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Mechanisms of Endothelial Dysfunction: Clues from Cyclosporine

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Abstract: There has been recent interest in the role of the kidney in the regulation of metabolic byproducts thought to be involved in the induction of endothelial dysfunction. The symptoms include increased blood pressure, decreased kidney function, increase in serum markers such as transforming growth factor beta, reactive oxygen species, asymmetric dimethylated arginine and an increase in the prothrombic and inflammatory state of the vasculature. Pathologies that exhibit this unique set of physiological findings include such diseases as diabetes, hypertension and other types of cardiovascular disease. Interestingly, the side effects of cyclosporine (CsA) treatment or more specifically, CsA induced nephropathy, exhibit many of the same type of symptoms. CsA was introduced as a way to inhibit the immune system for the purposes of increasing the long-term success of organ transplantations. However, as the CsA doses were increased to gain a higher level of immune system suppression, the greater the incidence of CsA induced side effects. When these are examined, there is a strong resemblance to the group of physiological findings in endothelial dysfunction. In what was supposed to be an immune system suppressant, CsA appears to have the ability, in high doses, to initiate an inflammatory response. This response seems to implicate a mechanism with its roots in the calcineurin/nuclear factor of activated T-cells (NFAT) pathway. Kidney dysfunction and hypertension are precursors to endothelial dysfunction and the calcineurin/NFAT pathway, which is disrupted by CsA, is strongly implicated in the process and may be a key to understanding the pathophysiology.

Key words: TGF- β , nitric oxide, reactive oxygen species, asymmetric dimethylated arginine

Introduction

There has been recent interest in the role of the kidney in the regulation of metabolic byproducts thought to be involved in the induction of endothelial dysfunction. Information is lacking on the possible mechanism behind the decrease in endothelial nitric oxide synthase (eNOS) that seems to underlie the group of symptoms typical of endothelial dysfunction. These symptoms include: increased blood pressure, decreased kidney function, increase in serum markers (e.g., transforming growth factor beta, reactive oxygen species and asymmetric dimethylated arginine) and an increase in the prothrombic and inflammatory state of the vasculature (Mezzano *et al.*, 2001; Endemann and Schiffrin, 2004; Li *et al.*, 2004a). Pathologies that exhibit this unique set of physiological findings include diseases such as diabetes, hypertension and other types of cardiovascular disease. Interestingly, the side effects of cyclosporine treatment, specifically Cyclosporine Induced Nephropathy (CIN), exhibit many of the same type of symptoms.

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Consideration of the side effects of cyclosporine (CsA) action on the human and mammalian organism may provide insight into a mechanistic understanding of the disease process known as endothelial dysfunction which impacts millions of people. The calcineurin/ nuclear factor of activated T-cells (NFAT) pathway may be key to understanding this pathophysiology.

Cyclosporine A

Background

The drug CsA and other calcineurin blocking agents have given new hope to transplant patients who would not have survived without powerful immune suppression. The clinical manifestations of CsA are well known to physicians and patients involved in organ transplantation. However, the focus of this paper is not on the role of CsA in organ transplantation, but on its role in CIN and the similarity to a newly recognized pathophysiology known as endothelial dysfunction.

CsA provides insight into the complex and dynamic role that the calcineurin/NFAT pathway has played in the homeostatic mechanisms of endothelial cells. Many of these lessons can give perspective into epithelial cell regulation and its role in the regulation of hemodynamic mechanisms and dependence on kidney physiology. These lessons have widespread potential to aid research into treatment for human pathologies such as diabetes, hypertension, heart disease and organ transplantation.

Developed in the late 1970's, CsA revolutionized the world of transplantation medicine during the 1980's. The introduction of CsA allowed dramatic increases in one year allograft survival rates of heart, lung, kidney and liver transplants; transplant successes, which were virtually unthinkable prior to the use of CsA. However, many of the mechanisms that make CsA an effective immunosuppressant also cause detrimental pathological conditions.

CsA, also known as Neoral (Novartis™) is an immunosuppressive cyclic protein (Fig. 1) that was derived from the fungus *Tolypocladium inflatum* or, in the case of Neoral, from the metabolites of *Beaveria nivea*. CsA is used in the treatment of solid organ and bone marrow transplants, as well as autoimmune diseases such as psoriasis and Ig A nephritic syndrome (Parhan, 2005).

