Restoration of CREB function ameliorates cisplatin cytotoxicity in renal tubular cells

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Aran I, Herbert J, Herbert Z, Safirstein RL. Restoration of CREB function ameliorates cisplatin cytotoxicity in renal tubular cells. Am J Physiol Renal Physiol 294: F577–F581, 2008. First published December 19, 2007; doi:10.1152/ajprenal.00487.2007.—We have shown that mouse proximal tubule cells (TKPTS) survive H2O2 stress by activating the CAMP-responsive element binding protein (CREB)-mediated transcription via the canonical EGFR-Ras/ERK pathway. By contrast, cisplatin activates EGFR/Ras/ERK signaling in TKPTS cells yet promotes cell death rather than survival. We now demonstrate that the cisplatin-induced activated EGFR/Ras/ERK signaling cascade fails to activate CREB-mediated transcription even in the presence of phosphorylated CREB. CREB-mediated transcription as well as survival was restored by the histone deacetylase (HDAC) inhibitor trichostatin A (TSA), an effective chemotherapeutic agent. Similar to severe oxidant stress, TSA-mediated survival could be abrogated by inhibition of CREB-mediated transcription. These studies confirm the importance of CREB-mediated transcription in the survival of renal cells subjected to either oxidant- or cisplatin-induced stress. The use of cisplatin and TSA in combined chemotherapy protocols may be an effective strategy to enhance cancer cell death and limit nephrotoxicity.

The CAMP-RESPONSIVE ELEMENT-binding protein (CREB) is important in the survival of a variety of cell types under different stress conditions (5, 8, 13, 15). Earlier, we found that during oxidant injury activation of the extracellular signal-regulated kinase (ERK) and consequent induction of CREB-mediated transcription is essential for survival of mouse proximal tubule cells (3, 4). CREB activation is mediated, at least in part, by the activated ERK (5, 20). By contrast, ERK contributes to the death of proximal tubule cells in cisplatin-induced nephrotoxicity (2). We interrogated possible sites of divergence between cisplatin- and oxidant-induced ERK activation and found that unlike oxidant stress, cisplatin-induced ERK-dependent phosphorylation of CREB was not followed by its transcriptional activation. Our studies now show that histone deacetylase (HDAC) inhibitors, which are known to reverse transcriptional repression and gene silencing induced by HDACs (18), is capable of restoring CREB transcriptional activation (6, 16). Importantly this report further establishes the role of CREB transcription in the survival of renal cells under stress.

MATERIALS AND METHODS

Cells and treatment. The immortalized mouse proximal tubule cell line (TKPTS) was a gift from Dr. E. Bello-Reuss (9) and was maintained as described previously (3, 7). Subconfluent cultures were treated with 25 μM cisplatin for the time points described in RESULTS.

Analysis of cell viability. Viable cell count was determined by trypan blue (Sigma, St. Louis, MO) exclusion in a hemocytometer. Cell morphology was determined by light microscopy using a Nikon Eclipse TE200 microscope equipped with Hoffman Modulation Contrast Optics. In addition, cell cycle analysis was performed by propidium iodide (PI) staining. TKPTS cells were collected after trypsinization and fixed in 70% ethanol overnight. After RNAse treatment, cells were incubated with 5 μg/ml PI and analyzed with a Becton-Dickinson FACSCalibur analyzer. The cell cycle profile was analyzed using CellQuest software. Cells residing in the subG1/G0 phase were considered to be apoptotic.

Adenoviral infection of TKPTS cells. The M1-CREB mutant adenovirus (19) was a gift from Dr. A. J. Zeleznik. The adenovirus (adGFP) adenovirus was provided by Dr. H. Kaneto (10). TKPTS cells grown in six-well-plates were infected with 25 multiplicity of infection (MOI) adenovirus at 37°C overnight and then treated with different agents, as described in RESULTS. For molecular analysis, total cell lysates from these cells were prepared as described below. The efficiency of infection was determined by a control adenovirus (adGFP) as described earlier (10). The efficiency of infection was ~80% (data not shown).

Protein isolation and Western blotting. Monolayers of TKPTS cells were lysed in RIPA buffer that contained 100 μg/ml phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), 100 mM sodium orthovanadate (Sigma), and 50 μl/ml of protease inhibitor cocktail (Sigma) as described earlier (3). Protein content was determined by using a Bio-Rad Protein Determination assay. Viable cell count was determined by trypan blue (Sigma, St. Louis, MO) exclusion in a hemocytometer. Cell morphology was determined by light microscopy using a Nikon Eclipse TE200 microscope equipped with Hoffman Modulation Optics. In addition, cell cycle analysis was performed by propidium iodide (PI) staining. TKPTS cells were collected after trypsinization and fixed in 70% ethanol overnight. After RNAse treatment, cells were incubated with 5 μg/ml PI and analyzed with a Becton-Dickinson FACSCalibur analyzer. The cell cycle profile was analyzed using CellQuest software. Cells residing in the subG1/G0 phase were considered to be apoptotic.


