CHAPTER VI
DISCUSSION

In this study, we investigated the ability of cadmium to consume NO liberated from NO donor DETANONOate. NO consumption by plasma of cadmium-exposed and control subjects was no different. These results suggest that cadmium does not interact with NO directly. Thereby, we hypothesized further that decreased blood nitrite levels in cadmium-exposed subjects could result from impaired synthesis by endothelium.

We proceed to confirm the function of endothelial cell in plasma of control and cadmium-exposed subjects using human thrombomodulin ELISA kits. The levels of thrombomodulin in plasma of cadmium-exposed subjects were higher than control subjects. Our data suggests that chronic cadmium exposure might cause endothelial dysfunction. In addition, the levels of thrombomodulin were also showed the correlation with urine cadmium and whole blood cadmium in subjects. A previous study shows that endothelial cell dysfunction was an early phase of atherosclerosis in children with idiopathic nephrotic syndrome. The levels of thrombomodulin increased in children with idiopathic nephrotic syndrome (16). Levels of thrombomodulin have been shown to increase in patients with localized atherosclerotic disease (carotid artery disease, iliac or femoral artery disease) (48). Normally, thrombomodulin is the multidomain integral membrane protein which constitutively expressed the lumenal surface of vascular endothelial cells. It serves as an anti-coagulation, anti-fibrinolytic and anti-inflammatory properties. For example, the possible mechanisms cause thrombomodulin releasing into blood circulation system such as oxidative stress. Therefore, oxidative stress is likely the one major problem to endothelial cell damage. A previous studies report that cadmium bind with sulfhydryl group and depleted antioxidant enzyme which induced increase ROS (49). Cadmium is also a potent inhibitor of electron transport chain process inside mitochondria by interfering the complexes II (succinate:ubiquinone oxidoreductase) and III (ubiquinol:cytochrome c oxidoreductase) resulting in stimulation of ROS production in mitochondria (37). Not

only oxidative stress occurred in chronic cadmium exposure but also chronic inflammation was associated with endothelial cell damage. Recently, cadmium has been shown to promote inflammation by interfering the integrity of endothelial cell layers through initiating facilitate pro-atherogenic serum components and leukocyte trafficking (39). Therefore, chronic cadmium exposure could cause endothelial cell dysfuction.

In order to prove that cadmium would reduce NO synthesis, we investigated the effects of cadmium on the NO production by HCAECs in vitro. The cytotoxicity of cadmium was evaluated by MTT assay. Cadmium at 100 μM incubated with HCAECs for 48 h, cadmium at 300 μM for 6 and 24 h caused significant endothelial cell death, and the 50% toxic concentration are 134 ± 2.2, 116.6 ± 1.4, and 63.5 ± 1.1 μM, respectively. Surprisingly, cadmium at low concentrations of 1, 3, 10 and 30 μM slightly promoted proliferation of HCAECs. This result was consistent with a previous report that cadmium at low concentrations (1-10 μM) seem to enhance the endothelial cell proliferation by suppression apoptosis pathway. Cadmium could inhibit the apoptosis by blocking procaspase-3 and caspase-9 in renal mesangial cells (9). Cadmium also induces metallothionein (MT) expression that bind to cadmium and forms cadmium-MT complex lead to detoxifying the toxicity of cadmium on vascular endothelial cell (50). Recently, cadmium was reported that at 4 μM increased human umbilical vein endothelial cell (HUVEC) permeability following 12 h exposure through p38-meadiated signaling pathway without inhibiting cell proliferation or viability (50).

At high concentration (> 100 μM) of cadmium caused cell death. Cadmium may induce apoptosis by the following mechanisms; 1) ER-mediated pathway by ER stress and calcium release from ER in the kidney, 2) mitochondria-mediated pathway by interfering directly or indirectly with electron transport chain in mitochondria, and 3) p53-dependent pathway by suppressing the Ube2d gene, decreasing p53 degradation and leading to accumulation of p53 (51). Cadmium could induce apoptosis through p38 MAPK pathway in brain microvascular endothelial cells followed by activation p53 (52). Cadmium also induces endothelial cell death through necrosis pathway (53).

Cadmium is taken up into endothelial cells and causes endothelial cell damage by disrupting cell-cell junction (6), increasing the vascular permeability and depleting glutathione and ATP (35). Cadmium also exerts cytotoxic effect on endothelial cell via indirect generation of ROS following its binding to protein sulfhydryl (-SH) and subsequent impaired antioxidant defense (49). Moreover, uncoupled eNOS is capable of producing ROS following depletion of substrate L-arginine and absence of cofactor such as tetrahydrobiopterin (BH_4). The uncoupled eNOS would produce ROS rather than NO. Superoxide may react with NO forming toxic peroxynitrite (54). Cadmium would decrease activity of dihydropteridine reductase (DHPR), an enzyme responsible for maintenance of tetrahydrobiopterin (BH4) level, leading to BH_4 imbalance and subsequence to eNOS uncoupling (55). Therefore, endothelial cell is one of the most likely targets of cadmium (18).