Mechanism of Action of Cyclosporine

CsA is thought to enter the cell via a mediated process, possibly by a cell membrane transporter protein (Takayama *et al.*, 1991). Once in the target cell, CsA owes its therapeutic action to inhibition of lymphokine production and general activation of T-helper cells, by arresting the T-cells in the immuno-incompetent G0--G1 phase of the cell cycle. These lymphocytes are responsible for the cell-mediated immune response by recognizing an antigen presenting cell and then activating B cells and their subsequent antibody production. T-cell arrest occurs through inhibition of the calcineurin/NFAT pathway. CsA inhibits calcineurin by blocking its phosphatase activity and preventing dephosphorylation of its downstream molecular targets, such as NFAT (Fig. 2). Although T-cell activation is blocked by CsA, there is no effect on phagocytic activity, enzyme secretion and chemotactic migration of granulocytes and macrophages (Borel, 1991; Novartis, 2004).

Normally the calcineurin/NFAT pathway in T-cells, when activated by an antigen presenting cell, causes translocation of the NFAT transcription factor to the nucleus after dephosphorylation by calcineurin. NFAT then binds to specific sites on DNA to activate gene transcription and synthesis of proteins required for T-cell activation. These processes are blocked by CsA which inhibits the cell signaling cascade (Fig. 2) (Hocherl *et al.*, 2002; Granja *et al.*, 2004; Jimenez *et al.*, 2004; Waters *et al.*, 2005).

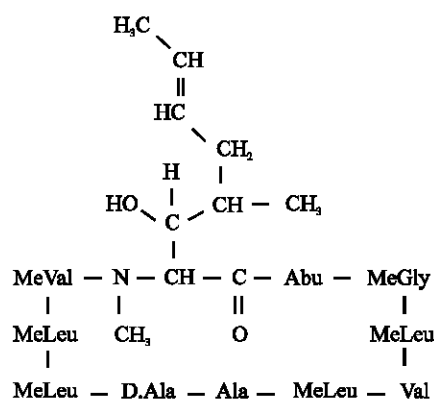


Fig. 1: The chemical structure of cyclosporine A, a cyclic protein derived from the metabolites of a fungus. Cyclosporine (mol. wt. 1203) is a general calcineurin inhibitor and is transported into cells via facilitative transport and metabolized via cytochrome P 450

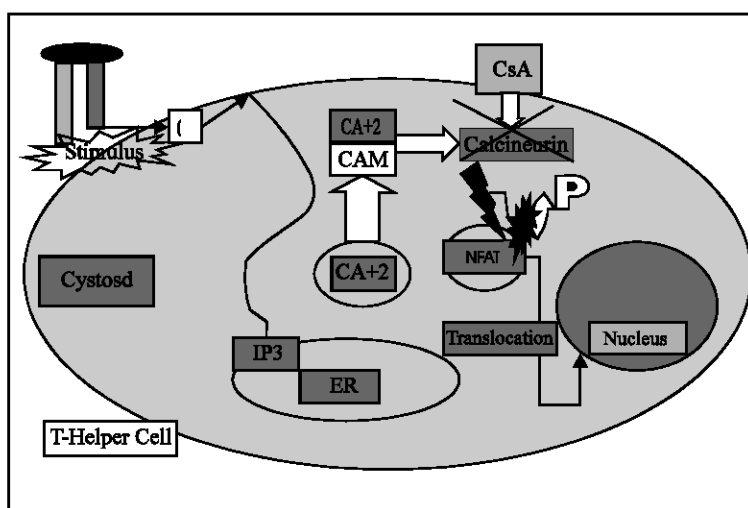


Fig. 2: The calcineurin/NFAT cell signaling pathway of activated T-helper cells. It is mediated via a G protein coupled receptor, inositol trisphosphate (IP3) and Ca^{2+} signaling, leading to activation of calcineurin by calmodulin (CAM). This pathway is well-defined in T-cells and recently has been discovered to play important regulatory roles in several other organs and tissues such as the brain, heart, kidneys, liver and the endothelium (Go *et al.*, 2004; Lopez-Rodriguez *et al.*, 2004; Puri *et al.*, 2004)

