

Review

Volume-sensitive Cl^- Channel as a Regulator of Acquired Cisplatin Resistance

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Abstract. *The platinum-based drug cisplatin (cis-diamminedichloroplatinum (II)) is widely used in cancer therapy. However, cancer cells can develop resistance after exposure to cisplatin. Recently, many studies have pointed to the involvement of plasma membrane ion channels in a cell's response to cisplatin. Our group has found that pretreatment with cisplatin enhanced the activity of volume-sensitive Cl^- channels in human epidermoid cancer KB cells; cisplatin-resistant KCP-4 cells derived from KB cells, on the other hand, lacked functional expression of these channels. This suggests that the activity of volume-sensitive Cl^- channels is an important factor in determining the sensitivity of cancer cells to cisplatin. Furthermore, when volume-sensitive Cl^- channel function was partially restored in cisplatin-resistant KCP-4 cells treated with a histone deacetylase inhibitor, KCP-4 cells exhibited a restoration of sensitivity to cisplatin; this increased sensitivity was inhibited by a volume-sensitive Cl^- channel blocker. We therefore propose that impaired activity of the volume-sensitive Cl^- channel is involved in the acquired cisplatin resistance of these cancer cells. In this review, we will outline the relationship between volume-sensitive Cl^- channels, cisplatin-induced apoptosis, and cisplatin resistance. Activating the volume-sensitive outwardly-rectifying Cl^- channel may be a new strategy in treating clinical cisplatin resistance.*

The platinum-based drug cisplatin (*cis*-diamminedichloroplatinum (II)) is an anticancer drug widely used in chemotherapy for a variety of cancers. Cisplatin forms intra- and interstrand adducts with DNA in cancer cells which trigger signaling pathways that lead to apoptosis (1-3).

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Key Words: Cl^- channel, cancer, cell volume, apoptosis, cisplatin, drug resistance, review.

However, it is well-known that cancer cells can become resistant to cisplatin; such acquired cisplatin resistance is a major problem in cancer chemotherapy. The mechanisms which are responsible for resistance to cisplatin have been classified into several categories, namely, reduced intracellular accumulation of cisplatin, detoxification of cisplatin, increased DNA repair or DNA tolerance, and disruption of apoptosis (1-5); however, resistance to cisplatin is a complex phenomenon which depends on the cell type and often involves several different mechanisms.

In the last ten years, many studies have demonstrated that ion channels involved in cell volume regulation have a pivotal role in apoptotic cell death (6, 7). Specifically, cell shrinkage induced by activation of ion channels has been found to be essential to the execution of apoptosis in a variety of cell types. In addition, we have recently demonstrated that the functional expression of volume-sensitive Cl^- channels, which are the most important component in cell volume regulation after cell swelling (8), correlated with lower cisplatin resistance (9). In this review, we will summarize recent reports about the relationship between ion channels and a cell's response to cisplatin.

Cisplatin Triggers Apoptosis

Cisplatin is a DNA-damaging reagent that attacks cancer cells by forming intra- and interstrand adducts with DNA. Severe, irreparable damage to DNA leads to cell death. Although the type of cell death is dependent on the concentration of cisplatin, cisplatin is thought to induce apoptosis at the chemotherapeutic concentration (10, 11). Apoptotic cell death is morphologically and biochemically characterized by cell shrinkage, cytochrome c release, caspase activation and DNA laddering. Cisplatin causes these phenomena in a variety of cells (12-15), consistent with the idea that it causes apoptosis. We have demonstrated that cisplatin-induced apoptosis associates with caspase-3 activation begins within 24 h of treatment in human epidermoid cancer KB cells (16). It has been reported that DNA damage caused by cisplatin

activates a variety of signal transduction pathways, including those involving AKT (v-akt murine thymoma viral oncogene homologue), p53 and MAPK (mitogen-activated protein kinase) / JNK (c-Jun NH₂-terminal kinase) / ERK (extracellular signal-regulated kinase) (2, 3). However, we are still far from a complete understanding of how cisplatin triggers apoptosis in cancer cells.

Ion Channels are Involved in Cisplatin-induced Apoptosis

Ion channels are known to have a role in the development of cancer. The roles of K⁺ channels, such as voltage-gated K⁺ channels and Ca²⁺-activated K⁺ channels, and Cl⁻ channels in the proliferation of cancer cells have been well summarized (17). In particular, because cell volume changes are necessary for tumor cell proliferation, ion channels involved in cell volume regulation are thought to have an important role in cancer growth. In addition to K⁺ channels, volume-activated Cl⁻ channels, which are involved in controlling cell volume, have recently been proposed as potential targets for cancer therapy (18, 19).