From our study, 0.1 μM cadmium reduced NO production in HCAECs via blocking the phosphorylation of eNOS at serine 1177 site whereas eNOS mRNA was unchanged. These findings are in agreement with a previous study using immortalized endothelial hybrid cell line (9). The reduction of NO synthesis by endothelial cells was observed at 4-12 h incubation with 0.1 μM cadmium. However, an *in vivo* study showed that cadmium could reduce eNOS protein levels and decrease vasodilation in response to acetylcholine in rats (8). The result from *in vivo* might reflect closed to human than our *in vitro* study. Because the different in the condition of incubation time and concentration of cadmium. However, the phosphorylation may see in animal model also.

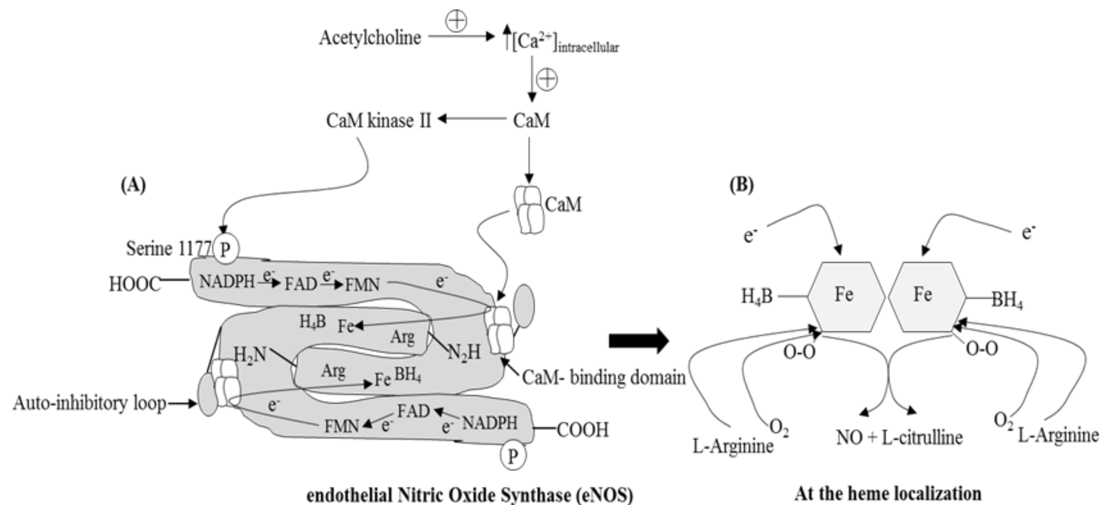

Figure 6.1 A scheme of eNOS activation. Initiation step, acetylcholine induced elevates of intracellular of calcium. Calcium activates calmodulin and form complex (calcium-calmodulin). Calcium/calmodulin activates calmodulin kinase II enzyme to phosphorylate on serine residue (1177). At the same time, calmodulin binds to CaM-binding domain and activates CaM-binding domain to transfer electron flux from reductase domain via NADPH to oxygenase domain (A). At the heme, electron is carried to iron located at oxygenase domain of eNOS (B). Electrons are used to reduce and activate oxygen molecules. To synthesize NO, L-arginine and oxygen are needed. Adapted from reference (22).

The serine residue at 1177 is a primary site for phosphorylation of eNOS (56). Phosphorylation at this site leads to increased eNOS activity. eNOS is an essential enzyme in regulation of blood flow by production of NO (57). eNOS activity may be regulated by a signaling cascade for regulation of eNOS mRNA (56), post-translational regulation by phosphorylation on multiple residues such as serine 617, 635 and 1177 which serve as activation sites for eNOS phosphorylation (57), and regulation of protein-protein interactions by interaction of eNOS with caveolin-1 which is a membrane-bound protein (ref). eNOS phosphorylation at these activation sites could be induced by acetylcholine, bradykinin, β2–adrenergic receptor agonist, thrombin, and ATP (56). In contrast, the phosphorylation at serine 116 and threonine 495 causes inhibition of eNOS activity.

In this study, we focused on the phosphorylation of eNOS at serine 1177 (activation site located in reductase domain). eNOS activity is modulated by kinases and phosphatases, and requires L-arginine and cofactors including tetrahydrobiopterin (BH4), iron, FMN, FAD and NADPH (56). eNOS phosphorylation at site serine 1177 occurs when intracellular calcium concentration is elevated.

Calcium binds to calmodulin (CaM), forming calcium-CaM complex which activates calmodulin kinase II (CaM kinase II) enzyme and stimulates CaM-binding domain. After CaM kinase II enzyme is activated, serine 1177 at reductase domain of eNOS is phosphorylated. At the same time, CaM-binding domain is stimulated, electron is transferred from reductase to oxidase domain (Figure 6.1) (56). C-terminal reductase domains of eNOS is responsible for transferring electron from NADPH to FAD and FMN, and finally to heme of oxygenase domains. At the heme of oxygenase domains, electrons are used to synthesize NO from O_2 and L-arginine in the presence of BH4 as cofactor.

eNOS activation may be mediated through calcium-dependent or calcium-independent pathways. Calcium-dependent pathways include eNOS activation by acetylcholine, bradykinin, VEGF-A, etc. On the other hand, shear stress produces calcium-independent eNOS activation. In calcium-dependent pathway, calcium-CaM complex binds to CaM-binding domain of eNOS. Simultaneously, calcium-CaM complex activates calmodulin kinase II, an enzyme that phosphorylates eNOS at serine 1177.

