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REVIEW

Cyclosporin A and cardioprotection: from investigative tool to therapeutic agent

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Ischaemic heart disease (IHD) is the leading cause of death and disability worldwide. The pathophysiological effects of IHD on the heart most often result from the detrimental effects of acute ischaemia–reperfusion injury (IRI) on the myocardium. Therefore, novel therapeutic targets for protecting the myocardium against acute IRI are required to reduce injury to the heart, preserve cardiac function and improve clinical outcomes in patients with IHD. In this regard, the mitochondrial permeability transition pore (mPTP) has emerged as a critical target for cardioprotection which is readily amenable to intervention at the time of myocardial reperfusion. The formation and opening of the mPTP at the onset of myocardial reperfusion is a major determinant of mitochondrial dysfunction and cardiomyocyte death in the setting of acute IRI. The seminal discovery in the late 1980s that mPTP opening could be pharmacologically inhibited by the immunosuppressive agent, cyclosporin A (CsA), has been fundamental in the elucidation of the critical role of the mPTP as a mediator of acute IRI and, therefore, a viable target for cardioprotection. Its initial role as an investigative tool was used to identify mitochondrial cyclophilin D to be a regulatory component of the mPTP. The mPTP as a viable target for cardioprotection has recently been translated into the clinical setting with CsA reducing myocardial infarct size in patients. In this article, we review the intriguing role of CsA as a tool for investigating the mPTP as a target for cardioprotection and its potential role as a therapeutic agent for patients with IHD.

Abbreviations

CsA, cyclosporin A; Cyp, cyclophilin; IHD, ischaemic heart disease; IRI, ischaemia–reperfusion injury; LV, left ventricular; mPTP, mitochondrial permeability transition pore; NF-AT, nuclear factor of activated T-cells; PPCI, primary percutaneous coronary intervention; PPI-ase, peptidyl-prolyl isomerase activity; SfA, Sanglifehrin A; STEMI, ST-segment elevation myocardial infarction

Introduction

Ischaemic heart disease (IHD) is the leading cause of death and disability worldwide. One major manifestation of IHD is an acute ST-elevation myocardial infarction (STEMI), the treatment of which is myocardial reperfusion using either primary percutaneous coronary intervention (PPCI) or thrombolytic therapy to limit the duration of acute myocardial ischaemia and thereby halt the subendocardial to epicardial progression of the developing myocardial infarct. Over recent years, the process of myocardial reperfusion has been optimized to limit the duration of acute myocardial ischaemia in STEMI patients, with the timely and rapid institution of PPCI, and the use of new and more effective anti-platelet and anti-thrombotic therapy to maintain the patency of the infarct-related coronary artery. However, the process of myocardial reperfusion, which is clearly essential to salvage viable myocardium, is paradoxically associated with the death of cardiomyocytes, which were previously viable at the end of ischaemia (Piper *et al.*, 1998). This phenomenon is termed 'lethal myocardial reperfusion injury', and its presence mitigates the benefits of myocardial reperfusion in STEMI patients (Braunwald and Kloner, 1985; Yellon and Hausenloy, 2007). Currently, there exists no effective therapy

for protecting cardiomyocytes against the detrimental effects of lethal myocardial reperfusion injury.

Therefore, novel therapeutic strategies capable of protecting the heart against lethal myocardial reperfusion injury when administered as an adjunct to PPCI are urgently required, in order to further reduce myocardial infarct size, preserve cardiac function and improve clinical outcomes in patients with IHD. In this regard, the mitochondrial permeability transition pore (mPTP) has emerged as a critical target for cardioprotection (Hausenloy and Yellon, 2003; Halestrap, 2009; Di Lisa *et al*., 2011). The formation and opening of the mPTP at the onset of myocardial reperfusion is a major determinant of mitochondrial dysfunction and cardiomyocyte death in the setting of acute ischaemia–reperfusion injury (IRI).

In the late 1980s, it was discovered that the opening of the mPTP could be pharmacologically inhibited by the immunosuppressive agent, CsA (Crompton *et al*., 1988). In this article, we review the intriguing role of CsA as an investigative tool for exploring the mPTP as a target for cardioprotection and its potential role as a therapeutic agent for patients with IHD. For a comprehensive account of the mPTP, the reader is directed to the following recent articles (Halestrap, 2009; Di Lisa *et al*., 2011).

The discovery of cyclosporin A as an immunosuppressant

Cyclosporin A (CsA) is a lipophilic cyclic peptide of 11 amino acids with a molecular weight of 1202 kDa, which was first discovered in 1970 as part of a screening programme for anti-fungal antibiotics first initiated in 1958 (Borel and Kis, 1991). Scientists working for Sandoz Ltd (now Novartis in Basel, Switzerland) isolated a fungus called *Tolypocladium inflatum* Gams in a soil sample obtained from Hardangervidda in Norway (Borel and Kis, 1991). This fungus was found to synthesize a number of neutral, lipophilic metabolites, which were later discovered to be novel cyclopeptides (termed cyclosporins). Although, these metabolites were found to have only modest anti-fungal activity, the metabolites known as 24–556, which comprised CsA and CsB, were found to mediate immunosuppression through a unique mechanism. This was by selectively inhibiting T lymphocyte proliferation without affecting somatic cell proliferation (Borel and Kis, 1991). CsA, which possessed the greater activity when compared to CsB, was then further developed as an immunosuppressive agent. Subsequently, CsA was approved by the US Food and Drug Administration for clinical use in 1983 to prevent graft rejection in transplantation and it is now widely used as an immunosuppressant and anti-rejection drug in solid organ transplantation (Van Buren *et al*., 1984).

