Mechanisms of Cadmium-Induced Proximal Tubule Injury: New Insights with Implications for Biomonitoring and Therapeutic Interventions

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List of non-standard abbreviations

ATSDR Agency for Toxic Substances and Disease Registry
GSK-3β Glycogen synthase kinase-3β
Kim-1 Kidney injury molecule-1
RT-PCR Reverse transcriptase polymerase chain reaction
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Abstract
Cadmium (Cd) is an important industrial agent and environmental pollutant that is a major cause of kidney disease. With chronic exposure, Cd accumulates in the epithelial cells of the proximal tubule resulting in a generalized reabsorptive dysfunction characterized by polyuria and low molecular weight proteinuria. The traditional view has been that as Cd accumulates in proximal tubule cells, it produces a variety of relatively non-specific toxic effects that result in the death of renal epithelial cells through necrotic or apoptotic mechanisms. However, a growing volume of evidence suggests that rather than merely being a consequence of cell death, the early stages of Cd-induced proximal tubule injury may involve much more specific changes in cell-cell adhesion, cellular signaling pathways, and autophagic responses that occur well before the onset of necrosis or apoptosis. In this commentary, we summarize these recent findings and we offer our own perspectives as to how they relate to the toxic actions of Cd in the kidney. In addition, we highlight recent findings suggesting that it may be possible to detect the early stages of Cd toxicity through the use of improved biomarkers. Finally, some of the therapeutic implications of these findings will be considered. Since Cd is, in many respects, a model, cumulative nephrotoxicant, these insights may have broader implications regarding the general mechanisms through which a variety of drugs and toxic chemicals damage the kidney.
Cd as an Environmental Health Problem. Cadmium (Cd) is a widespread environmental pollutant that is a major cause of kidney disease in many regions of the world. Cd is normally found at low concentrations throughout the lithosphere, but has become increasingly concentrated in the biosphere through mining, smelting, agricultural and industrial activities of humans. As a stable, divalent cation, Cd is not biodegradable and persists in the environment. Despite efforts by many countries and international agencies to reduce the usage of Cd, it continues to be a major public health problem, especially in emerging industrial nations where environmental controls are still being developed (Jarup and Akesson, 2009; Nordberg, 2004; Satarug et al., 2003; Teeyakasem et al., 2007).

Humans are typically exposed to Cd either in the workplace or through the ingestion of Cd-contaminated food or water (ATSDR, 2008; Jarup and Akesson, 2009; Satarug et al., 2010). Tobacco contains significant amounts of Cd, and smoking is a major source of exposure among the general population (ATSDR, 2008; Menke et al., 2009; Satarug and Moore, 2004). Depending on the dose, route and duration of exposure, Cd can damage various organs including the lung, liver, kidney and bone. Cd can also act as an endocrine disruptor, and it is carcinogenic. (for reviews see ATSDR, 2008; Byrne et al., 2009; Jarup et al., 1998; Joseph, 2009; Waalkes, 2003).

With the chronic, low-level patterns of Cd exposure that are commonly seen in human populations, the primary target organ of Cd toxicity is the kidney, where Cd causes a generalized dysfunction of the proximal tubule characterized by polyuria and increases
in the urinary excretion of glucose, amino acids, electrolytes (particularly Na\(^+\), K\(^+\) and Ca\(^{2+}\)) and low molecular weight proteins (ATSDR, 2008; Jarup, 2002). A growing volume of evidence indicates that adverse renal effects of Cd can result from even low levels of Cd exposure and that women, children and individuals with confounding health conditions, such as diabetes, may be especially susceptible (Akesson et al., 2005; ATSDR, 2008; Jarup, 2002; Navas-Acien et al., 2009; Nawrot et al., 2008; Satarug et al., 2003; Suwazono et al., 2010; Thomas et al., 2009).

**Evolving View of the Mechanisms of Cadmium Nephrotoxicity.** While the general effects of Cd on proximal tubule function have been well-documented, the specific molecular mechanisms that underlie these effects are not yet fully understood. It should be emphasized that the current uncertainties are not merely due to a lack of attention or information. Even though it is fashionable for authors of these types of reviews (including these authors) to state that “little is known” about the mechanisms of Cd toxicity, such statements are not completely true. In reality, a great deal is actually known regarding the basic molecular mechanisms by which Cd can alter renal epithelial cell function (for reviews see Jarup, 2002; Prozialeck, 2000; Thevenod, 2009). The problem is that much of this information has been generated from studies on renal epithelial cells in culture, and the relevance of many of these findings to the nephrotoxic effects of Cd *in vivo* remain unclear. This issue has been complicated by the unusual toxicokinetics of Cd in the body and by the ability of Cd to interact with a vast array of biological molecules. When using *in vitro* models, it is difficult, if not impossible, to mimic the conditions under which renal epithelial cells are exposed *in vivo* and to sort out the
relevant biological effects from the irrelevant. However, while the problems of identifying the mechanisms of Cd toxicity have certainly been formidable, they have not been insurmountable. By applying modern techniques of cellular and molecular biology to the study of in vivo model systems, investigators have, in fact, managed to obtain new insights into the molecular basis of Cd-induced proximal tubular injury.

