CHAPTER 4

DISCUSSION AND CONCLUSIONS

The major issue challenged in this study is whether caffeic acid phenethyl ester (CAPE) could prevent or ameliorate the functional and ultrastructural derangements of kidney mitochondria following cadmium exposure. The outcomes of the study provide evidence to demonstrate the mitochondrial protective effect of CAPE and further suggest that this benefit is mediated, at least in part, through its potential to prevent mitochondrial oxidative stress.

Cadmium is a potent environmental toxic compound that damage several organs of the body. Nephrotoxicity is the most serious complication of cadmium as it can progress to end-stage renal disease and death in the long run (68). Exposure to cadmium has been shown to induce apoptosis of the proximal tubular cells and, therefore, disrupt kidney function (69-71). Although there are numerous upstream pathways by which apoptosis is mediated, mitochondrion appears to be a merging point of apoptosis cascade (69, 72). A number of pro-apoptotic proteins such as cytochrome c, apoptosis-inducing factor (AIF) and procaspases have been shown to release from the intermembrane space of mitochondria into the surrounding cytosol following a cell death signal, and activate downstream pathways (72, 73). Accordingly, mitochondrial dysfunction is increasingly implicated as key event in the pathogenic cascade associated with cadmium-induced apoptotic cell death (72, 74).

In the present study, the rat kidneys were homogenized and underwent differential centrifugation for isolation of the mitochondria. The final fraction was examined by transmission electron microscope, which verified the presence of purified mitochondria in the pellet collected. Electron micrograph also demonstrated normal characteristics of healthy mitochondria like regular shape, intact membrane, dense matrix, and prominent cristae in almost all (>95%) of the harvested mitochondria. Thus, it is assumed that the mitochondria obtained by the isolation technique used in this study were well preserved. However, measurement of mitochondrial respiratory exchange ratio, if available, may be more precise method for evaluation of the mitochondrial quality.

Although there are several *in vitro* studies concerning the deleterious action of cadmium, the concentration of cadmium used in these investigations was variable, ranging from 10-100 μM, depending on the model and condition of the study (75-77). In the present work, the different concentrations of cadmium were initially tested and cadmium at 30 μM was selected based on the finding that it was the lowest concentration which clearly, consistently and moderately had an impact on mitochondria. As this dose of cadmium was corresponded to the range previously been published, it is reasonable to point out that the selected cadmium concentration was appropriate for using in the current study model and condition.

Using isolated rat kidney mitochondria as the model, the present study was able to disclose specific alterations in mitochondria caused by cadmium as indicated by an increased in mitochondrial ROS production, a swelling of mitochondria, and a decreased in mitochondrial membrane potential. Mitochondrial disruption was further

confirmed by electron micrograph showing destruction of mitochondrial ultrastructure after cadmium exposure. These results correlated well with earlier publications demonstrating cytotoxicity of cadmium in a variety of renal cell culture studies as well as significant renal structural and functional abnormalities subsequent to cadmium contamination in animal models (11, 78). Collectively, the current findings provide additional evidence to indicate that nephrotoxicity following cadmium exposure is, somewhat; the consequence of direct impact of cadmium at the mitochondrial level and that cadmium-induced mitochondrial damage contributes to renal cell death.

Mitochondrial oxidative phosphorylation is the major ATP synthetic pathway in eukaryotes. In this process, electrons liberated from reducing substrates are delivered to O_2 via a chain of respiratory H^+ pumps. These pumps (complexes I-IV) establish a H^+ gradient across the inner mitochondrial membrane, and the electrochemical energy of this gradient is then used to drive ATP synthesis by complex V (2, 32). Chemically, the stepwise reduction of O_2 proceeds via several reactive oxygen species (ROS). These ROS can damage cellular components such as proteins, lipids, and DNA, leading to cellular injury and death once the amounts accumulating within mitochondria exceed those required for microdomain cell signaling (79).

In the present study, cadmium-induced mitochondrial dysfunction was accompanied by the substantial rise in mitochondrial ROS production and the significant increase in malondialdehyde (MDA), a lipid peroxidation product. Several *in vivo* studies have also demonstrated the increased free radicals, lipid peroxidation,

lipid hydroperoxides and protein carbonyl contents in the kidneys following cadmium intoxication (5, 15, 22). Although cadmium itself is unable to generate free radicals directly, indirect formation of ROS and RNS involving the superoxide radical, hydroxyl radical and nitric oxide has all been reported in a variety of cells and tissues after exposure to cadmium (2, 6). Some experiments also confirmed the generation of non-radical hydrogen peroxide, which itself in turn may be a significant source of radicals via Fenton reaction (32). In addition, investigations using mitochondria isolated from heart, brain and liver revealed that cadmium can directly inhibit electron transfer and oxidative phosphorylation, mainly at complexes I and III, thereby enhancing the mitochondrial production of ROS (31). These studies along with the present observations indicate that an overproduction of ROS and, thus, mitochondrial oxidative stress could be an important mechanism underlying the pathogenesis of cadmium nephrotoxicity.