CsA and the cyclophilins

Within the cell, CsA has a high binding affinity for cyclophilins (Cyp; Handschumacher *et al*., 1984), proteins which possess enzymatic peptidyl-prolyl isomerase activity (PPI-ase), which is essential for protein folding *in vivo*. The binding of CsA to Cyp inhibits its PPI-ase activity. CypA is located within the cytosol and participates in the translocation of apoptosis-inducing factor to the nucleus and in protecting against oxidative stress. CypB resides within the endoplasmic reticulum and has a role in suppressing apoptosis associated with oxidative stress and altered Ca^{2+} metabolism. When CsA binds to CypA, it forms a drug–protein complex which binds to and inhibits calcineurin, a calcium and calmodulin-dependent phosphatase (Liu *et al*., 1991). The formation of this complex is required for its immunosuppressive effect as it in turn inhibits the translocation of a family of transcription factors, nuclear factor of activated T-cells (NF-AT), leading to reduced transcriptional activation of early cytokine genes for IL-2, TNF-α, IL-3, IL-4, CD40L, granulocyte-macrophage colony-stimulating factor and IFN-γ (Schreiber and Crabtree, 1992). Within the heart, calcineurin activation results in its translocation to the nucleus and its dephosphorylation of NF-AT, allowing it to transcribe genes implicated in inducing cardiac hypertrophy. The binding of CsA to CypD is fundamental to its inhibitory effect on the mPTP (please see next section).

Discovering the mitochondrial effects of CsA

In the early 1980s, the first reports were published demonstrating in human renal biopsies the presence of giant mitochondria containing disoriented cristae and paracrystalline inclusions in renal tubular cells, suggesting a nephrotoxic effect of CsA on mitochondrial function (Mihatsch *et al*., 1981; Verani, 1983). In 1985, Jung and Pergande (1985) then reported that *in vitro* treatment with CsA inhibited mitochondrial respiration in renal tubular cells. Following this in 1987, Fournier and co-workers (Fournier *et al*., 1987) made the intriguing observation that CsA not only inhibited mitochondrial respiration, but it also blocked the mitochondrial efflux of calcium, an effect which could be prevented by the calcium channel blocker verapamil (Sumpio *et al*., 1987). Based on the observation that CsA could inhibit mitochondrial calcium efflux (Fournier *et al*., 1987) and the observation that reversible opening of the mPTP could mediate mitochondrial calcium efflux (Al Nasser and Crompton, 1986a,b; Crompton *et al*., 1987), Crompton and co-workers (Crompton *et al*., 1988) first investigated whether CsA could inhibit the opening of the mPTP. They made the crucial observation that mPTP opening in isolated rat heart mitochondria induced by the addition of calcium and phosphate could be inhibited by CsA (60 ρmol CsA·mg^{-1} of mitochondrial protein), as evidenced by maintained mitochondrial calcium levels, preserved mitochondrial membrane potential and sucrose impermeability (Crompton *et al*., 1988). The inhibitory effect of CsA (150 ρmol CsA·mg^{-1} of mitochondrial protein) was confirmed in a subsequent study by an independent research group using isolated liver mitochondria (Broekemeier *et al*., 1989). It is interesting to note that the observed inhibitory effect of CsA on mitochondrial respiration noted in these early renal studies was for the most part neglected with most attention focused on the inhibitory

effects of CsA on mPTP opening. However, recent studies have now revisited the potential detrimental effects of CsA on mitochondrial function (see the section heading 'The effects of long-term CsA therapy').

The mechanism through which CsA inhibited mPTP opening was revealed in a subsequent study in 1990 by Halestrap and Davidson (1990), who reported that CsA inhibited mPTP opening by binding to CypD, a peptidyl-prolyl *cis-trans* isomerase in mitochondria. The evidence provided by that study in support of this finding included the following: it was already known that CsA bound to CypD within the cell (Fischer *et al.*, 1989); studies had shown that there existed a mitochondrial CypD (Tropschug *et al.*, 1988); the K_i of CsA for CypD and mPTP inhibition were similar at 5 nM; and, finally, the number of CsA-binding sites involved in mPTP inhibition in liver and heart mitochondria (about 125 $\rho mol \cdot mg^{-1}$ of protein) were very close to the amount of the CypD present in the mitochondrial matrix (Halestrap and Davidson, 1990). In this early study, the authors proposed that CsA, by binding to mitochondrial CypD, prevented it from associating with the inner membrane pore component of the mPTP, and inducing a conformational change in this component, which was believed at that time to be adenine nucleotide translocase (Halestrap and Davidson, 1990; Griffiths and Halestrap, 1991). A subsequent study in 1996 by Tanveer and co-workers confirmed the pharmacological target of CsA to be the 21 kDa human mitochondrial CypD protein (Tanveer *et al.*, 1996). Subsequent studies using mice deficient in mitochondrial CypD have confirmed that CypD is the pharmacological mitochondrial target of CsA and is an important regulator of the mPTP (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005).

