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Review

Novel roles for ceramides, calpains and caspases in kidney proximal tubule cell apoptosis: Lessons from *in vitro* cadmium toxicity studies

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ABSTRACT

Apoptosis is a tightly regulated physiological process, which can be initiated by toxic stimuli, such as cadmium (Cd^{2+}). Cd^{2+} (10–50 μM) induces a rapid increase in reactive oxygen species (ROS) (≥30 min) in a cell line derived from the S1 segment of rat kidney proximal tubule, without any apparent mitochondrial dysfunction. The sphingolipid ceramide is an important second messenger in apoptosis. Short exposure to Cd^{2+} (3 h) causes an increase in ceramides, which occurs downstream of ROS formation, and may interact with cellular components, such as endoplasmic reticulum and mitochondria. Following apoptosis initiation, execution must take place. The classical executioners of apoptosis are caspases, a family of cysteine proteases. However, increasing studies report caspase-independent apoptosis, which questions the essentiality of caspases for apoptosis implementation. With low micromolar Cd^{2+} concentrations (<10 μM), caspases are only activated after 24 h and not at earlier time points, which supports the notion of caspase-independent apoptosis. Due to increased cytosolic Ca^{2+} under pathological conditions, a role for the Ca^{2+}-dependent proteases, calpains, has emerged. Calpain activation by Cd^{2+} (3–6 h) seems to be regulated by ceramide levels, in order to induce apoptosis. Calpain and caspase substrates overlap but yield different fragments, which may explain their diverse downstream targets. Furthermore, calpains and caspases may interact with one another to enhance, as seen by Cd^{2+}, or diminish apoptosis.

In this review, we discuss novel roles for ceramides, calpains and caspases as part of Cd^{2+}-induced apoptotic signalling pathways in the kidney proximal tubule and their *in vivo* relevance to Cd^{2+}-induced nephrotoxicity.

Contents

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1. Introduction

Apoptosis is a physiological process, which in conjunction with autophagy, is important for the removal of damaged, unwanted, aged and superfluous cells in the body [1,2]. Perturbance of apoptosis execution leads to a disruption in tissue homeostasis and function and results in diseases such as cancer, neurodegenerative diseases and abnormal development. Generally, apoptosis signalling pathways can be subdivided into three major pathways: (1) the receptor-mediated or extrinsic pathway; (2) the mitochondrial or intrinsic pathway and (3) the endoplasmic reticulum (ER) pathway [3,4]. The receptor-mediated pathway involves binding of a death ligand to its receptor, which recruits death domains and activates caspase-8 and caspase-3/6 leading to cleavage of intracellular substrates and DNA condensation and/or fragmentation, a classical morphological change associated with apoptosis. The mitochondrial pathway is normally activated by other injurious compounds, such as reactive oxygen species (ROS) and Ca^{2+}. When stimulated, the mitochondria release proapoptotic factors, which normally reside in the intracellular space, and once in the cytosol, they can either activate caspase-9 and caspase-3 to induce apoptosis, such as cytochrome *c*, or they can cause apoptosis in a caspase-independent manner, such as apoptosis inducing factor (AIF) and endonuclease G. Cross-talk at the level of caspase-8 and the Bcl-2 family member, Bid, has been shown to exist between the extrinsic and intrinsic pathways [5]. The importance of the ER in apoptosis has been recently emerging, in particular the roles of Ca^{2+} release [6,7] and the unfolded protein response [8]. Increase in cytosolic Ca^{2+} causes toxicity to the cell by disrupting mitochondrial function and increasing the activity of enzymes, which are Ca^{2+}-dependent, for example, the calpains (discussed in further detail below). The unfolded protein response is activated through aggregation of misfolded proteins in the ER, which can be a result of cellular stresses, such as hypoxia and heavy metal ions [9]. The ER becomes stressed and upregulates a number of proteins, including chaperones and enzymes. This in turn leads to a commitment to apoptotic pathways and ultimately, cell death [10].

