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Protein Kinase Cδ Suppresses Autophagy to Induce Kidney Cell Apoptosis in Cisplatin Nephrotoxicity

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Abstract

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Nephrotoxicity is a major adverse effect in cisplatin chemotherapy, and renoprotective approaches are unavailable. Recent work unveiled a critical role of protein kinase Cδ (PKCδ) in cisplatin nephrotoxicity and further demonstrated that inhibition of PKCδ not only protects kidneys but enhances the chemotherapeutic effect of cisplatin in tumors; however, the underlying mechanisms remain elusive. Here, we show that cisplatin induced rapid activation of autophagy in cultured kidney tubular cells and in the kidneys of injected mice. Cisplatin also induced the phosphorylation of mammalian target of rapamycin (mTOR), p70S6 kinase downstream of mTOR, and serine/threonine-protein kinase ULK1, a component of the autophagy initiating complex. *In vitro*, pharmacologic inhibition of mTOR, directly or through inhibition of AKT, enhanced autophagy after cisplatin treatment. Notably, in both cells and kidneys, blockade of PKCδ suppressed the cisplatin-induced phosphorylation of AKT, mTOR, p70S6 kinase, and ULK1 resulting in upregulation of autophagy. Furthermore, constitutively active and inactive forms of PKCδ respectively enhanced and suppressed cisplatin-induced apoptosis in cultured cells. In mechanistic studies, we showed coimmunoprecipitation of PKCδ and AKT from lysates of cisplatin-treated cells and direct phosphorylation of AKT at serine-473 by PKCδ *in vitro*. Finally, administration of the PKCδ inhibitor rottlerin with cisplatin protected against cisplatin nephrotoxicity in wild-type mice, but not in renal autophagy–deficient mice. Together, these results reveal a pathway consisting of PKCδ, AKT, mTOR, and ULK1 that inhibits autophagy in cisplatin nephrotoxicity. PKCδ mediates cisplatin nephrotoxicity at least in part by suppressing autophagy, and accordingly, PKCδ inhibition protects kidneys by upregulating autophagy.

Keywords: cisplatin, apoptosis, nephrotoxicity

Cisplatin is one of the most widely used and most potent chemotherapeutic agents. It is used for the treatment of testicular, ovarian, breast, head and neck, lung, and many other types of cancers.[1,2]

However, the use of cisplatin is limited by its side effects in normal tissues, especially nephrotoxicity in kidneys.[3–7] Despite decades of research, effective renoprotective approaches during cisplatin chemotherapy remain unavailable. Our recent work revealed an important role of protein kinase C*δ* (PKC*δ*) in cisplatin-induced nephrotoxicity.[8,9] Notably, inhibiting PKC*δ* not only protected kidneys but enhanced the chemotherapeutic effects of cisplatin in several tumor models, opening a new avenue for renoprotection during chemotherapy.[8,9] However, the mechanism underlying the renoprotective effect of PKC*δ* inhibition is unclear.

Autophagy is a highly regulated cellular process of catabolism that degrades cytoplasmic constituents *via* the formation of autophagosome followed by its fusion with lysosome. Originally described as a cellular response to starvation, autophagy is now known to be crucial to the maintenance of cellular homeostasis and play important roles in animal development, physiology, and pathogenesis of a variety of diseases.[10–12] In cisplatin nephrotoxicity, autophagy is rapidly activated in kidney tubular cells and tissues.[13,14] Using renal tubule–specific Atg-knockout models, recent studies have further demonstrated autophagy as an important kidney protective mechanism.[15,16] However, it remains elusive how autophagy is regulated during cisplatin nephrotoxicity.

In view of these findings and questions, we hypothesized that PKC*δ* may play a regulatory role in autophagy during cisplatin nephrotoxicity and inhibition of PKC*δ* may protect kidney cells and tissues by upregulating autophagy. In support of this hypothesis, several studies have implicated PKC*δ* in the regulation of autophagy.[17–20] Nonetheless, whether PKC*δ* promotes or inhibits autophagy remains controversial. For example, Ann and colleagues[18] demonstrated that PKC*δ* mediated autophagy during acute hypoxic stress by phosphorylating/activating JNK1, whereas Ozpolat *et al.*[17] showed that PKC*δ* suppressed autophagy in pancreatic cancer cells by inducing tissue transglutaminase.

In this study, we have identified PKC*δ* as a critical negative regulator of autophagy in both *in vitro* and *in vivo* experimental models of cisplatin. Mechanistically, we show that PKC*δ* may directly bind and phosphorylate AKT at Serine-473, resulting in the activation of mammalian target of rapamycin (mTOR) to suppress ULK1 and autophagy. Moreover, PKC*δ* inhibitors lost their renoprotective effect in autophagy-deficient mice, supporting a role of autophagy in the effect of PKC*δ* inhibition.

Results

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Autophagy Is Induced during Cisplatin Treatment

We first verified that cisplatin induced autophagy *in vitro* in cultured rat proximal tubular cells (RPTC). In this experiment, we also observed the effect of chloroquine (CQ), which accumulates in lysosomes to raise pH resulting in the inhibition of lysosomal enzymes and the suppression of autophagic degradation. By this property, CQ is frequently used to block autolysosomal degradation to reveal upstream autophagic activation upon stimulation. In immunoblot analysis, cisplatin treatment for 6 hours induced the conversion of LC3I to LC3II, which was further enhanced by the presence of CQ (Figure 1, A and B). To visualize autophagsosome formation, the cells were transfected with GFP-LC3 and then treated with cisplatin in the presence or absence of CQ. As shown in Figure 1, C and D, cisplatin treatment increased the number of GFP-LC3 puncta, which was further increased by CQ, confirming autophagy induction in this experimental condition.

mTOR Is Activated to Suppress Autophagy during Prolonged Cisplatin Treatment

To investigate the regulatory mechanism of autophagy during cisplatin treatment, we initially focused on mTOR, a well documented negative regulator of autophagy. We hypothesized that mTOR might be inactivated during cisplatin treatment to induce autophagy. We first analyzed the status of mTOR activation

during cisplatin treatment by monitoring its phosphorylation on Ser2448. As shown in Figure 2A, cisplatin induced significant mTOR-Ser2448 phosphorylation at 6 hours and 16 hours of cisplatin treatment, whereas the phosphorylation was not obvious at 3 hours. Cisplatin also induced a similar pattern of phosphorylation of p70S6 kinase, a well established downstream target of mTOR (Figure 2A). A major mechanism for mTOR regulation of autophagy is through inhibitory phosphorylation of ULK1, a key component of the autophagy initiating complex.[21,22] Indeed, we found that cisplatin induced marked ULK1 phosphorylation at Ser757 at 6–16 hours, paralleling with the time course of mTOR activation, whereas total ULK1 was not significantly changed during cisplatin treatment (Figure 2A). To further confirm the role of mTOR in cisplatin-induced autophagy, we examined the effect of rapamycin, a specific pharmacologic inhibitor of mTOR. In immunoblot analysis, rapamycin attenuated p70S6 kinase phosphorylation and, notably, enhanced LC3II accumulation during cisplatin treatment (Figure 2B). Moreover, rapamycin significantly increased autophagic GFP-LC3 puncta formation during cisplatin treatment of GFP-LC3 transfected cells (Figure 2, C and D). These results suggested that mTOR activation may contribute significantly to the decrease of autophagy during the late stage of cisplatin treatment.

AKT Acts Upstream of mTOR for Autophagy Suppression during Cisplatin Treatment

To understand the mechanism of mTOR activation during cisplatin treatment, we focused on AKT, because: (*1*) AKT may act upstream of mTOR in cell signaling,[23] and (*2*) AKT is activated during cisplatin treatment of kidney tubular cells and tissues.[24,25] We verified AKT activation during cisplatin treatment of RPTC by detecting AKT phosphorylation at Ser473 and Thr308 (Figure 3A). Notably, AKT activation started within 0.5 to 1 hours of cisplatin treatment, reached peak levels at 3 hours, and decreased toward basal level by 24 hours. To determine the role of AKT on mTOR and autophagy regulation, we further examined the effects of VIII, a pharmacologic inhibitor of AKT. As shown in Figure 3B, VIII suppressed mTOR as indicated by the attenuation of 70-kDa ribosomal protein S6 kinase (p70S6K) phosphorylation. Concurrently, VIII induced LC3II in control as well as in cisplatin-treated cells (Figure 3, B and C). The autophagy-enhancing effect of VIII was further confirmed by GFP-LC3 puncta formation. Apparently, VIII further increased the formation of GFP-LC3 autophagic puncta (Figure 3, D and E**)**. Thus, inhibition of AKT led to the attenuation of mTOR activation and increased autophagy, suggesting that AKT may act upstream of mTOR to quench autophagy during prolonged cisplatin treatment.

AKT is well known for its prosurvival activity and as a result, by inhibiting AKT VIII, is expected to induce cell death. However, our above experiment (Figure 3) demonstrated that VIII could also activate the cytoprotective mechanism of autophagy. Does VIII induce or protect against cell death during cisplatin treatment? As shown in Supplemental Figure 1, VIII further increased apoptosis during 16 hours of cisplatin treatment of RPTC cells. We also analyzed the effect of VIII on cell proliferation (Supplemental Figure 2). Both VIII and cisplatin suppressed cell proliferation, and when added together they had additive effects. The results suggest that, although AKT may suppress cytoprotective autophagy, its overall cell biologic function is for cell survival and proliferation.