The mPTP as a mediator of IRI

In 1986, Crompton and co-workers were the first to propose that the mPTP may be a mediator of cell death in the setting of acute IRI (Al Nasser and Crompton, 1986a,b; Crompton *et al.*, 1987). In these early studies, Crompton's group (Crompton *et al.*, 1987) described the opening of the mPTP in isolated rat heart mitochondria in response to calcium ($\geq$1 μM), phosphate ($\geq$10 mM), oxidative stress and low ATP levels ($\leq$1 mM), conditions which are present during acute IRI. The discovery by the same research group in 1988, that CsA was a potent inhibitor of the mPTP (Crompton *et al.*, 1988), therefore provided the investigative tool required to test this proposition. The first study to report CsA protecting the heart against acute IRI was by Crompton's research laboratory in 1991 (Nazareth *et al.*, 1991). In that study, CsA, when administered at a narrow therapeutic concentration range (200 nM to 400 nM), was found to protect adult rat ventricular cardiomyocytes against necrotic cell death induced by lethal simulated IRI (Nazareth *et al.*, 1991). The reason for the narrow therapeutic range was unclear but may be attributed to the non-specific inhibitory effects of CsA on the cytosolic Cyp or inhibitory effects of CsA on mitochondrial respiration. Interestingly, the first experimental study to demonstrate a beneficial effect with CsA against acute IRI had actually been published 3 years earlier in 1988 (Hayashi *et al.*, 1988). In that study, it was reported that 3 days pre-treatment of dogs with CsA reduced ischaemic injury to the liver, an effect which was attributed to reduced lysosomal activity (Hayashi *et al.*, 1988).

CsA as an investigative tool for the mPTP

In 1992, Arteaga and co-workers (Arteaga *et al.*, 1992) were the first to demonstrate that *in vivo* treatment of rats with CsA (20 $mg \cdot kg^{-1}$) administered prior to 5 min of coronary artery ligation reduced reperfusion-induced arrhythmias and protected against myocardial necrosis as evidenced by decreased plasma levels of lactate dehydrogenase and creatinine kinase. The ability of CsA to protect against sustained myocardial IRI was first reported by Griffiths and Halestrap in 1993 (1993). These authors reported that CsA pre-treatment (at 200 nM but not at 1 μM) of isolated perfused rat hearts improved functional recovery and preserved myocardial ATP content following acute IRI (Griffiths and Halestrap, 1993). The effect of calcineurin inhibition as a mechanism for cardioprotection was excluded in this study by the demonstration that tacrolimus (a known calcineurin inhibitor) did not protect the heart against acute IRI (Griffiths and Halestrap, 1993). An important study by the same laboratory in 1995, investigated the timing of mPTP opening with respect to ischaemia and reperfusion (Griffiths and Halestrap, 1995). Using a novel method which allowed the detection and measurement of mPTP opening in the isolated perfused rat heart, based on the entrapment of titriated deoxyglucose within the mitochondria and later termed the Hot-DOG technique (Crompton, 1999), these authors were able to demonstrate that the mPTP remained closed during myocardial ischaemia and only opened in the first 2–3 min of myocardial reperfusion (Griffiths and Halestrap, 1995). It was postulated that mPTP opening was prevented during myocardial ischaemia by the strong inhibitory effect of the acidic pH (<7.0) induced by lactate accumulation and the failure of proton pumps to extrude hydrogen ions, and that the restoration of physiological pH in conjunction with further mitochondrial calcium and phosphate overload, oxidative stress and relative ATP depletion permitted the opening of the mPTP at the onset of myocardial reperfusion. A number of experimental studies have confirmed that the opening of the mPTP takes place in the first few minutes of myocardial reperfusion (Di Lisa *et al.*, 2001; Murata *et al.*, 2001) and that inhibiting its opening specifically at this time is cardioprotective (see next section) (Di Lisa *et al.*, 2001; Hausenloy *et al.*, 2002). Using real-time two-photon laser-scanning microscopy to detect continuous changes in mitochondrial membrane potential in the isolated perfused rat heart subjected to simulated IRI, Matsumoto-Ida and co-workers (Matsumoto-Ida *et al.*, 2006) also demonstrated *in situ* CsA-sensitive mPTP opening at reperfusion.

The critical observation that mPTP opening primarily occurred at the onset of myocardial reperfusion has positioned the mPTP as an important therapeutic target for limiting myocardial infarct size, which is readily amenable to intervention in patients presenting with an acute myocardial infarction (AMI) being treated with reperfusion therapies such as thrombolytic therapy and PPCI (see the section heading 'CsA as a potential therapeutic agent for acute myocardial infarction').

Crucially, the discovery that the mPTP was a critical mediator of acute IRI has resulted in a flurry of experimental studies implicating it as a target for cardioprotection elicited by ischaemic preconditioning (Hausenloy *et al.*, 2002; 2004; Javadov *et al.*, 2003) and post-conditioning (Argaud *et al.*, 2005b) and a diverse variety of pharmacological cardioprotective agents. Many of these studies have used CsA or its analogues to validate a wide variety of experimental models employed to detect and measure mPTP opening, thereby allowing the implication of mPTP inhibition as the cardioprotective mechanism.