The purpose of this commentary is to highlight some of the most significant findings in this evolving field of research. This will not be a comprehensive review, but will focus on recent in vivo findings showing that the early stages of Cd nephrotoxicity involve specific changes in proximal tubule cell-cell adhesion, cellular signaling cascades and autophagic responses that occur before the onset of necrosis or apoptosis of proximal tubule cells. We will also highlight recent findings suggesting that it may be possible to detect these early stages of Cd toxicity through the use of improved biomarkers, such as kidney injury molecule-1 (Kim-1). In discussing these topics, we will consider aspects of the toxicokinetics of Cd in vivo and some of the key pathologic events that are associated with the onset of proximal tubule injury. Finally, some of the potential therapeutic and mechanistic implications of these findings will be considered. In preparing the manuscript, we have tried to integrate and synthesize information from diverse sources to provide a concise over-view that will be of use to investigators in the Cd field. We have also tried to incorporate our own insights and perspectives that we have developed from working in the Cd field for many years. Since Cd is, in many respects a model cumulative nephrotoxin, these observations may have broader implications beyond the field of Cd toxicology.
Toxicokinetics of Cd in Vivo. Any discussion of the actions of Cd in the kidney must begin with consideration of the forms of Cd that exist under physiologic conditions and the distribution of these forms of Cd within the body. These topics have been the subject of several excellent reviews (Bridges and Zalups, 2005; He et al., 2009; Jin et al., 1998). However, they have often been overlooked in many of the in vitro studies on the cytotoxic actions of Cd, a fact that has greatly complicated the extrapolation of in vitro mechanistic findings to the actions of Cd in the whole kidney.

With respiratory exposure, Cd is efficiently absorbed from the lung; up to 40-60% of inhaled Cd reaches the systemic circulation. With oral exposure, the absorption of Cd from the gastrointestinal tract is considerably lower (only 5-10%). However, with long term exposure, even this low level of absorption from the gastrointestinal tract can lead to systemic accumulation of Cd and subsequent toxicities. The gastrointestinal absorption of Cd may be substantially higher in individuals with low body stores of iron, which is a factor that could contribute to individual variations in sensitivity to Cd exposure.

Once absorbed into the bloodstream, Cd is initially transported to the liver where it is taken up by hepatocytes and induces the synthesis of metallothionein, which binds Cd, and buffers its toxic effects in the cell. However, as the hepatocytes die off, either through normal turnover or as a result of Cd injury, the Cd-metallothionein complex can be released into the blood stream (Jin et al., 1998; Klaassen et al., 2009). Even though
the Cd-metallothionein complex is non-toxic to most organs, it can be filtered at the glomerulus and taken up by the epithelial cells of the proximal tubule. In this situation, Cd-metallothionein can have the paradoxical effect of facilitating the delivery of Cd from the liver to the kidney, and it has been suggested that Cd-metallothionein may actually mediate some of the toxic effects of Cd in proximal tubule (Klaassen and Liu, 1997). However, a great deal of evidence indicates that it is actually ionic Cd (Cd$^{+2}$), not Cd-metallothionein, that injures proximal tubule epithelial cells (Goyer et al., 1989; Klaassen et al., 2009). The fact that metallothionein-null animals are sensitive to Cd-induced proximal tubule injury provides compelling evidence that Cd-metallothionein does not play a critical role in directly mediating the nephrotoxic effects of Cd (Liu et al., 1998).

One especially important aspect of Cd disposition that has been overlooked frequently is that essentially all Cd in the plasma is bound to proteins or other molecules. The circulating Cd may either be tightly bound to specific metal binding proteins such as metallothionein (Klaassen et al., 2009; Klaassen and Liu, 1997), or may be loosely associated with molecules, such as albumin, amino acids or low molecular weight sulfhydryl compounds such as glutathione or cysteine (Bridges and Zalups, 2005; He et al., 2009). These interactions of Cd with proteins and low molecular weight compounds in plasma have greatly complicated efforts to identify the molecular mechanisms by which Cd is taken up by proximal tubule epithelial cells in vivo. Various studies to address this issue have shown that Cd can enter proximal tubule cells through a variety of mechanisms (He et al., 2009). As noted previously, circulating Cd-metallothionein can be filtered at the glomerulus and taken up by the epithelium of the proximal tubule.
in a process that involves megalin-mediated transport at the brush border (Klaassen et al., 2009; Squibb and Fowler, 1984). In addition, there is evidence for the uptake of lower molecular weight Cd-thiol conjugates (cysteine and glutathione) by proximal tubule cells (Bridges and Zalups, 2005). However, it is also important to note that the interaction between Cd and low molecular weight thiols is of a low enough affinity that Cd could dissociate from the thiol and bind to molecules on the cell surface and, in some cases, enter the cell. Indeed, there is evidence that Cd can enter renal tubular cells through a variety of channels and transporters for ions such as Ca$^{2+}$, Fe$^{2+}$ and Zn$^{2+}$ (Bridges and Zalups, 2005; He et al., 2009). Nebert and coworkers have recently provided compelling evidence that the ZIP8 family of metal ion transporters play especially important role in the cellular uptake of Cd in the kidney (He et al., 2009). Taken together, these findings suggest that with typical patterns of exposure, multiple mechanisms probably contribute to the uptake of Cd in the proximal tubule in vivo.

Another important consideration relates to the concentrations of Cd that are typically achieved in vivo (for review see Prozialeck and Edwards, 2010). The blood levels of Cd in non-exposed populations are typically less than 0.5 μg/L. Blood levels higher than 1.0μg/L are generally indicative of Cd exposure; levels higher than 5μg/L are considered hazardous. Urinary levels of Cd in non-exposed populations are normally below 0.5μg/g creatinine; values above 1-2 μg/g are indicative of exposure or elevated body burden. The critical urinary Cd concentration that is associated with the onset of renal injury is usually about 2-10 μg/g creatinine, which corresponds to a renal cortical Cd concentration of about 150-200 μg/g tissue (Jarup, 2002; Roels et al., 1979). These
levels of exposure need to be kept in mind when considering the possible relevance of *in vitro* studies to the action of Cd *in vivo*. Most *in vitro* studies typically have involved the exposure of cultured cells to low micromolar concentrations of Cd for less than 24 hours. While these concentrations are much higher than the concentrations of Cd in blood (5-10nM), they are well below the millimolar concentrations of Cd that are achieved in renal cortical tissue *in vivo*. It is also important to appreciate that individual cells and target molecules in the proximal tubule could actually be exposed to relatively high concentrations of Cd. For example, consider the situation in which a Cd-intoxicated proximal tubule cell lyses and releases its cytosolic contents, including Cd, into the local cellular environment. The localized concentrations of Cd in the immediate vicinity could easily exceed 1 mM. Even though all of this Cd would be bound to proteins or low molecular weight thiols, it could still undergo equilibrium interactions (dissociation and binding) with potential molecular targets in or on the adjacent cells. From a practical standpoint, it would be almost impossible to replicate these types of exposure conditions *in vitro*, a fact which further highlights the importance of *in vivo* models.