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Luciferase activity was determined by using a Luciferase Assay kit as suggested by the manufacturer (Promega, Madison, WI) under control conditions as well as 1, 6, and 12 h after incubation with cisplatin. This last time point was chosen because cells show no obvious damage at this time. The relative luciferase activity was measured and was normalized to the amount of activity detected for a cotransfected H9252-galactosidase plasmid.

Statistical analysis. Continuous variables are expressed as means ± SD. Means of multiple treatment groups were compared with controls by using of ANOVA with a post hoc test. A P value of <0.05 was considered statistically significant. All analyses were performed using a SigmaStat 3.5 software package.

RESULTS

Cisplatin increases serine 133 phosphorylation of CREB but not CREB-mediated transcription. CREB is a downstream target of ERK (5, 20), and we first determined whether serine 133-phosphorylated CREB (pCREB) occurred after cisplatin treatment. Western blots of cisplatin-treated TKPTS cells show prominent CREB phosphorylation at the indicated time points (Fig. 1, A and B). We next examined whether this increased phosphorylation would be accompanied by an elevated CREB-mediated transcription. Surprisingly, pCRE-Luc activity not only failed to increase but actually decreased 6 or 12 h after treatment with cisplatin (Fig. 1C). The most prominent changes were observed at 12 h posttreatment; thus we used this time point in further experiments. There is no indication of cell death 12 h after treatment (data not shown).

These results suggest that serine 133 phosphorylation of CREB itself is not sufficient for induction of CREB transcription and a consequent survival.

Fig. 1. Effects of cisplatin treatment on the cAMP-responsive element binding protein (CREB) phosphorylation and pCRE-Luc activity. A: TKPTS cells were treated with 25 μM cisplatin for the time points indicated. Serine 133-phosphorylated CREB together with the total CREB were determined by Western blotting. B: densitometric analysis of blots shown in A. Values are expressed as ratios of phospho-CREB and total CREB (means ± SD, n = 3). *P < 0.05, multiple comparison vs. control group by ANOVA with post hoc test. C: TKPTS cells were transiently transfected with a pCRE-Luc and a β-galactosidase plasmid for 24 h. Cells then were treated with 25 μM cisplatin for the time points indicated, and luciferase activity was determined together with the β-galactosidase. Luciferase values are normalized to β-galactosidase and expressed as percentage of control value (means ± SD; n = 3). *P < 0.05, multiple comparison vs. control group by ANOVA with post hoc test.

Fig. 2. Effects of trichostatine A (TSA) on cisplatin-induced CREB phosphorylation and CREB-mediated transcription. A: TKPTS cells were pretreated with 50 nM TSA 4 h before treatment with 25 μM cisplatin for 4 h. Cell lysates were prepared, and serine 133-phosphorylated CREB together with the total CREB were determined by Western blotting. B: densitometric analysis of blots shown in A. Values are expressed as ratios of phospho-CREB and total CREB (means ± SD, n = 3). *P < 0.05, multiple comparison vs. control group by ANOVA with post hoc test. C: TKPTS cells were transiently transfected with a pCRE-Luc and a β-galactosidase plasmid for 24 h. Cells then were treated with 25 μM cisplatin for 6 and 12 h, and luciferase activity was determined together with β-galactosidase. Luciferase values are normalized to β-galactosidase and expressed as a percentage of control value (means ± SD, n = 3). *P < 0.05, multiple comparison vs. control group by ANOVA with post hoc test.
TSA increases CREB phosphorylation, restores CREB-mediated transcription, and ameliorates cell death during cisplatin-induced cytotoxicity. Since TSA has been shown to increase CREB activity (6), we next determined whether TSA affects CREB phosphorylation and its transcriptional activity. Accordingly, TKPTS cells were pretreated with 50 nM TSA for 4 h and then treated with cisplatin for 4 h. CREB phosphorylation was determined by Western blotting. TSA alone increased CREB phosphorylation, which was even further increased in the presence of cisplatin, probably in an additive fashion (Fig. 2, A and B). Importantly, in the pCRE-Luc assay both TSA and the combination of TSA plus cisplatin significantly increased luciferase activity at the indicated time points (Fig. 2C). These results suggest that TSA treatment restores CREB-mediated transcription.

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**Fig. 3.** Effects of TSA on survival of TKPTS cells during cisplatin (CP) nephrotoxicity. TKPTS cells were pretreated with 50 nM TSA 4 h before treatment with 25 μM cisplatin for 24 h. Cells were trypsinized, and Trypan blue-negative (survival) cells were counted. Results are given as percentage of control (untreated or TSA-treated, respectively; means ± SD, n = 3). *P < 0.05, multiple comparison vs. control group by ANOVA with post hoc test.