CsA as a treatment for reducing experimental myocardial infarction

Experimental studies investigating the effect of CsA treatment on myocardial infarct size have administered this agent either prior to ischaemia, after the onset of ischaemia or at the time of myocardial reperfusion. The studies investigating CsA as an mPTP inhibitor have largely administered the drug at the time of myocardial reperfusion, when mPTP opening is believed to occur. In contrast, the earlier studies tended to administer the drug either prior to ischaemia or after the onset of ischaemia and attributed the cardioprotective effect of CsA to a variety of different mechanisms including calcineurin inhibition (Weinbrenner *et al.*, 1998), less leucocyte accumulation (Squadrito *et al.*, 1999) and a reduction in oxidative phosphorylation (Niemann *et al.*, 2002) (see Table 1), highlighting the non-specificity of CsA for the mPTP. In 2001, Di Lisa and co-workers (Di Lisa *et al.*, 2001) first implicated mPTP inhibition in the cardioprotective action of CsA by demonstrating that perfusion of an isolated rat heart with CsA throughout the ischaemia–reperfusion protocol reduced myocardial necrosis (measured by lactate dehydrogenase in the coronary effluent) and preserved mitochondrial NAD^+ levels (Di Lisa *et al.*, 2001).

In 2002, we were the first to demonstrate that the perfusion of isolated perfused rat hearts with CsA administered solely at the onset of myocardial reperfusion could limit myocardial infarct size, confirming that mPTP opening primarily occurred at the onset of myocardial reperfusion (Hausenloy *et al.*, 2002). Importantly, the calcineurin inhibitor, tacrolimus, did not limit myocardial infarct size when administered at the onset of myocardial reperfusion suggesting that mPTP inhibition, and not calcineurin inhibition, was the likely cardioprotective mechanism (Hausenloy *et al.*, 2002). Furthermore, in a subsequent study, Sanglifehrin A (SfA), a drug which inhibits mitochondrial CypD without affecting calcineurin activity, was also reported to be cardioprotective when administered at the onset of myocardial reperfusion (Hausenloy *et al.*, 2003). Interestingly, the cardioprotective effects of mPTP inhibition were completely lost if SfA was administered after the first 15 min of myocardial reperfusion had elapsed (Hausenloy *et al.*, 2003), underscoring the importance of intervening in the first few minutes to prevent mPTP opening. These studies have confirmed the role of the mPTP as a critical mediator of lethal myocardial reperfusion injury which is readily amenable to inhibition provided the therapeutic strategy is implemented prior to or at the onset of myocardial reperfusion before mPTP opening has occurred.

A number of experimental studies using both *ex vivo* and *in vivo* animal models (murine, rat, and rabbit) of IRI have subsequently confirmed the myocardial infarct size-limiting effects of CsA when administered at the time of myocardial reperfusion (these are summarized in Table 1). Interestingly, the narrow therapeutic dose range of CsA observed in the *ex vivo* studies has not been a major issue in the *in vivo* studies with beneficial effects of CsA observed at a range of concentrations from 1.0 to 10.0 $mg \cdot kg^{-1}$. Of interest, the recent studies using an *in vivo* porcine model of IRI have produced mixed results (Karlsson *et al.*, 2010; Skyschally *et al.*, 2010). The reason for this discordant finding is not known but may relate to variations in the design of the IRI model and the concentration of CsA used. Long-term benefits have been observed with mPTP inhibition at the time of myocardial reperfusion in a murine model of IRI. Gomez and co-workers (Gomez *et al.*, 2007) found that a single bolus of Debio-0125 (a CsA analogue which does not inhibit calcineurin) administered prior to myocardial reperfusion improved survival and preserved cardiac function at 30 days in mice following *in situ* myocardial infarction (MI).

In 2005, three independent laboratories provided genetic evidence implicating mitochondrial CypD to be a regulatory component of the mPTP (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005). Mice deficient in CypD were reported to be resistant to mPTP opening induced by either calcium or oxidative stress and sustained smaller myocardial infarct sizes when subjected to IRI (Baines *et al.*, 2005; Nakagawa *et al.*, 2005), underscoring the importance of the mPTP as a critical mediator of IRI and therefore a viable target for cardioprotection.