Much of the evidence for the role of the calcineurin/NFAT pathway comes from studies of NFAT knockout mice. NFAT has been shown to be expressed in the T-cells, brain, liver and kidney (primarily) as part of an adaptive mechanism to hyperosmotic stress (Gooch *et al.*, 2002; Go *et al.*, 2004; Lopez-Rodriguez *et al.*, 2004). Each of these organs and regions within, exhibit different isoforms of calcineurin and NFAT (Schinkel *et al.*, 1995; Tumlin, 1997; Gooch *et al.*, 2002). It is

believed that the survival of these tissues is dependent upon transport proteins that are regulated by the calcineurin/NFAT pathway. A good example of this is in kidney tubule epithelial cells normally exposed to hyperosmotic stress. The calcineurin/NFAT pathway regulates the transcription and translocation of transport proteins from the cytoplasm to the cell plasma membrane to facilitate transport of osmolytes, such as betaine, into the cells to balance extracellular hyperosmolarity (Go *et al.*, 2004). Independently of NFAT, calcineurin has been shown to regulate other proteins, such as aquaporin 2, by controlling the phosphorylation state (Jo *et al.*, 2001). Aquaporin 2 is a member of the family of water channel proteins required for water reabsorption in the kidney. NFAT knock out studies in mice showed the most severe effects in the kidneys. Mice that survived beyond the embryo stage showed atrophied, fibrotic and inflamed kidneys and had severely shortened life spans (Lopez-Rodriguez *et al.*, 2004).

Recent evidence supports the importance of the calcineurin/NFAT pathway in endothelial cells, where it has been implicated in the regulation of angiogenesis, although it has yet to be shown whether the link is direct or indirect. Studies in animal models with calcineurin inhibition (usually through CsA administration) show decreased ability for angiogenesis or a decrease in the animal's the ability to grow and develop new circulatory vessels (Rafiee *et al.*, 2004; Schrijvers *et al.*, 2004).

Side Effects of CsA Treatment

While CsA is a potent immune system suppressant because of its ability to block the calcineurin/NFAT pathway, the drug has a number of dose dependant side effects (Briggs, 2001; Shapiro, 2004). The normal dosing range of CsA is from 3.5 mg kg⁻¹, depending on the level of immunosuppression that is needed (Novartis, 2004). Factors that effect the treatment of patients with CsA and therefore their dosing regimen include the degree of HLA mismatch, insufficient immune system suppression, the number and severity of rejection episodes, the age of the donor or the patient, the existence of ischemic or reperfusion injury, side effect manifestations as a result of drug therapy and the presence of preexisting medical problems (Jurewicz, 2003). Dosing of CsA in the patient must be carefully monitored and a balance must be established between the need for proper immune system suppression and toxicity to the patient. In order to achieve this proper balance, the patient must be submitted to routine blood analysis and invasive biopsies which are used to develop a definitive diagnosis of either drug toxicity or acute allograft rejection (Brown *et al.*, 2001; Pefaur *et al.*, 2003; Li *et al.*, 2004a). As the dose of CsA is increased, there is a higher incidence of adverse effects, especially related to kidney physiology and health. The most common side effects include hypertension, decreased renal glomerular filtration rate, decreased renal blood flow, renal fibrosis, inflammation of renal glomeruli and medulla and decreased angiogenesis and vasculopathy (Schinkel *et al.*, 1995; Briggs, 2001; Justo *et al.*, 2003; Perez *et al.*, 2004; Jurewicz, 2003; Shapiro, 2004).

NFAT knockout studies have shown kidney pathology similar to that of human CIN. CIN patients and NFAT knockout animals both show altered kidney structure with abnormal anatomical landmarks, segments of kidney which show irregularities, tissue atrophy and inflammation of the renal cortex (Lopez-Rodriguez *et al.*, 2004). These studies provided valuable insight into the importance of NFAT in the kidneys, but were not focused on CIN and its possible role in endothelial dysfunction. The knockout studies do not prove causality and there is still much to learn about the causes of hypertension and vasculopathy that are also common in CIN. As will be shown there is evidence for a cyclic effect between kidney function (or dysfunction) and hemodynamic regulation. The decreased autoregulation of the vasculature, seen in CIN, has an intimate and potentiating effect on the observed pathology.

The cause of the symptoms found in CIN has been the focus of many lines of research. Emerging evidence has shown that the process is similar to what has been termed endothelial dysfunction. Endothelial dysfunction is a group of symptoms and physical findings which include increased blood serum levels of transforming growth factor beta (TGF- β) and asymmetric dimethylated arginine (ADMA), hypertension, renal failure and many forms of cardiovascular disease (Endemann and Schiffrin, 2004). Several of these factors are considered here as a possible link between the calcineurin/NFAT pathway and endothelial dysfunction.