Data from the present study also demonstrated a marked reduction in glutathione level in mitochondria exposed to cadmium. This suggests that cadmium-induced mitochondrial oxidative stress in the current study may not only attribute to an excessive mitochondrial ROS production but also an impairment of mitochondrial antioxidant defense system to overcome the formation of ROS. Glutathione (GSH) is the primary low molecular weight thiol in all aerobic cells. Mitochondrial GSH emerges as the main line of defense for the maintenance of the appropriate mitochondrial redox environment to avoid or repair oxidative modifications leading to mitochondrial dysfunction and cell death (80, 81). The importance of mitochondrial GSH is based not only on its abundance, but also on its versatility to counteract

various oxidant molecules and its role as a cofactor of several mitochondrial enzymes (80, 81). Changes in mitochondrial GSH concentration and its redox status have been associated with mitochondrial oxidative stress, causing alterations in mitochondrial calcium distribution, induction of the membrane permeability transition, activation of signaling pathways and expression of genes that regulate apoptosis (80-82). Numerous pathologies such as ischemia/reperfusion injury, liver diseases, and neurological disorders are also characterized by a consistent decrease in mitochondrial GSH (81, 83, 84).

Relating to cadmium nephrotoxicity, several *in vivo* experiments have shown a decrease in the renal GSH level upon cadmium exposure (16, 25). Studies have also demonstrated a high affinity of cadmium for thiol binding (24, 85). Since thiol groups are usually involved in the function of many enzymes, structure proteins and receptors, it has been suggested that the cadmium-thiol complexes possibly disturbed many functions of cells and that the depletion of intracellular thiols by cadmium is the prerequisite for ROS generation and disruption of kidney function (24, 82). Apart from GSH, the decreased activities of several thiol-based antioxidant enzymes like SOD, CAT, GPx and GST were also observed in the kidney of cadmium-intoxicated animals (16).

In the view of the above reports together with the data obtained in this study, it is likely that the interruption of cadmium with the electron transport chain and/or the modification of thiol-containing molecules could provoke a great increase of ROS within mitochondria. Thereafter, these molecules could initiate a series of events that alter mitochondrial functions, which in turn resulted in renal cell damage. This

suggestion was substantiated by recent studies in neuron and liver cell lines indicating that an increase in mitochondrial ROS is the first event in mitochondria exposed to cadmium and that is followed by mitochondrial membrane damage, which precedes cell death (5, 6, 34).

The change of mitochondrial permeability transition has been known to be a common and crucial mechanism in cell death (7, 82). Permeability transition is described as an abrupt increase of the inner membrane permeability to solutes with molecular masses of less than 1.5 kDa by the opening of the mitochondrial permeability transition pore (mPTP) (86, 87). The mPTP is a multiprotein complex expressed in the contact site between inner and outer membrane of mitochondria. It consists of hexokinase, voltage-dependent anion channel (VDAC), creatine kinase, the adenine nucleotide translocator (ANT) and cyclophilin D (CyP D) (82). The closing state of mPTP is crucial for maintaining the mitochondrial integrity (82, 88). Several studies have demonstrated the association between cadmium-induced mitochondrial damage and the induction of the permeability transition (9, 72, 82). Taken into consideration, it seems reasonable to propose that the accumulation of ROS up to a critical threshold level may induce a sudden transition in permeability of the highly selective inner membrane by the opening of the mPTP. The persistent opening of the mPTP causes the net flux of ions and thus osmotically obliged water into the mitochondrial matrix, resulting in mitochondrial matrix swelling, mitochondrial depolarization, and rupture of the outer mitochondrial membrane as observed in the present study. These mitochondrial events could further cause the

release of various apoptotic molecules from the intermembrane space that eventually leading to the damaging effects of cadmium.

There is also evidence showing that the outburst of ROS may trigger the opening of the inner membrane anion channel (IMAC) and allow the release of superoxide anion from the mitochondrial matrix, resulting in mitochondrial membrane depolarization (89). The release of ROS via IMAC opening could also activate the IMAC of its neighboring mitochondria (90). This may further elicit the increase of ROS within mitochondria and, consequently, exacerbate their damaging effects. On the whole, the findings reported herein reinforce the role of mitochondrial ROS and, thus, mitochondrial oxidative stress as a key element in mediating the renal toxic effects of cadmium.