In considering the use of *in vivo* models to study Cd nephrotoxicity, investigators must balance the need to be able to do the studies in a reasonably short time frame with the need to replicate the toxicokinetics of the long-term, low-level patterns of exposure that are common in humans. For example, even though humans are typically exposed to dietary Cd over many years or decades, it is simply not possible or practical to replicate this type of exposure in species such as rats or mice. Since, these species have shorter life spans, and for many practical reasons, exposure levels used in animal studies are usually higher, but shorter in duration, than those used in humans.
Cd is a classic cumulative nephrotoxicant. With higher levels of exposure, nephrotoxic effects occur more quickly than with lower levels of exposure. In commonly used animal models, there is a linear inverse relationship between the dose of Cd and the time of exposure causes onset of proximal tubule injury (i.e. doubling the dose produces effects in $\frac{1}{2}$ the time) (Prozialeck et al., 2007). However, higher doses of Cd can cause injury to organs other than the kidney, particularly the liver and gonads. For nephrotoxicity studies, one of the most useful approaches has involved the subcutaneous administration of moderate doses of Cd (0.4 – 0.8 mg/kg/day) for periods ranging from 4-12 weeks. However, even with this approach, slight differences in treatment protocols and methodologies can complicate comparisons of results from different laboratories. To address some of the key issues, our own research groups have been utilizing a treatment protocol that involves the subcutaneous administration of Cd to rats (0.6 mg/kg, 5 days per week for up to 12 weeks) (Prozialeck and Edwards, 2010; Prozialeck et al., 2007; Prozialeck et al., 2009a; Prozialeck et al., 2009b). This has been a widely used protocol in Cd research and accurately reflects key pathophysiological features of longer-term exposure in humans. Many of the major conclusions that we will be emphasizing are derived from studies employing this protocol. This treatment protocol was approved by the Institutional Animal Care and Use Committee of Midwestern University and the studies were carried out in accordance with the NIH Guide for the Care and Use of Animal Animals.

Role of Necrosis, Apoptosis and Autophagy in Cd Nephrotoxicity. Regardless of the uptake mechanisms that are involved, it is clear that over time Cd can accumulate in
the epithelial cells of the proximal tubule. The traditional view has been that when the tissue levels of Cd exceed a critical concentration of about 150 µg/g tissue, intracellular defenses such as metallothionein and glutathione are overwhelmed and the cells undergo injury and begin to die (Gobe and Crane, 2010; Prozialeck and Edwards, 2010). A very fundamental question that has only been addressed over the past 10-15 years concerns the relative roles of apoptotic, necrotic and autophagic mechanisms in Cd-induced proximal tubular cell death. This is an important issue because even though all 3 pathways can result in cell death, each of the pathways involves their own unique sequence of pathophysiologic events. (for review see Galluzzi et al., 2007). Apoptosis (or Type-I cell death) is characterized by mitochondrial depolarization, caspase activation, DNA fragmentation and cell shrinkage, followed by fragmentation of the cell into small, membrane-coated apoptotic bodies. Necrosis (Type III cell death) is characterized by swelling of mitochondria and other organelles, breakdown of the cell membrane and the leakage of cytosolic contents into the surrounding environment. Autophagy is the least understood of these so called “cell death” pathways. Autophagy is a programmed process that involves the internal phagocytosis of damaged proteins and cytosolic elements into double-membrane-coated vesicles known as autophagosomes, which in turn are broken down by lysosomes. While low levels of autophagy may actually represent a repair/survival mechanism to preserve cell function, persistent or high levels of autophagy may trigger cell death. The biochemical pathways leading to autophagic cell death may overlap, to a certain extent, with those leading to apoptotic death.
It has long been recognized that high nephrotoxic doses of Cd can cause proximal tubule necrosis. However, it has also been apparent that early manifestations of Cd-induced proximal tubule dysfunction occur well before the onset of necrosis. In the 1990’s, several investigators (Hamada et al., 1991; Tanimoto et al., 1993; Tanimoto et al., 1999; Yan et al., 1997) published results showing that the early stages of Cd nephrotoxicity were associated with an increase in the number of apoptotic cells in the proximal tubule. In each of these studies, there was no significant evidence of necrotic injury at the time that apoptosis was observed. The studies by Tanimoto were especially noteworthy because the authors also identified proximal tubule cells that appeared to be proliferating as part of the response to apoptotic injury. In another study, Aoyagi et al. (2003) noted an increase in the number of apoptotic cells in the renal cortex of Cd treated rats after 4 and 5 weeks of exposure, but that the level of apoptotic labeling was much less pronounced after 6 and 8 weeks of exposure. In more recent studies from our laboratory (Prozialeck et al., 2009a), we also found that Cd caused a low level of apoptosis in the proximal tubules of sub-chronically exposed rats. However, the onset of apoptosis appeared to occur after kidney-injury molecule-1 (Kim-1)-dependent tissue repair processes had already been activated suggesting that Cd can produce significant changes in the cells before the onset of apoptosis. In a very recent study utilizing an acute (only 5 days), intraperitoneal model of Cd exposure in the rat, Chargui et al. (2011), identified the activation of a variety of autophagic processes in the proximal tubule that occurred at a time when there was no evidence of apoptosis or general proximal tubule dysfunction. These studies, too, suggest that Cd is producing early toxic
effects within the cells that leads to activation of a repair process, in this case autophagy.