Endothelial Nitric Oxide Synthase

It has been well documented that there is a significant decrease in the levels of nitric oxide production in the vascular endothelium in disease processes consistent with endothelial dysfunction. This decrease in production has been the main focus of attention. The main cause of this decrease in production has yet to be discovered however, it is generally agreed that there is a mechanism which inhibits endothelial nitric oxide synthase (eNOS), the enzyme which catalyzes production of nitric oxide from arginine. The decrease in eNOS activity has very serious implications. As the dominant stimulus for vasodilatation, nitric oxide is a very important cell signal molecule in the regulation of vascular hemodynamics. It causes relaxation of vascular smooth muscle and dilation of the vasculature, it prevents platelet aggregation, it helps prevent localized inflammation by down-regulation of ICAMS and it aids angiogenesis (Vallance and Leiper, 2004; Endemann and Schiffrin, 2004).

Nitric oxide is an endogenous molecule which is utilized in paracrine cell signaling, smooth muscle relaxation and hemodynamic regulation of blood flow. Nitric oxide is produced during oxidation of arginine to citrulline, a reaction catalyzed by eNOS (Fig. 3). In the vasculature, the nitric oxide diffuses from the endothelium to the underlying smooth muscle cells where it binds to intracellular guanylate cyclase. This binding activates the cyclase which converts GTP to cyclic GMP, which acts to decrease cytosolic Ca^{2+} concentration. The result is relaxation of smooth muscle cells and dilation of the blood vessel. Nitric oxide production is controlled by several mechanisms including acetylcholine, histamine and shear stress forces against the endothelium caused by the flow of blood. Without eNOS, vessel

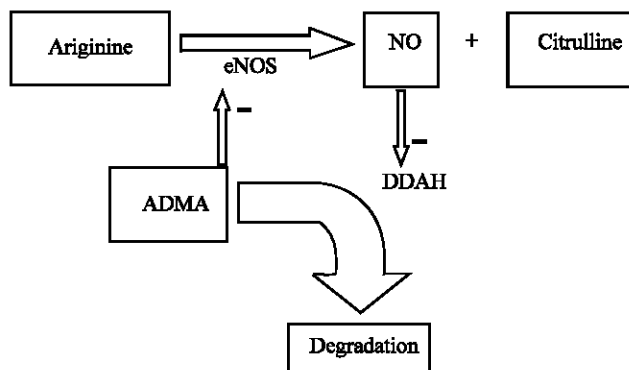


Fig. 3: The role of endothelial nitric oxide synthase (eNOS) in NO production, showing the auto-feedback regulation that occurs between asymmetric dimethylated arginine (ADMA) and eNOS, as well as NO and dimethylarginine dimethylaminohydrolase (DDAH). The inhibitory effect of ADMA on eNOS occurs in endothelial dysfunction and is postulated to be regulated by the calcineurin/NFAT pathway. This figure was modified from Vallance and Leiper (2004)

dilation does not occur, the sympathetic tone of the vasculature prevails and there is an increase in blood pressure and hypertension can be established. Chronic hypertension has potentially catastrophic consequences such as decreased microvascular growth and vasculopathy, which are especially damaging to organs such as the kidneys which receive approximately one quarter of the cardiac output in humans.

The root cause of eNOS inhibition has eluded researchers. There are some likely suspects, such as TGF- β , reactive oxygen species and ADMA, all of which are known to be inhibitors of eNOS and all of which are up-regulated in epithelial dysfunction and CIN.

TGF- β

TGF- β has important growth regulatory effects, especially in embryonic development. However, in adult animals, TGF- β is responsible for the development of fibrotic growth or scar tissue development. Among other effects, TGF- β has also been linked to the up-regulation of vascular endothelial growth factor receptors on vascular epithelial cells (Campistol *et al.*, 2001), which have been shown to bind phagocytic cells of the immune system that can cause inflammation. Clinicians and researchers who have investigated the pathology that is evident in CIN and endothelial dysfunction, have identified TGF- β as a key cytokine implicated in the pathogenesis of the kidney. Research to determine the causes and effects of the up-regulation of TGF- β and the apparent pathology it is thought to produce, shows that the cytokine plays a significant role in eliciting an inflammatory state. The exact mechanism behind stimulation and release of TGF- β is still unclear, but it appears that TGF- β may be a symptom, rather than a cause, of many of the problems associated with endothelial dysfunction.