Aside from physiological stimulants, apoptosis can be induced by exogenous compounds, such as poisons or environmental toxins. Depending on the compound, apoptosis may be targeted to one particular organ or a multitude of areas in the body. An example of a compound with multifaceted effects is the toxic heavy metal Cd^{2+}. In this short review, the signalling pathways activated by Cd^{2+} to mediate apoptotic cell death in renal proximal tubule (PT) cells will be examined. In particular, we discuss the connections between ROS, calpains, ceramides and caspases and their significance *in vivo*.

2. Cadmium nephrotoxicity

Cd^{2+} is an environmental pollutant which is causing increasing concern as thousands of tons are released from manufacturing industrial sites (in particular manufacture of Cd^{2+}–nickel batteries), contaminating water, plants and foodstuffs and eventually entering the food chain. Cd^{2+} has no known physiological properties but shares some toxic properties with other heavy metals. For the general population, i.e. non-occupational exposure, the principal Cd^{2+} intake is through the consumption of contaminated foods such as root vegetables, shellfish and offal, but also from inhalation of tobacco smoke [11]. Because Cd^{2+} has a long biological half-life (10–25 years) and low rate of excretion, the body becomes a 'sink' and Cd^{2+} accumulates until a threshold is reached and causes toxicity to many vital organs including the lungs, liver and kidneys [12,13]. Traditionally, the threshold Cd^{2+} concentration for nephrotoxicity was set at around 200–300 μg/g wet tissue weight in occupationally exposed individuals [14–16]. However, studies on non-occupationally exposed individuals have demonstrated that renal tubule dysfunction occurs at Cd^{2+} concentrations as low as 50 μg/g wet tissue weight, suggesting that the threshold for the general population is much lower than originally thought [17–19]. In fact, it is thought that up to 7% of the general population may have significant Cd^{2+}-induced kidney alterations due to chronic exposure with low kidney Cd^{2+} levels (50 μg/g) [19]. Moreover, a recent study in mice suggests that the critical Cd^{2+} concentration in the kidney cortex even needs to be set at a lower level [20]. Interestingly, the manifestation of renal dysfunction does not seem to be solely dependent on the kidney Cd^{2+} concentration, indicating that genetic disposition, and possibly other factors, may play a role in determining Cd^{2+}-induced nephrotoxicity [21,22].

Once in the blood plasma, Cd^{2+} binds to albumin and is widely distributed in the body but primarily accumulates in the liver where production of detoxifying metal-binding proteins, metallothionein (MT), is induced. In the liver, Cd^{2+} is found mainly as a complex bound with high affinity to MT (Cd^{2+}–MT). Cd^{2+} may also be excreted with bile as a Cd^{2+}–glutathione complex. Cd^{2+}–MT then redistributes to the kidney [23] following its release into the circulation. There it is readily filtered in the glomerulus due to the small molecular weight of Cd^{2+}–MT (~6–7 kDa) and taken up by tubular endocytosis in the PT cells that involves receptor-mediated endocytosis via the multi-ligand polyspecific receptors, megalin/cubulin [24–26]. In PT cells, Cd^{2+} is thought to be released from the MT moiety in late endosomes/lysosomes and transported into the cytosol, most likely via the divalent metal transporter DMT1 [27–29]. Cytosolic Cd^{2+} then induces expression of MT in PT cells to neutralise its toxic potency. Once the balance of

detoxifying proteins and Cd^{2+} is aberrant, and free Cd^{2+} is no longer detoxified, it results in toxicity of the PT.

