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Cyclosporine A and Rapamycin induce *in vitro* cholesteryl ester transfer protein activity, and suppress lipoprotein lipase activity in human plasma

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ABSTRACT

Purpose: Cyclosporine A (CsA), Rapamycin (RAPA), Tacrolimus (FK-506) and Mycophenolate mofetil (MMF) are immunosuppressants that are widely used in solid organ transplant patients. However, some of these drugs have been reported to cause dyslipidemia in patients. Our aim was to determine the effects of these drugs on *in vitro* cholesteryl ester transfer protein (CETP), hepatic lipase (HL) and lipoprotein lipase (LPL) activity within human plasma.
Methods: We measured CETP activity in human normolipidemic plasma with and without drug treatment, by measuring the incorporation of labeled cholesteryl ester into lipoproteins. To further confirm the result, we also measured recombinant CETP (rCETP) activity with and without drug treatment. We measured HL and LPL activity in post-heparin normal human plasma in the presence and absence of the drugs by measuring the release of fatty acids from radiolabeled triolein.
Results: We found an increase in CETP activity in human normolipidemic plasma and rCETP treated with CsA and RAPA. By contrast, CETP activity was not altered significantly in the presence of FK-506 and MMF. LPL activity in post-heparin normal human plasma was suppressed following the co-incubation with CsA, RAPA, FK-506 or MMF whereas HL activity remained unaffected.
Conclusions: The increase in CETP activity and suppression in LPL activity following CsA and RAPA treatment observed in the present study may be associated with elevated LDL cholesterol levels and hypertriglyceridemia seen in patients administered these drugs.

1. Introduction

Cardiovascular disease (CVD) is a major cause of both graft loss and patient death in transplant patients. In fact, mortality rates due to CVD in transplant recipients are greater than in the general population (Ojo, 2006). In addition to common risk factors such as hypertension and diabetes mellitus, there are other specific transplantation risk factors such as acute rejection episodes and the use of immunosuppressive drugs that contribute to the development of CVD in transplant patients. However, the relationship of the use of different immunosuppressants and the magnitude of CVD risk factors is still under considerable debate. Some of the immunosuppressants, like Tacrolimus (FK-506) and Mycophenolate mofetil (MMF), have been reported to have a more favourable cardiovascular risk profile than Cyclosporine A (CsA) and Rapamycin (RAPA), but the effects and the mechanisms of how these drugs contribute to the observed cardiovascular profile have not been completely elucidated (Fellstrom, 2001; Rabkin et al., 2002; Cosio and Larson, 2003; Kramer et al., 2003; Boots et al., 2004).

Corticosteroids have often been used in transplantation patients, but a number of undesirable effects have been observed including osteoporosis, obesity, and atrophy of the skin and vessel wall (Boots et al., 2004). Alternative treatments that have been widely used include CsA, RAPA, FK-506 and MMF (Table 1). However, some of these drugs have been reported to cause hypercholesterolemia and hypertriglyceridemia in transplant patients, especially CsA and RAPA (Mathis et al., 2004). The mechanisms by which CsA and RAPA alter plasma lipid levels are not well understood.

Previous studies have suggested that CsA-administered transplantation patients, who exhibit plasma dyslipidemia may have an elevated cholesteryl ester transfer protein (CETP) level (Gueguen et al., 2004). CETP is a hydrophobic glycoprotein that is secreted mainly from the liver and circulates in the plasma, bound mainly to HDL (Tall, 1993). Plasma CETP facilitates the transfer of cholesteryl

Abbreviations: CsA, Cyclosporine A; RAPA, Rapamycin; FK-506, Tacrolimus; MMF, Mycophenolate mofetil; CETP, cholesteryl ester transfer protein; HL, hepatic lipase; LPL, lipoprotein lipase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

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Table 1
Summarized comparison of some properties of different immunosuppressive drugs that we used in our study