TGF- β is responsible for much pathology, such as synthesis, proliferation and degradation of the extracellular matrix, tubulointerstitial fibrosis and glomerular sclerosis (Campistol *et al.*, 2001). TGF- β appears to be stimulated by reactive oxygen species (ROS) and ROS increases are common in CsA treatment and in pathologies associated with endothelial dysfunction. TGF- β is also increased by the renin-angiotensin system (Li *et al.*, 2004b) and is released from kidney endothelial tissue during the apoptosis (Ling *et al.*, 2003) that results from a decreased ability to adapt to osmotic stress (Lopez-Rodriguez *et al.*, 2004). There is an increase in the transcription factor AP-1, which regulates TGF- β transcription and a subsequent increase in TGF- β mRNA in the cells treated with CsA (Saggi *et al.*, 2004). This increase in TGF- β levels has been shown to inhibit nitric oxide production by eNOS and appears to be a cause of increased endothelin production (Campistol *et al.*, 2001). There is some argument whether there is a causative effect of the increase in TGF- β and the symptoms found in endothelial dysfunction and CIN. This stems from evidence gathered in several studies which show that TGF- β up-regulation and release is in fact preceded by hypertension, decreased renal blood flow and a decreased rate of glomerular filtration, suggesting that there may be other causative influences (Waiser *et al.*, 2002).

It has been known that eNOS is inhibited in patients who are in various stages of kidney failure and exhibit increased levels of serum and urine TGF- β (Goumenos *et al.*, 2002). Since TGF- β is a known eNOS inhibitor, treatment strategies has focused on blocking or attenuating TGF- β production. Several strategies include antibody therapy to block TGF- β action (Ling *et al.*, 2003). However, many of the most successful and practical treatments focused on countering the influence of the renin-angiotensin system or, in other words, treating the resultant hypertension that occurs as a result of the inability of the vasculature to dilate when nitric oxide is reduced (Campistol *et al.*, 2001;

Waiser *et al.*, 2002). This hypertension occurs prior to the release of large quantities of TGF- β , so it appears likely that another factor inhibits eNOS prior to up-regulation and release of TGF- β .

Reactive Oxygen Species

ROS have been studied for a possible role in CIN and endothelial dysfunction because ROS could be produced directly or indirectly through metabolism of CsA. ROS are known to stimulate TGF- β (Waiser *et al.*, 2002), which in turn interferes with nitric oxide generation through inhibition of the eNOS co-factor tetrahydrobiopterin. Generation of ROS after CsA injection was seen more frequently in the earlier formulations of the drug and in some current generic brands which have poor absorption rates so the drug remains in the blood longer. The studies on the role of CsA in ROS generation showed that there is indeed a correlation between the administration of the drug, ROS formation and eNOS inhibition (Krauskopf *et al.*, 2002). However, as with TGF- β , a direct link between the CsA and ROS formation was not found. A recent study with cultured cells investigated ROS formation both directly from CsA administration and indirectly from CsA metabolism by cytochrome P-450, a common pathway for oxidative metabolism. ROS were not produced directly by CsA addition, nor as a by-product of CsA metabolism, even when using doses at the upper end of the therapeutic range (Krauskopf *et al.*, 2002).

ROS generation has also been implicated in pathologies related to endothelial dysfunction, independently of CsA treatment. For example, studies on diabetes induced nephropathy looked to ROS generation as a possible explanation for the decrease in NO production and vasculopathy and the increase in TGF- β release (Gooch *et al.*, 2002). However, the conclusions are often invalidated by controlled testing and a mechanism of action has not been elucidated.

Asymmetric Dimethylated Arginine

Methylated arginines are the products of post-translational modification of proteins via the addition of methyl groups by enzymes known as protein arginine methyl transferases (Fig. 4). These enzymes are found in the nucleus and are thought to be responsible for processing and controlling RNA transcription. Methylated arginines are released into the cytoplasm as part of normal protein turnover. These methylated arginines take one of three major forms, asymmetric dimethylated arginines (ADMA), monomethylated arginines (MMA) and symmetric dimethylated arginines (SDMA), with ADMA being the most prevalent (Fig. 4). In the cytoplasm the methylated arginines are degraded by the enzyme dimethyl arginine dimethylaminohydrolase (DDAH) (Fig. 4).

There is a strong correlation between serum ADMA levels and an increase in systemic blood pressure. One of the consequences of high levels of circulating ADMA is that it can inhibit eNOS by competing with arginine for binding sites on the enzyme, thereby blocking its ability to oxidize arginine to nitric oxide and citrulline (Fig. 3) (Vallance and Leiper, 2004). This competition can cause a decrease in NO production and lead to a vasoconstrictive state which is common both in diseases associated with endothelial dysfunction and as a side effect of CsA treatment. There is also a correlation between increased ADMA levels and kidney dysfunction, hypercholesterolemia, glucose tolerance, atherosclerosis and aging (Nijveldt *et al.*, 2002; Endemann and Schiffrin, 2004; Vallance and Leiper, 2004). In fact, ADMA has been shown to be a significant predictor of death in critically ill patients studied in intensive care units. Increases in ADMA levels were associated with the onset of multiple organ failure (Nijveldt *et al.*, 2004).

