Close Association Between the Reduction in Myocardial Energy Metabolism and Infarct Size: Dose-Response Assessment of Cyclosporine

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ABSTRACT

Cyclosporine protects the heart against ischemia/reperfusion injury, but its effect on cardiac metabolism is largely unknown. We assessed cyclosporine-induced metabolic changes in the rat heart prior to occlusion using magnetic resonance spectroscopy (MRS) and correlated effects with infarct size in a coronary occlusion/reperfusion model. The two study groups were cyclosporine and cyclosporine + coronary occlusion (n = 20/group). Rats were pretreated with cyclosporine (5, 10, 15, and 25 mg/kg/day) or the vehicle by oral gavage for 3 days (n = 4/dose). On day 4, hearts of rats in the cyclosporine group were excised, and extracted cell metabolites were measured using 1H and 31P MRS. The second group was subjected to 30 min of coronary artery occlusion followed by 24 h of reperfusion. Infarct size and area at risk were measured using a double staining method. In the cyclosporine group, cyclosporine reduced cardiac energy metabolism (ATP: r = −0.89, P < 0.001) via depression of oxidative phosphorylation and the Krebs’ cycle in a dose-dependent manner. The decrease of ATP levels was positively correlated with changes of NAD+ (r = 0.89), glutamate (r = 0.95), glutamine (r = 0.84), and glucose concentrations (r = 0.92, all P < 0.002). It was inversely correlated with lactate (r = −0.93, P < 0.001). In the coronary occlusion group, cyclosporine dose dependently reduced the ratio [area of infarct/area of the left ventricle] (r = −0.86, P < 0.01), with 15 mg/kg/day being the most effective cyclosporine dose. The reduction in infarct size correlated with the reduction in oxidative phosphorylation (ATP: r = 0.97; NAD+: r = 0.82, P < 0.01). The reduction in cardiac energy metabolism before occlusion may be the cause of myocardial preservation during ischemia/reperfusion.

The calcineurin inhibitor cyclosporine has been widely used as an immunosuppressant after organ transplantation (Kahan, 1989) and for the treatment of immune diseases (Faulds et al., 1993) for more than 2 decades. However, its clinical use is limited by a narrow therapeutic index and toxicity, mainly to kidney, liver, and the central nervous and cardiovascular systems (Kahan, 1989). Recent studies have indicated that cyclosporine significantly inhibits energy metabolism in brain (Serkova et al., 2001, 2002), kidney (Henke et al., 1992), and liver (Zhong et al., 2001), all primary target organs of cyclosporine toxicity. Although cyclosporine causes neurotoxicity (Kahan, 1989; Gijtenbeek et al., 1999) and was shown to significantly inhibit brain mitochondrial energy metabolism under normoxic conditions (Serkova et al., 2001), paradoxically, cyclosporine protects mitochondrial energy metabolism during ischemia and reperfusion in cell cultures and animal models (Uchino et al., 1998; Serkova et al., 2002). Although a substantial effort has been made to assess the neuroprotective effects of cyclosporine against stroke, only relatively few reports have evaluated the potential of cyclosporine to prevent ischemic/reperfusion damage in other organs, such as the heart. This is surprising since cyclosporine only poorly penetrates the blood–brain barrier, limiting its clinical application as a neuroprotective agent (Begley et al., 1990).

Recently, it has been shown that cyclosporine readily distributes into cardiac tissue, reaching concentrations greater than in blood (Serkova et al., 2000). Previous in situ and in vitro studies have suggested that cyclosporine has both detrimental and beneficial effects on the heart (Paul et al., 1991, 1992; Tatou et al., 1996; Rao et al., 1998; Park et al., 1999).
For example, cyclosporine may protect the heart against toxicity of drugs such as doxorubicin (Adriamycin) and cyclophosphamide (Al-Nasser, 1998a,b), prevent cardiac hypertrophy (Force et al., 1999), and diminish ischemia/reperfusion injury (Griffiths and Halestrap, 1993; Halestrap et al., 1997; Massoudy et al., 1997; Weinbrenner et al., 1998; Squadrito et al., 1999). As of today, the changes of myocardial cell metabolism caused by cyclosporine under normoxic conditions have not yet been described in detail. If, as in the brain, cyclosporine reduces mitochondrial oxidative energy production prior to cardiac occlusion, it could have a significant impact on ischemia/reperfusion injury in the infarcted heart. It has been previously shown that depletion of energy sources prior to cardiac ischemia produces physiological preconditioning, which is beneficial for postischemic outcome (Murry et al., 1986; Garnier et al., 1996). It was our goal to study the effects of cyclosporine on cardiac metabolism in vivo using magnetic resonance spectroscopy (MRS) to determine whether there is any correlation between the infarct size and the status of cardiac metabolism, and to find the optimum cyclosporine dose for cardiac protection against ischemia/reperfusion injury.