The primary target of Cd^{2+}-induced nephrotoxicity is the S1 segment of the PT because it is the first opportunistic site of reabsorption following filtration from the glomerulus. In addition, PT cells of the S1 segment possess a variety of transporters, metabolising enzymes and receptors, which are essential for Cd^{2+} uptake into the cell [30–32]. Cd^{2+}–MT is taken up via receptor-mediated endocytosis, but the major uptake pathways for free Cd^{2+} or Cd^{2+}–glutathione complexes are not yet known. Possible mechanisms could be other transporters for essential metals, such as Cu^{2+} or Fe^{2+}, or the recently identified ZIP8 transporter [33,34]. Significant evidence points towards Cd^{2+}-induced cell death of PT cells *in vitro* and *in vivo*, which disrupts normal kidney function and causes a Fanconi-like syndrome with polyuria, proteinuria, glucosuria, aminoaciduria and phosphaturia. The type of cell death induced by Cd^{2+} differs, depending on the concentration, exposure time and cell type. Generally speaking, acute exposure to high concentrations of Cd^{2+} (>50 μM) leads to necrosis, whereas following exposure to low Cd^{2+} concentrations, apoptotic cell death predominates. Similar effects are seen both *in vitro* [35,36] and *in vivo* [37–41]. Apoptotic cell death induced by chronic intoxication with low concentrations of Cd^{2+} or Cd^{2+}–MT *in vivo* [39,40,42] can be mimicked in a cell culture model of kidney PT, the WKPT-0293 Cl.2 immortalised cells derived from the S1 segment of rat kidney PT, by exposing cells to low Cd^{2+} concentrations (≤50 μM) for 3–24 h ([36] and unpublished data).

It is well established that Cd^{2+} has ROS-inducing properties even though it is not a Fenton metal [43,44]. The general dogma of increased ROS seems to be a direct effect on mitochondrial function which is followed by release of proapoptotic factors and, in some cases, caspase activation [45–48]. Increases in ROS in response to Cd^{2+} have been previously measured in cultured PT cells [49] and were detected already after 1 h exposure to Cd^{2+} (10–50 μM) (unpublished data). However, there is a lack of evidence for Cd^{2+}-induced apoptosis mediated by mitochondrial damage at early time points (see below). Thévenod and Friedmann [49] demonstrated that Cd^{2+}-induced oxidative stress leads to degradation of the Na^+/K^+ ATPase as part of the toxic effects caused by Cd^{2+}. The source of increased ROS in response to Cd^{2+} does not seem to be damaged mitochondria since ATP levels were not disturbed in a similar time course (Lee and Thévenod, unpublished observations). Thus, it is thought that Cd^{2+} displaces other Fenton metals, such as Fe^{2+}, from their binding sites, which in turn induces ROS formation [50]. Other documented apoptotic mechanisms that could be initiated by Cd^{2+} include release of Ca^{2+} from intracellular stores [51], alterations in sphingolipid metabolism [52], and/or depletion of ROS scavengers such as glutathione [53].

The apoptosis pathways activated by Cd^{2+} have been researched intensively in cell lines derived from kidney as well as other vital organs affected by Cd^{2+} exposure, such as liver and lung. Increasing evidence from us and others display the involvement of ceramide formation [52], calpains [54–56], caspases [56–58], mitochondria [35,36,47] and the Bcl-2 family of proteins [54,59,60]. It is also becoming increasingly apparent that the contribution of each individual event as well as the sequence of multiple events are highly dependent on Cd^{2+} concentration, duration of Cd^{2+} exposure and target tissues/cells [35,54,56].

3. Ceramides: role in cell death

Sphingolipids are ubiquitous components of cellular membranes and play important roles in intracellular signalling and membrane structure. Ceramides, the structural backbone of sphingolipids/sphingomyelin, can be directly converted to other forms by specific enzymes. They are present intracellularly in a highly dynamic pool and can change its form depending on the requirements of stimuli sent to the cell [61]. As shown in Fig. 1, the ceramide pool is balanced by the generation of new ceramides via the *de novo* synthesis pathway and the removal of ceramides via the degradation pathway resulting in the formation of sphingosine and sphingosine-1-phosphate, which function as pro- and anti-apoptotic signalling factors, respectively. Ceramide can be converted to other sphingolipids, such as sphingomyelin, or additional sugar groups can be added to the ceramide structure to yield glycosphingolipids, such as galactosylceramide and glucosylceramide.