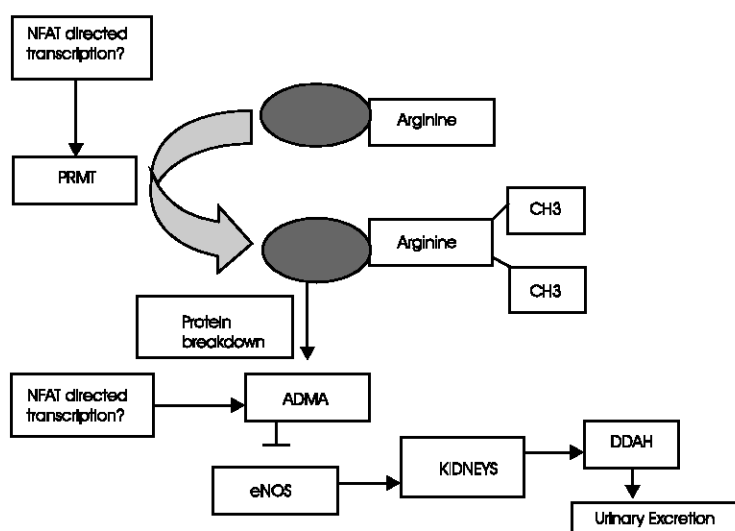


Fig. 4: The role of protein arginine N-methyltransferases (PRMT) and dimethylarginine dimethylaminohydrolase (DDAH) in protein breakdown within a cell. PRMT marks proteins for degradation, especially in the nucleus and DDAH converts asymmetric dimethylated arginines (ADMA) into non-reactive methyl arginines. This highlights the role that the calcineurin/NFAT pathway could play in regulation of protein turnover in cell. It also suggests a potential role in disease pathways, such as endothelial dysfunction, via increased ADMA and increased inhibition of endothelial NO synthase (eNOS). This figure was modified from Nijveldt *et al.* (2004)

Under normal circumstances DDAH clears the ADMA and other methylated arginines from the circulation as the blood is filtered through the kidneys (Nijveldt *et al.*, 2004), where levels of DDAH are relatively high. It may be possible that as levels of ADMA increase, the level of DDAH activity is no longer adequate and some ADMA remains intact to inhibit nitric oxide production. This is exhibited in experiments on human subjects where injection of ADMA resulted in a marked increase in vasoconstriction and a decrease in cardiac output (Nijveldt *et al.*, 2002; Vallance and Leiper, 2004).

There is a feedback loop between DDAH and nitric oxide (Fig. 3) (Vallance and Leiper, 2004). Nitric oxide can inhibit DDAH which, in turn, increases the levels of ADMA so that eNOS activity is suppressed. This allows for a self-regulating system where nitric oxide and ADMA levels keep each other in check and hemodynamic controls are maintained. However, much remains unknown about the role of ADMA in disease processes, what causes it to increase and whether ADMA is the sole cause of the pathophysiology in endothelial dysfunction.

Conclusions

CsA was introduced as a way to inhibit the immune system for the purpose of increasing the long-term success of organ transplants. However, as the CsA doses increased to gain a higher level of immune system suppression, the incidence of CsA induced side effects also increased. When the list of these side effects is examined, there is a strong similarity with the findings in endothelial

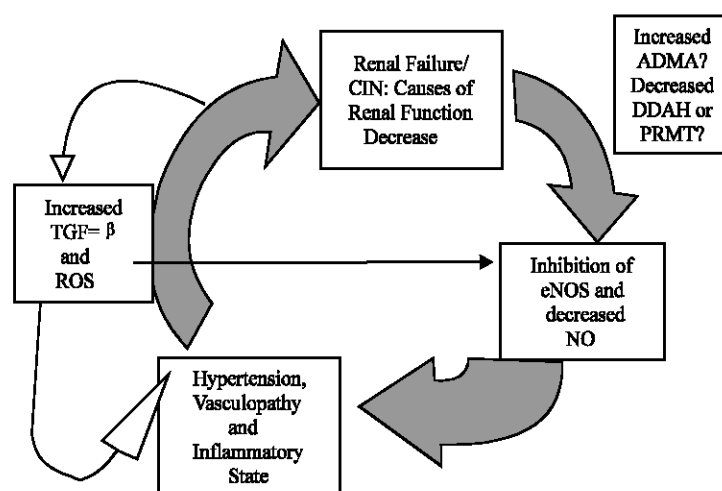


Fig. 5: The proposed cycle of events that leads to endothelial dysfunction, a group of physiological findings that occurs in cyclosporine induced nephropathy (CIN) and other disease processes. This cycle is complicated with many interrelated aspects, yet in some cases, there appears to be a link to a possible defect of the calcineurin/NFAT cell signaling pathway. (ADMA, asymmetric dimethylated arginine; DDAH, dimethylarginine dimethylaminohydrolase; PRMT, protein arginine N-methyltransferases; eNOS, endothelial NO synthase; ROS, reactive oxygen species; TGF- β , transforming growth factor- β .)