Almost all animal cells can restore their original volume after volume changes due to osmolarity imbalances across the plasma membrane. The cell volume regulation after cell swelling or shrinkage is called regulatory volume decrease (RVD) or regulatory volume increase (RVI), respectively (8). During these processes, ion movements provide a driving force for water. That is, upon cell swelling, KCl efflux from cells is induced mainly by activation of K⁺ and Cl⁻ channels; cell shrinkage, on the other hand, causes cotransporter-mediated NaCl influx as well as hypertonicity-induced cation channel (HICC)-mediated Na⁺ influx after cell shrinkage (20, 21). Thus, ion movements are essential for inducing cell volume changes. It is well known that the Cl⁻ efflux necessary for RVD is commonly mediated by the volume-sensitive outwardly-rectifying (VSOR) Cl⁻ channel expressed in most cells (8, 22, 23). In addition to its volume regulatory role, it has recently become clear that the VSOR Cl⁻ channel also has an important role in apoptosis (24).

A major hallmark of apoptosis is normotonic cell shrinkage called apoptotic volume decrease (AVD) that occurs at an early phase preceding caspase activation, DNA laddering, and cell fragmentation (24, 25). It has been demonstrated that ion channels are involved in AVD; since the RVD process was facilitated in cells undergoing apoptosis and AVD was inhibited by blockers of K⁺ or Cl⁻ channels, it has been suggested that AVD occurs by appropriating components normally used in the RVD mechanism (24, 26). We have previously demonstrated that VSOR Cl⁻ channels are activated even upon normotonic conditions by the apoptotic inducers staurosporine, tumor necrosis factor (TNF) α , Fas ligand, and H₂O₂ in human

epithelial HeLa cells (27). In addition, similar Cl⁻ currents are observed in other types of cells treated with staurosporine (28), Fas ligand (29), or H₂O₂ (30, 31). On the other hand, apoptotic inducers are reported to activate a variety of K⁺ channel types (32-39). These findings have led to the concept that AVD is caused by coupled activation of K⁺ channels and VSOR Cl⁻ channels and that the VSOR Cl⁻ channel is a common fundamental component of the AVD process. Importantly, since prevention of AVD induction by ion channel blockers suppressed subsequent biochemical events such as caspase activation and DNA fragmentation, resulting in inhibition of mitochondrial pathway-mediated or death-receptor pathway-mediated apoptosis in epithelial, lymphoid and neuronal cells (25), it appears that AVD is one of the determinant factors in apoptotic cell death.

What is the role of ion channels in cisplatin-induced apoptosis? We recently found that not only caspase-3 activation, but also a decrease in cell viability induced by cisplatin was inhibited by a stilbene-derivative Cl⁻ channel blocker, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) in human epidermoid cancer KB cells, as shown in Figure 1. In addition, VSOR Cl⁻ channel currents were found to be prominently augmented when cells were pretreated with cisplatin for 12 h (16). The currents were blocked by application of DIDS (16). These findings suggest that activation of the VSOR Cl⁻ channel is involved in cisplatin-induced apoptosis. As for K⁺ fluxes, it has been reported that amphotericin B, a K⁺ ionophore, influences the cytotoxicity of cisplatin (40-42). Marklund and coworkers demonstrated that amphotericin B combined with bumetanide, a blocker of the Na⁺-K⁺-2Cl⁻ cotransporter, remarkably augmented caspase-3 activity and nucleosome formation induced by cisplatin (43, 44). Investigating the role of K⁺ channels in cisplatin-induced apoptosis, Liang and coworkers found that Ca²⁺-activated, voltage-dependent big conductance K⁺ (BK) channels were activated in cisplatin-treated fibrocytes and that blockade of BK channels by tetraethyl ammonium (TEA), a non-specific K⁺ channel blocker, inhibited cisplatin-induced apoptosis (45). We found that blockage of the Ca²⁺-activated, intermediate conductance K⁺ (IK) channel inhibits cisplatin-induced apoptosis in KB cells (E. Lee and Y. Okada, unpublished data). These results suggest that K⁺ efflux, mediated by activation of Ca²⁺-activated K⁺ channels, is important for cisplatin-induced apoptosis. Since cisplatin treatment enhances the activity of VSOR Cl⁻ and causes the activation of K⁺ channels, there is a strong possibility that AVD occurs in, and is necessary for, cisplatin-induced apoptosis.