Accumulating evidence implicates ceramides as important second messengers involved in cell growth, death, differentiation and senescence, including apoptosis [62–64]. Targets of ceramides in apoptotic signalling pathways include mitochondria (and increased oxidative stress), jun kinases (JNK), lysosomal cathepsin D, Ca^{2+} homeostasis, p38 mitogen activated protein kinase (MAPK), protein phosphatases, Bcl-2 family members and protein kinase Cζ (reviewed in

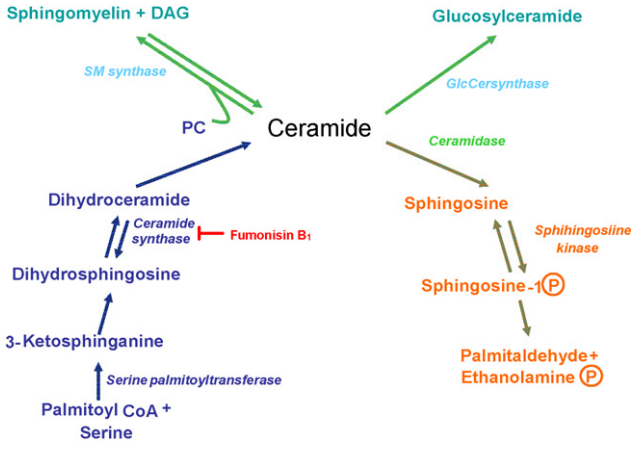

Fig. 1 – Schematic of ceramide metabolic pathways. Ceramide exists in a dynamic pool in the intracellular space depending on the cell's needs. Ceramide can be degraded to sphingosine by ceramidase via the degradation pathway. *De novo* synthesis of ceramide takes place through metabolism of palmitoyl CoA and serine, from which dihydroceramide is generated by ceramide synthase. Ceramide can also be metabolised to other forms such as sphingomyelin, by sphingomyelin (SM) synthase, and glucosylceramide (GlcCer). DAG, diacylglycerol; PC, phosphatidylcholine.

[62,65,66]). It is generally accepted that ceramides have a detrimental effect on mitochondrial integrity and function during apoptosis induction [65,67,68]. The exact mechanisms are somewhat unclear but there appears to be a common consensus on the ability of ceramide to form large protein permeable channels in mitochondrial membranes to advocate the release of proapoptotic proteins into the cytosol and activation of subsequent caspase cascades [69,70]. This hypothesis is further supported by data which show that direct application of ceramide analogues can cause release of cytochrome *c* from mitochondria [71,72].

A few studies in PT cells have implicated ceramides to be an "acute renal stress reactant" that increase in response to diverse ischemic or toxic insults [73–75]. Up until recently, there had been no studies investigating the role of ceramides in Cd^{2+}-induced PT cell death. But we showed for the first time in cultured PT cells that ceramides are increased by Cd^{2+} as early as after 3 h exposure and this continued to increase as a function of time up to 24 h [52]. The formation of ceramide following Cd^{2+} exposure could be a significant finding because this event may be implicated in Cd^{2+}-induced apoptosis. Indeed, we observed that depletion of ceramide using a low non-toxic concentration of fumonisin B_1, a fungal mycotoxin which blocks *de novo* ceramide formation through ceramide synthase inhibition (Fig. 1) [76], attenuated total intracellular ceramide levels and consequently, apoptotic cell death induced by Cd^{2+} [52]. There are two possibilities for the sequence of events in terms of ceramide and ROS: (1) Cd^{2+}-induced ROS formation results in subsequent ceramide generation; and/or (2) Cd^{2+} may induce ceramide generation which could in turn act on mitochondria to induce damage and elicit the increased formation of ROS, followed by release of proapoptotic mitochondrial factors, concluding with the activation of caspases and ultimately apoptosis [77–79]. Preliminary experiments by our laboratory suggests that ceramides are not upstream of ROS since preincubation with fumonisin B_1 did not result in attenuation of Cd^{2+}-induced ROS formation (Fig. 2 and Lee and Thévenod, unpublished observations). Other documented effects of ceramide increase include non-mitochondrial targets such as cytosolic Ca^{2+} increase [52,80], cathepsin D [81,82], ERK kinase [83] and, more recently, calpains [52].

