



ADVERSE EFFECTS OF IMMUNOSUPPRESSANTS

Effect of Cyclosporine and Sirolimus on Fatty Acid Desaturase Activities in Cultured HEPG₂ Cells

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ABSTRACT

The aim of the present work was to evaluate the influence of cyclosporine (CsA) and sirolimus (SRL) on fatty acid (FA) desaturase activities. These enzymes (named $\Delta 9$, $\Delta 6$, and $\Delta 5$ desaturases) catalyze reactions leading to the biosynthesis of n-9, n-6, and n-3 FA families. n-3 FA family, derived from alpha-linolenic acid, is involved in the prevention of vascular events, which appear after successful kidney transplantation. Five groups of HepG₂ cells in culture were treated with either CsA (1 $\mu\text{g}/\mu\text{L}$ and 2 $\mu\text{g}/\mu\text{L}$) or SRL (10 ng/mL and 20 ng/mL) for 3 days, including a control group without immunosuppressive treatment. We studied the incorporation and metabolic conversion of radioactive [1-¹⁴C]palmitic, linoleic, and eicosatrienoic acids. We also analyzed fatty acid composition. The distribution of radioactive metabolic products after incubation of these cells with [1-¹⁴C]palmitic acid revealed a decrease in $\Delta 9$ desaturase activity in the presence of each immunosuppressive drug: CsA = 0.61 ± 0.01 ; SRL = 0.59 ± 0.04 versus control = 0.79 ± 0.05 ($P < .01$). We observed a significant increase in $\Delta 6$ and $\Delta 5$ desaturase activities under the influence of the immunosuppressive drugs: radiolabeled linoleic acid (CsA: 0.93 ± 0.04 ; SRL: 1.02 ± 0.03 vs control 0.60 ± 0.03 ; $P < .01$) and eicosatrienoic acid (CsA: 1.12 ± 0.02 ; SRL: 1.07 ± 0.01 vs control 0.75 ± 0.01 ; $P < .01$). In conclusion, CsA and SRL modulated the biosynthesis of polyunsaturated FAs, decreasing $\Delta 9$ desaturase and increasing $\Delta 6$ and $\Delta 5$ desaturase activities.

DYSLIPIDEMIA is common among patients after a successful organ transplantation. Particularly, it has been associated with vascular complications and graft atherosclerosis.¹ Previous reports have suggested that administration of cyclosporine (CsA) and sirolimus (SRL) in combination with steroids is the major cause of lipid abnormalities.^{2,3} It is well known that immunosuppressive therapies induce serum lipid abnormalities with total serum cholesterol and triglyceride enhancement. Furthermore, we have previously reported that these treatments also altered membrane neutral lipids and fatty acid (FA) profiles of

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various tissues in immunosuppressed, transplanted rats.⁴ Polyunsaturated fatty acids (PUFAs) are structural components of membrane phospholipids implicated in the pathogenesis of atherosclerosis as precursors of eicosanoids, inflammatory responses, and platelet aggregation. McHugh et al⁵ observed encouraging results in a clinical trial wherein kidney graft recipients were prescribed diets enriched with PUFAs added to the immunosuppressive therapy. However, follow-up studies have not been published. Nowadays, it is known that PUFAs produce immunomodulatory effects promoting vasodilatation and reducing endothelial cell activation.⁶

It is known that the biosynthesis of unsaturated FA in mammals occurs in the endoplasmic reticulum of cells through an alternative sequence of desaturation and chain elongation.^{7,8} The enzymes responsible for these insaturation reactions are the $\Delta 9$, $\Delta 6$, and $\Delta 5$ desaturases. They are membrane-bound enzymes that require an electron transport chain for NADH, which is catalyzed by the NADH-cytochrome *b₅* reductase and cytochrome *b₅*. The $\Delta 9$ desaturase, considered a lipogenic enzyme, introduces a cis-double bond at the $\Delta 9$ position of saturated FA, resulting in the production of monounsaturated fatty acids. The main substrates are palmitoyl (16:0) CoA and stearoyl (18:0) CoA, which are converted into palmitoleoyl (16:1) CoA and oleoyl (18:1) CoA, respectively.⁹ The $\Delta 6$ and $\Delta 5$ desaturases that introduce a cis-double bond at the $\Delta 6$ and $\Delta 5$ position are the key enzymes in PUFAs biosynthesis using linoleic acid (n-6 series) or α -linolenic acid (n-3 series) as precursors. The objective of the present study was to examine the influence of CsA and SRL on desaturase activities using a line of cultured human hepatoma cells (HepG2).