Protecting the heart in other settings using CsA

The majority of experimental studies investigating the cardioprotective effects of CsA have focused on reducing myocardial infarct size using an animal model of IRI, which most closely recapitulates the acute IRI experienced by the STEMI patient undergoing PPCI. However, CsA has also been reported to protect the heart against acute IRI in other experimental settings such as neonatal cardioplegic arrest and reperfusion (Oka *et al.*, 2008; Leung *et al.*, 2011) and resuscitated cardiac arrest (Cour *et al.*, 2011). By measuring the mitochondrial entrapment of titriated glucose at reperfusion following cardioplegic arrest, Leung and co-workers (Leung *et al.*, 2011) demonstrated that in a neonatal rabbit model of cardioplegic arrest and reperfusion, CsA-sensitive mPTP opening occurs at the time of myocardial reperfusion, providing preliminary experimental evidence that treatment with CsA or another mPTP inhibitor may be beneficial in terms of reducing perioperative myocardial injury in neonates undergoing corrective cardiac surgery for congenital heart disease. In another study, Cour and colleagues (Cour *et al.*, 2011) found that administering either intravenous CsA (5 $mg \cdot kg^{-1}$) or N-methyl-4-isoleucine cyclosporin (NIM811) (2.5 $mg \cdot kg^{-1}$) at the time of reperfusion following a 15 min period of primary asphyxial

Table 1

The major experimental studies investigating the effect of CsA on myocardial infarct size

Study	IRI Model	CsA dose and timing	Effect	Notes
CsA administered prior to or after the onset of myocardial ischaemia				
Weinbrenner *et al.*, 1998	*Ex vivo* rabbit heart LAD 30 min I 2 h R	100 nM or 750 nM CsA either 10 min prior or 10 or 20 min after onset of I	↓IS from 29 to 10% of area at risk except when CsA given 20 min after onset of I	Tacrolimus administered prior to I similarly cardioprotective
Squadrito *et al.*, 1999	*In vivo* rat heart LAD 30 min I 48 h R	0.25, 0.5 or 1 mg·kg^{-1} CsA given 5 min after onset of I	The most effective dose was 1 mg·kg^{-1} with ↓IS from 57 to 12% of area at risk.	Less leucocyte accumulation, and reduces TNF-α, ICAM-1 and myocardial myeloperoxidase expression.
Niemann *et al.*, 2002	*In vivo* rat heart LAD 30 min I 24 h R	Daily pretreatment with CsA for 3 days (5, 10, 15, 25 mg·day^{-1}) prior to I	The most effective dose was 15 mg·kg^{-1} with ↓IS from 29 to 7% of LV area.	Cardioprotective effect associated with reduced oxidative phosphorylation and ATP content.
Di Lisa *et al.*, 2001	*Ex vivo* rat heart LAD 30 min I 2 h R	200 nM CsA present throughout perfusion protocol.	↓myocardial necrosis as measured by LDH in coronary effluent	Associated preservation of mitochondrial NAD^+ content.
Xie and Yu, 2007	*In vivo* rat heart LAD 30 min I 180 min R	10 mg·kg^{-1} CsA given 10 min prior to I	↓IS from 49 to 30% of area at risk.	
CsA administered at the onset of myocardial reperfusion				
Hausenloy *et al.*, 2002	*Ex vivo* rat heart LAD 35 min I 2 h R	200 nM CsA for the first 15 min of reperfusion	↓IS from 45 to 25% of the area at risk.	Tacrolimus administered at R not cardioprotective
Hausenloy *et al.*, 2003	*Ex vivo* rat heart LAD 35 min I 2 h R	1 μM SfA for the first 15 min of R	↓IS from 44 to 24% of the area at risk.	No cardioprotection when SfA administered after 15 min of R
Argaud *et al.*, 2005a	*In vivo* rabbit heart LAD 35 min I 2 h R	10 mg·kg^{-1} of CsA or NIM811 given 1 min prior to R	↓IS from 60 to 24% with CsA and 25% with NIM811 of the area at risk.	NIM811 a CsA derivative which does not inhibit calcineurin was equally cardioprotective
Lim *et al.*, 2007	*In vivo* murine heart LAD 30 min I 2 h R	10 mg·kg^{-1} CsA or 25 mg·kg^{-1} SfA given 5 min prior to R	↓IS from 48 to 32% and 29%, respectively, of area at risk.	
Skyschally *et al.*, 2010	*In vivo* pig heart LAD 90 min hypoperfusion 2 h R	5 mg·kg^{-1} CsA given 5 min prior to R	↓IS from 34 to 24% of area at risk.	Note this study using myocardial hypoperfusion as opposed to complete regional myocardial ischaemia.
Karlsson *et al.*, 2010	*In vivo* pig heart LAD 45 min I 2 h R	10 mg·kg^{-1} CsA	No difference in IS	Potential worsening of IS when CsA administered with isoflurane.
Huhn *et al.*, 2010	*In vivo* rat heart (Zucker obese) LAD 25 min I 2 h R	5 or 10 mg·kg^{-1} CsA given 1 min prior to R	No difference in IS	However, CsA not demonstrated to be effective in Zucker lean rat.

IRI, ischaemia–reperfusion injury; I, ischaemia; R, reperfusion; CsA, cyclosporin A; SfA, Sanglifehrin A; LAD, left anterior descending coronary artery; IS, infarct size; NIM811, N-methyl-4-isoleucine cyclosporin.

in situ cardiac arrest in rabbits improved survival, reduced myocardial necrosis and inhibited mPTP opening in isolated cardiac mitochondria. Again, this experimental study provides preliminary evidence that the administration of CsA or another mPTP inhibitor may be potentially beneficial in successfully resuscitated cardiac arrest patients.