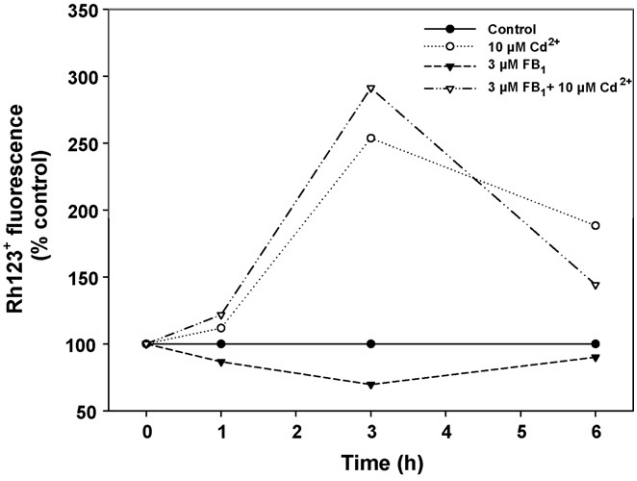

Fig. 2 – Cd^{2+}-induced oxidative stress is upstream of ceramide formation. WKPT-0293 C1.2 cells (6.5×10^4) were seeded into 24-well plates and grown for 2 days prior to treatment with 10 μM Cd^{2+} in serum-free medium. Proximal tubule cells were depleted of ceramides by preincubation with fumonisin B_1 (FB_1) (Calbiochem, San Diego, CA), which inhibits ceramide synthase (see Fig. 1). Following subsequent Cd^{2+} treatment, reactive oxygen species production was determined by incubating with dihydrorhodamine 123 (DHR123) (Axxora GmbH, Lörrach, Germany), which becomes oxidised to fluorescent rhodamine 123$^+$. At each time point, medium was replaced with Hank's buffered salt solution + 2.5 μM DHR123 + 1 μM PSC833 (Novartis Pharma, Basel, Switzerland), which prevents efflux of the dye, and incubated in the dark for 30 min at 37 °C. Cells were lysed with RIPA buffer and intracellular fluorescence was determined in a Berthold Mithras LB40 microplate reader. Graph is representative of three to four experiments.

4. Roles of calpains and caspases in apoptosis

Characteristic of cell death execution is the awakening of dormant proteases and mass cleavage of intracellular proteins to breakdown cellular integrity and function. A number of protease families are involved in the execution process. These include the caspases and calpains, which are discussed further below, as well as the cathepsins and granzymes [84–86].

4.1. *Calpain-dependent mechanisms of apoptosis*

Classically, the caspases are regarded as the apoptosis executioners. However, other classes of proteases have also been identified, such as the ubiquitous physiological Ca^{2+}-dependent cysteine proteases, calpains, which participate in many cellular functions aside from apoptosis, including necrosis, proliferation and cell adhesion and motility [85,87–89]. Although the role of calpains in PT cell injury and death by necrosis/oncosis has been well documented [90,91] the exact mechanisms and consequences of their actions in PT apoptosis remain unclear. Calpains are comprised of two subunits: a common small ~20 kDa regulatory subunit and a large catalytic ~80 kDa subunit, which varies in structure depending on the isoform. Dormant calpains are widely distributed across the intracellular space where they are kept inactivated by their physiological inhibitor, calpastatin. When stimulated, the calpain protein undergoes autolysis and allows the dissociation of the large subunit from the small subunit. This results in a truncated large subunit which is catalytically active and has a lower requirement for Ca^{2+}. The high *in vitro* Ca^{2+} requirements for calpain isoforms I and II (μM and mM, respectively) have been questioned since cytosolic Ca^{2+} levels never reach this activation threshold. However it is becoming increasingly apparent that many factors are able to decrease the Ca^{2+} requirement for calpain activation such as binding of phospholipids and interactions with other proteins [92–94].