Cancer cells can develop resistance to cisplatin; in cells with acquired cisplatin resistance, cisplatin no longer induces apoptosis. The evidence outlined above suggests

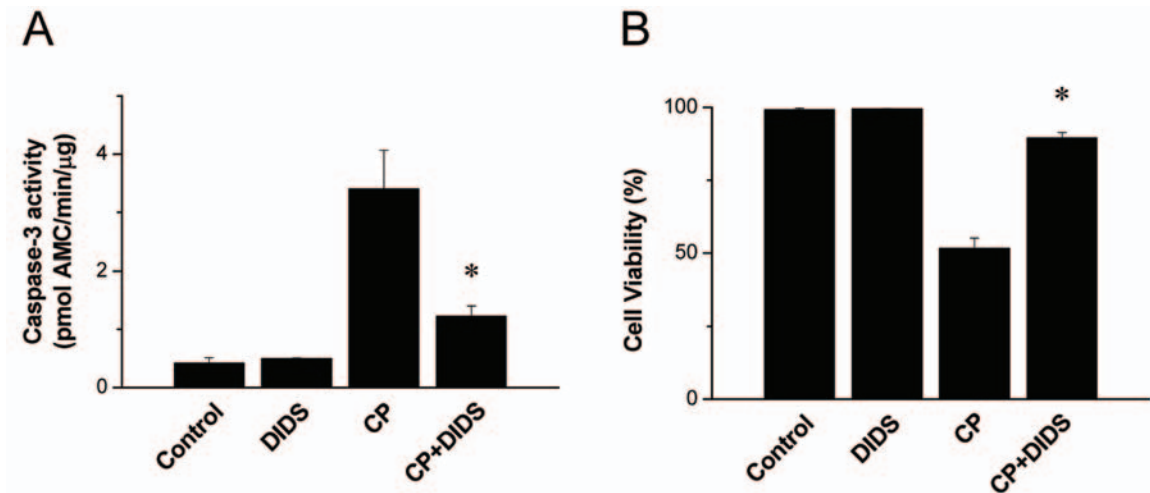


Figure 1. Cisplatin induced apoptosis in KB cells. Each bar represents the mean \pm S.E.M. from 4 independent experiments. A, Caspase-3 activity 18 h after cisplatin treatment. B, Cell viability 24 h after cisplatin treatment. CP, cisplatin; *Significant difference ($p < 0.05$) between data obtained in the absence and presence of 100 μ M DIDS in cisplatin-pretreated KB cells.