dysfunction. In what was supposed to be an immune system suppressant, CsA appears to have the ability, in high doses, to instigate an inflammatory response. This response seems to implicate a mechanism with its roots in the calcineurin/NFAT pathway.

The calcineurin/NFAT pathway is an important cell signaling pathway in many different tissues and organs, especially the kidney. This is seen through the devastating effects of knockout studies in animals and the side effects of CsA treatment on renal function. The kidneys, which are the starting point for endothelial dysfunction, receive one quarter of the cardiac output and are responsible for maintenance of ionic, pH, fluid and nutrient homeostasis. In addition, these remarkably complex organs are the sites of a wide range of hemodynamic controls, such as the renin-angiotensin system and enzymes such as DDAH. It is not surprising that patients with endothelial dysfunction frequently exhibit some stage of renal failure.

The development of CIN may provide insight into the sequence of events that lead to endothelial dysfunction. Even at therapeutic levels of CsA, there is a frequent occurrence of hypertension, decreased renal blood flow and decreased glomerular filtration rate within the first months of CsA treatment. Hypertension has often been suspected as a predictor of underlying disease processes, including metabolic disorder and endothelial dysfunction. This suggests that a process beginning with inhibition of eNOS and the subsequent decreased synthesis of nitric oxide may precipitate the cycle of disease events (Fig. 5).

Many different possible causes of eNOS inhibition have been considered and include (as discussed here) TGF- β , ROS and ADMA. Of these, TGF- β has been studied the most and it is easy to see that TGF- β is influential especially in the formation of scar tissue and inflammation i.e., renal fibrosis.

However, the effects appear to be a result of the development of hypertension and vasculopathy. Therefore, TGF- β is unlikely to be the root cause of the findings in endothelial dysfunction and CIN. Like TGF- β , it appears that the generation of ROS is a secondary result of a cycle of pathology initiated by another culprit. ROS generation is a product of the inflammation and decreased blood flow that result from inhibition of NO production.

ADMA inhibits eNOS, causes vasoconstriction and is highly correlated with the disease processes of endothelial dysfunction. The same is true for TGF- β , ROS and several other metabolic byproducts correlated with this disease. However, ADMA is unique because its degradation by DDAH first requires extraction from the circulation and accumulation in the kidneys.

Perspectives for Future Research

There are two possible scenarios to connect the calcineurin/NFAT pathway to ADMA and eNOS inhibition. First, calcineurin isoforms are important cell regulators of plasma membrane transport proteins, such as aquaporin 2 and may regulate localization of these proteins to the plasma membrane by controlling the phosphorylation state as part of protein complexes (Jo *et al.*, 2001). Calcineurin may have a similar action on transport proteins which are responsible for the uptake of ADMA in renal tubules. If this is the case, an inherited or acquired defect of the calcineurin/NFAT pathway, such as inhibition by CsA, could block the ability of this transport protein to localize to the cell membrane and the result would be an increase in plasma ADMA levels (Fig. 6). Alternatively, pharmacological inhibition or genetic and/or structural defects of calcineurin would block NFAT activation and prevent transcriptional activation and synthesis of the transport protein.

An additional possibility to examine relates to the possibility of DDAH control by NFAT. Methylated arginines are a part of normal cell protein turnover and these methylated arginines, including ADMA, are degraded by DDAH. As a transcription factor NFAT could regulate DDAH and/or PRMT synthesis in renal epithelial cells (Fig. 7). In this case, a genetic or acquired defect of the calcineurin/NFAT pathway would prevent the synthesis of sufficient DDAH and/or PRMT in the kidney, preventing normal protein turnover, as highlighted in the diabetic kidney (Gooch *et al.*, 2002, 2004) and possibly resulting in an increase in plasma ADMA and other metabolites which depend on transporter proteins for renal clearance.