When cultured kidney PT cells are treated with low micromolar concentrations of Cd^{2+}, calpains are activated at short time points (3–6 h) and are no longer active after longer exposure times (>8 h) [56]. Upon activation calpains cleave a large range of substrates including cytoskeletal proteins, other proteases and transcription factors. In contrast to the caspases, which degrade their substrates extensively, calpains make limited cuts in their substrates resulting in stable protein fragments that can have different functions from the intact protein. This culminates in specific changes of cellular function that are associated with calpain activation [85,95]. During apoptotic cell death, calpains have been reported to be implicated in a cross-talk signalling pathway with caspases. However there is some discrepancy. In some studies, calpains are shown to be responsible for cleaving and inactivating caspases [96,97], whereas other studies report the requirement of calpain-mediated proteolysis of caspases in order for their activation [56,98]. In our model for Cd^{2+}-induced apoptosis in PT cells, we found that calpains were partly responsible for activating caspases to induce cell death since calpain inhibitors attenuated caspase-3 activity and cell death [56]. Other documented downstream targets of calpain activation include Bax [54], mitochondria [99] and, as mentioned above, caspases [98].

The sequence of events leading to calpain activation has not been extensively researched, although an increase in cytosolic Ca^{2+} is to be expected [100]. We have recently shown that ceramide can function as an inducer of calpain activity. Depletion of ceramide levels in cultured PT cells using fumonisin B_1 completely abolished Cd^{2+}-induced calpain activation after 6 h [52]. The mechanism seems to be an increase of cytosolic Ca^{2+} caused by ceramide, which has been reported by us as well as by others [52,80,81,101]. However, when using Cd^{2+} as a stimulus, the method of measuring cytosolic Ca^{2+} levels is hampered by the availability of suitable Ca^{2+}-sensitive fluorescent dyes. Because Ca^{2+} and Cd^{2+} have similar ionic radii, Cd^{2+} also binds to traditional Ca^{2+} probes and chelators, such as Fura-2 and BAPTA, hence it is difficult to distinguish between Ca^{2+}- and Cd^{2+}-induced effects [102,103]. The same applies for Ca^{2+} chelation using BAPTA; Cd^{2+} would also be removed. Therefore, the data of many reports detailing the effect of Cd^{2+} on intracellular Ca^{2+} homeostasis [47,104,105] should be taken with care.

4.2. *Caspases: the classical executioners*

Cysteinyl aspartate-specific proteases, commonly known as caspases, are the mammalian analogues of the *Caenorhabditis elegans* death protease CED-3. They are synthesised as dormant proenzymes in the cytoplasm that are activated by proteolysis following a death signal. But caspases are not restricted to the cytosol; they are also present in the intermembrane space of mitochondria and are released by apoptotic stimuli. Subsequently, they acquire the ability to cleave important intracellular substrates, such as inhibitors of apoptosis and structural cytoskeletal proteins (e.g. lamins, fodrin, gelsolin), that result in the structural disassembly, morphological changes and DNA condensation/fragmentation identified with apoptosis [106–108].

Currently, there are around 15 known caspase members but only a small subset is involved in the proteolytic cascade leading to apoptotic cell death with other caspase members involved in eliciting inflammation [109]. The caspases can be separated into two major groups: the initiator caspases (-8, -9, -10) and the effector or executioner caspases (-3, -6, -7). Effector procaspases lack the ability to self-activate and appear to require cleavage by activated initiator caspases. The intrinsic enzymatic property of caspases could be perilous as auto-activation may occur spontaneously, thus an additional protective mechanism must be in place in order to prevent apoptosis happening by mistake. Indeed, there are physiological inhibitors of caspases ubiquitously expressed in all cell types. Inhibitor of apoptosis proteins (IAPs) are conserved family of proteins which regulate apoptotic function by inhibiting the caspases (reviewed in [110]). Different initiator caspases are activated by differing upstream pathways, but they seem to converge onto the effector caspases (-3, -6, -7) where the number of active protease molecules is amplified. For example, stimuli that bind to death receptors activate caspase-8 and -10 by death effector domains whereas stimuli following the mitochondria-dependent pathway activate caspase-9 via the recruitment of procaspase-9 into the apoptosome complex with Apaf-1 and cytochrome *c* [111]. Once the caspases are active, they increase the activity of the caspase-activated DNase (CAD) to degrade DNA in high molecular weight fragments [112].