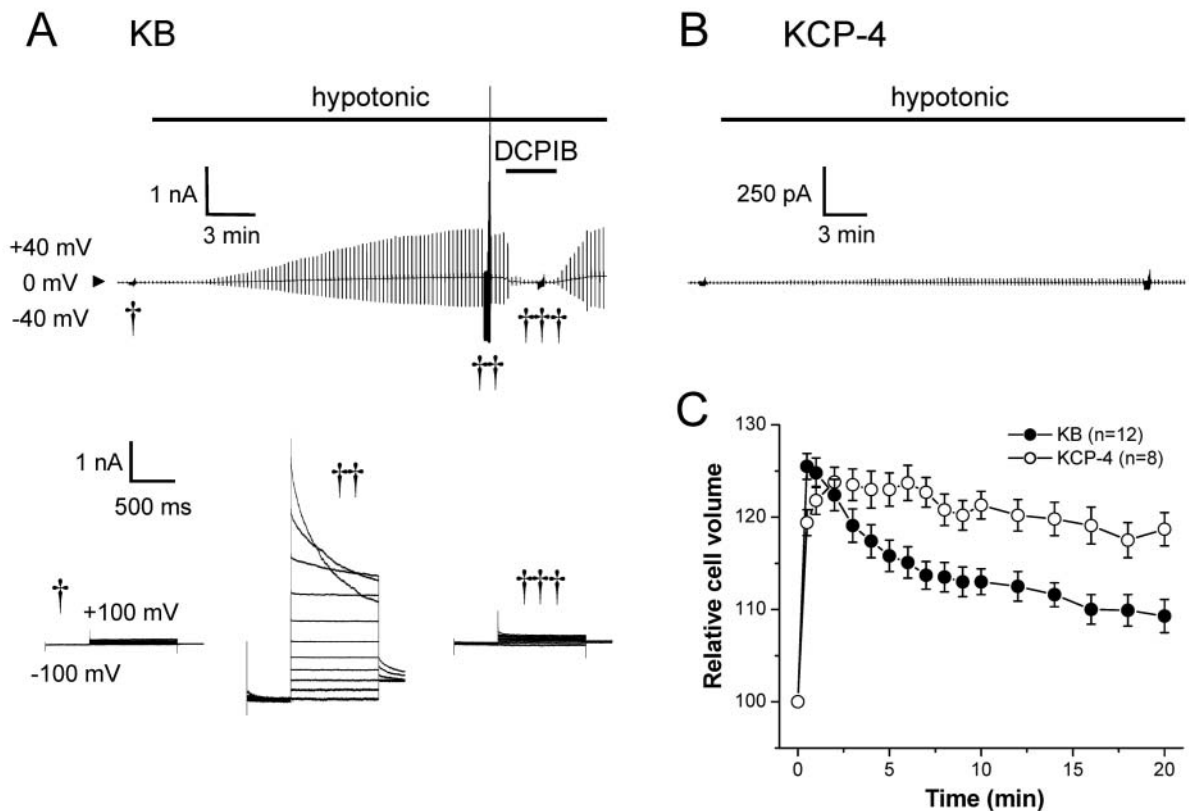


Figure 2. Parental KB cells exhibit functional expression of VSOR Cl⁻ currents and cell volume regulation (RVD) under hypotonic conditions but cisplatin-resistant KCP-4 cells do not. A, A representative whole-cell recording of VSOR Cl⁻ currents in KB cells before and during application of hypotonic solution in the absence or presence of 10 μ M DCPIB. Step pulses from -100 to +100 mV in 20-mV increments were applied at time points indicated by daggers. B, A representative whole-cell recording of VSOR Cl⁻ currents in KCP-4 cells before and during application of hypotonic solution. C, Cell volume changes during application of hypotonic solution in KB and KCP-4 cells, measured with a Coulter-type cell counter. Each symbol represents the mean \pm S.E.M. Data in the two groups were significantly different starting at 4 min of hypotonic exposure.