PKC*δ* Is Activated during Cisplatin Treatment to Activate mTOR and Suppress Autophagy

Our recent work demonstrated a rapid activation of PKC*δ* during cisplatin treatment of RPTC and mice. Moreover, pharmacologic and genetic suppression of PKC*δ* afforded remarkable renoprotective effects.[8] Because autophagy is an important mechanism of renoprotection in kidney injury including cisplatin nephrotoxicity,[26] we hypothesized that PKC*δ* inhibition may protect *via* autophagy. To test this possibility, we first confirmed PKC*δ* activation during cisplatin treatment of RPTC by immunoblot analysis of its phosphorylation (Figure 4A). To determine the involvement of PKC*δ* in cisplatin-induced autophagy, we examined the effects of dominant-negative PKC*δ* (PKC*δ*-KD) and catalytically active PKC*δ* (PKC*δ*-CF). As shown in Figure 4B, LC3II accumulation during cisplatin treatment was suppressed by PKC*δ*-CF, but

increased by PKC*δ*-KD. Consistently, in GPF-LC3-transfected cells PKC*δ*-CF suppressed, whereas PKC*δ*-KD enhanced, GFP-LC3 puncta formation (Figure 4, C and D). To understand the underlying mechanism, we examined whether PKC*δ* suppressed autophagy through the activation of mTOR and consequent inhibitory phosphorylation of ULK1. As shown in Figure 4E, cisplatin treatment led to phosphorylation of mTOR, p70S6K, and ULK1, which was further increased by PKC*δ*-CF, whereas PKC*δ*-KD was inhibitory. Together, the results suggested that PKC*δ* might contribute to the activation of mTOR resulting in the suppression of ULK1 and autophagy.

We further monitored the effects of PKC*δ*-KD and PKC*δ*-CF on apoptosis during cisplatin treatment. In morphologic analysis, cisplatin induced significant apoptosis in RPTC, which was increased by PKC*δ*-CF and suppressed by PKC*δ*-KD (Figure 5, A and B). The morphologic analysis was supported by the results of caspase activity assay (Figure 5C). It was concluded that PKC*δ* might suppress autophagy to facilitate apoptosis.

PKC*δ* Binds to and Phosphorylates AKT at Ser473

To investigate the mechanism whereby PKC*δ* activates mTOR, we first postulated that PKC*δ* might interact with and directly phosphorylate mTOR. However, coimmunoprecipitation assay did not show the binding of PKC*δ* to mTOR in control or cisplatin-treated cells (data not shown). We then hypothesized that PKC*δ* may regulate mTOR indirectly through AKT, because AKT was shown to play a role in mTOR activation during cisplatin treatment (Figure 3). As shown in Figure 6A, AKT phosphorylation at both Ser473 and Thr308 was induced during cisplatin treatment. PKC*δ*-CF further increased p-AKT(S473), whereas PKC*δ*-KD had opposite effects. Interestingly, neither PKC*δ*-CF or PKC*δ*-KD affected pAKT(T308), suggesting a site specific regulation of AKT by PKC*δ*. We further assessed whether AKT physically interacted with PKC*δ* by coimmunoprecipitation. Anti-PKC*δ* precipitated both PKC*δ* and AKT (Figure 6B). Anti-AKT also pulled down both AKT and PKC*δ* (Figure 6C). Reciprocal co-IP of these two proteins was consistently detected at 6 hours of cisplatin treatment (Figure 6, B and C). It was unclear whether PKC*δ* could directly phosphorylate AKT. To test this possibility, we incubated AKT with recombinant active PKC*δ* in a kinase reaction buffer. As shown in Figure 6D, this incubation led to evident phosphorylation of AKT at Ser473, but not at Thr308. This test tube experiment, together with the observation of cisplatin-treated cells (Figure 6A), suggested that PKC*δ* may directly phosphorylate AKT at Ser473 resulting in AKT activation during cisplatin treatment.

Restoration of Cisplatin-Induced Kidney Injury in PKCδ-Null Mice by CQ

To demonstrate the role of PKC*δ* in autophagy regulation *in vivo*, we compared PKC*δ*-null (PKC*δ*−/−) mice and their wild-type (PKC*δ*+/+) littermates for the response to cisplatin treatment. In wild-type mice, cisplatin induced severe renal failure as shown by high levels of blood urea nitrogen (BUN) (152 mg/dl) and serum creatinine (1.6 mg/dl) at day three of treatment. In contrast, PKC*δ*−/− mice showed significantly better renal function, with 85 mg/dl BUN and 0.9 mg/dl serum creatinine (Figure 7, A and B). Notably, inhibition of autophagy with CQ restored cisplatin injury in PKC*δ*−/− mice (Figure 7, A and B), supporting a role of autophagy in the observed kidney injury resistance of these mice. Consistently, PKC*δ*−/− mice showed a better renal histology and less apoptosis than wild-type mice after cisplatin treatment, and again CQ restored kidney tissue damage and apoptosis in PKC*δ*−/− mice (Figure 7, C and D). At day three of cisplatin treatment, the tissue damage score was 3.1 for wild-type mice, 0.99 for PKC*δ*−/− mice, and 2.8 for PKC*δ*−/− mice with CQ, respectively (Figure 7F). This observation was further verified by counting apoptotic cells in kidney cortical and outer medulla regions (Figure 7G).

PKC*δ*-Null Mice Show High Renal Autophagy under Control and Cisplatin Treatment Conditions

To examine autophagy in kidney tissues, we used electron microscopy. Three days after cisplatin injection, numerous vacuoles appeared in some proximal tubular cells and some of the vacuoles contained cytoplasmic materials, indicative of autophagic vesicles (Figure 8A). For quantification, we counted the cells with autophagic vesicles (Figure 8B). After cisplatin treatment, 23% of renal tubular cells were autophagic in wild-type mice, and 44% in PKCδ−/− mice. CQ further increased it to 66% (Figure 8B). To verify this observation, we collected kidney tissues for immunoblot analysis of LC3 (Figure 8C). PKCδ−/− tissue showed significantly higher LC3 than wild-type mice, and there was a notable increase of LC3-II in renal tissues of wild-type mice after cisplatin injection, which was further increased in PKCδ−/− mice with cisplatin. Together, these results suggested that enhanced autophagy may be responsible for the resistance of PKCδ-null mice to cisplatin nephrotoxicity.

Cisplatin-Induced AKT/mTOR Activation in Kidney Tissues Is Blocked in PKCδ−/− Mice

We further determined the involvement of AKT/mTOR in PKCδ signaling *in vivo* during cisplatin nephrotoxicity. In wild-type mice, cisplatin induced p-AKT-S473, p-mTOR-Ser2448, p-p70S6K-T389, and p-ULK1-S757 in kidney tissues (Figure 9), indicative of the activation of the AKT/mTOR/ULK1 pathway for autophagy suppression. Notably, this pathway was significantly less active in PKCδ−/− tissues at both control and cisplatin treatment conditions (Figure 9). These *in vivo* data were consistent with our cell culture results (Figures 4 and 5), suggesting that AKT/mTOR was downstream of PKCδ in autophagic signaling during cisplatin nephrotoxicity.

The Renoprotective Effect of Rottlerin Is Lost in Kidney Autophagy–Deficient Mice

Our recent work demonstrated a critical role of PKCδ in cisplatin nephrotoxicity and inhibitors of PKCδ showed remarkable renoprotective effects.[8] The results presented thus far in this study have suggested that PKCδ may activate AKT/mTOR to suppress renal autophagy to contribute to cisplatin-induced injury and cell death. To further investigate this *in vivo*, we determined whether the renoprotective effect of rottlerin, a pharmacologic PKCδ inhibitor, depended on autophagy in renal tubules. For this purpose, we tested a conditional knockout model in which Atg7 was ablated specifically from kidney proximal tubule cells (PT-Atg7-KO).[15] Essentially, the effects of rottlerin on cisplatin-induced kidney injury in PT-Atg7-KO and wild-type littermate mice were examined and compared. Without treatment, PT-Atg7-KO mice and their wild-type littermates showed similarly low levels of BUN and serum creatinine, indicating normal renal function. Three days after cisplatin injection, wild-type mice developed moderate renal failure as indicated by 104 mg/dl BUN and 0.8 mg/dl serum creatinine, which was significantly attenuated by rottlerin (Figure 10). In sharp contrast, cisplatin induced more severe renal failure in PT-Atg7-KO mice with 148 mg/dl BUN and 1.3 mg/dl serum creatinine (Figure 10, A and B). Importantly, rottlerin could not ameliorate cisplatin-induced renal failure in PT-Atg7-KO mice. Histopathologic analysis confirmed that, compared with wild-type, cisplatin induced much severer kidney tissue damage in PT-Atg7-KO mice with widespread, extensively damaged proximal tubules, and notably, rottlerin reduced kidney tissue damage in wild-type mice, but not in PT-Atg7-KO mice (Figure 10C). In quantification, wild-type mice had a tissue damage score of 1.2 that was reduced to 0.5 by rottlerin, whereas PT-Atg7-KO mice had a score of 2.1 that was not affected by rottlerin (Figure 10E). This observation was further verified by counting apoptotic cells in kidney tissues (Figure 10, D and F), showing that rottlerin was effective in reducing apoptosis in wild-type mice, but not in PT-Atg7-KO mice. Together, these results suggested that the renoprotective effect of PKCδ inhibition was at least partly mediated by autophagy.

Discussion

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Nephrotoxicity is a major side effect in cisplatin chemotherapy that limits the use and efficacy of cisplatin in cancer patients. PKCδ was recently identified as a novel regulator of cisplatin nephrotoxicity.[8]

Remarkably, inhibitors of PKC*δ* protected against cisplatin-induced kidney tubular cell death and tissue damage while enhancing chemotherapeutic effects in tumors, supporting their potential of clinical applicability.[8] However, it was unclear as to how PKC*δ* mediates cisplatin nephrotoxicity and how PKC*δ* inhibitors protect kidney cells and tissues. In this study, we have demonstrated that PKC*δ* suppresses autophagy during cisplatin treatment of kidney cells *in vitro* and mouse kidneys *in vivo*, contributing to cisplatin nephrotoxicity. Our results further suggest that PKC*δ* inhibitors may protect kidneys by upregulating autophagy in renal tubular cells. Mechanistically, the results have, for the first time, demonstrated the evidence of direct phosphorylation and activation of AKT by PKC*δ*. Upon activation, AKT further activates mTOR to induce the inhibitory phosphorylation of ULK1, resulting in the suppression of autophagy. As a result, this study has unveiled a novel pathway of PKC*δ*/AKT /mTOR/ULK1 that is responsible for autophagy suppression during cisplatin treatment of kidney cells and tissues, contributing to cisplatin nephrotoxicity during chemotherapy. Accordingly, blockade of this pathway, *e.g.,* by inhibiting PKC*δ*, maintains autophagy during cisplatin treatment to afford renoprotection.