Although the caspases are the ultimate step to apoptosis execution, their absolute requirement during apoptosis is debatable due to a growing number of studies reporting the occurrence of a caspase-independent apoptotic cell death [56,113–116]. Even in the absence of caspase activation,

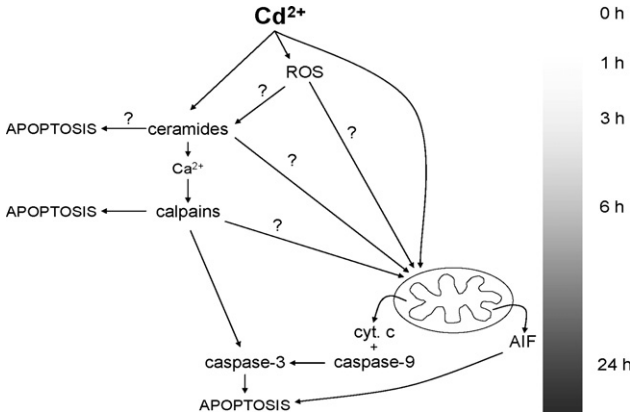

Fig. 3 – Model for apoptotic pathways induced by Cd^{2+}. Following uptake into a proximal tubule cell, Cd^{2+} quickly causes ROS formation followed by increased ceramide formation. Ceramides may cause an increase in cytosolic Ca^{2+}, which in turn would activate the Ca^{2+}-dependent proteases, calpains. Calpains cleave intracellular substrates to induce apoptosis. Following longer exposure to Cd^{2+}, mitochondrial damage occurs. Proapoptotic factors from the intermembrane space initiate caspase-dependent and -independent apoptosis. Unknown interactions and/or mechanisms are indicated by questions marks.

changes in DNA morphology, such as fragmentation, are still observed. In studies of Cd^{2+}-induced apoptosis, caspase-9 and -3 were only activated by low micromolar concentrations (10 μM Cd^{2+}) after 24 h exposure and not at any other earlier time points tested, which is in contrast to calpain activation [56]. Higher Cd^{2+} concentrations (50 μM) did not increase caspase activity suggesting that apoptosis execution differs depending on the Cd^{2+} concentration. How could Cd^{2+} activate the mitochondrial apoptotic pathway after 24 h exposure? Besides ROS, ceramide formation and calpain activation, we and others have shown that Cd^{2+} has direct effects on mitochondria (Fig. 3), as demonstrated in *in vitro* studies on mitochondria isolated from rat kidney cortex [36,117] as well as from rat liver [118,119]. Mitochondrial swelling and subsequent cytochrome *c* release was induced by Cd^{2+} in a concentration-dependent manner. In our experiments, the hypothesised mitochondrial permeability transition pore [120] was not involved in Cd^{2+}-induced mitochondrial swelling [36], in converse to findings by others [121,122]. Rather, Cd^{2+} uptake via the Ca^{2+} uniporter and activation of aquaporin-8 was the principle mechanism for expansion of the mitochondrial matrix [36]. As well as mitochondrial swelling, Cd^{2+} induces K^+ cycling, which results in swelling and contraction of energised mitochondria, dissipation of the mitochondrial membrane potential and breakdown of the pH gradient via targeting of the mitochondrial K^+/H^+ exchanger [117].