Materials and Methods

Experimental Design. All animal protocols were reviewed and approved by the University of California, San Francisco, Committee on Animal Research, and animal care was in agreement with the National Institutes of Health guidelines for ethical animal research (National Institutes of Health publication 80-123, revised 1985). For our studies, we used young adult male Sprague-Dawley rats (6 months old). Body weight (280–320 g) was monitored daily, and no change occurred during the cyclosporine treatment. Rats were randomly assigned to two groups of cyclosporine and cyclosporine + coronary occlusion (n = 20/group). In both groups, cyclosporine (doses 0, 5, 10, 15, and 25 mg/kg/day, n = 4) was administered as its Neoral formulation (Novartis Pharma AG, Basel, Switzerland) diluted in milk by daily oral gavage for 3 days.

Effect of Cyclosporine on Cardiac Energy Metabolism. To assess cyclosporine-induced metabolic changes, we quantified key cell metabolites in perchloric acid (PCA) and lipid extracts using MRS. On day 4, 4 h after the last cyclosporine dose, rats were anesthetized, and the beating hearts were excised and immediately frozen in liquid nitrogen. Frozen hearts were weighed, homogenized in a mortar and the mitochondria were harvested by 2 ml. The reaction mixture was incubated for 60 min at 95°C. After cooling to 0°C, 2 ml of butanol/pyridine (1:1 v/v) were added, and the solution was shaken vigorously and then centrifuged at 4000 rpm for 5 min. The organic phase was used to measure absorption in a photometer at 532 nm or fluorescence with excitation set to 515 nm and 5 min. The organic phase was used to measure absorption in a photometer at 532 nm or fluorescence with excitation set to 515 nm.

Effect of Cyclosporine on Infarct Size in Rats after Coronary Occlusion and Reperfusion. On day 4, 4 h after the last cyclosporine dose, rats in the cyclosporine + coronary occlusion group were subjected to 30 min of coronary artery occlusion followed by 24 h of reperfusion. The rats were anesthetized with a single dose of 50 mg/kg ketamine and 1.4 mg/kg xylazine injected intraperitoneally. The trachea was exposed, and a tracheotomy was performed. The rats were ventilated using a constant-volume ventilator with a volume of 1 to 2 ml/100 g of body weight at a rate of 65 to 70 strokes/min. The chest was opened through the fourth intercostal space. The pericardium was opened, and the main branch of the left coronary artery was occluded for 30 min in 2 mm below the left atrial appendage by an intramural stitch with a 6.0 polypropylene suture. Subsequently, the suture was removed and the chest was closed; the animals were allowed to recover from anesthesia. Animals were monitored for 2 h after completion of surgery.

After 24 h of reperfusion, the coronary artery was reoccluded, and 0.2 ml of phthalalocyanine blue dye was injected into the tail vein. This dye imparts a blue color to normally perfused myocardium, but the territory of the occluded artery (area at risk) remains unstained (Saeed et al., 1989). After the animal was sacrificed, the left ventricle was transversely sliced into three 2-mm thick slices. Both upper and lower surfaces of the stained three slices (apex, center, and base of the left ventricle) were photographed with a flatbed scanner connected to a computer and quantified. Then the three slices were incubated in 2% triphenyltetrazolium chloride saline solution for 8 min at 37°C. After 2% triphenyltetrazolium chloride staining saline solution, viable myocardium stains brick red, whereas infarcted regions stain pale white. Both faces of each slice were then photographed again. Epicardial and endocardial contours of normal and postischemic myocardium were traced manually. The fractions of area at risk and infarct size to total slice surface area were measured by planimetry from the two sets of photographs (Saeed et al., 1999).