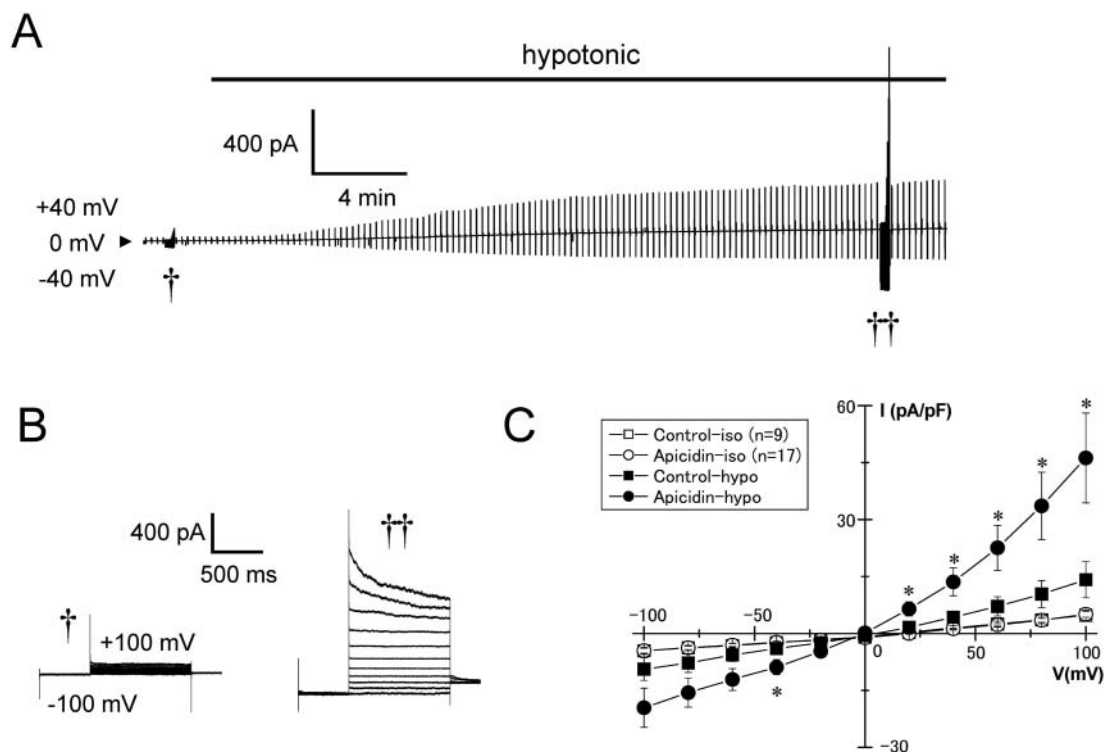


Figure 3. Restoration of VSOR Cl⁻ currents in cisplatin-resistant KCP-4 cells pretreated with 1.6 μM apicidin for 24-36 h. A, A representative whole-cell recording of VSOR Cl⁻ currents before and during application of hypotonic solution. B, Step pulses from -100 to +100 mV in 20-mV increments were applied at time points indicated by daggers. C, I-V relationships for VSOR Cl⁻ currents. Each symbol represents the mean ± S.E.M. *Significantly different ($p < 0.05$) compared to currents recorded in vehicle-treated KCP-4 cells.

that the VSOR Cl⁻ channel is involved in cisplatin-induced apoptosis. To examine whether it might be involved in cisplatin resistance, we measured volume-sensitive Cl⁻ currents in a cisplatin-resistant cell line, the KCP-4 cell line, which is derived from KB cells (46). In KB cells, osmotic swelling induced whole-cell currents, as shown in Figure 2A. The current exhibited moderate outward rectification and time-dependent inactivation at positive potentials. It was inhibited by the non-specific Cl⁻ channel blockers DIDS and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), as well as by the specific VSOR Cl⁻ channel blockers phloretin (47) and 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB) (48). These biophysical and pharmacological properties are consistent with those of VSOR Cl⁻ channels (8, 9, 16). However, our previous study (9) surprisingly showed that VSOR Cl⁻ currents were undetectable in cisplatin-resistant KCP-4 cells, as shown in Figure 2B, although the cells exhibited osmotic swelling to the same extent. As expected from the absence of VSOR Cl⁻ currents and in contrast to KB cells, cisplatin-resistant KCP-4 cells did not show RVD, the process by which most animal cells recover after osmotic swelling (Figure 2C).

Since almost all animal cells are known to have VSOR Cl⁻ channel activity (8), this is a unique phenotype. This finding suggests that the loss of VSOR Cl⁻ channel function contributes to the acquisition of cisplatin resistance in KCP-4 cells, possibly by impairing the mechanism of AVD and preventing the apoptosis that would normally occur in response to cisplatin treatment.

The Restoration of Functional VSOR Cl⁻ Channels Causes a Decrease in Cisplatin Resistance

A cisplatin-resistant cancer cell line derived from a cisplatin-sensitive parental cancer cell line is often used as a model of acquired cisplatin resistance. Because of the fact that the cisplatin-resistant cell line originates from the parental cell line, the two lines are closely related and it is reasonable to expect that the profile of genes expressed is very similar except for the genes involved in cisplatin resistance. The absence of VSOR Cl⁻ current in cisplatin-resistant KCP-4 cells is therefore likely to result from the alteration of a gene (or genes) involved in cisplatin resistance.