PKC*δ* has been implicated in the regulation of a variety of cellular processes, ranging from apoptosis to cell survival, migration, and proliferation.[27–31] Notably, the role of PKC*δ* depends on the cellular context and experimental conditions. As a result, apparently conflicting observations have been reported and the cellular function of PKC*δ* was described to be “complex and enigmatic”.[30] Of much relevance to this study, PKC*δ* is known to be proapoptotic in some cell types and tissues (*e.g.,* kidneys and HeLa cells[8,32]), but it is prosurvival in multiple cancer cell lines.[33–37] It remains elusive as to how PKC*δ* regulates apoptosis. This study suggests that PKC*δ* may contribute to cisplatin-induced kidney cell apoptosis and nephrotoxicity by suppressing autophagy. First, PKC*δ* activation suppressed autophagy, whereas PKC*δ* inhibition upregulated autophagy during cisplatin treatment, indicating clearly that PKC*δ* negatively regulates autophagy under the experimental condition (Figure 4). Second, autophagy regulation by PKC*δ* was accompanied by changes in apoptosis (Figures 4 and 6). For example, blockade of PKC*δ* with a dominant negative mutant led to increased autophagy during cisplatin treatment (Figure 4) and a marked decrease in apoptosis (Figure 6). In addition, we and others have demonstrated autophagy as a critical protective mechanism during cisplatin treatment of kidney cells *in vitro* and nephrotoxicity *in vivo*. Finally, in this study the protective effect of rottlerin (PKC*δ* inhibitor) was diminished in kidney tubule–specific autophagy deficient mice (Figure 9), indicating that the effect of PKC*δ* inhibitors depends on autophagy. Together, these results have identified autophagy suppression as the key mechanism whereby PKC*δ* mediates cell injury and death.

Regulation of autophagy by PKC*δ* has been reported recently, but the results from these studies are not consistent. In 2008, Ann and colleagues demonstrated a role of PKC*δ* in autophagy activation in the model of acute hypoxic stress, where PKC*δ* was shown to promote JNK1-mediated Bcl-2 phosphorylation leading to the dissociation and release of Beclin-1 from Bcl-2 for autophagy.[18,38] Consistently, more recent work by Shahnazari *et al.* showed that PKC*δ* was essential to autophagy activation for bacterial clearance from mammalian cells.[20] In sharp contrast, PKC*δ* was suggested to suppress autophagy in pancreatic ductal carcinoma cells by inducing tissue transglutaminase 2.[17,39] In addition, inhibition of PKC*δ* by Safingol (synthetic L-threo–stereoisomer of sphinganine) led to the activation of autophagy in HCT116 colorectal cancer cells.[19] The exact cause of the discrepancy between these few studies is unclear. This study demonstrates an autophagy suppressive role of PKC*δ*. In both *in vitro* cell culture and *in vivo* mouse models of cisplatin nephrotoxicity, blockade of PKC*δ* led to upregulation of autophagy (Figures 4, 7), suggesting that, upon activation, PKC*δ* suppresses autophagy during cisplatin treatment of kidney cells and tissues. Thus the role of PKC*δ* depends on, and may change according to, the cellular context and experimental conditions.[18]

This study provides significant insights into the signaling pathways that contribute to the regulation of cisplatin-induced kidney cell injury and nephrotoxicity. In 2008, we and Kaushal’s group first reported

autophagy during cisplatin treatment of kidney tubular cells.[13,14] By electron microscopy and immunoblotting of LC3, we further suggested autophagy *in vivo* in kidneys after cisplatin injection in mice.[13] These and subsequent studies, especially those using renal tubule–specific autophagy-deficient mouse models,[15,16] have demonstrated convincing evidence for a renoprotective role of tubular autophagy in cisplatin nephrotoxicity. Consistently, autophagy has been demonstrated to be a major renoprotective mechanism in kidney injury induced by renal ischemia-reperfusion.[15,40–42] Despite these functional studies, there is a significant lack of understanding of the signaling pathways and/or regulatory mechanisms that govern autophagy in renal tubular cells in AKI conditions. In this study, mTOR did not decrease to account for initial autophagy activation after cisplatin treatment; instead, it was activated as a negative regulation mechanism of autophagy by phosphorylating ULK1 (Figure 2). Upstream of mTOR, our results suggest the signaling relay from AKT (Figure 3). Interestingly, PKC*δ* was shown to be further upstream of AKT (Figure 6). Therefore, we have elucidated a novel signaling pathway consisting of PKC*δ*/AKT /mTOR/ULK1 that is activated during cisplatin nephrotoxicity to suppress autophagy. In view of the protective role of autophagy, it is suggested that this signaling pathway is important to the occurrence of tubular cell apoptosis and nephrotoxicity during cisplatin therapy. It is noteworthy that AKT activation occurred earlier than mTOR during cisplatin treatment (Figures 2 and 3). One possibility is that AKT may phosphorylate mTOR at early time-points and p-mTOR may last to later time-points; in this case, later p-mTOR and the associated autophagy decrease still depend on PKC*δ*-AKT signaling. Nonetheless, autophagy decrease at a late stage of cisplatin treatment may also involve other mechanisms.

Regulation of AKT/mTOR by PKC*δ* has been reported previously. For example, PKC*δ* inhibitors suppressed AKT/mTOR and associated signaling in a murine model of allergic asthma.[43] The latest work by Dai *et al.* further suggested that PKC*δ* might regulate the proliferation and migration of human gastric adenocarcinoma cells *via* the AKT/mTOR/p70S6 kinase pathway.[44] Despite these findings, whether PKC*δ* can regulate AKT directly *via* phosphorylation remains unclear. Our current data provide evidence that PKC*δ* may directly phosphorylate AKT during cisplatin treatment of kidney cells and tissues (Figure 6). In RPTC cells, cisplatin induced AKT phosphorylation at Ser473, which was further increased by active PKC*δ* and attenuated by dominant negative PKC*δ*. Our co-IP analysis also suggested the interaction of PKC*δ* with AKT and, notably, the interaction increased during cisplatin treatment (Figures 5C and 6B). Moreover, incubation of AKT with active PKC*δ* induced specific AKT phosphorylation at Ser473 (Figure 6D). Thus, PKC*δ* may regulate AKT by directly phosphorylating AKT at Ser473 under this experimental condition. Obviously, such a conclusion requires further in-depth investigation to substantiate. If proven true, it may have significant implications for the understanding of AKT regulation. It is known that AKT activation depends on its phosphorylation at Thr308 in the T-loop close to the catalytic core of the kinase domain and Ser473 near the carboxyl terminus.[45] Phosphoinositide-dependent kinase 1 (PDK1) is responsible for Thr308 phosphorylation, but the molecular basis of Ser473 phosphorylation remains elusive. One possibility is that, after Thr308 phosphorylation and initial activation, AKT may autophosphorylate Ser473. However, the autophosphorylation hypothesis has been seriously challenged. For example, in PDK1-deficient cells, AKT is not phosphorylated at Thr308 and shows low activity, but Ser473 phosphorylation can still be induced by insulin.[46] Although this and other studies[47] suggest the existence of a distinct kinase-PDK2 that is responsible for Ser473 phosphorylation and full activation of AKT, the identify of PDK2 remains a mystery. This study suggests that PKC*δ* may be a candidate of PDK2 that is responsible for AKT phosphorylation at Ser473 during cisplatin treatment of kidney cells and tissues. Further investigation needs to extend this finding to verify if PKC*δ* phosphorylates AKT-Ser473 under other experimental conditions.

In conclusion, this study has delineated a novel signaling pathway consisting of PKC*δ*, AKT, mTOR, and ULK1, which is activated in experimental models of cisplatin nephrotoxicity and negatively regulates autophagy. In this pathway, PKC*δ* may directly interact with and phosphorylate AKT at serine-473,

resulting in full activation of this kinase. AKT then phosphorylates and activates mTOR to induce inhibitory phosphorylation of ULK1, leading to autophagy suppression. Suppression of autophagy by this pathway contributes to kidney cell death and tissue damage in cisplatin nephrotoxicity. Accordingly, the study further suggests that PKCδ inhibitors protect kidneys during cisplatin treatment at least in part by blocking this pathway to facilitate autophagy, a major protective mechanism in kidney cells.

Concise Methods

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Reagents

The following primary antibodies were used: anti-LC3 from Novus Biologicals (Littleton, CO); anti–β-actin and anti-ULK1 from Sigma-Aldrich (St. Louis, MO); anti-cyclophilin B from Abcam Inc. (Cambridge, MA); anti-PKCδ, anti-AKT, anti–phospho-AKT(S473), anti–phospho-AKT(Thr308), anti-mTOR, anti–phospho-mTOR(S2448), anti-p70S6K, anti–phospho-p70S6K (T389), and anti–phospho-ULK1(S757) from Cell Signaling Technology (Danvers, MA). All secondary antibodies for immunoblot analysis were from Thermo Fisher Scientific (Vernon Hills, IL). Carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD.AFC) and 7-amino-4-trifluoromethyl coumarin (AFC) were from Enzyme Systems Products (Livermore, CA). Recombinant active PKCδ and recombinant inactive AKT were purchased from R&D Systems (Minneapolis, MN) and BD Biosciences (San Jose, CA), respectively. AKT inhibitor VIII was from Calbiochem (San Diego, CA). Cyquant-NF cell proliferation assay kit was from Invitrogen (Carlsbad, CA). Unless indicated, all other reagents (cisplatin, rapamycin, *etc.*) were from Sigma-Aldrich.

Experimental Models of Cisplatin Nephrotoxicity

PKCδ–/–mice were generated by targeted gene deletion as described previously[48] by Dr. Robert Messing's laboratory at the University of California, San Francisco, CA. The PT-Atg7-KO mouse model was established by breeding Atg7flox/flox mice[49] with PEPCK-Cre transgenic mice obtained from Dr. Volker Haase (University of Pennsylvania, Philadelphia, PA).[50] The transgenic mouse lines were mainly in C57 background. All animals were housed in the animal facility of Charlie Norwood Veterans Affairs Medical Center. Animal experiments were conducted with the approval of and in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Charlie Norwood Veterans Affairs Medical Center and Medical College of Georgia. The RPTC rat kidney proximal tubule line was described previously.[13,40] For *in vivo* study, mice were injected with a single dose of 30 mg/kg cisplatin to induce kidney injury, as described previously.[8,13,40] Control animals were injected with saline. For *in vitro* study, RPTC were incubated with 20 μM cisplatin, which induced significant apoptosis as indicated previously.[8,13,40]

Analysis of Renal Function and Histology

Renal failure or loss of renal function was indicated by serum creatinine and BUN using commercial kits as previously described.[8,13,40] For histology, kidney tissues were fixed with 4% paraformaldehyde for paraffin embedding and H&E staining. Tissue damage was scored by the percentage of renal tubules with cell lysis, loss of brush border, and cast formation (0, no damage; 1, <25%; 2, 25%–50%; 3, 50%–75%; 4, >75%).