There are several aspects of these studies that merit special attention. First, in all of the studies in which apoptotic cells were identified, the onset of apoptotic cell death appeared to coincide with the onset of proximal tubule dysfunction, as evidenced by polyuria and/or proteinuria. However, it is also noteworthy that in each of these studies, the number of proximal tubule cells that were actually undergoing apoptosis were quite low (i.e. well below 5%). The vast majority of proximal tubular cells were largely unaffected by Cd and/or appeared to dedifferentiate and proliferate as part of the repair process. The fact that only a small percentage of renal cells are being affected by Cd could greatly complicate efforts to identify the biochemical mechanisms by which the effects are occurring because it can be technically difficult to identify any possible Cd-induced biochemical changes in a few cells that are located in a sea of cells that are not being affected by Cd. However, it is also apparent that Cd causes some sort of injury that triggers this low level of apoptosis. In this context, the studies by Prozialeck at al. (2009a) and Chargui (2011) are especially significant in that they clearly show that Cd is producing detectable effects, such as upregualtion of Kim-1 and induction of autophagy, in proximal tubule cells before there is evidence of apoptosis or proximal tubule dysfunction.

The key question that has yet to be resolved is, how is Cd causing the initial injury to proximal tubule cells? Studies over the past 10 years have yielded some insights.
general, these studies have implicated 3 possible early response mechanisms in the proximal tubule. These include: disruption of cadherin-mediated cell-cell adhesion, modulation of intracellular signaling cascades and induction of oxidative stress.

**Cadherin Cell Adhesion Molecules as Potential Targets of Cd Toxicity.** One of the earliest toxic effects of Cd that is evident in proximal tubule cells, both *in vitro* and *in vivo*, involves the disruption of cadherin-mediated cell-cell adhesion (for reviews see Prozialeck, 2000; Prozialeck et al., 2003; Prozialeck and Edwards, 2007). The cadherins represent a family of calcium-dependent cell adhesion molecules that are usually localized at adherens junctions in epithelial cells (Prozialeck and Edwards, 2007). The extracellular domain of the cadherin contains Ca-binding sites and the adhesive regions. The intracellular domain is bound to β-catenin which is bound to α-catenin, which links the entire complex to the actin cytoskeleton. β-catenin also functions as a regulator of gene expression through the wingless/ Wnt nuclear signaling pathway (Prozialeck and Edwards, 2007; Thevenod, 2009). When β-catenin is released from the junctional complex into the cytosol, it may either be targeted for proteosomal degradation in a process that involves the adenomatous polyposis coli (APC) gene product and the serine/threonine kinase GSK-3β, or it can enter the nucleus, where it can bind to TCF-LEF-1 transcription factors and alter the expression of genes involved in apoptosis and cell-cycle control (Prozialeck and Edwards, 2007; Thevenod et al., 2007; Thevenod, 2009).
The finding that the cadherins are targets of Cd toxicity stemmed from observations by Prozialeck and Niewenhuis (1991) who found that exposing cultured renal epithelial cells to 5-20 μM Cd for 1-4 hours caused the cells to separate from each other and change from epitheloid to rounded, an effect that coincided with the loss of E-cadherin from the cell-cell contacts. Subsequent studies showed that Cd had similar effects on E- and N-cadherin junctions in many types of epithelial cells and on VE-cadherin junctions in vascular endothelial cells (Prozialeck, 2000). The ability of Cd to disrupt cadherin-dependent cell-cell junctions has been confirmed by many other laboratories and is now generally accepted as one of the primary actions of Cd on epithelial cells (for review, see Prozialeck and Edwards, 2007). It is also noteworthy that even though Cd was the first nephrotoxic agent that was found to disrupt cadherin-mediated cell-cell adhesion, it is now recognized that this is a primary effect of many nephrotoxic substances including mercury, bismuth and aminoglycoside antiobiotics (for reviews see Parrish and Prozialeck, 2010; Prozialeck and Edwards, 2007). This further illustrates how results of studies on an environmental pollutant such as Cd can have implications that are relevant to the nephrotoxic actions of drugs and therapeutic agents.

While much of the original work showing that cadherins are targets of Cd toxicity involved studies on epithelial cells in culture, it was less clear whether or not Cd can disrupt cadherin-dependent cell junctions in vivo. This issue was complicated by the unexpected finding that proximal tubule epithelial cells exhibit different patterns of cadherin expression in vivo than they do in vitro. Most proximal tubule-derived cell lines, including the cell lines that were used for our in vitro studies (for review see
Prozialeck, 2000), primarily express E-cadherin, which is the main cadherin expressed in most types of epithelial cells. However, when we tried to visualize E-cadherin in rat kidney, we found that the predominant cadherin in the proximal tubule was not E-cadherin but N-cadherin (Prozialeck et al., 2003; Prozialeck et al., 2004). Once this issue was resolved, we were able to show that Cd caused pronounced alterations in the pattern of N-cadherin localization in the proximal tubule without affecting E-cadherin in other nephron segments. Photos showing the effects of Cd ion N-cadherin localization in the S-3 segment of the proximal tubule are shown if Figure 1. As may be seen, the N-cadherin labeling in the control sample was highly concentrated along the basolateral cell surface and at the lateral cell-cell contacts. By contrast the sample from the Cd-treated animal shows a marked reduction in the labeling at the lateral cell-cell contacts. In addition, the labeling along the basolateral surface was much more diffuse than in the control samples. It is important to emphasize that these changes in N-cadherin localization were widespread and were readily apparent in all of the samples from the Cd-treated animals that were examined. Other investigators have shown that at about this same stage of inquiry significant changes in the microvilli and actin cytoskeleton (Sabolic et al., 2001; Sabolic et al., 2006). Together, these findings suggest that the cadherins or their associated catenins or cytoskeletal elements may be key early targets of Cd toxicity.

