four (A, B) and five (C) determinations. The asterisks indicate significant differences between cells treated with cisplatin in the absence and the presence of the kinase inhibitors (A, B, and C), or between cells transfected with GFP and MKK6(K82A) or MKK6(Glu) plasmid. (C) All other conditions were as in Figures 1 and 2.
Our present results, showing that cisplatin decreases p38-MAPK phosphorylation, seem to contrast with earlier publications describing activation of p38-MAPK in a serum-fed, proliferating murine tubular cell line (Arany et al., 2004; Ramesh and Reeves, 2005, 2006). To determine whether the differences observed in p38 activation could be related to the culture conditions, additional experiments were carried out in which p38-MAPK phosphorylation and the effect of p38-MAPK inhibitor were comparatively analyzed in exponentially growing and confluent/serum-starved NRK-52E cells. In accordance with previously published reports, cisplatin stimulated p38-MAPK phosphorylation in growing cells (Fig. 5A, left blot). Under these cultures condition, SB203580 prevented p38-MAPK phosphorylation and decreased cisplatin-provoked apoptosis (Fig. 5B, upper panel). Of note, growing cells are more sensitive than growth-arrested cells to cisplatin toxicity, as revealed by the higher number of apoptotic cells (Fig. 5B).

**Apoptosis Executioner Pathways**

One of the effects of cisplatin is the capacity to disturb mitochondrial metabolism (Brady et al., 1990). In addition, it is accepted that most chemotherapeutic drugs induce apoptosis throughout activation of the “intrinsic” (mitochondrial) executioner pathway (Ortiz et al., 2003). For these reasons we examined the disruption of transmembrane potential (ΔΨm), as well as the modulation of factors critical for the regulation of the intrinsic pathway. This included the release of cytochrome c from mitochondria to the cytosol, required for apoptosome assembly; and the expression of the Bcl-2 family member proteins Bcl-2 and Bcl-XL (anti-apoptotic), and Bax and Bid (pro-apoptotic). The results are indicated in Figure 6. It was observed that treatment of confluent/serum-deprived cells with cisplatin alone caused ΔΨm dissipation in a subpopulation of cells, as determined by the decrease in rhodamine 123-derived fluorescence, and this effect was exacerbated by SB203580 (Fig. 6A). In addition, treatment with cisplatin alone caused cytochrome c release from mitochondria, as measured by immunoblot using cytosolic extracts, decreased total Bcl-XL expression, and increased total Bax expression, and these effects were potentiated by cotreatment with SB203580 (Figs. 6B, C). Cotreatment with cisplatin plus the p38-MAPK inhibitor slightly caused Bid cleavage/activation, having as a criterion the decreased amount of the 21-kDa proform (Fig. 6C). The expression of Bcl-2 remained below detection levels (result not shown). As a rule, the changes in mitochondria-related regulatory events were already detected at hour 6, coincident with the downregulation of p38-MAPK phosphorylation (Fig. 6D), and preceding apoptosis execution and activation of executioner caspase-3 (Fig. 6E).