Analysis of Apoptosis

Apoptosis in kidney tissues was analyzed by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit from Roche Diagnostics (Indianapolis, IN), as described in our previous work.[8,13,40] Apoptosis in cell cultures was analyzed by standard methods, including cell morphology and caspase activity. For morphologic analysis,

cells were stained with Hoechst33342. The cells showing typical morphologic features, including cellular and nuclear condensation and fragmentation, were counted to determine the percentage of apoptosis. Caspase activity was measured by an enzymatic assay as previously described.[8,13,40] Briefly, cells were lysed with a buffer containing 1% Triton X-100. The lysates of 25 μg protein were added to an enzymatic assay buffer containing 50 μM DEVD.AFC for 60 minutes at 37°C. Fluorescence at excitation 360 nm/emission 535 nm was measured with a GENios plate-reader (Tecan US Inc.). Free DEVD.AFC was used to plot a standard curve, and, using the standard curve, the fluorescence reading from the enzymatic reaction was converted into the nanomolar amount of DEVD.AFC liberated per mg protein per hour as a measure of caspase activity.

Transfection of RPTC

Various PKC plasmids were obtained from Dr. Jae-Won Soh (Inha University, Inchun, Republic of Korea) and Dr. Fushin Yu (Wayne State University, Detroit, MI). The GFP-LC3 plasmid was a generous gift from Dr. Yoshimori (National Institute of Genetics, Mishima, Japan). The RPTC line was obtained from Dr. U. Hopfer (Case Western Reserve University, Cleveland, OH) and maintained for experiments as described previously.[51] Cells were plated at 0.5×10^6 cells per 35 mm dish to reach 50%–60% confluence after overnight growth. The cells were then transfected with 1 μg PKC plasmids (PKCδ-KD, PKCδ-CF) using Lipofectamin 2000 (Invitrogen). To identify the transfected cells for analysis, 0.2 μg pEGFP-C3 was cotransfected. The cells were subjected to experimental treatment after 24 hours of transfection.

Analysis of Autophagy after GFP-LC3 Transfection

GFP-LC3 puncta formation was examined to show autophagy in RPTC cells as described previously.[8,13,40] Briefly, cells were plated on a coverslip to reach 50% confluence for transfection with 1 μg of GFP-LC3 by using Lipofectamine reagent (Invitrogen). The cells were then maintained in culture medium for 24 hours to reach 80%–90% confluence for cisplatin treatment. At the end of incubation, cells were fixed with 4% paraformaldehyde and examined by fluorescence microscopy to collect images and evaluate GFP-LC3 puncta.

Coimmunoprecipitation of PKCδ and AKT

Kidney tissue and cell lysates were collected with the immunoprecipitation lysis buffer and subjected to immunoprecipitation using an anti-PKCδ or anti-AKT antibody. The resultant precipitates were analyzed by gel electrophoresis and immunoblotting for AKT and PKCδ.

Test Tube Analysis of AKT Phosphorylation by PKCδ

Recombinant active PKCδ (0.1 μg) and recombinant inactivate AKT1 (0.1 μg) were suspended in the kinase reaction buffer containing 20 μM ATP for 5, 15, and 60 minutes of incubation at 30°C. After the incubation, 2% SDS was added to terminate the reaction. The samples were then subjected to gel electrophoresis and transferred to PVDF membrane for immunoblot analysis of AKT, phospho-AKT(S473), and phospho-AKT(Thr308).

Immunoblot Analysis

Kidney tissue and cell lysates were lysed in 2% SDS buffer containing protease inhibitor cocktail and nuclease. Protein concentration was determined with BCA reagent from Thermo Fisher Scientific. Equal amounts (50–100 μg) of protein were loaded in each lane and separated on SDS-polyacrylamide electrophoresis gel followed by immunoblot analysis by standard method.

Statistical Analyses

Qualitative data shown in this study, including immunoblots and cell and tissue images, are representative of at least three separate experiments. Quantitative data are expressed as mean±SD. *t* test was used to determine the statistical significance in the differences between two groups. One-way ANOVA followed by Tukey *post-hoc* test was used to compare multiple treatment groups. Two-way ANOVA was used to assess the statistical significance of the differences between multiple treatment groups at different time points. Statistical analysis was performed using GraphPad Prism version software (GraphPad Software, La Jolla, CA). $P<0.05$ was considered to reflect significant differences.

Disclosures

Go to:

None.

Supplementary Material

Go to:

Supplemental Data:

Acknowledgments

Go to:

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Footnotes

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Figures and Tables

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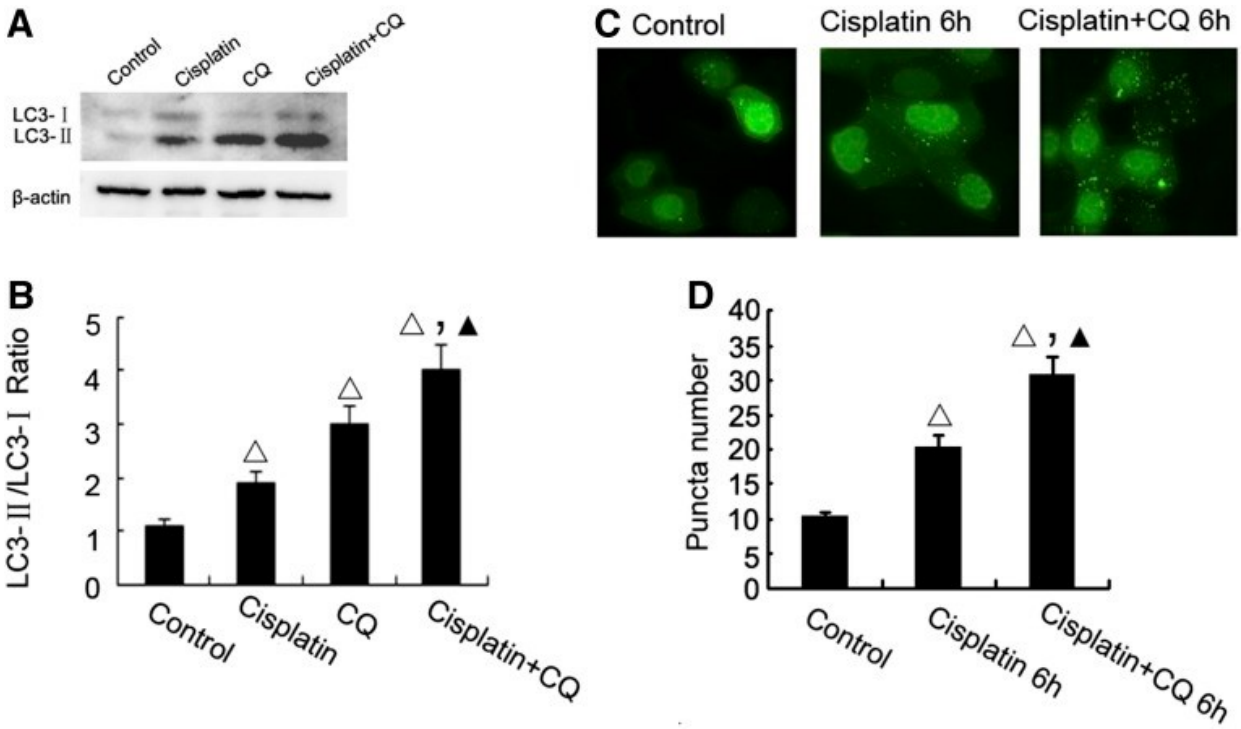
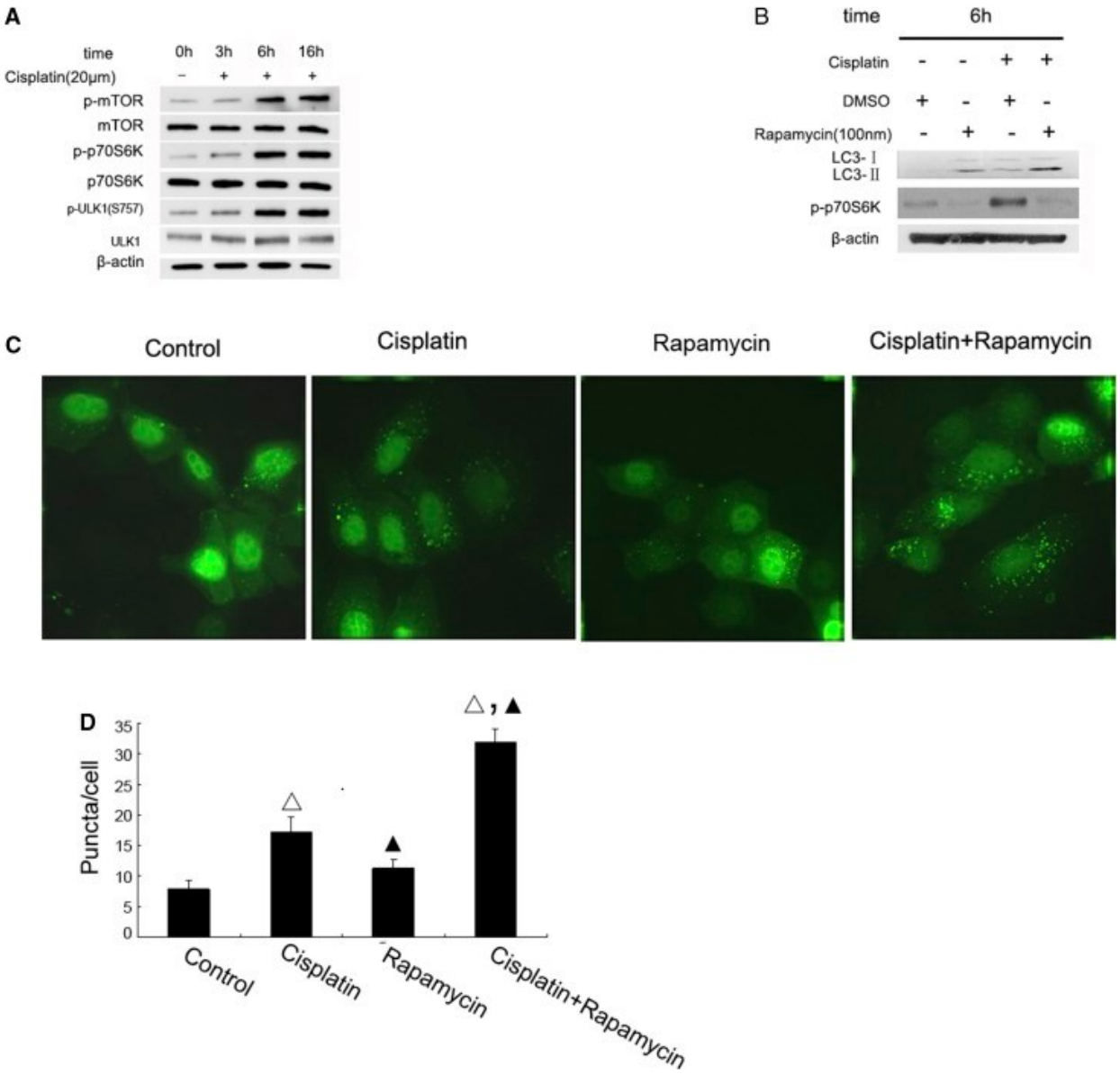
Figure 1.