Since the cadherins play a critical role in establishing and maintaining the epithelial polarity that is essential for the normal functioning of the proximal tubule (Molitoris and Marrs, 1999; Prozialeck and Edwards, 2007), we hypothesized that the Cd-induced loss
of N-cadherin-mediated adhesion in the proximal tubule might lead to changes in epithelial polarity and barrier function. As a first step to address this issue, we examined the effects of Cd on the localization on Na⁺, K⁺-ATPase in the proximal tubule. Under normal circumstances, this transport protein is localized at the basolateral surface of the epithelial cells, where it plays a key role in sodium and fluid reabsorption. It is thought that the cadherin dependent cell-cell junctions serve as a “fence” to confine Na⁺, K⁺-ATPase to the basolateral cell surface. The images at the right of Figure 1 show that Cd does, in fact, cause alterations in the localization of Na⁺, K⁺-ATPase in the proximal tubule. Note that in the control kidney, Na⁺, K⁺-ATPase is localized along the basolateral surface of the epithelial cells of the proximal tubule. By contrast, in the sample from the Cd-treated animal, the Na⁺, K⁺-ATPase labeling is present over the entire cell surface and, in some areas, appears on the apical surface. These changes in Na⁺, K⁺, ATPase localization are similar to those described previously by Sabolic et al. (2006). These findings suggest that Cd-induced changes in cadherin dependent cell-cell adhesion may result in changes in epithelial polarity that are similar to those that have been described in ischemic kidney injury (for reviews, see Molitoris and Marrs, 1999).

The finding that the early stages of Cd-induced proximal tubule injury are associated with changes in N-cadherin localization raises important questions regarding the mechanisms by which Cd is producing these effects. Is Cd altering the genetic expression of N-cadherin or is altering the function of the molecule, either directly or indirectly?
These findings also raise the important question as to whether the disruption of N-cadherin-mediated adhesion results in the activation of β-catenin-regulated gene expression. This is potentially very significant because β-catenin is a key regulator of a variety of genes that are involved in cell cycle control, cell differentiation and apoptosis (for review, see Thevenod, 2009; Thevenod and Chakraborty, 2010).

Previous studies from our laboratories and more recent studies by Thevenod and coworkers have provided evidence that the disruption of cadherin-mediated adhesion by Cd results in the nuclear accumulation of β-catenin and activation of β-catenin-regulated gene expression (Chakraborty et al., 2010; Prozialeck et al., 2002; Thevenod et al., 2007; Thevenod, 2009; Thevenod and Chakraborty, 2010). The study by Chakraborty et al. (2010) is particularly significant. Using an in vivo mouse model of long-term Cd ingestion, the investigators showed that Cd caused the up regulation in the expression of specific Wnt ligands and receptors that coincided with increases in the expression of several β-catenin-regulated genes including: c-myc, cyclin D1 and Abcb1.

To further address these issues we have utilized real time RT-PCR techniques to analyze the effects of Cd on the patterns of gene expression in the renal cortex. Specific genes that were examined included: N-cadherin, E-cadherin, VE cadherin and β-catenin along with a panel of β-catenin-regulated genes (cyclin-D1, matrilysin, fibronectin, c-myc and C-jun), the Cd-binding protein metallothionein, and a panel of stress response genes (superoxide dismutase, glutathione-S-transferase, heme oxygenase and NADPH oxidase). Results of these studies are summarized in Table 1. Note that Cd
differentially affected the expression of E-cadherin, VE-cadherin and N-cadherin. There were no effects on the expression of E-cadherin or VE-cadherin at either 6 weeks or 12 weeks, or N-cadherin at 6 weeks. However, at 12 weeks, the expression of N-cadherin mRNA by about 50%. The fact that the expression of N-cadherin changes in response to Cd exposure, while the expression of E-cadherin and VE-cadherin do not, is consistent with our findings that N-cadherin is a target of Cd toxicity. Moreover, the fact that there is no change in N-cadherin mRNA or protein levels (protein data not shown) at 6 weeks, a time at which changes in the localization of N-cadherin in the proximal tubule are readily apparent, suggests that the initial effects of Cd involve either direct effects on N-cadherin or its associated molecules, or actions on one of the signaling pathways that regulate the function of N-cadherin.

It is also noteworthy that Cd had no effect on the expression of β-catenin even though immunofluorescence (Figure 1) and Western blot analyses (not shown) revealed a pronounced redistribution of β-catenin from the cell borders to the cytosol. Moreover, Cd had no effect on the mRNA expression of several β-catenin responsive genes cyclin D1, fibronectin, and c-jun, but did increase the expression of c-myc and the matrix metalloproteinase matrilysin. This indicates that even though Cd causes the breakdown of the N-cadherin/β-catenin complex in the proximal tubule, it only results in partial activation of β-catenin-regulated gene expression.

Another interesting finding from the mRNA analyses is that even though Cd caused a marked increase in the expression of metallothionein, it had little or no effect on the
expression of various stress response elements such as heme oxygenase, glutathione-S-transferase and superoxide dismutase. This suggests that at the time Cd-induced changes in N-cadherin localization are occurring, the renal epithelial cells are not undergoing a generalized stress response and, at most, may only be undergoing a very mild level of oxidative stress. Again, this suggests that Cd is probably acting on specific molecular targets within the epithelial cells although the targets have yet to be identified.