Under physiologic condition, endothelial cells are exposed to hemodynamic forces such as laminar shear stress. In calcium-independent pathways, shear stress induces eNOS phosphorylation via phosphoinoside 3-kinase (PI3K)/Akt and adenylate cyclase (AC)/ protein kinase A (PKA) pathways (58). PI3K/Akt phosphorylates at serine 615 and 1177, whereas AC/PKA phosphorylates at serine 633 and 1177. Most agonists induce eNOS phosphorylation through both calcium-dependent and calcium-independent pathways. For example, VEGF, bradykinin and estrogen also induce eNOS phosphorylation through calcium-independent pathway (activated PI3K/Akt and phospholipase C-γ (PLC-γ) pathway).

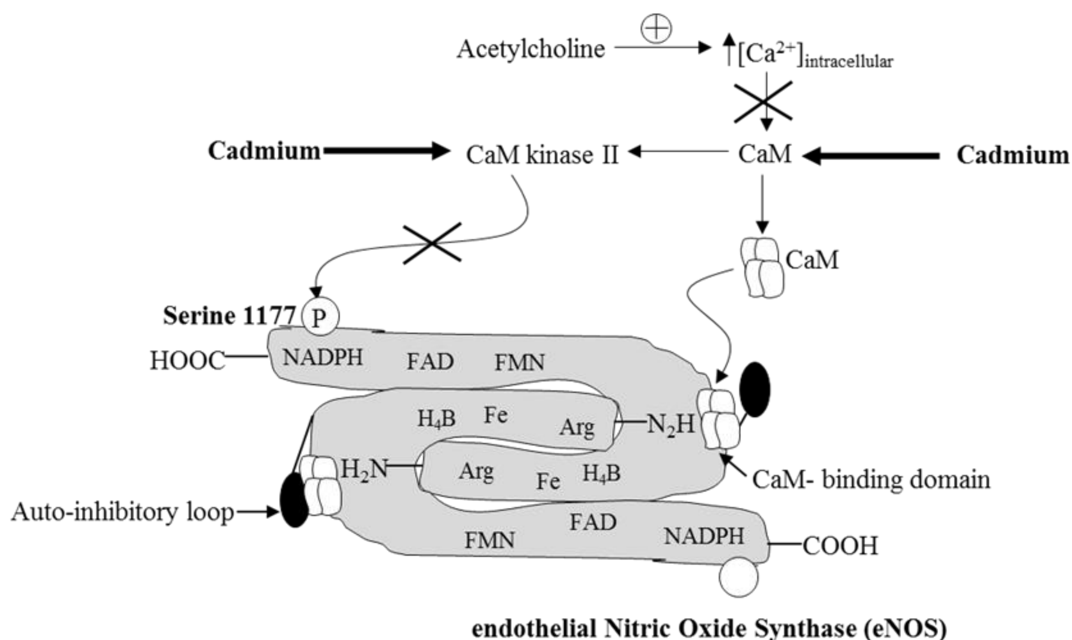

Figure 6.2 A sketch of hypothetic events that cadmium may inhibits CaM kinase II enzyme lead to impairment of eNOS phosphorylation. Adapted from reference (22).

Acetylcholine induces eNOS phosphorylation after its binding to acetylcholine receptor (AchR) on plasma membrane. Acetylcholine interacts with acetylcholine receptor lead to activation of phospholipase C-γ (PLC-γ). PLC- γ produces IP3 which increases the cytoplasmic calcium levels. Then, calcium binds to CaM. Calcium-calmodulin complex activates CaM kinase II enzyme to phosphorylate on serine 1177. At the same time, calcium-calmodulin complex binds to CaM-binding domain of eNOS, promoting the alignment of reductase and oxygenase domains for NO synthesis. Our study showed that cadmium could inhibit acetylcholine-induced NO synthesis by suppression eNOS phosphorylation at serine 1177. Thus, cadmium would inhibit CaM kinase II enzyme (Figure 6.2). It is unknown whether cadmium could bind to CaM and then cause inactivation of CaM. In addition, the effect of cadmium on acetylcholine-induced phosphorylation at threonine 495 (inhibition site) remained to be investigated.

Other divalent metals may have effect on eNOS. Sodium arsenite at concentrations higher than 10 μM could attenuate eNOS phosphorylation at serine 1177 site and decrease Akt and eNOS protein levels, thus leading to decreased eNOS

activity in porcine aortic endothelial cells (59). Moreover, methyl mercury has been report to inhibit NO production and eNOS activity in HUVEC (60) which may be associated with chronic exposure to mercury induced hypertension in patient subjects (61). In addition, Lead (Pb) has been shown to reduce NO bioavailability in rats resulting in hypertension (62)