	Cyclosporine A (CsA)	Rapamycin (RAPA)	Tacrolimus (FK-506)	Mycophenolate mofetil (MMF)
Property	Calcineurin inhibitor	Molecular target of Rapamycin (mTOR) inhibitor	Calcineurin inhibitor	Inhibitor of inosine monophosphate dehydrogenase
Mechanism	Inhibits lymphokine and IL release, thus reduced the function of effector T cells	Blocks signal transduction of T cells and inhibits cell cycle from G_1 to S	Inhibits T-lymphocyte signal transduction and IL-2 transcription	Inhibits synthesis of guanosine nucleotides in T and B lymphocytes
Dosage	10–14 mg/(kg day)	1–7 mg/(kg day)	0.05–0.3 mg/(kg day)	1 g twice daily
Physiological blood concentrations	0.5–1.25 mg/L	8–40 μg/L	15–30 ng/mL	1–38 μg/mL
Major side effects	Nephrotoxicity, dyslipidemia, hypertension	Dyslipidemia, hypertension, anemia	Nephrotoxicity, hyperglycemia, hypertension	Gastrointestinal intolerance; haematological aberrations

ester (CE) from high-density lipoprotein (HDL) to apolipoprotein (apo) B-containing lipoproteins with reciprocal transfer of triglycerides (Qiu et al., 2007).

The fact that hypertriglyceridemia is more frequently observed after certain immunosuppressive treatments may be caused by changes in the synthesis and elimination of triglycerides. By hydrolyzing triglycerides, lipoprotein lipase (LPL) and hepatic lipase (HL) play a critical role in plasma triglyceride clearance. A study described by Vaziri et al. (2000) showed marked reductions of both skeletal muscle and adipose tissue lipoprotein lipase abundance in CsA-treated rats, thus suggesting lipoprotein lipase deficiency. A pilot study in renal transplant patients receiving RAPA treatment suggests that hyperlipidemia induced by RAPA is the result of reduced catabolism of apoB100-containing lipoproteins (Hoogeveen et al., 2001). Despite the role of CETP and lipases in lipid metabolism, the effect of CsA and RAPA on these proteins is not well understood.

The objective of this study was to investigate the effects of the immunosuppressive drugs; CsA, RAPA, FK-506 and MMF on CETP and LPL and HL activity *in vitro*. This was accomplished by assessing the CE transfer activity of CETP, activity of LPL and HL *in vitro* in human normolipidemic plasma and post-heparin human plasma, respectively, following incubation of these different drugs.

2. Methods

2.1. Materials

Radiolabeled CE [1α,2α(*n*)-^{3}H-cholesteryl oleate, specific activity 68.8 mCi/mg], dissolved in toluene solution, was purchased from Amersham Biosciences (Piscataway, NJ, USA). Tris–HCl, sodium chloride, sodium bicarbonate, sodium bromide, sodium azide, sodium phosphate, sodium hydroxide, ethylenediaminetetraacetate (EDTA), potassium phosphate monobasic, potassium chloride, bovine serum albumin and CsA were purchased from Sigma Chemical Company (St. Louis, MO). T150 buffer was made from 50 mM Tris–HCl, 150 mM NaCl, 0.02% NaN_3, 0.01% disodium EDTA at pH 7.4. RAPA, FK-506 and MMF were purchased from Tecoland Corporation (Edison, NJ).

Normolipidemic human plasmas (total plasma cholesterol concentrations in the range of 135–150 mg/dL) were obtained from Bioreclamation (Hicksville, NY). Post-heparin normal human plasma was a gift from St. Paul's Atherosclerosis Specialty Lab (Vancouver, BC, Canada) and used as received. TP2, a monoclonal antibody that specifically binds to the CE-binding site of CETP, was obtained from the Ottawa Heart Institute (Ottawa, Ont., Canada). Recombinant CETP (rCETP) was purchased from Roar Biomedical Inc. (New York, USA). Plasma and lipoprotein total cholesterol and triglycerides were determined using enzymatic assays obtained from Sigma Diagnostics (St. Louis, MO).

2.2. Lipoprotein separation

The human normolipidemic plasma was separated into very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and lipoprotein-deficient plasma (LPDP) fractions by density gradients and ultracentrifugation as has been previously described (Havel et al., 1955; Ramaswamy et al., 1997). Human plasma (3 mL) was placed in the centrifuge tubes and its density was adjusted to 1.25 g/mL by adding sodium bromide. Discontinuous density gradients were constructed by underlayering the following NaBr solutions: 1.006 g/mL, 1.063 g/mL and 1.21 g/mL, respectively. Plasma sample was then spun at 40,000 rpm for 18 h at 15 °C in a SW41 Ti rotor to separate the fractions (L8-30 M; Beckman, Toronto, Ont., Canada). The HDL fractions were further dialyzed against 0.9% saline solutions at 4 °C overnight. The molecular weight cut-off of the dialysis tubing used was 500 Da. After dialysis, these fractions were filtered through a 0.2-μm filter.