Cd and Cellular Signaling Cascades. These findings strongly suggest that alterations in N-cadherin function and epithelial polarity represent very early events in the pathophysiology of Cd-induced proximal tubule. However, the specific molecular mechanisms that mediate these effects have yet to be elucidated. Results of studies utilizing renal epithelial cells in culture and polypeptide analogs of E-cadherin have shown that Cd can interact with the extracellular Ca\(^{2+}\) binding domains on the molecule and alter its adhesive properties (for review, see Prozialeck, 2000). This mechanism appears to account for the ability of Cd to disrupt cadherin-dependent cell-cell junctions \textit{in vitro}, when cells are exposed to micromolar concentrations of free Cd. However, it is less clear whether or not this mechanism can explain the actions of Cd on N-cadherin in the proximal tubule, where the epithelial cells would be exposed to unknown concentrations of Cd in the form of Cd-protein or Cd-thiol conjugates (Bridges and Zalups, 2005). Results of our own \textit{in vivo} mechanistic studies to date have shown that at the time the initial Cd-induced changes in N-cadherin localization, and Kim-1 expression are occurring, there is no evidence of necrosis, and only minimal evidence of oxidative stress or apoptosis in the proximal tubule (Prozialeck and Edwards, 2010;
Prozialeck et al., 2003; Prozialeck et al., 2009a; Prozialeck et al., 2009b). Again, these findings strongly suggest that Cd may exert relatively specific effects on one of the many signaling pathways that regulate cadherin-mediated cell-cell adhesion in the proximal tubule. Indeed, there is a large volume of literature showing that Cd can affect a variety of cellular signaling pathways in epithelial cells (for review, see Thevenod, 2009). Some of the specific pathways that have been shown to be affected by Cd include: protein kinase C, cAMP, NO, MAP kinases (ERK ½, P38, JNK, etc), NF-κβ, p53 and wnt/beta-catenin. However, with the exception of the recent studies by Chakraborty et al. (2010), essentially all of these studies have involved the use of in vitro models and exposure of the cells to µmolar concentrations of Cd. Again, the relevance of these reported effects to the actions of Cd in the intact kidney are not clear. There is currently very little information on the possible mechanisms by which Cd alters N-cadherin function in the intact kidney. Further studies are needed to resolve this issue.

**Cd and Oxidative Stress.** One final aspect of Cd nephrotoxicity that merits special discussion is the role of oxidative stress in the pathophysiologic processes. Cd is not a Fenton metal, and as a stable divalent cation, it does not undergo redox cycling. However, Cd is clearly able to induce oxidative stress, and this mechanism has long been thought to play a role in Cd-induced kidney injury (for reviews, see Gobe and Crane, 2010; Liu et al., 2009). Rather than directly causing oxidative stress, Cd appears to act indirectly by binding to intracellular thiols such as glutathione, and/or interfering with the actions of various enzymes that protect against oxidative stress. Through
these indirect mechanisms, Cd can greatly amplify the actions of normal oxidative processes within the cell, which results in oxidative stress. While oxidative stress has long been thought of as a relatively non-specific mechanism of cellular injury, it is now appreciated that oxidative stress, particularly at low-moderate levels, may actually trigger the activation of specific oxidative signaling pathways (Liu et al., 2009; Thevenod, 2009). It is noteworthy that many of these so called oxidative signaling pathways have also been shown to be modulated by Cd exposure (Thevenod, 2009). However, at the present time, the cause-effect relationship between the signaling effects of Cd and the development of oxidative stress are not clear. Our own studies (Prozialeck et al., 2003, along with data in Table 1), indicate that at the time the initial changes in N-cadherin localization, Kim-1 expression and even apoptosis begin to occur in rat kidney, there is little evidence of oxidative stress. However, some of those studies involved the analysis of total non-protein thiols and thiobarbituric acid reactive substances, and the expression of stress-response genes, which are all relatively crude and late markers of oxidative stress. Additional in vivo studies utilizing more sensitive and specific indicators of oxidative stress, are needed to resolve this issue.

**Emerging Model of Cd Induced Proximal Tubule Injury.** From these observations, we have developed a model of Cd-induced proximal tubular injury that resembles a model of ischemic kidney injury that was first proposed over 20 years ago (Molitoris and Marrs, 1999). This emerging Cd model is summarized in Figure 2. Under normal circumstances, the tubular epithelial cells are attached to each other and the basement membrane/extracellular matrix through specialized junctional complexes. Over time, the
proximal tubule begins to accumulate Cd that eventually begins to affect epithelial cell function. These early effects appear to involve mild oxidative stress, disruption of cellular signaling cascades and alterations in cell adhesion. These effects, in turn, trigger autophagic responses in the cells. If the level of injury is mild, the autophagic response may be sufficient to repair damage. However, if the injury is more severe, apoptosis and/or autophagic cell death can occur. This in turn, triggers epithelial to mesenchymal transformation of surviving cells and a proliferative repair response. If the injury to cells is widespread and severe, repair processes are inadequate resulting in necrosis of the proximal tubule cells.

**Cd and Glomerular Injury.** While the proximal tubule is the primary target of Cd-induced kidney injury, there is evidence that Cd, particularly at higher levels of exposure, can also affect the glomeruli (Xiao et al., 2009). Changes in classic markers of glomerular dysfunction such as serum or urinary creatinine are generally not seen during the early or mild stages of Cd-induced kidney injury (Prozialeck and Edwards, 2007; Prozialeck et al., 2009a). However, several investigators have reported associations between Cd exposure and alterations (either increased or decreased) creatinine clearance (Bernard, 2004; Mueller et al., 1998; Navas-Acien et al., 2009; Trzcinka-Ochocka et al., 2004). Some studies have also shown increased urinary excretion of albumin during the early stages of Cd toxicity (Haswell-Elkins et al., 2008; Mueller, 1993; Mueller et al., 1998), which is classically interpreted as a marker of glomerular damage. Navas-Acien et al. (2009) have recently reported significant alterations in glomerular filtration along with albuminuria in subjects exposed to Cd and
Pb. At present, the relative contributions and relationship of glomerular injury and proximal tubule injury to these reported increases in urinary albumin excretion remain unclear.