**Intracellular GSH Content, Platinum Accumulation, and DNA Platination**

It is known that cisplatin cytotoxicity depends on intracellular GSH content (Troyano et al., 2001; Zhang et al., 2001) and references therein). In addition, we recently demonstrated that MAPK inhibitors may cause intracellular GSH depletion (Ramos et al., 2006). For these reasons, we measured intracellular GSH in confluent/serum-deprived NRK-52E cells treated with cisplatin, alone and in the presence of p38-MAPK inhibitors. The results in Figure 7A indicate that the GSH content was not affected by treatment with SB203580 alone, was slightly decreased by cisplatin alone, and was decreased to a higher extent by cotreatment with cisplatin plus SB203580. Of note, the decrease was already observed at 6 h
of treatment, preceding the timing of apoptosis execution. To corroborate the importance of GSH decrease for apoptosis potentiation, control assays were carried out using BSO, a selective inhibitor of \( \gamma \)-glutamylcysteine synthetase, the rate-limiting enzyme for GSH biosynthesis. It was observed that BSO decreased the intracellular GSH content (Fig. 7B, left panel), and accordingly potentiated apoptosis generation by cisplatin (Fig. 7B, right panel). Taken together, these results suggest that p38-MAPK inhibitors potentiate cisplatin-provoked apoptosis by downregulating intracellular GSH. Of note, the opposite assay, namely preventing GSH depletion by addition of exogenous GSH or the GSH-increasing agent N-acetyl-L-cysteine, could not be carried out because these agents may directly interact with cisplatin in solution. In spite of GSH depletion, we did not detect significant modifications in intracellular peroxide accumulation, as measured using the fluorescent probe dichlorodihydrofluorescein diacetate, and apoptosis induction was not affected by the hydrogen peroxide scavenger catalase and the antioxidant butylated hydroxyanisole (results not shown).

GSH is a critical factor in the cisplatin detoxification machinery (Zhang et al., 2001). In addition, it has been reported that protein kinase inhibitors may affect cisplatin uptake by the cells (Amran et al., 2005; Basu and Evans, 1994). Moreover, it is generally accepted that the toxicity of cisplatin is primarily the consequence of its capacity to bind DNA (Fuertes et al., 2003; Jamieson and Lippard, 1999). For these reasons, experiments were carried out to measure intracellular platinum content, as an indicator of drug accumulation, as well as the amount of platinated DNA, as an indication of cisplatin-DNA binding. It was observed that cotreatment of confluent/serum-deprived NRK-52E cells with SB203580 increased the intracellular platinum accumulation, an effect which was already detected at hour 6 (Fig. 8A). In addition, the p38-MAPK inhibitor caused an even higher increase in the amount of DNA-bound platinum (Fig. 8B). These results suggest that p38-MAPK inhibition potentiates cisplatin-caused toxicity in renal tubule cells by increasing intracellular drug accumulation that, among other effects, leads to increased cisplatin-DNA binding.

FIG. 5. Differential response of exponentially growing and growth-arrested NRK-52E cells. Exponentially growing and confluent/serum-starved cells were left untreated (Cont) or treated for 24 h with 200\( \mu \)M cisplatin, in the absence or the presence of SB203580. (A) Changes in p38-MAPK phosphorylation. (B) Cell cycle distribution. The numbers inside the flow cytometry profiles indicate the frequency (%) of apoptotic cells (Ap) and cells at S plus G2, in relation to the total cell population. All other conditions were as in Figures 1–3.

DISCUSSION

The present work indicates that treatment with cisplatin induces death by apoptosis in NRK-52E and other kidney epithelial cells, which is in full agreement with earlier reports from our laboratory and others (Amran et al., 2005; Kaushal et al., 2001; Lee et al., 2001; Lieberthal et al., 1996; Park et al., 2002). Although a detailed study of apoptosis regulatory mechanisms was beyond the scope of this work, we observed that cisplatin caused mitochondrial dysfunction, as indicated by \( \Delta \Psi \mathrm{m} \) dissipation, and activated the mitochondrial executioner pathway, as indicated by the decrease in Bcl-XL expression, increase in Bax expression and release of cytochrome c to the cytosol. Of note, confluent/serum-deprived NRK-52E cells are less sensitive to cisplatin toxicity than proliferating cells, as measured by the frequency of apoptosis at equimolar concentrations (Amran et al., 2005; and results in Fig. 5). There are multiple examples indicating inverse relationship between growth activity and toxicity in cells treated with cisplatin or other chemotherapeutic drugs. For instance, human carcinomas are often refractory to chemotherapy, and one of the of the possible reasons is the presence of a high proportion of growth-arrested ("Q") cells in the tumor core, due to poor oxygen and nutrient supply (Jackson, 1989). Also, quiescent (G0/G1-arrested) thymocytes, which represent the major cell population in the immature rat thymus, are much less sensitive to cisplatin-provoked
apoptosis than the minor subpopulation of proliferating (G1-S-G2/M) cells (Evans et al., 1994). Although the growth arrest experimentally obtained by contact inhibition and/or growth factor deprivation may not be properly considered as genuine quiescent state, nonetheless confluent/serum-deprived NRK-52E cells also underwent G1 accumulation, with almost total disappearance of the S and G2/M subpopulations (see Figs. 1A and 5B). This altered cell cycle distribution may adequately explain the relative resistance to cisplatin-provoked apoptosis. In fact, as indicated above it is normally accepted that the main determinant of cisplatin toxicity is the formation of DNA adducts, and it is also known that nonrepaired DNA cross-links are mainly lethal in cells traversing S phase (i.e., undergoing DNA replication) and G2/M (Eastman, 1990; Evans et al., 1994; and references therein). Nonetheless, because cisplatin also causes other deleterious effects (e.g., mitochondrial dysfunction, cytoskeleton disruption), a limited occurrence of apoptosis in confluent/serum-deprived cells is still possible.