2.3. Isolation of CETP

CETP was isolated from human lipoprotein-deficient normolipidemic plasma as previously described (Morton and Zilversmit, 1982). Citrated human plasma was made lipoprotein-deficient by the dextran sulfate–$MnCl_2$ precipitation procedure of Burstein et al. (1970).

2.4. Radiolabeling of plasma lipoproteins

Human LDL fractions were labeled by the lipid dispersion technique as previously described (Morton and Zilversmit, 1982, 1983). Human plasma was incubated with [^{3}H]-CE (17.5 μCi) overnight at 37 °C. Then the radiolabeled LDL fractions were isolated from the plasma by ultracentrifugation as previously described and further dialyzed against 0.9% saline solutions at 4 °C overnight. The molecular weight cut-off of the dialysis tubing used was 1000 Da. After dialysis, these fractions were filtered through a 0.2-μm filter.

2.5. CETP radioactive assay

The drugs concentrations used in all our experiments were chosen below and above the blood concentrations in the body (Table 1). However, for CsA, the higher drug concentrations were used, because CsA is highly lipophilic, is known to distribute in lipoproteins, and could potentially accumulate in atherosclerotic foam cells (Wasan et al., 2002; Jin et al., 2004a). CE transfer activity between lipoproteins by CETP was measured using the method that has been previously described (Morton and Zilversmit, 1982; Wasan et al., 1998). Briefly, 10 μg (total cholesterol) of radiolabeled LDL and unlabeled HDL were incubated with delipidated plasma as the source of CETP and with or without the drug in T150 buffer

for 90 min at 37 °C. TP2, a monoclonal antibody specific against the CE-binding site on CETP, was used as a positive inhibition control in this experiment. Enzymatic assay kit from Sigma Diagnostics (St. Louis, MO) was used to determine total and lipoprotein cholesterol concentrations. In order to measure the CE transfer from [^{3}H]-LDL to HDL, the donor particle was precipitated with the addition of PO_4^{3-} and Mn^{2+} prior to the scintillation counting for the radioactivity. The CETP activity which is represented as the amount of CE transferred is calculated as the following:

$$\%\,\text{of CE transferred} = \frac{[^3\text{H}]\text{-sample} - [^3\text{H}]\text{-blank}}{[^3\text{H}]\text{-total}} \times 100$$

where [^{3}H]-sample is the radioactivity in the sample, [^{3}H]-blank is the radioactivity in the absence of CETP and [^{3}H]-total is the total radioactivity without precipitating out donor particles. The data were expressed as a percentage of control.

2.6. CETP fluorescence assay

In the CETP fluorescence assay, CETP activity was measured with a commercial kit as directed (Roar Biomedical Inc., New York, NY). Incubation of donor and acceptor with a CETP source results in the CETP-mediated transfer of fluorescent neutral lipid from donor to acceptor. The CETP-mediated transfer is determined by the increase in fluorescence intensity as the fluorescent neutral lipid is removed from donor to acceptor. The amount of fluorescent substrate transferred was expressed as a percentage of control.

2.7. Lipase activity assay

In order to assess LPL and HL activity, we used a triolein emulsion containing radiolabeled [^{3}H]-triolein as described previously (Hill et al., 1998). Post-heparinized normal human plasma was used for the assay. Total lipase activity was measured in the presence of apoC-II at a physiological salt concentration. LPL activity was represented as total lipase activity subtracted by salt-resistant lipase activity (HL).

2.8. Statistical analysis

Differences in the mean values of CETP, HL and LPL activities in the controls and drug-treated plasmas were compared using the one-way ANOVA test (INSTAT; GraphPad, San Diego, CA, USA). Critical differences were assessed by the Tukey *post hoc* tests. A difference in the mean values was considered to be significant if $p < 0.05$. Data were presented as mean ± S.D.