MATERIALS AND METHODS

Chemicals

The following radioactive FA were used: [¹⁻¹⁴C]palmitic (57.0 mCi/mmol, 98% pure) and [¹⁻¹⁴C]linoleic (58.0 mCi/mmol, 99% pure) from Dupont-NEN (Boston, Mass, USA) and [¹⁻¹⁴C]eicosa-8,11,14-trienoic (55.0 mCi/mmol, 99% pure) from American Radiolabeled Chemicals, Inc (St Louis, Mo, USA). The FA were stored in benzene under nitrogen atmosphere at -20°C .

Cell Culture and Treatment

Stock cultures of the human hepatocarcinoma cell line, HepG2 (American Type Tissue Culture Collection, Rockville, Md, USA), were maintained at 37°C in Eagle's minimal essential medium (MEM) with 10% heat-inactivated fetal bovine serum, as described previously.¹⁰ At the beginning of the experiment, 35 flasks (70 cm² each) were seeded with 2.5×10^6 cells per flask in 15 mL of Eagle's MEM containing 10% fetal calf serum. Three days later, when the cells were in the logarithmic growth phase, the medium was replaced by minimum Essential medium zinc-option (IMEM-Zo), and the flasks were separated into five groups ($n = 7$). During 3 days, four groups received daily various concentrations of CsA or SRL as reported by Rayyes et al¹¹ and Sahin et al,¹² respectively, who used the same immunosuppressive drugs and cell line. In general, these doses were based on immunosuppressive drug

concentration in the blood of patients maintained with once-daily administration.¹³⁻¹⁵ The doses are not toxic compared with those concentrations observed in patients, because both drug concentrations (those used in patients and in culture cells) are equivalent. These groups were: group A = CsA 1 $\mu\text{g}/\mu\text{L}$; group B = CsA 2 $\mu\text{g}/\mu\text{L}$; group C = SRL 10 ng/mL; and group D = SRL 20 ng/mL. The drugs were diluted in IMEM-Zo medium. An untreated fifth group maintained in IMEM-Zo medium without the addition of immunosuppressive drugs was used as a control. Fresh IMEM-Zo medium was replaced every 24 hours. On the basis of this experiment, we selected the highest nontoxic dose that produced changes in FA profile.

In a second experiment that was performed on the third day of immunosuppressive treatment, the medium was washed three times with 5 mL of cold phosphate buffered saline solution (295 mOsm/kg H₂O, pH 7.4) and the cells were incubated in 15 mL of IMEM-Zo medium supplemented with 2 $\mu\text{g}/\mu\text{L}$ of CsA or 20 ng/mL of SRL and 10 nmol of [¹⁻¹⁴C]palmitic or [¹⁻¹⁴C]linoleic or [¹⁻¹⁴C]eicosa-8,11,14-trienoic acids for 24 hours ($n = 7$ flasks per group). Radioactive FA were added as their sodium salts bound to delipidated albumin in a ratio of 2 nmol of FA to 1 nmol of albumin. At the end of the experiment, the attached cells were washed three times with 5 mL of ice-cold saline solution, detached from the growing surface mechanically with a rubber-tipped spatula, and pelleted at 500g for 10 minutes. The sedimented cells were resuspended in 5 mL of the same solution. An aliquot of the resulting suspension was used to determine cellular protein content.¹⁶ The remaining cell material was centrifuged in the same way, the supernate discarded and the pellet processed for lipid analysis.

Cellular Lipid Extraction and Separation

Lipids were extracted from the pellets using the method of Folch et al.¹⁷ Fatty acids were converted to their corresponding methyl esters (FAME) by transesterification with trifluoride methanol,¹⁸ before analysis using a Hewlett-Packard Model 5840-A gas liquid chromatograph equipped with flame-ionization detector (Hewlett-Packard, Calif, USA).

An aliquot of the organic phase was used to measure total radioactivity incorporated into the cell using a Wallac 1214 Rack-beta liquid scintillation counter (Pharmacia, Turku, Finland) with 97% efficiency for ¹⁴C. To determine the metabolic elongation-desaturation of the incubated radiolabeled FA, the distribution of radioactivity among the obtained FAME was analyzed using radiochromatograph (Varian Star 3400-CX) in tandem with a proportional gas detector (GL-Ram, Inus System Inc), following the methods described in a previous study.¹⁹

Statistical Analysis

Results were compared with respective controls using either Student *t* test, or one-way analysis of variance as appropriate.

RESULTS

Examination of the total FA composition of HepG₂ cells revealed a high content of oleic acid (Table 1). Treated groups that received a high concentration of immunosuppressive drugs showed a decreased relative percentage of 16:1 n-7, linoleic and arachidonic acids compared to the control group. In addition, the same groups revealed enhanced PUFAs.