Protecting other organs against IRI using CsA

CsA has also been reported to protect other organs against acute IRI such as the liver, brain, kidney and so on. Interest-

ingly, the protective effects of CsA against IRI were actually first observed in the liver in 1989 (Kawano *et al.*, 1989) when compared to the heart in 1991 (Nazareth *et al.*, 1991). In that earlier study, 4 days pre-treatment with CsA (10 mg·kg^{-1}/day) reduced hepatic necrosis following a sustained episode of liver ischaemia (Kawano *et al.*, 1989). The first experimental study to report beneficial effects against IRI with CsA treatment in the brain was by Shiga and co-workers (Shiga *et al.*, 1992) in 1992. These authors found that 5 days treatment with CsA (15 mg·kg^{-1}/day) reduced cerebral infarct size following 1 h of middle cerebral artery occlusion followed by 24 h reperfusion (Shiga *et al.*, 1992). In that study, the neuroprotective effect was attributed to the immunosuppressive effect of CsA. Using a similar experimental model of cerebral IRI, Matsumoto and co-workers (Matsumoto *et al.*, 1999) demonstrated that mPTP inhibition at the time of myocardial reperfusion was neuroprotective. A number of studies have confirmed these findings, but genetic evidence for the involvement of the pharmacological target of CsA, mitochondrial CypD, as a mediator of cerebral IRI was provided in a study published by Schinzel and co-workers (Schinzel *et al.*, 2005), in which mice deficient in CypD were reported to sustain small cerebral infarcts following IRI compared to wild-type littermates. Although CsA therapy in solid organ transplantation had been associated with nephrotoxicity, at lower doses, it has been reported to protect kidneys against acute IRI. In 2001, Yang and co-workers (Yang *et al.*, 2001) first reported that a single intravenous bolus of CsA (3 mg·kg^{-1}) protected the kidney against acute lethal IRI, an effect which was attributed to the activation of heat shock protein 70.

Co-morbidities and mPTP inhibition by CsA

For a novel cardioprotective strategy to be successfully translated into the clinical setting, it is necessary to demonstrate that it is effective in the presence of certain co-morbid conditions which exist in patients with IHD. The presence of diabetes, hypertension with left ventricular (LV) hypertrophy, hypercholesterolaemia and age can negatively impact on the efficacy of endogenous cardioprotective strategies such as ischaemic preconditioning and post-conditioning (Ferdinandy *et al.*, 2007). It has been shown that in the presence of diabetes, both the rat heart (Tsang *et al.*, 2005) and human myocardium (Ghosh *et al.*, 2001) are resistant to standard ischaemic preconditioning protocols and that a more intense ischaemic preconditioning stimulus is required to confer cardioprotection. Interestingly, a recently published experimental study has suggested that mPTP inhibition using CsA may be ineffective in the pre-diabetic normoglycaemic Zucker obese rat (Huhn *et al.*, 2010). These authors found that these animals were resistant to the myocardial infarct size-limiting effects of CsA (5 or 10 mg·kg^{-1}) administered at the onset of myocardial reperfusion (Huhn *et al.*, 2010). Unfortunately, this study did not demonstrate that CsA dose used could limit myocardial infarct size in the Zucker lean rat, and it also did not demonstrate that CsA was ineffective in inhibiting mPTP opening in the pre-diabetic heart.

Further studies are required to determine whether the presence of co-morbidities influences the cardioprotective effect of CsA and if these findings confirmed the underlying mechanisms that need to be explored. The ongoing large, multicentre, randomized clinical outcome studies investigating CsA as an adjunct to PPCI in AMI patients may shed some light on whether the presence of co-morbidities such as diabetes, age, hypertension and hypercholesterolaemia impact on CsA cardioprotection in the clinical setting.

The effects of long-term CsA therapy

The problems with using CsA as a potential cardioprotective agent include its adverse effects and its non-selectivity for the mPTP. These adverse effects which include nephrotoxicity, hypertension, hyperlipidaemia, neurotoxicity, hepatotoxicity, anorexia, nausea, vomiting, paraesthesia, hypertrichosis, gingival hyperplasia and tremor are most often associated with chronic CsA therapy. The administration of a single bolus of CsA in STEMI patients would be expected to have minimal adverse effects, and, indeed, none were reported in the recent proof-of-concept clinical study by Piot and colleagues (Piot *et al.*, 2008). Experimental studies have investigated the effect of prolonged CypD ablation using either long-term therapy with CsA or a genetic CypD ablation. However, it must be appreciated that CsA has many other intracellular effects, and, therefore, any effects observed with long-term treatment with CsA may not necessarily be due to CypD inhibition. Furthermore, the majority of studies using genetic CypD ablation have used whole-body knockout of CypD, and, therefore, any effects induced by CypD ablation may be the result of CypD ablation in non-cardiac cells or tissue. Oie and co-workers (Oie *et al.*, 2000) investigated the effect of chronic CsA therapy (50 mg·kg^{-1}/day) for 2 weeks following *in situ* permanent occlusion of the left anterior descending coronary artery in the rat as a model of ischaemic heart failure. Interestingly, by inhibiting compensatory LV hypertrophy presumably through calcineurin inhibition, post-MI remodelling was worsened with increased LV dilatation and impaired LV systolic function (Oie *et al.*, 2000). In this study, the detrimental effects of CsA on calcineurin inhibition may have outweighed any potential effects of long-term CypD inhibition. In this regard, we have recently found that mice deficient in CypD had improved survival, less cardiomyocyte hypertrophy and interstitial fibrosis and improved cardiac function after 28 days following permanent occlusion of the left anterior descending coronary artery in a murine model of ischaemic heart failure (Lim *et al.*, 2010). In an earlier study by Nakayama and colleagues (Nakayama *et al.*, 2007), mice deficient in CypD were also found to be resistant to the heart failure induced by calcium overload in a genetic murine model over-expressing sarcolemmal L-type calcium channel. However, the same research group has recently reported that CypD ablation may be detrimental in some situations. They found that although mice deficient in CypD did not have a cardiac phenotype under basal conditions, they were more susceptible to pressure overload and exercise-induced LV hypertrophy and heart failure (Elrod *et al.*, 2010). This detrimental effect of CypD ablation was attributed to inhibition of mitochondrial calcium efflux, causing mitochondrial calcium overload and a change in metabolic substrate preference to glycolysis over fatty acids,