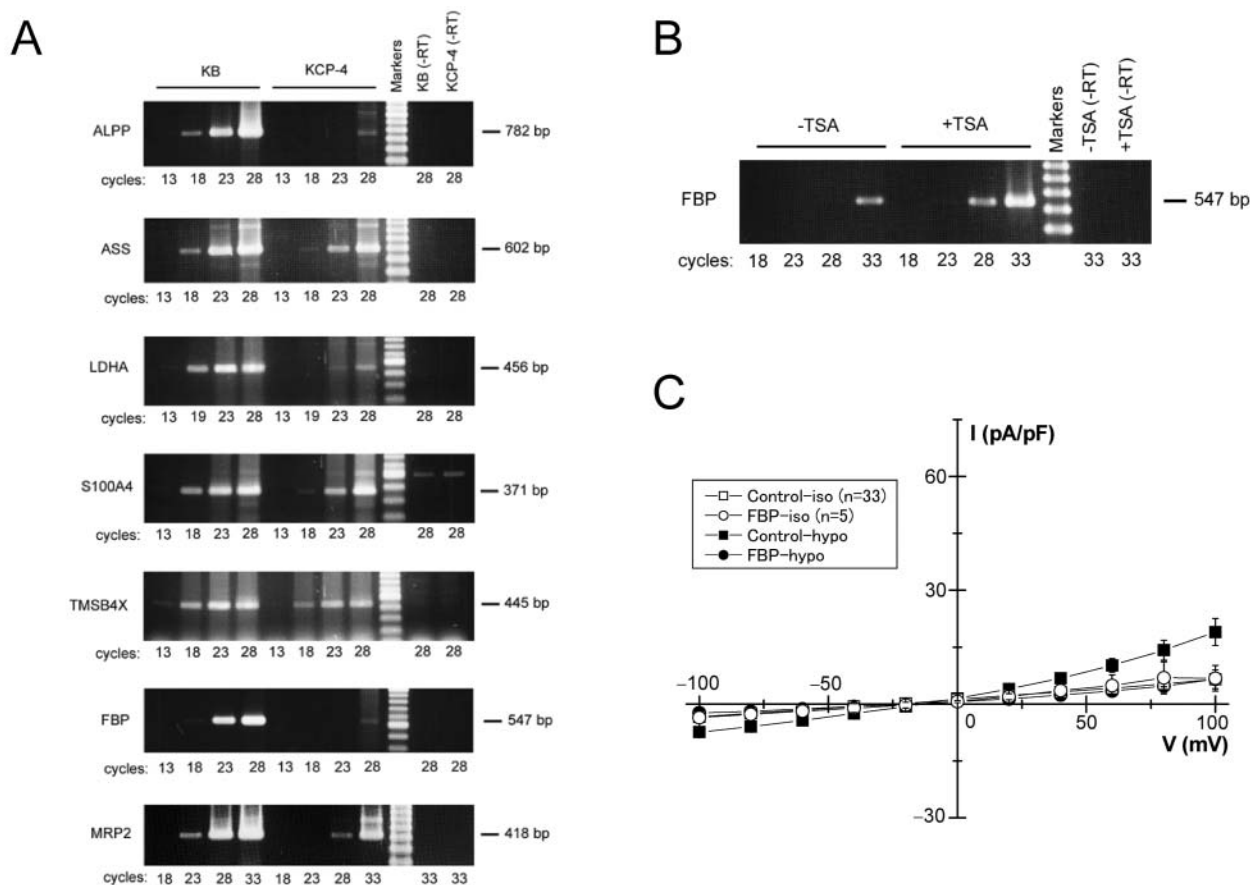


Figure 4. Gene expression differences in KB, KCP-4 and TSA-treated KCP-4 cells. *A*, Semi-quantitative RT-PCR results showing that the expression levels of 7 genes were lower in KCP-4 cells compared to KB cells. Aliquots (5 μ l) from PCR reactions were taken at the cycles indicated. PCR product sizes in base pairs (bp) are shown. (-RT), negative controls in which reverse transcriptase was excluded from the cDNA synthesis reaction. Markers, 100 bp DNA ladder. See text for gene abbreviations. *B*, Semi-quantitative RT-PCR result showing that FBP transcript levels were upregulated in KCP-4 cells pretreated with 400 nM TSA. *C*, I-V relationships for VSOR Cl⁻ currents in mock- and FBP-transfected KCP-4 cells. A plasmid construct containing the open reading frame of FBP in the multiple cloning site of pIRES2-EGFP (Clontech) was used for transient transfections, carried out 24-36 h before recordings. Each symbol represents the mean \pm S.E.M.

To begin investigating whether this is the case, we tested, in KCP-4 cells, the effects of a drug known to cause global changes in gene expression. We found that treatment with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, partially restored VSOR Cl⁻ currents in the cells (9). HDACs are known to play a fundamental role in regulating gene expression (49, 50); their inhibition by drugs, such as TSA, results in the restoration of silenced genes (50, 51). Although there is evidence that HDAC inhibitors enhance sensitivity to anticancer drugs including cisplatin (52), the gene expression changes that may be responsible for the increased cytotoxicity are not well understood.

We show here that apicidin, an HDAC inhibitor structurally unrelated to TSA (51, 53), is also able to restore VSOR Cl⁻ currents in cisplatin-resistant KCP-4 cells (Figure

3). The currents showed time-dependent inactivation at positive potentials (Figure 3B) and moderate outward rectification (Figure 3C). These results support the idea that the restoration of VSOR Cl⁻ channel activity by TSA is due to changes in HDAC activity, which cause alterations in gene transcription, and not to a direct interaction of the drug with the channel protein.