The most obvious caspase-independent mechanisms for cell death are proapoptotic factors released from mitochondria, which have endonuclease activity, such as AIF and endonuclease G, and other proteases. However, in our studies, we observed neither mitochondrial damage nor the release of AIF at early time points (0–8 h) [56]. So the question remains as to how DNA degradation occurs in the absence of caspase activation and release of mitochondrial factors. Recent studies report that DNA degradation can be a caspase-independent process. Ajiro et al. [123] have shown in isolated non-apoptotic nuclei that cleavage of DNA is Ca^{2+}-dependent and is sensitive to intracellular K^+ concentration. Some studies suggest a role for serine proteases as the mediator of DNA fragmentation [124]. Others have shown that the inhibitor of CAD could be directly degraded by enzymes other than caspases, namely granzyme B, to enhance CAD activity [125,126] or that DNA fragmentation can occur in the absence of CAD inhibitor cleavage [127,128]. Still, how DNA degradation takes place in the absence of caspase activation and release of proapoptotic factors from the mitochondria is not entirely clear.

5. *In vivo* relevance of *in vitro* studies

The data from our laboratory demonstrate roles for ceramides, calpains and caspases as part of a Cd^{2+}-induced apoptosis signalling cascade were generated in an immortalised cell line derived from the S1 segment of rat kidney PT. Hence the legitimate question arises as to whether the data obtained *in vitro* in this cell culture model reflect chronic Cd^{2+} nephrotoxicity *in vivo*. Though there is no doubt that chronic exposure to low Cd^{2+} concentrations induces apoptotic cell death of kidney PT *in vivo* (see Section 2 for its discussion), two specific issues need to be addressed: (1) Are the Cd^{2+} concentrations tested on PT cells in culture (10–50 μM) comparable to the Cd^{2+} concentrations found in renal proximal tubules, renal cortex or kidney *in vivo* in animals or humans chronically exposed to this metal *in vivo*? (2) Are caspases, calpains and ceramide involved in cadmium-induced apoptosis of renal PT *in vivo*? Most *in vivo* studies of chronic Cd^{2+} nephrotoxicity in humans and experimental animals indicate a threshold level of 50 μg/g kidney tissue wet weight for the development of signs of kidney dysfunction and PT damage [19,20]. These values are of similar magnitude as the concentrations of $CdCl_2$ used in our PT cell culture model to induce apoptosis, namely 2–10 μg/ml [36,52,56]. So far, apoptotic cell death of kidney PT mediated by ceramide and/or calpains has not been investigated *in vivo*. Interestingly, involvement of caspase-3 has been described in several animal models of chronic Cd^{2+} nephrotoxicity [41,42]. This evidence supports the notion that PT cell culture models are a useful tool to identify apoptotic (and necrotic) signalling mechanisms involved in chronic Cd^{2+} nephrotoxicity and to develop working hypotheses that can be subsequently tested *in vivo*.

6. Conclusions

Apart from physiological apoptotic stimuli, an increasing number of toxic substances can induce cell death resulting in a disturbance of organ and bodily function. The environmental pollutant, Cd^{2+}, affects multiple organs in the body and disrupts function by inducing apoptosis, in particular the kidney proximal tubule. Apoptosis involves a tightly regulated sequence of events, which differs depending on the stimulus as well as the time of exposure. Following short-term exposure to Cd^{2+} (up to 6 h), ROS, ceramides and cytosolic Ca^{2+} are increased and calpains are activated (Fig. 2 and [52]). Although ceramides are still elevated after 24 h, long-term exposure to Cd^{2+} (24 h) results in a different signalling pathway. Mitochondrial damage occurs leading to release of proapoptotic factors from the intermembrane space culminating in caspase activation and apoptosis (Fig. 3). Cross-talk between the two pathways for short- and long-term Cd^{2+} exposure has been observed at the calpain–caspase level [52,56]. Still, other players may be involved in the apoptotic pathway induced by Cd^{2+} and how these affect our current observations remains to be investigated.

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