resulting in inadequate energy reserves in times of increased demand (Elrod *et al.*, 2010). This observation supports the earlier findings made by Fournier in 1987 (Fournier *et al.*, 1987) and later confirmed in cardiomyocytes in 1992 (Altschuld *et al.*, 1992) that CsA inhibited mitochondrial efflux of calcium. These early findings suggested that under normal conditions, the mPTP may provide a mechanism for the mitochondrial release of solutes such as calcium or it may provide a port of entry into mitochondria for essential cytosolic components (Gunter and Pfeiffer, 1990). However, a recent study did not find evidence for a Na^+-independent CsA-sensitive mitochondrial calcium efflux in guinea pig cardiac mitochondria (Wei *et al.*, 2011). Instead, these authors found that high concentrations of CsA (IC_{50} = 2 μM) may actually inhibit the mitochondrial Na^+-Ca^{2+} exchanger and result in mitochondrial calcium overload through this mechanism (Wei *et al.*, 2011). Clearly, further study is required to elucidate the conditions under which CypD ablation is beneficial or detrimental. The use of CsA in this regard will be difficult given its non-specific effects. In terms of a potential clinical therapy, only single dosing with CsA should be considered and, in fact, this is all that is required when using CsA as an adjunct to PPCI in STEMI patients.

Discovering more specific mPTP inhibitors

Although CsA prevents mPTP opening by inhibiting mitochondrial CypD, it has a variety of non-specific effects related to its inhibitory actions on other intracellular cyclophilins such as CypA and CypB. CsA binds to CypA to inhibit calcineurin, the mechanism underlying its immunosuppressive effects and its inhibitory actions on LV hypertrophy. Researchers have tried to overcome these non-specific effects of CsA using several different approaches: (i) by using non-immunosuppressive analogues of CsA which do not inhibit calcineurin such as NIM811 (Argaud *et al.*, 2005a), Cs29 (Argaud *et al.*, 2004) or Debio-0125 (Gomez *et al.*, 2007); (ii) by demonstrating that tacrolimus, a calcineurin inhibitor, does not have the effect elicited by CsA (Griffiths and Halestrap, 1993; Hausenloy *et al.*, 2002); (iii) by using SfA (Clarke *et al.*, 2002; Hausenloy *et al.*, 2003), an inhibitor of mitochondrial CypD which does not inhibit calcineurin; or (iv) by modifying CsA so it is more selective for mitochondrial CypD and less selective for cytosolic CypA (Malouitre *et al.*, 2010). Crompton's laboratory conjugated CsA with a lipophilic triphenylphosphonium cation which enabled it to preferentially accumulate in mitochondria according to the mitochondrial membrane potential resulting in a greater degree of CypD inhibition and less CypA inhibition (Malouitre *et al.*, 2010). This approach was found to result in more potent mPTP inhibition in neuronal cells (Malouitre *et al.*, 2010). Despite intensive investigation, the actual molecular identity of the pore component(s) of the mPTP remains unknown with both the adenine nucleotide translocase (Kokoszka *et al.*, 2004) and the voltage-dependent anion channel (Baines *et al.*, 2007) reported not to be obligatory components of the mPTP. The identification of the actual molecular components of the mPTP would provide novel targets for mPTP inhibition and a novel therapeutic strategy for cardioprotection. Finally, there are several new drugs currently under investigation as potential novel mPTP inhibitors including TRO4303 (Schaller *et al.*, 2010) and GNX (S. Plyte, unpubl. data). Schaller and co-workers (Schaller *et al.*, 2010) have shown that TRO4303 limits myocardial infarct size in an in vivo rat model of IRI and was found to inhibit mPTP opening in intact adult rat cardiomyocytes but not in isolated mitochondria (Schaller *et al.*, 2010). The drug, GNX, has also been reported to reduce *in situ* myocardial infarct size when administered at the time of myocardial reperfusion, and it has been found to inhibit mPTP opening in isolated cardiac mitochondria in a CypD-independent manner (Plyte, unpubl. data). The mechanism through which GNX inhibits the mPTP is currently unknown and is being investigated.