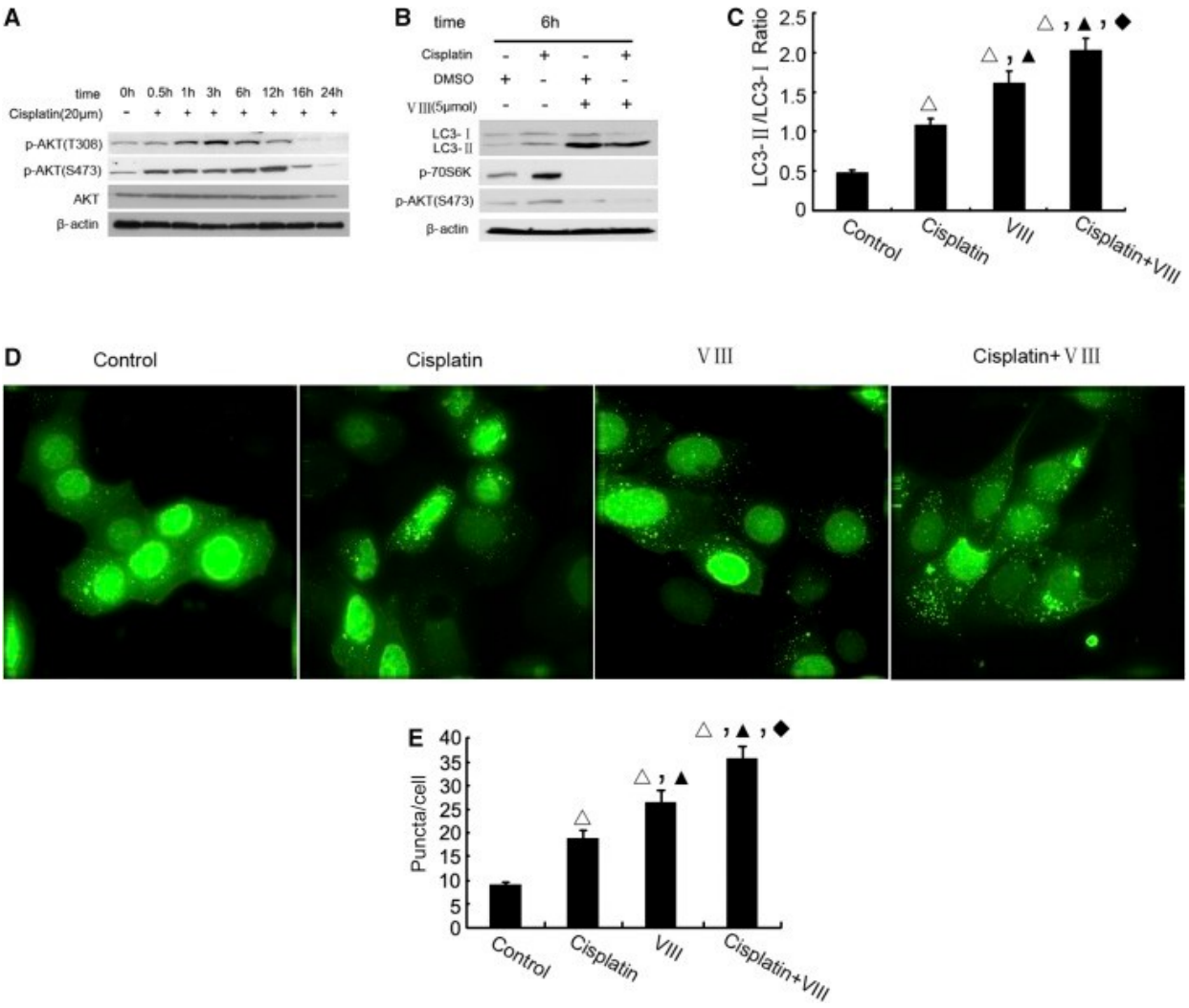
Cisplatin-induced autophagy in RPTC cells. (A) LC3-II formation during cisplatin treatment. RPTC were incubated with 20 μM cisplatin for 6 hours in the absence or presence of 50 μM CQ to collect whole cell lysates for immunoblot analysis of LC3 and also β-actin as a protein loading control. (B) For densitometry, the LC3-II signal was divided by the LC3-I signal of the same sample to determine the ratio. (C) Representative cell images showing punctate GFP-LC3 distribution after cisplatin treatment. RPTC transiently transfected with GFP-LC3 were incubated with 20 μM cisplatin for 6 hours in the absence or presence of CQ and then examined by confocal microscopy. (D) The number of GFP-LC3 puncta per cell in each of the conditions described in (C) was assessed. Data in (B, D) are expressed as mean±SD (n=4); Δ, statistically significantly different from the control group without cisplatin treatment; ▲, statistically significantly different from cisplatin treatment group; P<0.05. Data in panels (A, C) are representative of at least four separate experiments. The results show a rapid induction of autophagy by cisplatin RPTC cells.

Figure 2.



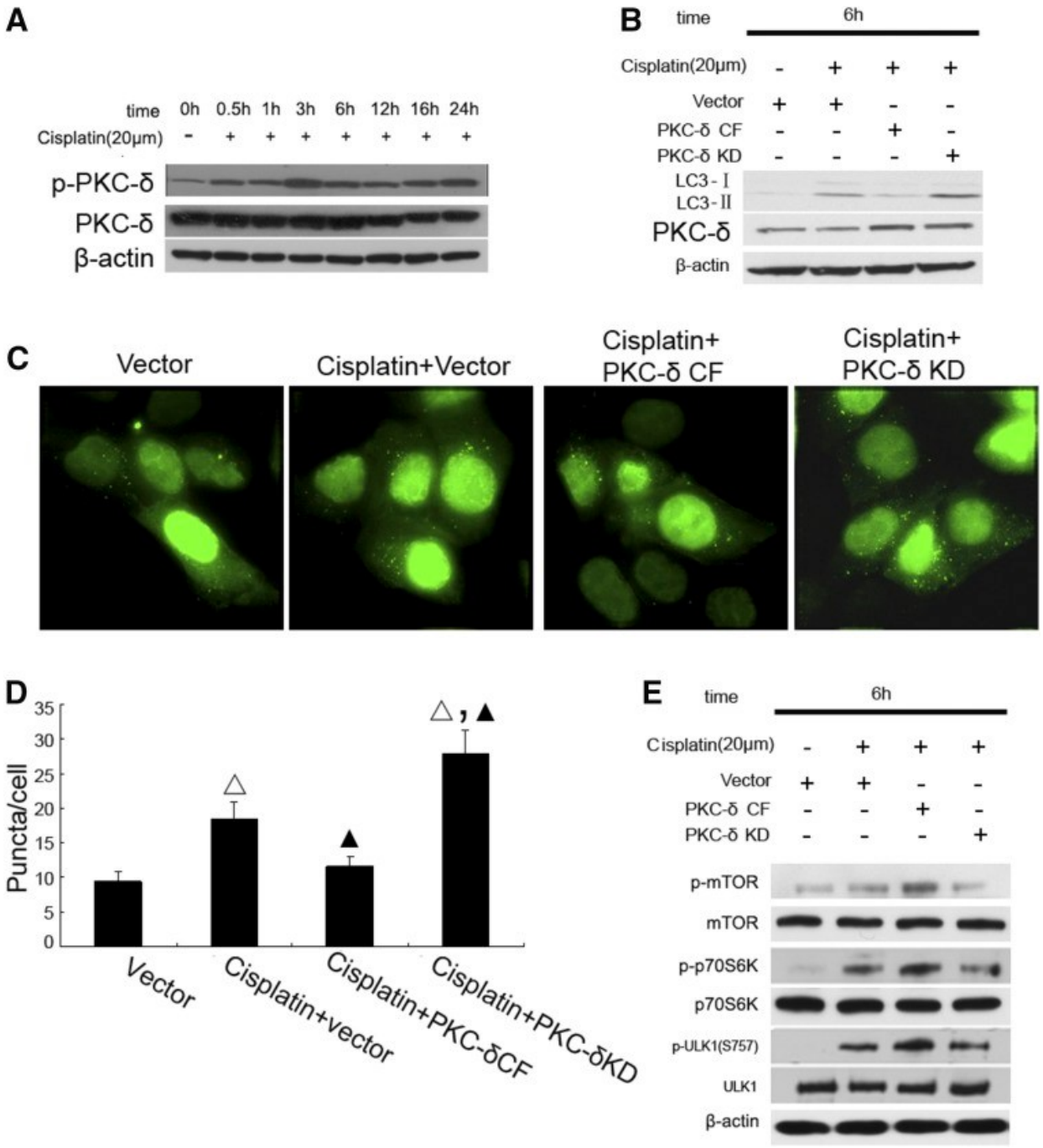
mTOR is activated to suppress autophagy during cisplatin treatment. (A) The time course of cisplatin-induced mTOR activation. RPTC were incubated with 20 μM cisplatin for 0–16 hours to collect whole cell lysates for immunoblot analysis of total and phosphorylated mTOR, p70S6k, and ULK1, and also β-actin as a protein loading control. (B) Inhibition of mTOR by rapamycin increased LC3-II formation during cisplatin treatment. RPTC were incubated with 20 μM cisplatin with or without 0.1 μM rapamycin for 6 hours to collect whole cell lysates for immunoblot analysis of LC-3, p-p70S6k, and β-actin. (C) Representative cell images showing punctate GFP-LC3 distribution after cisplatin with or without rapamycin treatment. RPTC transiently transfected with GFP-LC3 were incubated with 20 μM cisplatin with or without rapamycin for 6 hours and then examined by confocal microscopy. (D) The number of GFP-LC3 puncta per cell in each of the conditions described in (C) was assessed. Data in (D) are expressed as mean±SD (n=4); Δ, statistically significantly different from the control group (P<0.05); ▲, statistically significantly different from cisplatin treatment group (P<0.05). Data are representative of at least four separate experiments. The results show that mTOR is activated during cisplatin treatment to suppress autophagy in RPTC cells.