The role of MAPKs as regulators of tumor cell resistance to cisplatin-provoked apoptosis has been a subject of extensive investigation. The current information indicates that cisplatin activates p38-MAPK, and this activation normally exerts a pro-death role in tumor cells (reviewed by Brozovic and Osmak, 2007). Less information is available on the role of MAPK in cisplatin-induced nephrotoxicity. A p38-MAPK inhibitor attenuated cisplatin-provoked nephrotoxicity in mice (Ramesh and Reeves, 2005). Moreover, other groups reported that cisplatin caused p38-MAPK activation in serum-fed TKPTS murine tubular cells (Arany et al., 2004; Ramesh and Reeves, 2006) which, as judged by the presence of a G2 peak in flow cytometry diagrams (Arany et al., 2004), were proliferating cells. Thus, our findings showing that treatment of growth-arrested cells with cisplatin decreases p38-MAPK phosphorylation, and that enforced kinase downregulation potentiates apoptosis execution and activation of mitochondrial-regulatory events, represents the first known evidence of an inverse relationship between p38-MAPK activation and apoptosis related to cell cycle distribution and apoptosis induction.
induction in cisplatin-treated renal tubule cells. Of note this observation (obtained in four cell lines derived from different animal species) must be specifically linked to the restrictive (confluence/serum deprivation) growth conditions, because cisplatin treatment of nonconfluent serum-fed NRK-52E cells did not decrease but stimulated p38-MAPK activation, as reported by other authors. The factors that decide the growth-dependent differential kinase response remain to be determined. In vivo, multiple cell types and systemic mediators contribute to nephrotoxicity. For instance, both TNFα and toll-like receptor 4 (TLR4) contribute to leukocyte recruitment and renal dysfunction (Zhang et al., 2007, 2008). TLR4 was hypothesized to activate p38-MAPK, which in turn promotes TNFα synthesis, and more than half renal p38 activation during cisplatin-provoked nephrotoxicity in vivo was TLR4-dependent (Zhang et al., 2008). Although there are several potential endogenous ligands for TLR4, the ligand(s) that contribute to cisplatin nephrotoxicity have not been characterized (Zhang et al., 2008). Hence, a possible explanation for the disparate growth-dependent p38-MAPK response observed in our experiments is that noncharacterized ligands present in serum or synthesized in response of serum may facilitate TLR4, leading to kinase activation.