CsA as a potential therapeutic agent for acute myocardial infarction

The first step towards translating mPTP inhibition from a laboratory-based phenomenon into a potential therapeutic cardioprotective strategy in the clinical setting was made in 2003 by Schneider and co-workers (Schneider *et al.*, 2003) who were the first to demonstrate that human heart tissue was amenable to mPTP inhibition. These authors demonstrated that the pre-treatment of slices of human right atrial appendage tissue, harvested from IHD patients undergoing coronary artery bypass graft (CABG) surgery, with CsA (200 nM) reduced myocardial necrosis following a sustained lethal period of simulated ischaemia and reperfusion (Schneider *et al.*, 2003). We confirmed these findings in a subsequent study in which it was demonstrated the administration of CsA (200 nM) at the onset of myocardial reperfusion improved the recovery of baseline contractile function following an episode of simulated ischaemia in human right atrial trabeculae isolated from IHD patients undergoing CABG surgery (Shanmuganathan *et al.*, 2005). Crucially, in the latter study, it was also shown in isolated human atrial cardiomyocytes that treatment with CsA reduced cell death following an episode of sustained simulated IRI, and that CsA was able to inhibit mPTP opening in human atrial cardiomyocytes (Shanmuganathan *et al.*, 2005).

In 2008, in a landmark, small proof-of-concept clinical study, Piot and co-workers (Piot *et al.*, 2008) were the first to demonstrate that a single intravenous bolus of CsA (2.5 $mg \cdot kg^{-1}$) administered 10 min prior to PPCI could limit myocardial infarct size in STEMI patients as measured by total serum creatinine kinase and late gadolinium enhancement cardiac magnetic resonance imaging. It is important to appreciate that, in this study, not all STEMI patients were eligible for recruitment and the patients were carefully selected in order to increase the chances of observing a benefit with an intervention applied at reperfusion (Piot *et al.*, 2008). Patients with significant coronary flow in the infarct-related coronary artery, with significant coronary collateralization to the area at risk or with circumflex coronary artery occlusions were all excluded (Piot *et al.*, 2008). In addition to successfully translating mPTP inhibition from the laboratory to the clinical setting, the result of this clinical study highlighted the role of

the mPTP as a critical mediator of IRI and confirmed the existence of lethal myocardial reperfusion injury in man (Hausenloy and Yellon, 2008). Importantly, there were no adverse effects with CsA therapy such as renal dysfunction or hypertension despite high blood levels of CsA in the first few hours following PPCI. The beneficial effects of CsA on myocardial infarct size reduction were confirmed in a follow-up study by the same authors, although no difference in cardiac function was noted (Mewton *et al.*, 2010). Importantly, the latter study reported no adverse effects on LV remodelling in patients receiving CsA at the time of PPCI (Mewton *et al.*, 2010).

Large multicentre randomized clinical trials are now underway which have been designed to investigate whether CsA administered at an adjuvant to PPCI can improve clinical outcomes in IHD patients presenting with a STEMI. Other potential clinical settings of acute IRI in which CsA may be beneficial are in CABG surgery or PCI, as a pharmacological agent for reducing peri-operative or peri-procedural myocardial injury respectively. Furthermore, it may be investigated in cardiac arrest patients in whom circulation has been restored. Finally, in the setting of cardiac transplantation, it may be given as a cardioprotective as well as an immunosuppressive agent. However, it must be appreciated that the preclinical experimental studies supporting a role for CsA as a cardioprotective agent in these different clinical settings of IRI are lacking, with the majority of studies supporting a role for CsA reducing MI size in experimental models of IRI.

Whether CsA will be a viable therapeutic agent for IHD patients in the future is unclear, given its potential adverse effects. Clearly, safer, more efficacious mPTP inhibitors need to be developed if this therapeutic strategy for cardioprotection is going to benefit patients with IHD.

Conclusion

The seminal discovery in the 1980s that the immunosuppressant CsA could inhibit the opening of the mPTP has been fundamental in implicating the mPTP as a viable target for cardioprotection, which can be modulated at the time of myocardial reperfusion. Its use as a tool for investigating the mPTP resulted in the identification of mitochondrial CypD as a regulatory component of the mPTP. Furthermore, its ability to inhibit mPTP opening when administered at myocardial reperfusion has been recently demonstrated in a proof-of-concept clinical study to reduce myocardial infarct size in STEMI patients undergoing PPCI. Large, multicentre, randomized clinical trials are now underway to determine whether a single bolus of CsA administered to STEMI patients as an adjunct to PPCI can improve clinical outcomes. Inhibition of mPTP opening using either CsA or other mPTP inhibitors may also be beneficial in other clinical settings of acute IRI such as during cardiac surgery, following cardiac arrest or at the time of cardiac transplantation. In addition, mPTP inhibition may also provide a novel therapeutic strategy for protecting non-cardiac organs against acute IRI such as in patients presenting with stroke or in patients undergoing organ transplantation or surgery. However, because of the potential adverse effects and non-selectivity of CsA for the mPTP, more specific and safer novel mPTP inhibitors need to be identified and developed in order to implement mPTP inhibition as a therapeutic cardioprotective strategy.

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Conflict of interest

None declared.

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