Figure 3.



AKT acts upstream of mTOR for autophagy suppression during cisplatin treatment. (A) The time course of cisplatin-induced AKT activation. RPTC were incubated with 20 μM cisplatin for 0–24 hours to collect whole cell lysates for immunoblot analysis of total and S473 or T308 site phosphorylated AKT. (B) Inhibition of AKT by VIII increased LC3-II formation during cisplatin treatment. RPTC were incubated with 20 μM cisplatin alone or together with 5 μM VIII for 6 hours to collect whole cell lysates for immunoblot analysis of LC-3, p-p70S6k, p-AKT S473, and also β-actin as a protein loading control. (C) For densitometry, the LC3-II signal was divided by the LC3-I signal of the same sample to determine the ratio. (D) Representative cell images from confocal microscopy showing punctate GFP-LC3 distribution. (E) The number of GFP-LC3 puncta per cell in each of the conditions described in (D) was assessed. Data in (C, E) are expressed as mean±SD (n=4); Δ, statistically significantly different from the control group (P<0.05); ▲, statistically significantly different from cisplatin treatment group (P<0.05); ♦, statistically significantly different from VIII group (P<0.05). Data are representative of at least four separate experiments. The results suggest that AKT is upstream of mTOR involved to suppress cisplatin induced autophagy.

Figure 4.



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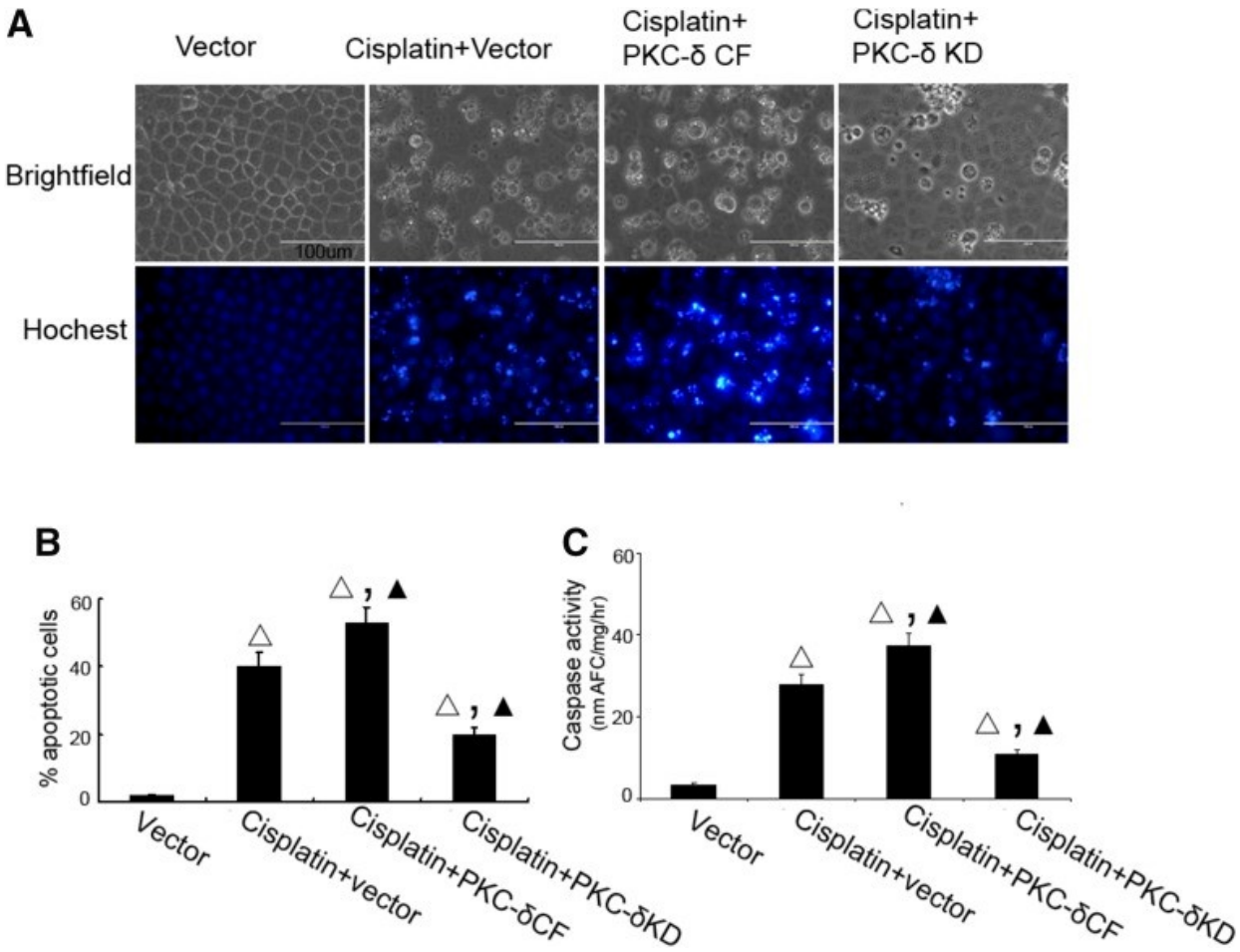
PKC*δ* is activated early during cisplatin treatment to induce mTOR for autophagy suppression. (A) The time course of cisplatin-induced PKC*δ* activation. RPTC were incubated with 20 *μ*M cisplatin for 0–24 hours to collect whole cell lysates for immunoblot analysis of total and phosphorylated PKC*δ*, and also *β*-actin as a protein loading control. (B) PKC*δ*-KD increased, whereas catalytically active PKC*δ* fragment (PKC*δ* CF) blocks, cisplatin-induced LC3-II formation. RPTC were transfected with PKC*δ*-KD or PKC*δ*-CF, and then treated with 20 *μ*M cisplatin for 6 hours to collect whole cell lysates for immunoblot analysis of LC-3 and PKC*δ*, and also *β*-actin as a protein loading control. (C) Representative cell images showing punctate GFP-LC3 distribution. RPTC were cotransfected with GFP-LC3 and another indicated plasmid (empty vector, PKC*δ*-KD, or PKC*δ*-CF), and then treated with 20 *μ*M cisplatin for 6 hours. (D) The number of GFP-LC3 puncta per cell in each of the conditions described in (C) was assessed. (E) Regulation of mTOR during cisplatin treatment by PKC*δ*. RPTC were transfected with PKC*δ*-KD or PKC*δ*-CF, and then treated with 20 *μ*M cisplatin for 6 hours to collect whole cell lysates for immunoblot analysis of total and phosphorylated mTOR, p70S6k, and ULK1. Data in (D) are

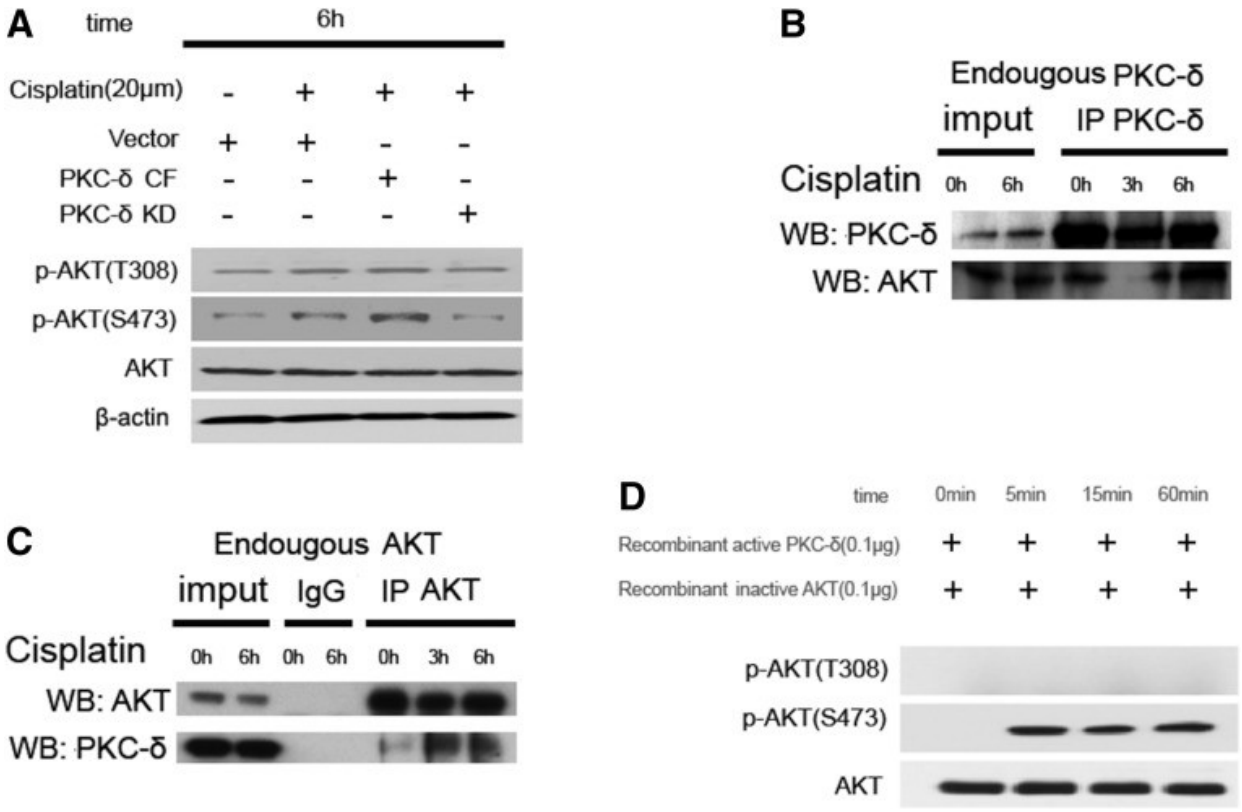
expressed as mean±SD (n=4); Δ, statistically significantly different from the Vector group (P<0.05); ▲, statistically significantly different from Cisplatin+vector group (P<0.05). Data are representative of at least four separate experiments. The results suggest that PKCδ is activated during cisplatin treatment to induce mTOR to suppress autophagy.