With TSA treatment, cisplatin-resistant KCP-4 cells became sensitive to cisplatin, as observed in a cell viability assay (9). The increased sensitivity to cisplatin resulting from TSA treatment correlated with increased caspase-3 activation, suggesting that cisplatin induced apoptosis in TSA-treated KCP-4 cells. In addition, it was found that the Cl⁻ channel blocker DIDS blocked not only the decrease in cell viability but also the increase in caspase-3 activity resulting from cisplatin treatment of these cells (9). These

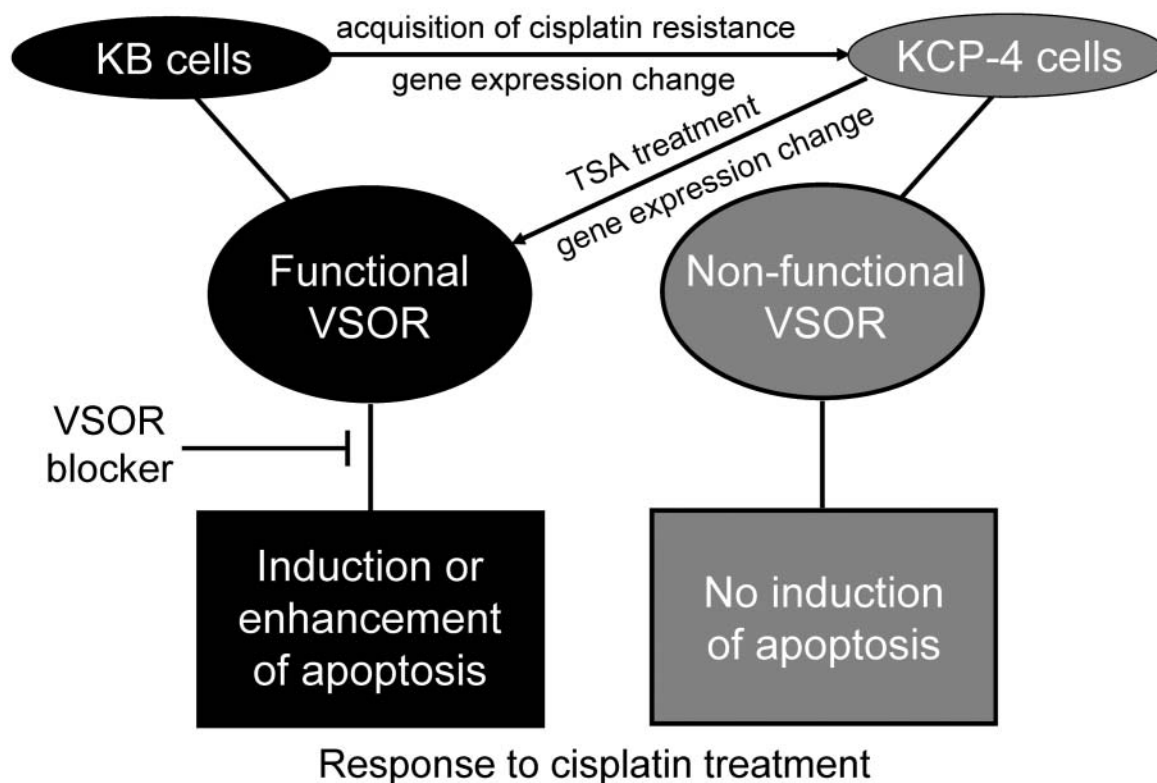


Figure 5. A schematic summarizing of the relationship between VSOR Cl^- channels and the response of KB and KCP-4 cells to cisplatin treatment.

results provide further evidence that the activity of VSOR Cl^- channels is important for cisplatin-induced apoptosis and suggest that the loss of VSOR Cl^- channel activity contributes to the development of the cisplatin-resistant phenotype.

What Genes are Altered in Acquired Cisplatin Resistance?

It is clear that gene expression changes are important in the loss of VSOR Cl^- channel function and the acquisition of cisplatin resistance in KCP-4 cells. To identify the changes that might be responsible, we have investigated the differences in gene expression between KCP-4 cells and the parental KB cells, by a subtractive hybridization method. Using semi-quantitative RT-PCR, we have confirmed that the expression of 5 genes identified by subtractive hybridization – placental alkaline phosphatase (ALPP), argininosuccinate synthetase (ASS), lactate dehydrogenase (LDHA), S100 calcium binding protein A4 (S100A4) and thymosin β 4 (TMSB4X) – were reduced in cisplatin-resistant KCP-4 cells, as shown in Figure 4A (upper 5 images). In addition, because multidrug resistance-associated protein 2 (MRP2), also called cMOAT or ABCC2, was previously found to be down-regulated in KCP-4 cells (54) and folate binding protein

(FBP), also called folate receptor, was down-regulated in cisplatin-resistant cell lines derived from KB cells (55, 56), we examined their gene expression levels. Consistent with the previous reports, these genes were expressed at lower levels in cisplatin-resistant KCP-4 cells (Figure 4A; bottom 2 images). Although MRP2 is known to work as a drug efflux protein at the plasma membrane (57), the transcript expression level of MRP2 appears to indicate that it is not a contributor to acquired cisplatin resistance in cisplatin-resistant KCP-4 cells.