The present investigation demonstrated that treatment with CAPE prior to cadmium not only ameliorated mitochondrial dysfunction, but also restored the rise in ROS, MDA and the fall in GSH associated with cadmium. These results implied that the beneficial outcomes of CAPE may be related to its ability to maintain redox equilibrium within mitochondria and subsequently block all ROS-mediated mitochondrial damage. CAPE is well documented for its protection against oxidant-induced renal injury in various disease conditions, including nephrotoxicity caused by xenobiotics (40, 46, 47). A large number of studies have demonstrated the capability of CAPE to scavenge ROS and exert its antioxidant properties by chelating the transition metals, suppressing lipid peroxidation, inhibiting xanthine oxidase, NADPH oxidase, and nitric oxide synthase (19, 20, 42). Evidence has shown that the presence of *ortho*-dihydroxyl functionality in the catechol ring along with the polarity,

hydrophobicity and stability of CAPE are responsible for its potent antioxidative and free radical scavenging activities (91). There are also reports of CAPE to enhance the cellular antioxidants such as reduced glutathione, and antioxidant enzymes such as SOD, CAT, GPx and GST (40, 43-45).

According to the present findings, it is possible that the chelating property of CAPE may enhance the elimination of cadmium, which might reduce the cadmium burden with displacement of metal cofactors and/or cadmium binding with enzymes. In addition, the capability of CAPE to react with free radicals or with highly reactive byproducts of lipid peroxidation as well as enhancement of mitochondrial thiol pools may diminish or reverse oxidative modification of antioxidant enzymes, thereby ameliorating the oxidant-mediated mitochondrial damage following cadmium exposure. In line with this scheme, recent study has demonstrated that CAPE prevented the impairment of mouse brain and liver mitochondria after *in vitro* anoxia-reoxygenation in a concentration-dependent fashion. The protection by CAPE has shown to be associated with its antioxidant activity to limit mitochondrial membrane fluidity, decrease mitochondrial lipoperoxidation, reduce mitochondrial protein carbonylation, enhance mitochondrial GSH level, and block the release of cardiolipin and cytochrome c from mitochondria (55).

It is of interest to note that all doses of CAPE used in the present investigation seemed to be advantageous, but the greatest therapeutic benefit was apparent at the highest (10 μM) concentration examined. This corresponded to the dose previously been reported to protect against oxidant-induced injury in diverse organ systems under different pathogenic conditions (20, 40, 41, 92, 93). While

CAPE at this dose completely overcomes the oxidative injury and the dissipation of mitochondrial membrane potential, it can only partially reduce the swelling of mitochondria. This suggests that ROS-induced mPTP opening may not be the only mechanism underlying mitochondrial swelling triggered by cadmium. Consistent with this finding, earlier publications have shown that inhibitors of the mPTP such as cyclosporin A and bongkrekic acid do not consistently abolish cadmium-induced swelling of mitochondria (11, 77, 94). Moreover, the aquaporin 8 (AQP8) water channels have recently been identified on the inner membrane of kidney mitochondria (95). It is postulated that cadmium may enter the matrix space to activate AQP8 and water influx resulted in osmotic swelling, breakdown of mitochondrial membrane potential, and release of cytochrome c (11, 77, 94). Thus, data obtained from the present study provide more evidence for the possible involvement of the mPTP-independent mechanism and/or the ROS-independent mPTP opening in mediating the swelling of cadmium-exposed mitochondria.

Although the present results underscore the antioxidative protection of CAPE, the data obtained are not sufficient to point out where CAPE is really acting. Also, the protection by CAPE may lie in some other mechanisms apart from its antioxidant activity. It is conceivable that CAPE may antagonize effects of cadmium by direct binding to cadmium (Figure 4-1, ❶) or interfering with the uptake of cadmium into mitochondria via the mitochondrial calcium uniporter (MCU) (Figure 4-1, ❷), thereby decreasing the available cadmium to accumulate within mitochondria. Otherwise, it may be able to directly block the inhibitory effects of cadmium on the individual complexes of the mitochondrial electron transport chain