Figure 5.



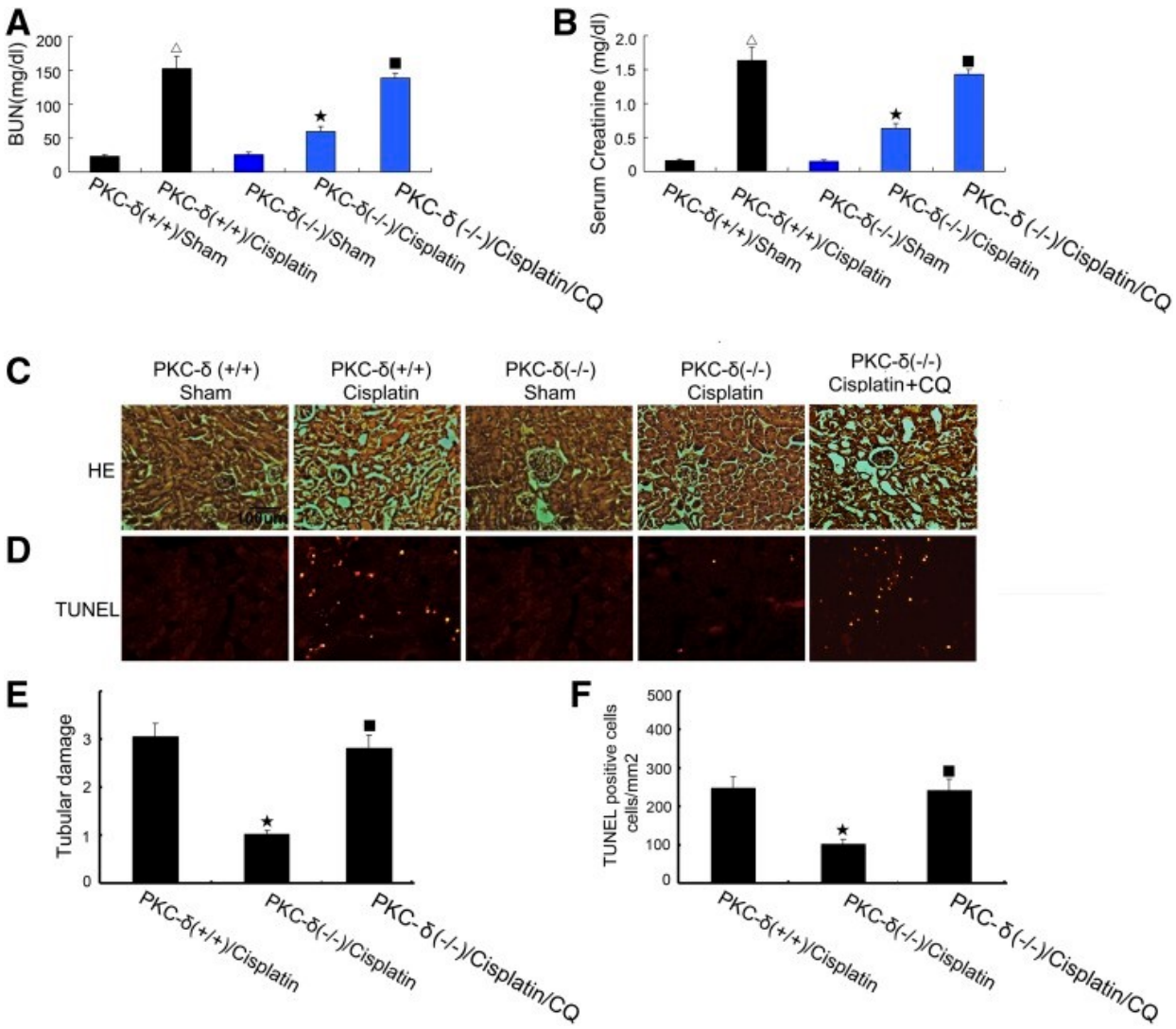
PKC*δ* mediates AKT phosphorylation on Ser473. (A) AKT phosphorylation at S473 (but not T308) was suppressed by PKC*δ*-KD. RPTC were transfected with PKC*δ*-KD or PKC*δ*-CF, and then treated with 20 *μ*M cisplatin for 6 hours to collect whole cell lysates for immunoblot analysis of p-AKT-S473, p-AKT-T308, and total AKT, and also *β*-actin as a protein loading control. (B, C) Reciprocal coimmunoprecipitation of AKT and PKC*δ*. RPTC were incubated with 20 *μ*M cisplatin for 0–6 hours to collect whole cell lysates for immunoprecipitation using anti-PKC*δ* (B) or anti-AKT (C). The immunoprecipitates were analyzed for AKT and PKC*δ* by immunoblotting. (D) PKC*δ* can phosphorylate AKT on Ser473 *in vitro*. Recombinant active PKC*δ* (0.1 *μ*g per reaction) were incubated with 0.1 *μ*g recombinant inactive AKT for 0–60 minutes. Reaction products were analyzed by immunoblotting.

Figure 6.



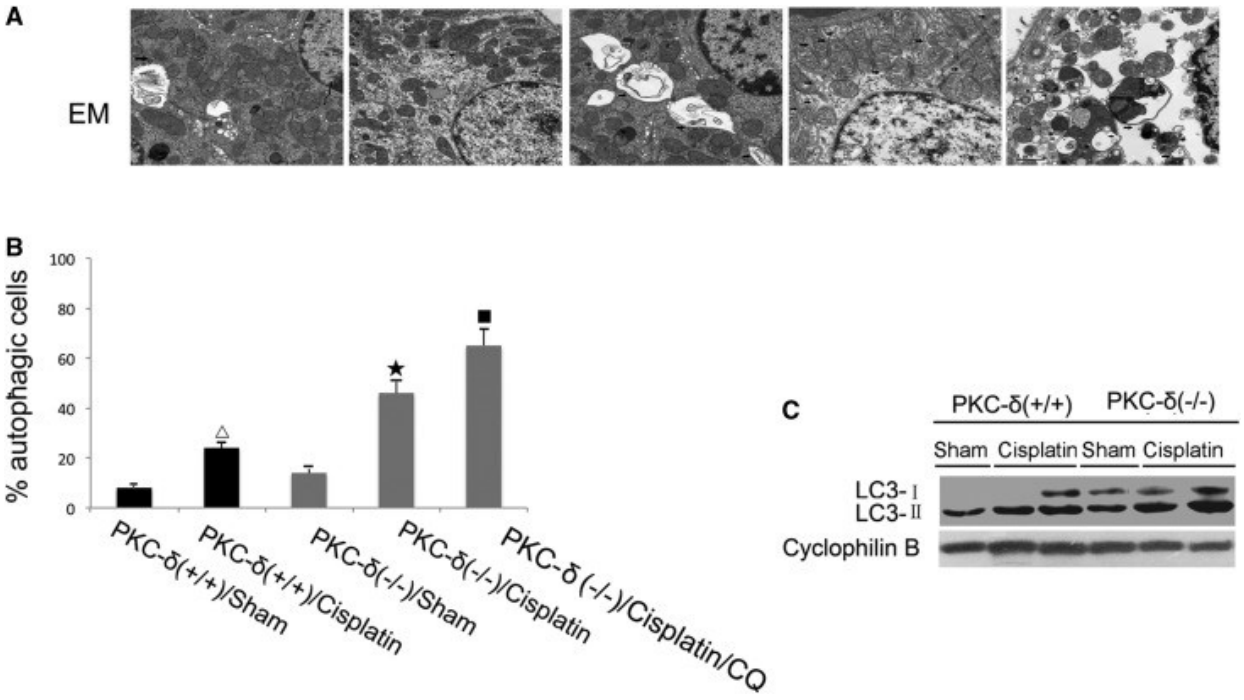
Effects of PKCδ inhibition on cisplatin-induced apoptosis in RPTC. (A–C) RPTC were transfected with PKCδ-KD or PKCδ-CF, and then treated with 20 μM cisplatin for 16 hours. (A) Morphology. After treatment, cells were stained with Hoechst33342 to record cellular and nuclear morphology by phase-contrast and fluorescence microscopy. Bar, 100 μm. (B) Percentage of apoptosis assessed by counting the cells with typical apoptotic morphology. (C) Caspase activity. Cell lysate was collected for enzymatic assay of caspase activity. Δ, statistically significantly different from the Vector group (P<0.05); ▲, statistically significantly different from Cisplatin+vector group (P<0.05). Data are representative of at least four separate experiments.

Figure 7.