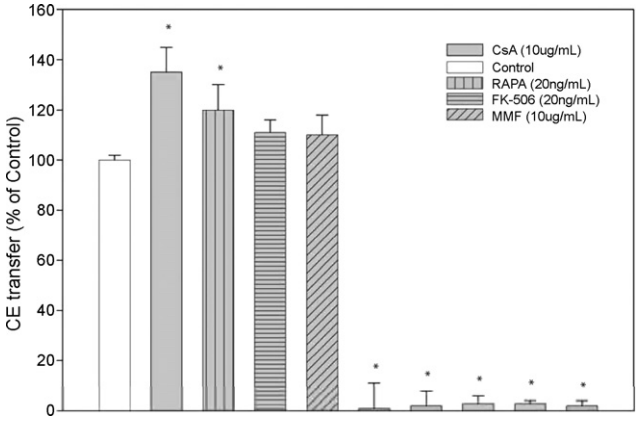

Fig. 1. CsA and RAPA significantly induced CETP activity in human normolipidemic plasma, at average and maximum blood concentrations, while FK-506- and MMF-treated plasmas were not significantly different, compared to untreated plasma. The TP2, monoclonal antibody against the CE-binding site, significantly inhibited CETP-mediated transfer of cholesteryl esters (CEs) in the untreated plasma and the drug-treated plasma, respectively ($n = 3$, $^*p < 0.001$). Values expressed as mean ± S.D.

3. Results

3.1. Influence of CsA, RAPA, FK-506 and MMF on CETP activity

As shown in Fig. 1, the *in vitro* human normolipidemic plasma CETP activity was significantly increased in CsA- or RAPA-treated (90 min) samples compared to the control condition. However, at maximum blood concentrations, FK-506 and MMF did not result in a significant difference in plasma CETP activity relative to untreated plasma. When the samples were pre-treated with TP2 for 90 min prior to the incubation with the drugs, all samples showed a significant decrease in CETP activity (Fig. 1). Fig. 2A and B shows that CsA and RAPA significantly induce *in vitro* CETP activity in a concentration-dependent manner, respectively.

3.2. Influence of CsA, RAPA, FK-506 and MMF on rCETP activity

Similarly, we assessed the effects of CsA, RAPA, FK-506 and MMF on the *in vitro* activity of rCETP using the CETP fluorescence assay.

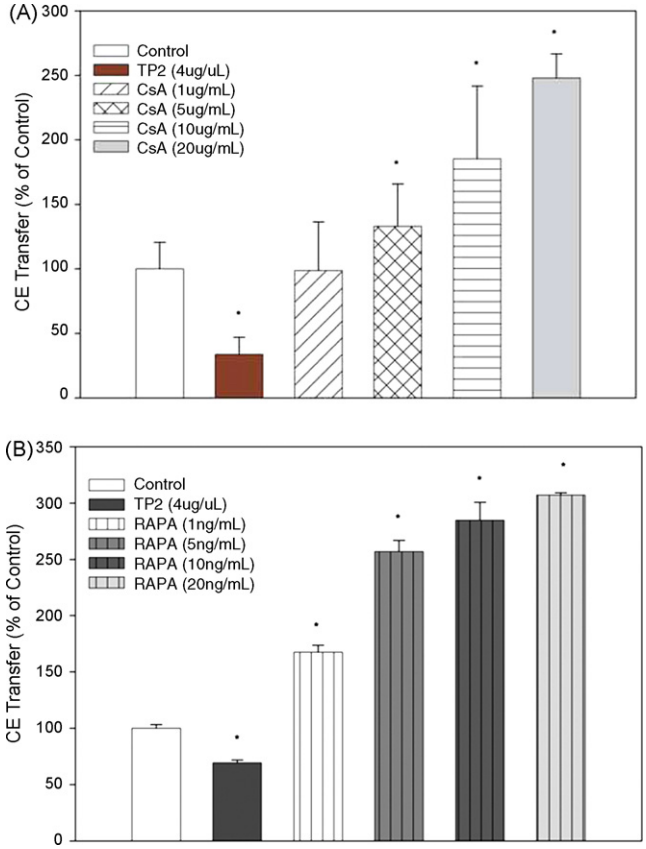

Fig. 2. (A) CsA significantly increased CETP activity within human normolipidemic plasma, after the incubation for 90 min, in a concentration-dependent manner, relative to the control ($n = 6$, $^*p < 0.001$ vs. control). The inhibition by TP2, a monoclonal antibody against the CE-binding site, showed that the transfer of CE was due to a facilitated transfer by CETP, not to a spontaneous transfer among lipoprotein classes. Values expressed as mean ± S.D. (B) RAPA significantly induced CETP activity in human normolipidemic plasma, in a concentration-dependent manner, compared to untreated plasma ($n = 3$, $^*p < 0.001$ vs. control). The co-incubation with TP2, a monoclonal antibody against the CE-binding site, significantly inhibited the CE transfer (data not shown). Values expressed as mean ± S.D.