Whether or not ADMA has a key role, the fact remains that kidney dysfunction and hypertension are precursors to endothelial dysfunction and the calcineurin/NFAT pathway is strongly implicated in the process. This is highlighted with research on patients with diabetic nephropathy where there is a strong correlation with endothelial dysfunction. Though this is a product of increased activation of calcineurin rather than inhibition, many of the consequences are the same; patients exhibit renal deficiency, hypertension, increased serum TGF- β and ADMA and renal scarring (Gooch *et al.*, 2002; Kelly *et al.*, 2005). This could be viewed as a naturally occurring protein over-expression study, where upregulation of calcineurin leads to a detriment in protein synthesis which inhibits the physiological functions of the cell. Could this also produce an increase in serum ADMA levels by blocking the normal regulation of transport proteins or by saturating DDAH with the increased byproducts of protein production? Much more research into the calcineurin/NFAT pathway and its role in kidney physiology is needed to answer this question.

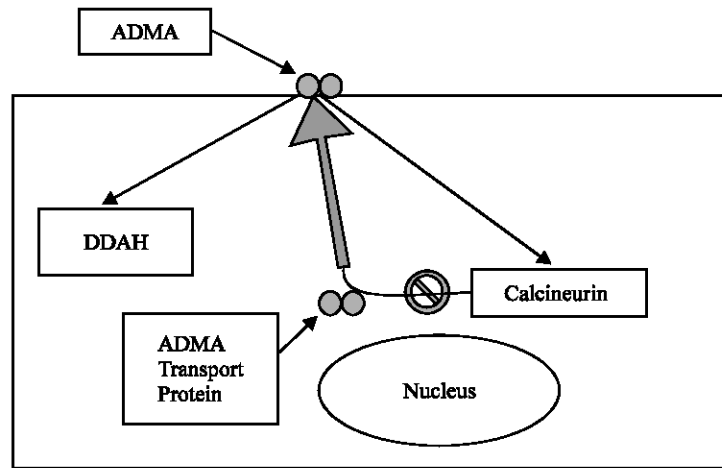


Fig. 6: Theoretical pathway that could link the calcineurin/NFAT pathway to ADMA regulation and eNOS inhibition, whereby calcineurin is responsible for the localization of the ADMA transporter to the cell membrane of a kidney tubule epithelial cell. Calcineurin has been implicated as part of protein complexes responsible for transporter localization to the membrane. A defective calcineurin could disrupt this complex formation and disrupt proper localization and/or transporter stability in the membrane. (ADMA, asymmetric dimethylated arginine; DDAH, dimethylarginine dimethylaminohydrolase)

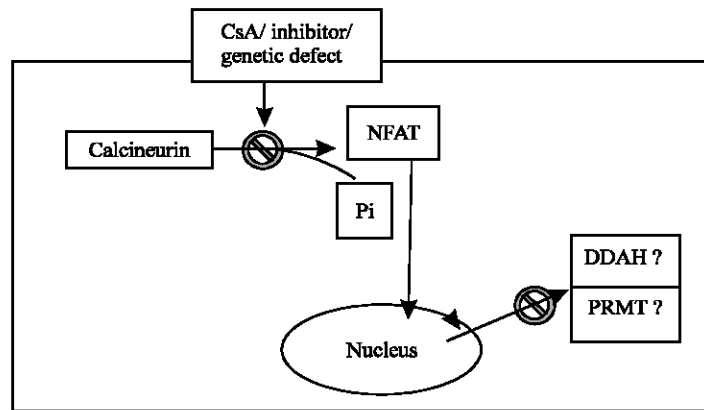


Fig. 7: Possible link between the calcineurin/NFAT pathway and eNOS inhibition, a main cause of endothelial dysfunction and cyclosporine induced nephropathy (CIN), where cyclosporine (CsA) or another chemical inhibitor or genetic defect blocks the transcription of DDAH or PRMT. One mechanism is depicted where CsA blocks dephosphorylation of NFAT by calcineurin, preventing its translocation to the nucleus. The loss or decreased transcription of DDAH or PRMT could lead to improper protein turnover and decreased renal clearance of ADMA from plasma. (ADMA, asymmetric dimethylated arginine; DDAH, dimethylarginine dimethylaminohydrolase; PRMT, protein arginine N-methyltransferases)

The calcineurin/NFAT pathway seems to play an important role in the onset of eNOS inhibition and endothelial dysfunction, as highlighted with the disruption of the pathway during CsA treatment and other disease processes. An improved understanding of the nuances of the calcineurin/NFAT pathway in various organs and tissues could lead to specific therapeutic targets, taking advantage of the various isoforms of the cell signaling pathway and advance the treatments for renal diseases and immune system suppression.

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