It was recently reported that the p38-MAPK inhibitor SB203580 caused nonsignificant increase in cisplatin uptake (approximately 15%) in serum-fed proliferating murine TKPTS tubular cells (Ramesh and Reeves, 2005). Our present results indicate that cotreatment with SB203580 significantly increased intracellular cisplatin accumulation and DNA binding in confluent/serum-deprived NRK-52E cells. Thus, increased cisplatin bioavailability might explain at least in part the exacerbation of drug-provoked apoptosis by the kinase inhibitor. Although on the ground of these results we may reasonably conclude that p38-MAPK somewhat regulates the overall cisplatin transport inside/outside the cell, the identification of the exact target(s) of regulation is a difficult task, due to the great complexity and still incomplete knowledge of the cisplatin transport system(s). A possible explanation is that cisplatin overaccumulation is the consequence of defective GSH-dependent drug detoxification, a process that involves the formation of GSH/cisplatin conjugates and their extrusion from the cell by the multidrug-resistance associated protein. In this regard, the level of intracellular GSH was reported to be the

![FIG. 7. Intracellular GSH content. (A) Intracellular GSH level, as determined by monochlorobimane derivation, in confluent/serum-deprived NRK-52E cells treated for the indicated time periods with 200 μM cisplatin alone, SB203580 alone, and the combination of both agents. The results are expressed in relation to untreated cells (approximate GSH content, 22 nmol/10⁶ cells), which received the arbitrary value of one. (B) Intracellular GSH content at hour 16 (left panel), and frequency of apoptotic cells at hour 24 (right panel), in cell cultures treated with 1 mM BSO, alone and in combination with cisplatin. The asterisks indicate significant differences in relation to untreated cells, or between the indicated pair of treatments. All other conditions were as in Figures 1 and 3.](image1)

![FIG. 8. Platinum uptake and DNA platination. (A) Intracellular platinum content, and (B) amount of DNA-bound platinum, in confluent/serum-deprived NRK-52E cells treated for the indicated time periods with 200 μM cisplatin, in the absence and the presence of SB203580. The asterisks indicate significant differences between the indicated pair of treatments. All other conditions were as in Figures 1 and 3.](image2)
rate-limiting step in the glutathione-based detoxification machinery in cisplatin-treated HepG2 human hepatocarcinoma cells (Zhang et al., 2001). In support of this possibility, in our experiments cisplatin plus SB203580 caused a decrease in intracellular GSH content, and GSH in turn regulated cisplatin toxicity, as demonstrated by the increase in cisplatin-provoked apoptosis in BSO-cotreated cells. Nonetheless, it has been demonstrated that xenobiotic-induced stress activate MAPK pathways, which in turn cause ARE/EpRE (antioxidant/electrophile response element)-mediated transcription of Phase II drug metabolizing genes (Kong et al., 2001). Thus, an alternative or complementary explanation is that p38-MAPK downregulation leads to a decrease in the levels of metabolizing enzymes required for cisplatin conjugation. Moreover, we may not exclude that protein kinases might also directly regulate the cisplatin transport by mechanisms other than the GSH-detoxification machinery. As an example, phorbol ester-provoked PKC activation was reported to potentiate cisplatin accumulation in HeLa cells by stimulating the rate of drug uptake (Basu and Evans, 1994). In addition, the fact that in our experiments the increase in DNA platination was higher than the increase in intracellular platinum content suggest that p38-MAPK may directly affect the formation or removal of cisplatin-DNA adducts, independently of intracellular drug availability.

In summary, the present results indicate that cisplatin induces apoptosis in confluent/serum-deprived kidney tubular cells with lower efficacy than in exponentially growing cells, a result which may be explained by the altered cell cycle phase distribution. Under these restrictive growth conditions cisplatin down-regulates p38-MAPK phosphorylation/activation, and enforced p38-MAPK inhibition exacerbates apoptosis induction. Depletion of intracellular glutathione and possibly decrease in glutathione-dependent detoxifying enzymes, and increased intracellular cisplatin accumulation and DNA binding, might explain this unusual regulatory action of p38-MAPK. This information may be relevant to better understand the relative role of cisplatin itself or putative additional serum-derived factors in the clinical problem of cisplatin nephrotoxicity.

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