Measurement of Cyclosporine Concentrations in Blood and Heart Tissues. At the time of sacrifice of the cyclosporine + coronary occlusion group, blood samples (0.25 ml, anticoagulated with 360 MHz, and a standard presaturation pulse program was used for water suppression. The other parameters were 40 accumulations, 90° pulse angle, 0-DB power level, 7.35 µs of pulse width, 10 ppm of spectral width, and 12.85 s of repetition time. Trimethylsilyl propionic-2,3,3,3-d4 acid (0.6 mmol/l) was used as an external standard for the quantification of metabolites based on 31P MR spectra. Chemical shifts of spectra were referenced to trimethylsilyl propionic-2,3,3,3-d4 acid at 0 ppm. For 31P MRS analysis of PCA extracts, 100 mmol/l EDTA were added for complexation of divalent ions, resulting in significantly narrower line width of 31P MR spectra. The pH was adjusted to 7 using KOH and HCl. The following NMR parameters with a composite pulse decoupling program were used: 145.7-MHz operating 31P frequency, 800 accumulations, 90° pulse angle, 12-DB power level for 31P channel, 9 µs of pulse width, 35 ppm of spectral width, and 2.0 s of repetition time. The chemical shift of phosphocreatine at −2.33 ppm was used as a shift reference. The absolute concentrations of phosphocreatine calculated from 31P MRS were used for metabolite quantification of 31P IR spectra.

In addition, lipid peroxidation (LPO) was measured in cardiac tissues using the malondialdehyde/ thiobarbituric acid (TBA) test (Mihara and Uchiyama, 1978). Three hundred fifty µl of tissue homogenate (100 mg/100 µl extraction buffer) were added to a solution containing 15 mM SDS, 1 mM EGTA, 2.25 mM butylated hydroxytoluene, and 20 mM 2-TBA. Twenty percent acetate acid (pH adjusted with NaOH to 1.5) was added, resulting in a final volume of 2 ml. The reaction mixture was incubated for 60 min at 95°C. After cooling to 0°C, 2 ml of butanol/pyridine (1:1 v/v) were added, and the solution was shaken vigorously and then centrifuged at 4000 rpm for 5 min. The organic phase was used to measure absorption in a photometer at 532 nm or fluorescence with excitation set to 515 nm and emission set to 555 nm. 1,1,3,3-Tetraethoxypropan was used as a standard.

Measurement of Cyclosporine Concentrations in Blood and Heart Tissues. At the time of sacrifice of the cyclosporine + coronary occlusion group, blood samples (0.25 ml, anticoagulated with 31P NMR spectroscopy were carried out as described previously (Serkova et al., 2001). For each extract, 1 g of heart tissue was used. The samples were centrifuged, the aqueous phase was removed, and the samples were neutralized using KOH and centrifuged once again. The lipid fraction was extracted from the pellets remaining after the aqueous extraction step. The pellets were dissolved in 4 ml of 0.01 M phosphate buffer (pH 7) and the solution was neutralized. Both the aqueous extract and the redissolved pellet were lyophilized overnight. The lyophilisates of the aqueous extracts were redissolved in 0.45 ml of deuterium oxide (D2O) and adjusted to pH 7 using DCl and NaOD. The lipid fraction was extracted from the lyophilisates of the redissolved pellet by the addition of 1 ml of deuterated chloroform/methanol mixture (CDCl3/CD3OD; 2:1 v/v). After centrifugation, the supernatants were analyzed by MRS.