To determine whether changes in any of these genes might contribute to the restoration of VSOR Cl^- channel function in TSA-treated KCP-4 cells, we compared their expression in TSA-treated and untreated KCP-4 cells. Of the 7 genes examined, only FBP was restored after a 24 h treatment with TSA, as shown in Figure 4B. This result suggests the possibility that a reduction in the levels of FBP is responsible for the loss of VSOR Cl^- channel function. However, the possibility that a reduction in FBP by itself accounts for the loss of function is unlikely, because overexpression of FBP did not restore VSOR Cl^- current in cisplatin-resistant KCP-4 cells (Figure 4C). To clarify how the channel function is reduced in KCP-4 cells and restored by TSA treatment, a more complete examination of the

gene expression profiles of parental KB and cisplatin-resistant KCP-4 cells, and TSA-treated KCP-4 cells, is therefore necessary. While the expression levels of the 7 genes examined here were confirmed to be reduced in cisplatin-resistant KCP-4 cells, the roles of these genes in acquired cisplatin resistance are not yet clear.

Conclusion and Perspectives

Resistance of cancer cells to cisplatin treatment is a major problem in cancer therapy. Some types of cancer cells have high intrinsic resistance to cisplatin. On the other hand, acquired cisplatin resistance can be developed through chronic exposure to cisplatin. Although a number of mechanisms are known to contribute to cisplatin resistance, the role of ion channels in cisplatin resistance is only beginning to be understood. A better understanding of their role could potentially lead to new strategies for treating cisplatin-resistant cancer types.

Here, we have summarized the role of VSOR Cl⁻ channels in cisplatin-induced apoptosis and cisplatin resistance (Figure 5). The observation that parental cancer KB cells have functional VSOR Cl⁻ channels whose activity is augmented by cisplatin pretreatment and whose blockage leads to reduced cisplatin sensitivity, along with the exciting finding that cisplatin-resistant KCP-4 cells have virtually no functional VSOR Cl⁻ channels at all, strongly suggest that the channel is an essential part of the mechanism of cisplatin-induced apoptosis in these cells. As VSOR Cl⁻ channels are known to be part of the mechanism of AVD, a requisite process in apoptotic induction (6, 7, 24, 25), such a conclusion seems quite reasonable. Further evidence supporting a role of the channel in the response to cisplatin is that cisplatin-resistant KCP-4 cells gained sensitivity to cisplatin when the activity of VSOR Cl⁻ channels was partially restored by pretreatment of the cells with TSA, an HDAC inhibitor. Although much is unknown about how HDAC inhibitors enhance the sensitivity of cancer cells to anticancer drugs, we propose that restoration of VSOR Cl⁻ channel activity may be one mechanism by which they exert this effect (Figure 5).

VSOR Cl⁻ channels have not been molecularly identified yet, although several candidates, including P-glycoprotein (58), pICln (59) and ClC-3 (60), all considered unlikely now, have been proposed. A thorough comparison of gene transcripts, or protein products, in cisplatin-resistant KCP-4 cells and KB cells could be a strategy for identifying the molecule corresponding to the VSOR Cl⁻ channel, in addition to other molecules involved in cisplatin resistance. A similar comparison could be carried out using TSA-treated and untreated KCP-4 cells. Besides determining the identity of the VSOR Cl⁻ channel molecule, finding channel activators could be of clinical importance. Currently, pharmacological activators of the channel are not available;

because activation of the VSOR Cl⁻ channel may be a strategy for combatting cisplatin resistance, identifying activators could be a step towards more effective therapies.

Acknowledgements

The authors thank Dr. N. Takahashi, Dr. T. Numata and M. Ohara for technical assistance, and T. Okayasu for secretarial assistance. This work was supported by Grants-in-Aid for Scientific Research from MEXT and JSPS.

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Received September 13, 2007

Revised November 14, 2007

Accepted December 17, 2007