Table 2
Corresponding to the previous result shown for human normolipidemic plasma, CsA and RAPA also increased CE transfer activity of recombinant CETP (rCETP)

Activity (% of control)	% of Control ± S.D.					
	Control	Cyclosporin (10 μg/mL)	Rapamycin (20 ng/mL)	FK-506 (20 ng/mL)	Mycophenolate mofetil (10 μg/mL)	TP2 (4 μg/μL)
CE transfer by rCETP	100 ± 1	118 ± 6*	140 ± 1*	110 ± 3	101 ± 8	11 ± 3*

No significant difference was observed in FK-506- and MMF-treated samples, compared to the control. TP2, a monoclonal antibody against the CE-binding site, was also shown to inhibit the CE transfer of rCETP ($n = 3$, $^*p < 0.001$). Values expressed as mean ± S.D.

Table 3
At chosen concentrations, CsA, RAPA, FK-506 and MMF did not significantly change the hepatic lipase activity in post-heparin normal human plasma, relative to control ($n = 4$, $^*p < 0.001$ vs. control)

Activity (% of control)	% of Control ± S.D.				
	Control	Cyclosporin (20 μg/mL)	Rapamycin (20 ng/mL)	FK-506 (20 ng/mL)	Mycophenolate mofetil (10 μg/mL)
Hepatic lipase activity	100 ± 12	114 ± 5	105 ± 5	101 ± 8	118 ± 10

Values expressed as mean ± S.D.

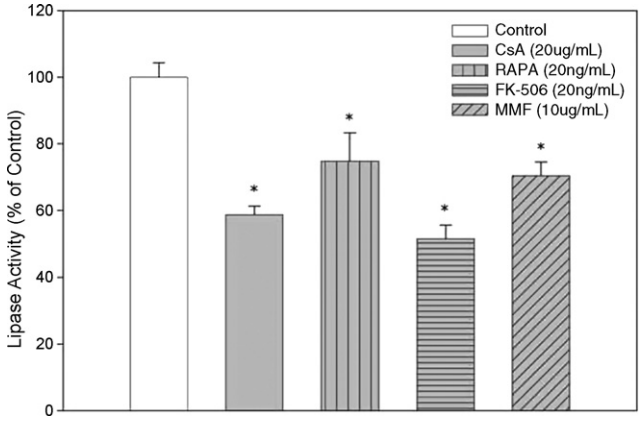

Fig. 3. At average and maximum blood concentrations, CsA, RAPA, FK-506 and MMF significantly suppressed lipoprotein lipase activity in post-heparin normal human plasma, compared to untreated plasma ($n = 4$, $^*p < 0.001$ vs. control). Values expressed as mean ± S.D.

As can be observed in Table 2, the effects of these drugs on the rCETP activity corresponded to the results shown for human normolipidemic plasma. Similarly, co-incubation of rCETP with TP2 resulted in a significant reduction of the CE transfer activity by rCETP.

3.3. Influence of CsA, RAPA, FK-506 and MMF on HL and LPL activity

Using post-heparin normal human plasma, Fig. 3 shows the effects of CsA, RAPA, FK-506 and MMF on the *in vitro* activity of LPL. LPL activity was decreased significantly when co-incubated with CsA, RAPA, FK-506 and MMF at each chosen concentration (Fig. 3). However, no differences in *in vitro* HL activity were observed for the same drugs incubations compared to untreated plasma (Table 3).

4. Discussion

Previous studies have suggested that immunosuppressive-administered transplantation patients, who exhibit plasma dyslipidemia including hypercholesterolemia and hypertriglyceridemia, might have some changes in their lipid metabolism (Gueguen et al., 2004). However, available data on the mechanisms of immunosuppressive-induced dyslipidemia are limited. The present study was undertaken to investigate the effects of CsA, RAPA, FK-506 and MMF on the *in vitro* activity of key lipid regulatory enzymes including CETP, HL and LPL.

We have previously demonstrated that the transfer of CsA between HDL and LDL is partially facilitated through CETP CE and TG transfer activities (Kwong and Wasan, 2003). Since CETP facilitates the exchange of CE from CE-rich lipoproteins for TG from TG-rich lipoproteins, these data suggested that the presence of CsA may affect the transfer of neutral lipids between lipoproteins. However, the interaction or the effect of other immunosuppressive drugs on CETP has not been addressed previously.