(Figure 4-1, ❸), apart from its effect to enhance mitochondrial GSH level (Figure 4-1, ❹). Given that cadmium can directly cause the mPTP opening possibly via binding to vicinal thiol groups of ANT (82), CAPE may act directly on the VDAC (Figure 4-1, ❺) or ANT (Figure 4-1, ❻) to block the binding of cadmium or probably occupy the active site of CyP D (Figure 4-1, ❼) and form a VDAC-ANT-CyP D-CAPE complex to block the mPTP opening. Alternatively, CAPE may possibly minimize mitochondrial swelling via its action on AQP8 (Figure 4-1, ❽). Furthermore, it is widely accepted that calcium is capable of acting at several levels within mitochondria to regulate mitochondrial function (96). Cadmium-induced cytotoxicity has been linked to the ability of the metal to produce intracellular calcium overloading and nephrotoxicity has also been shown to be associated with many calcium-dependent processes (75). It is assumed that CAPE may exert its protective effect by controlling mitochondrial calcium homeostasis. This speculation arises from recent reports showing the potential of CAPE to prevent cerebellar granule neurons against glutamate-induced neurotoxicity and isolated brain mitochondria against hypoxic-ischemic brain injury by directly inhibiting calcium-induced mitochondrial swelling and cytochrome *c* release (54, 92). On the basis of the present findings, however, it can only be inferred that CAPE could directly act on the mitochondria to alleviate cadmium-induced mitochondrial injury through inhibiting ROS generation, decreasing lipid peroxidation and/or maintenance of the antioxidant glutathione content within the mitochondria. More specific approaches, such as the use of MCU inhibitor, specific inhibitor of ETC, mPTP blockers, and/or AQP8

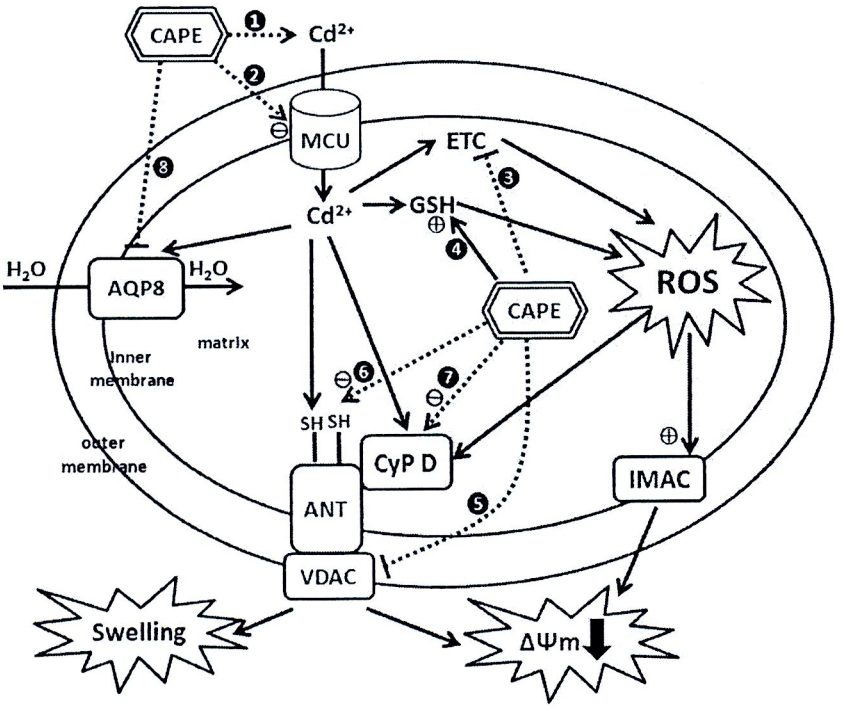

Figure 4-1 Proposed mitochondrial protective mechanisms by caffeic acid phenethyl ester (CAPE). ❶ CAPE may bind to cadmium (Cd^{2+}). ❷ CAPE may block the entry of Cd^{2+} via mitochondrial calcium uniporter (MCU). ❸ CAPE may block the effect of Cd^{2+} on electron transport chain (ETC). ❹ CAPE may enhance mitochondrial glutathione (GSH) level. ❺ CAPE may act directly on the voltage-dependent anion channel (VDAC). ❻ CAPE may inhibit Cd^{2+} acting on thiol group (SH) of the adenine nucleotide translocator (ANT). ❼ CAPE may inhibit Cd^{2+} acting on cyclophilin D (CyP D) or directly bind to CyP D. ❽ CAPE may inhibit water (H_2O) uptake via the aquaporin 8 (AQP8) water channel. ROS, reactive oxygen species; IMAC, inner membrane anion channel; $\Delta\Psi m$, mitochondrial membrane potential change.

inhibitor, are needed to address the actual site of action and the exact mechanisms underlying the protection afforded by CAPE in this model.

In conclusion, the present investigation indicates that cadmium can directly deteriorate the structure and function of mitochondria in the kidney. This detrimental effect of cadmium is associated with changes in mitochondrial redox status. The findings reported herein also provide new insight regarding the antioxidant potential of CAPE at the mitochondrial level and its therapeutic benefits in the setting of nephrotoxicity caused by cadmium.