MRS experiments based on 1H and 31P nuclear magnetic resonance (NMR) spectroscopy were carried out as described previously (Serkova et al., 1996). In brief, all one-dimensional MR spectra of tissue PCA aqueous and lipid extracts were recorded on an AMX 300 spectrometer and processed using WINNMR software (Bruker, Karlsruhe, Germany). A 5-mm HX-inverse probe was used for all experiments. For proton MRS, the operating frequency was 360 MHz, and a standard presaturation pulse program was used for water suppression. The other parameters were 40 accumulations, 90° pulse angle, 0-DB power level, 7.35 µs of pulse width, 10 ppm of spectral width, and 12.85 s of repetition time. Trimethylsilyl propionic-2,3,3,3-d4 acid (0.6 mmol/l) was used as an external standard for the quantification of metabolites based on 31P MR spectra. Chemical shifts of spectra were referenced to trimethylsilyl propionic-2,3,3,3-d4 acid at 0 ppm. For 31P MRS analysis of PCA extracts, 100 mmol/l EDTA were added for complexation of divalent ions, resulting in significantly narrower line width of 31P MR spectra. The pH was adjusted to 7 using KOH and HCl. The following NMR parameters with a composite pulse decoupling program were used: 145.7-MHz operating 31P frequency, 800 accumulations, 90° pulse angle, 12-DB power level for 31P channel, 9 µs of pulse width, 35 ppm of spectral width, and 2.0 s of repetition time. The chemical shift of phosphocreatine at −2.33 ppm was used as a shift reference. The absolute concentrations of phosphocreatine calculated from 31P MRS were used for metabolite quantification of 31P MR spectra.
Effect of Cyclosporine on Myocardial Energy Metabolism. ATP, ADP, phosphocreatine, NAD+/NADH, and water-soluble phospholipids were quantified based on \(^{31}\)P MR spectra (Fig. 1). The following cell metabolites could be detected and were quantified using \(^1\)H MRS of heart PCA extracts: 1) glycolysis intermediates lactate and alanine; 2) Krebs’ cycle products glutamate, glutamine, and succinate; 3) high-energy phosphates creatine/phosphocreatine; 4) osmotolites taurine; and 5) glutathione. Three days of cyclosporine oral treatment significantly reduced cardiac high-energy phosphates as indicated by a dose-dependent decrease of ATP and phosphocreatine concentrations as well as by reduction of the [ATP/ADP] and [phosphocreatine/creatine] ratios surrogate markers for (Table 1). The concentration of NAD+/NADH, surrogate markers for oxidative phosphorylation, was decreased after treatment (Table 1) and correlated positively \((r = 0.89, P < 0.002)\) with the ATP levels. Furthermore, cyclosporine significantly reduced Krebs’ cycle intermediates by as much as 35% (glutamate at a dose of 25 mg/kg/day) (Table 1). A positive correlation with the ATP concentrations was found for both glutamate \((r = 0.95, P < 0.001)\) and glutamine \((r = 0.84, P < 0.002)\). In parallel, the concentrations of lactate, the end product of anaerobic glycolysis, were significantly increased in all treatment groups (Table 1) and inversely correlated with ATP concentrations \((r = -0.93, P < 0.001)\). The increase in lactate production was associated with a decrease in glucose concentrations that correlated with the reduction in high-energy phosphate concentrations \((ATP; r = 0.92, P < 0.001)\).

The following lipids were quantified based on \(^1\)H MR spectra: fatty acids, polyunsaturated fatty acids (PUFA), triacylglycerol (TAG), cholesterol, and cholines. Cyclosporine had no statistically significant effect on the metabolite patterns of TAG and cholines. However, cyclosporine significantly increased cholesterol concentrations up to 3.8 \(\mu g/ml\) or 141% of controls after treatment with 25 mg/kg/day (Table 2) and reduced the concentrations of total fatty acids and of PUFA at the highest dose of cyclosporine, indicating a reduction of the fatty acid pool (Table 2). It should be noted that only the highest cyclosporine dose (25 mg/kg/day) increased LPO (Table 2).

Effect of Cyclosporine on Infarct Size in Rats. Cyclosporine at doses of 10 and 15 mg/kg/day significantly reduced the size of infarct relative to the area at risk \((P < 0.03)\). At a...
Dose-dependent metabolic changes in the rat heart after treatment with cyclosporine for 3 days calculated based on $^1$H and $^{31}$P MR spectra of PCA extracts.

The concentrations are micromoles per gram of wet weight for the vehicle control group (except ratios) and percentage of the control for cyclosporine-treated groups. Data is presented as means ± standard deviations ($n = 4$).

**TABLE 1**

Dose-dependent changes in the lipid composition of rat heart tissue after treatment with cyclosporine for 3 days calculated from $^1$H MR spectra of lipid extracts.

Concentrations are micromoles per gram of wet weight for the vehicle control group (except ratios) and percentage of the control for cyclosporine-treated groups. Data is presented as means ± standard deviations ($n = 4$).

**TABLE 2**

Dose-dependent changes in the lipid composition of rat heart tissue after treatment with cyclosporine for 3 days calculated from $^1$H MR spectra of lipid extracts.

Concentrations are micromoles per gram of wet weight for the vehicle control group (except ratios) and percentage of the control for cyclosporine-treated groups. Data is presented as means ± standard deviations ($n = 4$).

Cyclosporine Blood and Cardiac Tissue Concentrations. Cyclosporine doses were significantly correlated with cyclosporine concentrations in blood ($r = 0.81, P < 0.001$), in normal myocardial tissue ($r = 0.86, P < 0.001$), and in the infarcted tissue ($r = 0.87, P < 0.001$) but not in the area of risk (Fig. 3). A significant inverse correlation was evident between the cyclosporine dose (blood concentration) and the percentage of area of infarct/area of risk as well as the percentage of area of infarct/area of the left ventricle with $r = -0.81, P < 0.01$ and $r = -0.86, P < 0.01$, respectively. If the 25 mg/kg/day was excluded from the analysis, the correlation coefficients improved to $-0.86$ and $-0.91$, respectively. In addition, there was a significant negative correlation between the cyclosporine dose and ATP ($r = -0.89, P < 0.01$), NAD$^+/NADH$ ($r = -0.94, P < 0.001$), glutamate concentrations ($r = -0.83, P < 0.02$), and a significant positive correlation with the lactate concentrations ($r = 0.87, P < 0.002$).