**Implications for Biomonitoring and Therapeutic Interventions.** The finding that the early toxic effects of Cd in the proximal tubule may involve relatively specific changes in cell-cell adhesion and cellular signaling cascades has very important implications for biomonitoring Cd-exposed populations and for the potential treatment of Cd nephrotoxicity. The monitoring of at risk populations for early signs of Cd nephrotoxicity has posed special challenges (for reviews, see (Bernard, 2004; Prozialeck and Edwards, 2010). As a result of the tendency of Cd to accumulate in tissues such as the liver and kidney, monitoring of blood and urinary Cd often provides an incomplete reflection of the level of exposure and kidney disease. Because of these problems, investigators have utilized various biomarkers to characterize the severity of Cd-induced kidney disease. Some of the urinary biomarkers that have been used for this purpose include the Cd-binding protein metallothionein, low molecular weight proteins such as β-2 microglobulin, proximal tubule derived enzymes such as NAG and even Cd itself (Bernard, 2004). While these markers have been used to monitor Cd toxicity in humans and experimental animals, several problems remain. Most significantly these current markers only identify relatively late stages of Cd-induced kidney injury. By the time these markers appear in the urine, the injury to the kidney is generally considered to be irreversible and untreatable. Thus, there is a need for better early biomarkers of Cd-induced kidney injury.
One novel marker that has shown exceptional promise in preclinical studies is kidney injury molecule-1 (Kim-1). Kim-1 is a transmembrane protein that is not detectable in normal kidney but is expressed at high levels in the proximal tubule after ischemic or toxic injury (Vaidya et al., 2008). Kim-1 acts as a regulator of cell adhesion and endocytosis in regenerating cells of the injured tubule as they reform a functional epithelial barrier. This process is associated with the proteolytic cleavage of the ectodomain of Kim-1 into the urine. The ectodomain is stable in urine and has been shown to be a sensitive marker of renal injury induced by a variety of agents (Vaidya et al., 2008).

In studies utilizing a rat model of Cd-induced kidney injury, Kim-1 outperformed traditional urinary markers (Prozialeck et al., 2007; Prozialeck et al., 2009a; Prozialeck et al., 2009b). Kim-1 was detected in the urine 4-5 weeks before onset of proteinuria, and 2-5 weeks before the appearance of other markers such as metallothionein and CC-16. Other studies showed that the Cd-induced increase in Kim-1 expression occurred at a time when there was little or no evidence of either necrosis or apoptosis of proximal tubule epithelial cells (Prozialeck et al., 2009a). The fact that Kim-1 can be detected at a time before lethal injury to proximal tubule epithelial cells has occurred may be especially significant. Perhaps, with earlier detection via Kim-1, it may be possible to reverse, or at least more effectively treat, Cd-induced kidney injury. In light of this possibility, studies on the utility of Kim-1 as marker of Cd toxicity in humans are certainly warranted.
It should be emphasized that even though Kim-1 shows considerable promise as an early biomarker of Cd toxicity, there are still several important questions that need to be resolved. For example, how does Kim-1 expression change with higher levels of Cd exposure and how does it change when Cd exposure is stopped? Likewise, from a mechanistic perspective, it is unclear how the disruption of cadherin-mediated adhesion might be related to the activation of Kim-1 expression. Ichimura et al., (2008) have shown that Kim-1 expressing cells “phagocytize” debris from necrotic and apoptotic cells through the binding of Kim-1 to phosphatidylserine residues on damaged cells. However, it is unclear how these findings relate to the action of Cd in the proximal tubule. At the time Kim-1 is expressed in the proximal tubule, there is no evidence of necrosis and only modest evidence of apoptosis (Prozialeck et al., 2009a). At this same time, there are widespread alterations in the localization of N-cadherin (Prozialeck et al., 2003). Perhaps, the loss of N-cadherin mediated cell-cell adhesion is a key event in triggering the expression of Kim-1. Moreover, it is unclear how these disruption of cell-cell adhesion and the expression of Kim-1 might be related to the onset of oxidative stress and/or the disruption of cellular signaling cascades. Additional studies are needed to resolve these issues.

An important caveat that needs to be considered is that even with the use of a very sensitive biomarkers such as Kim-1, Cd exposure is not evident until some sort of toxic injury has occurred. One of the more intriguing aspects the studies summarized here is that some of the findings suggest that it may be possible to detect evidence of Cd
exposure before toxic cellular injury occurs. For example, Cd acts by affecting cellular signaling cascades, it may be possible to detect such alterations in function before the cytopathologic cascade of injury starts. One possible avenue of research might be to monitor the effects of Cd on changes in phosphorylation status of proteins or the identification of phospho-protein residues in urine. In addition, recent studies suggest that posttranslational modifications of protein expression may also play a role in Cd-induced injury. For example, our own recent studies have revealed that Cd causes changes in the patterns of micro RNA expression in renal epithelial NRK-52E cells (De La Fuente et al., 2011) and in rat kidney (unpublished observation). Determining how such Cd-induced alterations in micro RNA expression might influence protein expression and kidney function would also seem to be an interesting area for future research.