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**Fig. 4.** Effects of TSA treatment on cell cycle progression and caspase-3 activity. TKPTS cells were pretreated with 50 nM TSA 4 h before treatment with 25 μM cisplatin for 24 h. A: cell cycle progression was determined by FACS analysis. The number of dead (subG1/G0) cells are given as a percentage of total cell number. B: cell lysates were prepared after 24-h treatment and subjected to SDS-PAGE. The amount of activated (cleaved) caspase-3 was determined by Western blotting. Densitometric data are shown. *P < 0.05, multiple comparison vs. control group by ANOVA with post hoc test.
Next, we determined whether TSA pretreatment affects cisplatin-induced cell death. TKPTS cells were pretreated with 50 nM TSA 4 h before treatment with 25 μM cisplatin for 24 h, and cell counts were determined. As seen in Fig. 3, TSA pretreatment significantly increased cell survival after treatment with cisplatin (from 41 to 79%). TSA alone inhibited cell growth (~30% compared with the untreated cells); thus, for a better comparison, we used untreated cells as controls for cisplatin treatment and TSA-treated cells for TSA plus cisplatin. Surprisingly, TSA treatment did not affect cell viability or cell cycle progression (Fig. 4A). FACS analysis also confirmed the effect of TSA on cisplatin-induced cell death: TSA pretreatment significantly decreased the number of dead (subG0/G1) cells induced by cisplatin from 56.3 to 29.5% without affecting cell cycle distribution (Fig. 4A). Also, TSA pretreatment significantly inhibited cisplatin-mediated caspase-3 activation (Fig. 4B), further demonstrating the prosurvival role of TSA.

Inhibition of CREB attenuates TSA-induced CREB-transcription and survival during cisplatin-induced nephrotoxicity. To demonstrate the role of TSA-induced and CREB-mediated transcriptional events in survival during cisplatin-induced cytotoxicity, TKPTS cells were infected with 25 MOI of a mutant (M1-CREB) adenovirus (19) for 18 h. The M1-CREB mutant competes with endogenous CREB for binding to CREB-responsive elements. As a control, TKPTS cells were infected with the adGFP adenovirus. The M1-CREB mutant attenuated TSA- and TSA+CP-induced pCRE-Luc activity while adGFP did not (Fig. 5).

Also, M1-CREB but not adGFP attenuated TSA-induced survival after treatment with cisplatin (Fig. 6). M1-CREB and adGFP alone did not affect cell survival significantly, as described elsewhere (3, 4). These data demonstrate a direct link between CREB transcriptional activation and survival similar to that observed after oxidant injury (4).

**DISCUSSION**

Activated CREB is crucial for survival of neural, muscle, or other cell types during a variety of stress conditions including hypoxia and DNA damage (5, 8, 13, 15). We showed that TKPTS cells survive oxidant stress by activating CREB-mediated transcription via the canonical EGFR-Ras/ERK pathway (4). Here, we found that even though cisplatin increases phosphorylation of CREB (Fig. 1, A and B) it does not activate CREB-mediated transcription (Fig. 1C). In this regard, cisplatin would seem to be similar to LPS-induced stress in hepatocytes, where LPS treatment increases CREB phosphorylation.
but CREB-mediated reporter activity actually falls (21). In those studies, it is thought that the inhibition is served by cytokine activation of c-Jun. A similar situation may exist in cisplatin nephrotoxicity, as c-Jun is prominently expressed during cisplatin exposure (17).

Aware of the effect of cisplatin on reducing histone acetylation in cancer cells (14) and the effect of HDAC inhibitors such as TSA to enhance CREB-driven transcription (6, 16), we sought to determine the effect of TSA on CREB-mediated transcription and whether such a reversal would enhance survival in cisplatin-treated cells.

Pretreatment of TKPTS cells with TSA restored pCRE-luc activity and increased the survival of cells exposed to cisplatin (Figs. 2–4). Inhibition of CREB function via a mutant CREB adenovirus (19) significantly diminished both TSA-induced CREB-mediated transcription (Fig. 5) and survival (Fig. 6) during cisplatin nephrotoxicity. These experiments proved that TSA-induced survival is mediated through CREB-mediated transcriptional events. Presumably, the restoration of CREB transcription is mediated either by a gain of an activating cofactor or by the loss of a transcriptional regulator at target CRE sites (14). The inhibition of caspase-3 activation by restoring CREB transcription places this pathway upstream of caspase-3 activation (Fig. 4B) in the proapoptotic pathway initiated by cisplatin.

Another important implication of our findings is the fact that the HDAC inhibitors increase antitumor effects of DNA-damaging agents such as cisplatin (12). As we now show that TSA abrogates cisplatin-induced cytotoxicity in renal cells (Figs. 4–6), this difference between renal cells and cancer cells may be exploited clinically to enhance the therapeutic index of cisplatin when both drugs are combined.

In summary, the HDAC inhibitor TSA increased CREB-dependent transcription and consequent survival of renal tubular cells probably by reversing CREB-mediated gene repression during cisplatin-induced cytotoxicity in renal tubular cells. Identifying the mechanism by which TSA activates CREB-mediated transcription may provide additional potential targets to ameliorate the nephrotoxicity of cisplatin and other cellular stressors that damage kidney cells.

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