**Discussion**

The main result of the present study is that cyclosporine inhibits cardiac high-energy phosphate and Krebs' cycle me-
of mitochondrial energy homeostasis, can produce the protective effect on myocardium against prolonged ischemia/reperfusion damages (Murry et al., 1986; Garnier et al., 1996). It can be speculated that by inducing metabolic changes similar to hypoxia, cyclosporine may also have ischemic preconditioning effects. Besides direct depression of mitochondrial respiration, another possible mechanism, such as induction of heat shock protein expression as shown for the kidney (Yang et al., 2001), may be involved in cyclosporine-induced pharmacological preconditioning. However, the potential contribution and the exact mechanism of cyclosporine-induced preconditioning will require further assessment.

This study showed the salutary effect of cyclosporine on infarct size in rats subjected to 30 min of coronary occlusion. Pretreatment with cyclosporine for 4 days was based on the rationale that protective and/or adaptive mechanisms, such as pharmacological preconditioning, induced by cyclosporine may depend on changes of gene expression that may take hours to days for full effect. We found that within 24 h of 30 min of coronary artery occlusion, cyclosporine pretreatment produced a dose-dependent reduction in infarct size relative to the area at risk and to the area of the left ventricle. Our evaluation of different doses suggested maximum protective effect at doses between 10 and 15 mg/kg/day, with a fall-off at higher doses (25 mg/kg). This finding is in good agreement with those of Griffiths and Halestrap (1993) who observed that in isolated perfused hearts the cardioprotective effect was dose-dependent with an apparent reversal of the protective effect at higher doses. Furthermore, the ratio [area of infarct/area at risk] in the occlusion group strongly correlated with the decrease of cellular ATP and NAD⁺ concentrations in control rats. Doses of 10 and 15 mg/kg/day cyclosporine produced inhibition of mitochondrial energy production prior to occlusion, resulting in a decrease in infarct size after 24 h of reperfusion. At 25 mg/kg/day cyclosporine, the positive effect of cyclosporine appears to be antagonized, possibly due to an increased lipid peroxidation.

Other effects of cyclosporine, such as inhibition of the immune response following ischemia/perfusion injury, have also been described, which may contribute to the beneficial effect of cyclosporine on ischemia/reperfusion injury (Squadrito et al., 1999). Griffiths and Halestrap (1993) found that cyclosporine keeps the mitochondrial nonspecific pores closed during reperfusion. This effect was not dependent on calcineurin inhibition but mostly likely due to inhibition of cyclophilin D, since a nonimmunosuppressive cyclosporine derivative was equally as effective as cyclosporine. Weinbrenner et al. (1998) found evidence that cyclosporine protects the ischemic rabbit heart by inhibiting the calcium-calmodulin-dependent protein phosphate 2B, calcineurin. Massouy et al. (1997) reported that cyclosporine acts as a cardioprotective agent by an endothelin-dependent mechanism. None of these studies pretreated animals with cyclosporine or were based on isolated hearts; in the only in vivo study reported (Squadrito et al., 1999), cyclosporine was administered intravenously 15 min after coronary occlusion. We also measured cyclosporine concentrations in the remote nonischemic myocardium, the area at risk, and the infarcted area by using a highly specific and sensitive, validated HPLC/MS assay. Since cyclosporine oral bioavailability is known to be erratic and variable (Oellerich et al., 1995), we also measured cyclosporine blood concentrations. Our study
suggested a good correlation between the oral cyclosporine dose and cardiac tissue concentrations as well as a good correlation between blood and cardiac tissue concentrations. The cyclosporine doses and blood concentrations that produced significant reduction of the area of infarction are comparable with those obtained in/desired in patients after heart transplantation (Oellerich et al., 1995). In conclusion, cyclosporine, in a dose-dependent fashion, induced depression of the cardiac energy metabolism before occlusion and decreased infarct size in a rat coronary occlusion model. The depression of cardiac energy metabolism induced by cyclosporine correlated with the reduction in infarct size and suggests that cyclosporine pretreatment produces an energetic milieu similar to that achieved by ischemic preconditioning. Further studies are needed to test the effect of cyclosporine on cardiac function and the severity of myocardial injury.

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References


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