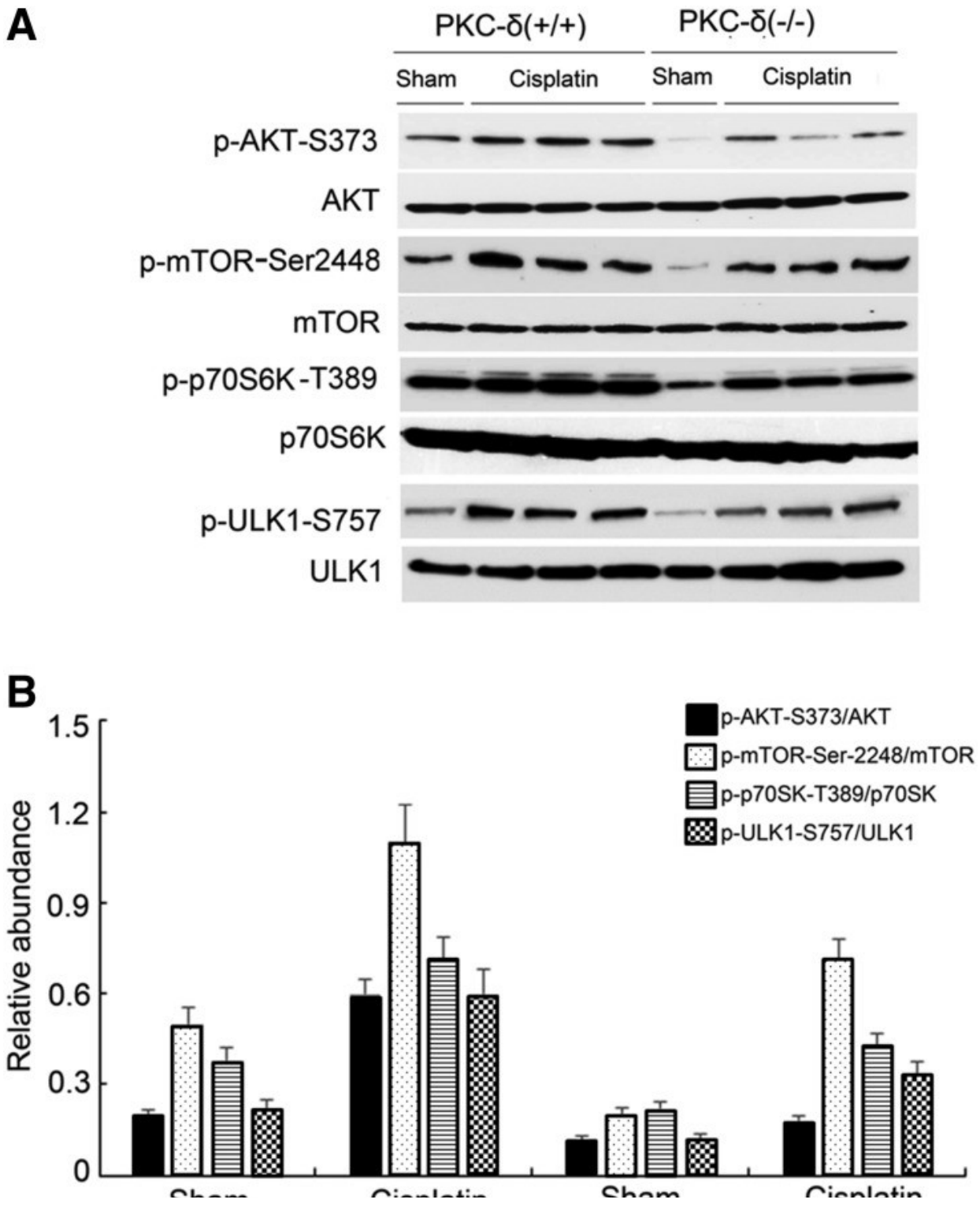
Restoration of cisplatin-induced kidney injury in PKC*δ*-null mice by CQ. (A–D) Wild-type and *PKCδ−/−* littermate mice (male, 8–10 weeks of age) were injected with 30 mg/kg cisplatin in the absence or presence of 50 mg/kg per day CQ. At day three, the animals were euthanized to collect blood samples to measure BUN (A) and serum creatinine (B). Kidney tissues were collected for H&E staining of histology (C), TUNEL assay of apoptosis (D), and tubular damage and TUNEL-positive apoptosis were also semiquantified (E, F). *Δ*, statistically significantly different from the Sham group without cisplatin treatment ($P<0.05$); ★, statistically significantly different from *wild-type* mice with cisplatin treatment group ($P<0.05$); ■, statistically significantly different from *PKCδ−/−* mice with cisplatin treatment group ($P<0.05$).

Figure 8.



PKC*δ*-null mice show higher renal autophagy. Wild-type and *PKCδ−/−* mice were injected with 30 mg/kg cisplatin. (A, B) Kidney tissues were collected for electron microscopy analysis to determine the percentage of tubular cells with notable autophagic vesicles. (B) Kidney tissues were analyzed for immunoblot of LC3 and cyclophilin B (loading control). *Δ*, statistically significantly different from the Sham group without cisplatin treatment ($P<0.05$); ★, statistically significantly different from *wild-type* mice with cisplatin treatment group ($P<0.05$); ■, statistically significantly different from *PKCδ−/−* mice with cisplatin treatment group ($P<0.05$). Data are representative of at least four separate experiments.

Figure 9.



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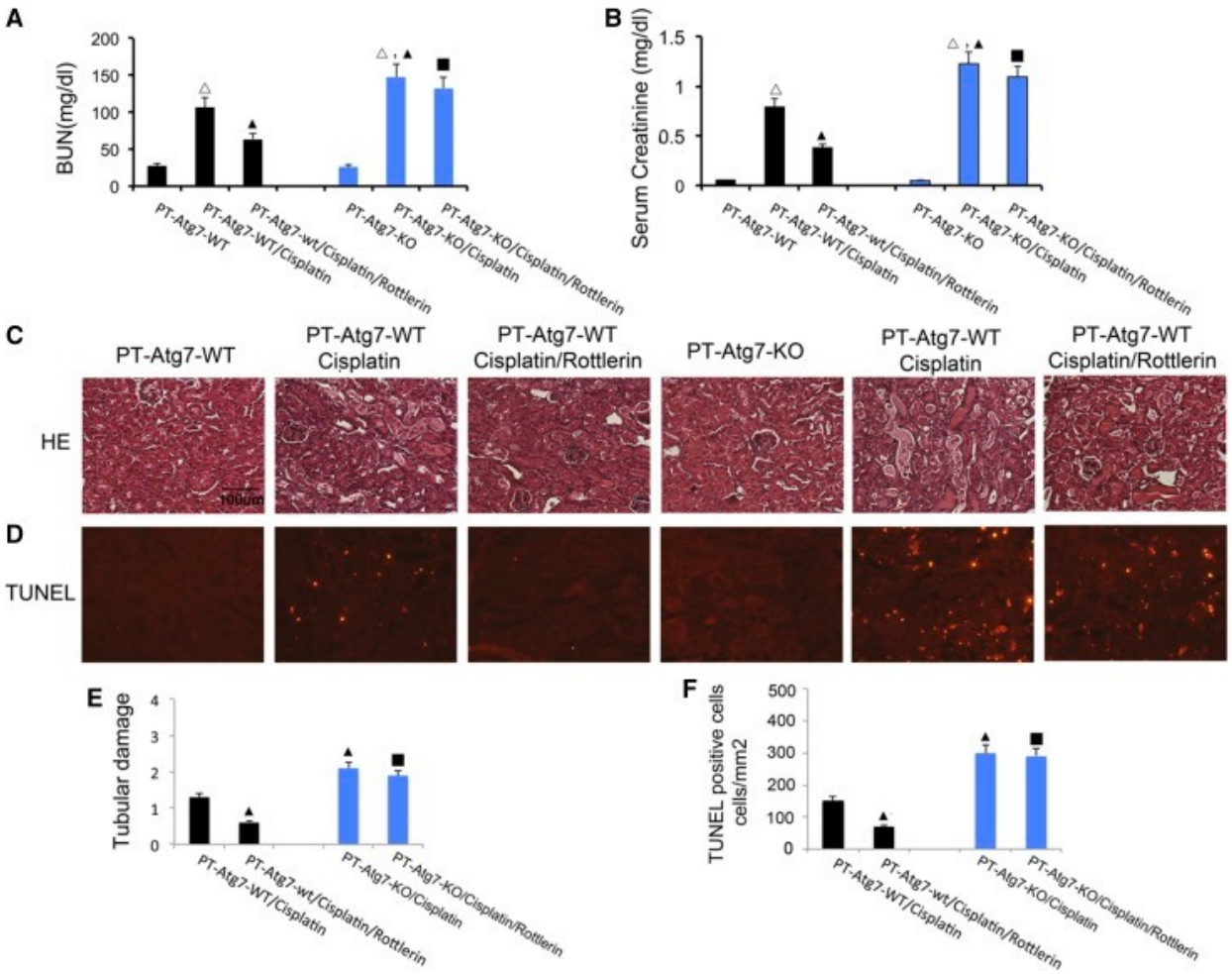
Cisplatin-induced AKT/mTOR activation in kidney tissues is blocked in PKC*δ*−/− mice. Wild-type and PKC*δ*−/− mice were injected with 30 mg/kg cisplatin to collect whole kidney lysates for immunoblotting analysis of total and phosphorylated AKT, mTOR, p70S6k, and p-ULK1. (A) Representative immunoblots. (B) Densitometry analysis of proteins signals on immunoblots.

Figure 10.



The renoprotective effect of rottlerin is lost in kidney autophagy deficient mice. PT-Atg7-WT and *PT-Atg7-KO* mouse littermates (male, 8–10 weeks of age) were injected with 30 mg/kg cisplatin in the absence or presence of 10 mg/kg/day rottlerin. Blood samples were collected at 3 days to measure BUN (A) and serum creatinine (B). Kidney tissues were collected for H&E staining of histology (C) and TUNEL assay of apoptosis (D). Tubular damage and apoptosis were also semiquantified (E, F). *Δ*, statistically significantly different from the control group without cisplatin treatment ($P<0.05$); ▲, statistically significantly different from PT-Atg7-WT/cisplatin group ($P<0.05$); ■, not different from *PT-Atg7-KO*/cisplatin group ($P>0.05$). Data are representative of at least four separate experiments.