With respect to treatment, there are currently no proven effective treatments for Cd-induced kidney disease. Traditional chelating agents that are effective in treating poisoning with other metals, either do not mobilize Cd from intracellular stores or they have the paradoxical effects of facilitating the delivery of Cd to the kidney and actually increasing the level of kidney injury. As noted previously, the studies summarized here strongly suggest that Cd appears to specifically affect cadherin-mediated cell adhesion and cellular signaling pathways well before the onset of apoptosis or necrosis in the proximal tubule. These findings along with early detection with novel biomarkers such as Kim-1 suggest that it may be possible to utilize pharmacologic agents to modulate or
even halt these pathophysiologic processes before they become irreversible. It is our hope that this review will serve as a framework for future studies in this area.
Acknowledgements

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Authorship Contributions

Overall direction of project: Prozialeck

Review of literature: Prozialeck and Edwards

Design, performance and analysis of original experiments: Prozialeck and Edwards
References


Prozialeck WC and Niewenhuis RJ (1991) Cadmium (Cd2+) disrupts Ca(2+)-dependent cell-cell junctions and alters the pattern of E-cadherin immunofluorescence in LLC-PK1 cells. *Biochemical and Biophysical Research Communications* **181**:1118-1124.


Footnote to Title

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**Titles and Legends for Figures**

**Figure 1.** Effects of Cd on the Localization of N-Cadherin, β-Catenin and Na⁺, K⁺-ATPase in Proximal Tubule.

Adult male Sprague-Dawley rats were treated with Cd (0.6 mg/kg, subcutaneously) 5 days per week for 6 weeks, while control animals received saline vehicle alone. The kidneys were removed and processed for the immunofluorescent visualization of the molecules of interest as described previously (Prozialeck et al., 2003). The images of N-cadherin and β-catenin labeling originally appeared in the publication by Prozialeck et al. (2003) and are shown here with permission from the publisher. These particular images show the patterns of labeling in the S-3 segment of the proximal tubules in the inner cortex, near the outer stripe of the medulla. The original magnification was approximately 410 X.

**Figure 2.** Schematic Diagram Summarizing the Toxic Effects of Cd in the Proximal Tubule.
Table 1. Real Time RT-PCR Analyses of the Effects of Cd on Gene Expression in the Renal Cortex.

<table>
<thead>
<tr>
<th>Gene Category</th>
<th>Specific Protein</th>
<th>Cd 6 week (% of Control) (n=7)</th>
<th>Cd 12 week (% of Control) (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Adhesion/ Scaffolding Molecules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-cadherin</td>
<td>101±16</td>
<td>30±5*</td>
<td></td>
</tr>
<tr>
<td>E-cadherin</td>
<td>114±7</td>
<td>101±25</td>
<td></td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>103±18</td>
<td>117±29</td>
<td></td>
</tr>
<tr>
<td>β-catenin</td>
<td>129±19</td>
<td>69±27</td>
<td></td>
</tr>
<tr>
<td>β-Catenin Regulated Genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>80±10</td>
<td>73±13</td>
<td></td>
</tr>
<tr>
<td>c-myc</td>
<td>154±16*</td>
<td>69±20</td>
<td></td>
</tr>
<tr>
<td>Matrilysin</td>
<td>210±20*</td>
<td>534±77*</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>90±20</td>
<td>120±20</td>
<td></td>
</tr>
<tr>
<td>c-jun</td>
<td>68±18</td>
<td>119±35</td>
<td></td>
</tr>
<tr>
<td>Metallothionein</td>
<td>970±155*</td>
<td>1512±329*</td>
<td></td>
</tr>
<tr>
<td>Metal-Binding Proteins and Stress Indicators</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Super oxide dismutase</td>
<td>72±12</td>
<td>36±13*</td>
<td></td>
</tr>
<tr>
<td>Glutathione-S--transferase</td>
<td>117±15</td>
<td>78±8</td>
<td></td>
</tr>
<tr>
<td>Heme oxygenase</td>
<td>75±11</td>
<td>162±29</td>
<td></td>
</tr>
<tr>
<td>Alpha-1-acid glycoprotein</td>
<td>190±24*</td>
<td>142±11*</td>
<td></td>
</tr>
<tr>
<td>NADPH oxidase</td>
<td>173±8*</td>
<td>172±28</td>
<td></td>
</tr>
<tr>
<td>Biomarker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kim-1</td>
<td>611±158*</td>
<td>2434±193*</td>
<td></td>
</tr>
</tbody>
</table>

Adult male Sprague Dawley rats were treated with Cd 0.6 mg/kg, subcutaneously 5 days per week for 6 or 12 weeks, while control animals received saline vehicle alone. Samples of renal cortex were analyzed for patterns of gene expression using standard real time RT-PCR techniques performed by Jie Liu in the laboratory of Dr. Michael Waalkes at the NCI/NIEHS. Results are expressed as % of control values and represent the mean ± SEM from 5-7 replicate samples. Results of these studies were originally presented at the 2006 Society of Toxicology Meeting and some of the data was included in portions of Prozialeck et al., 2007; Prozialeck et al., 2009a. An * denotes...
that levels of expression in the Cd-treated animals was significantly different from control values (p < 0.05) as determined by Students’ t-tests.
Figure 1.

Control, N-cadherin

Control, β-catenin

Control, Na⁺, K⁺-ATPase

Cd-treated, N-cadherin

Cd-treated, β-catenin

Cd-treated, Na⁺, K⁺-ATPase
Figure 2.

Early low level Cd exposure results in:
1. oxidative stress
2. alteration of signaling cascades
3. alterations in cell adhesion
4. damaged proteins and other molecules

Mild Injury
Cell detachment, autophagic and apoptotic cell death

Moderate Injury
Cell detachment, autophagic and apoptotic cell death

Activation of Autophagic Non-proliferative Repair

Non-injured cells dedifferentiate, migrate to the denuded area of basement membrane and proliferate to reform the epithelial barrier.

Cell separation and activation of apoptosis/autophagic death cascades results in breakdown of cell-cell and cell-substrate adhesion leading to loss of polarity,

Severe Injury
Proximal Tubule Necrosis And Irreversible Damage