In our study, we have demonstrated that CsA and RAPA induced CE transfer activity by CETP in a concentration-dependent manner. By contrast, we did not find any significant difference in amounts of CE transferred between untreated and FK-506- or MMF-treated plasma, suggesting that FK-506 and MMF do not affect the CE transfer activity of CETP. This was further confirmed by the observation of similar results using rCETP.

In order to determine if CsA and RAPA increased CE transfer by CETP specifically, the CE-binding site on CETP was blocked by using the TP2 monoclonal antibody (Swenson et al., 1988). We observed that when CETP was co-incubated with TP2 and CsA or RAPA, there was a significant reduction in the CETP activity. In addition, based on the overall structure of CETP (Qiu et al., 2007), we know that there is no direct interaction between CsA or RAPA and the CE-binding site within the CETP molecule. We hypothesize that CsA and RAPA may bind to site(s) separate from the CE-binding site on CETP, causing a conformational change that enhances its CE transfer activity.

Lipoprotein lipase deficiency has been associated with hypertriglyceridemia and impaired chylomicron and VLDL clearance, as well as triglyceride enrichment of various lipoproteins (Vaziri et al., 2000). The result of our study has shown significant reductions in *in vitro* LPL activity of CsA-, RAPA-, FK-506- and MMF-treated plasma. This observation may correspond to a reduction in the hydrolysis of triglycerides contained within triglyceride-rich lipoproteins and the observed hypertriglyceridemia in patients receiving these drugs.

Interestingly, although we observed significant decrease in the lipoprotein lipase activity in CsA-, RAPA-, FK-506- and MMF-treated plasma, the hepatic lipase activity appeared unaffected. By contrast, a previous study reported an increase in VLDL and LDL level in CsA-treated patients that was associated with a decreased activity of HL (Derfler et al., 1991).

However, FK-506, which we had shown to affect the lipoprotein lipase, but not the CETP, rarely causes dyslipidemia. The two calcineurin inhibitors, CsA and FK-506, seem to have different effects

on lipid metabolism. The effect of FK-506 on lipid metabolism is similar to that of CsA, although the rise in total cholesterol levels may be less pronounced (Steinmuller et al., 1994). Current study also supports improvement in total and LDL cholesterol levels upon conversion from CsA to FK-506 (Copley et al., 1998). Besides that, it has also been reported that patients on FK-506 showed an increased risk of new-onset diabetes after transplantation (NODAT) as compared to CsA, only in the presence of high TG levels (Porrini et al., 2008). CsA, significantly increases incidence and prevalence of high TC and LDL-C, while FK-506 causes a greater degree of glucose homeostasis alteration (Boots et al., 2004; Vincenti et al., 2007).

However, since these studies were performed *in vitro*, there are multiple factors that would also lead to drug-induced dyslipidemia, specifically the influence on fatty acid homeostasis in the body. Other possible factor is the activation of peroxisome proliferator-activated receptors (PPARs), which may lead to enhanced fatty acid uptake and oxidative fatty acid metabolism. *In vitro* RAPA treatment on macrophages showed concentration- and/or time-dependent effects of PPARγ (Mathis et al., 2006). In addition, Jin et al. had found the opposing effect of FK-506 and CsA on CD36 and PPARγ in macrophages. Further, the regulation of CD36 and PPARγ observed with these drugs may suggest a direct effect of the immunosuppressant on PPARγ expression through a yet unknown mechanism (Ricote et al., 2000; Jin et al., 2004b). PPARγ activation can lead to upregulation of CD36, which results in accumulating of cholesterol by macrophages, yet PPARγ activation also stimulates ABCA1, which then prevents atherosclerosis through enhanced efflux (Mathis et al., 2006).

In conclusion, we have demonstrated that CsA and RAPA can induce *in vitro* CETP activity and, along with FK-506 and MMF, suppress LPL activity. Increased CE transfer by CETP could result in the enrichment of the apolipoprotein (apo) B-containing lipoproteins (VLDL and LDL) with cholesteryl esters, which is also a proatherogenic step in reverse cholesterol transport. On the other hand, reduction in LPL activity may lead to an elevated triglyceride, low total cholesterol and a pronounced decrease in HDL-cholesterol plasma levels.

Since many of the post-transplant patients who are administered these drugs experience dyslipidemia, including hypertriglyceridemia and hypercholesterolemia, these results may provide a possible explanation as to why it occurs. Further clinical study will be necessary in order to evaluate the clinical implications of our *in vitro* findings. Our study investigating the effects of these immunosuppressive drugs on the CETP and LPL activity in a population of renal transplant patients is currently being conducted.

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