Table 1. Percent Distribution of Total Fatty Acids From HepG2 Human-Hepatoma Cells

Fatty Acid	Control	CsA 1 µg/µL	CsA 2 µg/µL	SRL 10 ng/mL	SRL 20 ng/mL
16:0	22.0 ± 1.3	23.4 ± 1.3	26.0 ± 0.8	24.5 ± 1.8	24.6 ± 0.4
16:1 n-9	16.7 ± 1.4	15.4 ± 1.2	16.0 ± 0.7	14.0 ± 1.2	15.5 ± 0.9
16:1 n-7	2.2 ± 0.01	1.9 ± 0.01*	1.5 ± 0.01*	1.6 ± 0.02*	1.7 ± 0.01*
18:0	12.4 ± 1.2	11.6 ± 0.5	9.6 ± 0.4	12.2 ± 1.0	13.8 ± 1.2
18:1 n-7	17.8 ± 0.5	19.2 ± 1.0	19.0 ± 0.5	18.0 ± 0.7	18.3 ± 1.2
18:1 n-9	21.9 ± 1.2	21.6 ± 1.1	21.5 ± 0.5	22.4 ± 0.9	19.5 ± 0.8
18:2 n-6	3.0 ± 0.02	2.8 ± 0.01	2.2 ± 0.06*	2.6 ± 0.01	2.3 ± 0.1*
20:3 n-6	1.0 ± 0.01	1.0 ± 0.01	0.9 ± 0.01	0.9 ± 0.04	0.9 ± 0.01
20:4 n-6	2.0 ± 0.02	2.1 ± 0.04	1.5 ± 0.01†	1.7 ± 0.1	1.8 ± 0.02‡
22:4 n-6	0.1 ± 0.00	0.3 ± 0.06*	0.4 ± 0.00*	1.0 ± 0.06*	0.3 ± 0.01*
22:5 n-6	0.3 ± 0.01	0.3 ± 0.01	0.5 ± 0.01*	0.4 ± 0.01	0.8 ± 0.04‡
22:5 n-3	0.3 ± 0.01	0.1 ± 0.03	0.6 ± 0.01*	0.3 ± 0.01	0.5 ± 0.01*
22:6 n-3	0.3 ± 0.04	0.3 ± 0.02	0.3 ± 0.01	0.2 ± 0.0	0.3 ± 0.01

Results are given as means ± standard errors (n = 7). CsA, cyclosporine; SRL, sirolimus.
*P < .05; †P < .001; ‡P < .01 vs control.

To test the effects of CsA and SRL on the ability of HepG₂ cells to incorporate and metabolize labeled FA precursors from the medium, the cells were incubated with a specific radiolabeled FA precursor simultaneously with the immunosuppressive drug. The addition of CsA to the incubation media enhanced the uptake of palmitic acid, whereas it decreased the uptake of 20:3 n-6 acid with respect to the control group. No changes were seen in the linoleic acid uptake. In the presence of SRL, an increment in the incorporation of 18:2 n-6 was observed when compared with the control values (Fig 1).

The distribution of radioactive metabolic products after incubation of HepG₂ cells with [1-¹⁴C]palmitic acid, is shown in Fig 2. Palmitic acid was desaturated at the Δ9 position to palmitoleic acid (16:1 n-9). Then, this acid was further elongated to oleic acid (18:1 n-9). However, 16:0 might be firstly elongated to 18:0, and then desaturated at the 9 position, giving rise to oleic acid. Compared to the controls, the addition of CsA or SRL diminished biosynthesis of 18:0 and 18:1 n-9. These results were better expressed when we used a conversion index: [product/

substrate = Σ (16:1 n-9 + 18:0 + 18:1 n-9)/16:0], since it showed lower values in both treated groups compared with controls: control 0.79 ± 0.05 versus CsA 0.61 ± 0.01; versus SRL 0.59 ± 0.04 (P < .01 and P < .05, respectively). This result indicated a probable inhibition of Δ9 desaturase activity by both immunosuppressive drugs.

When HepG₂ cells were incubated with [1-¹⁴C]linoleic acid, Δ6-desaturase catalyzed the first and rate-limiting step in the transformation of the radiolabeled precursor, linoleate to arachidonate. Linoleic acid was desaturated at the 6 position generating 18:3 n-6 acid, which was then elongated to 20:3 n-6. Then, Δ5 desaturase introduced a double bond at the 5 position, giving rise to 20:4 n-6 (Fig 3). Analyzing the conversion index in this n-6 FA series [Σ (18:3 + 20:3 + 20:4/18:2)], we demonstrated that immunosuppressive-treated cells showed an increment of the conversion index as a consequence of enhanced Δ6 and Δ5 desaturase activities: CsA 0.63 ± 0.04; SRL 1.02 ± 0.03 compared with controls 0.60 ± 0.03 (P < .01).

Figure 4 shows that the HepG₂ cell line actively converted eicosatrienoic acid (n-6) into arachidonate, evidenc-

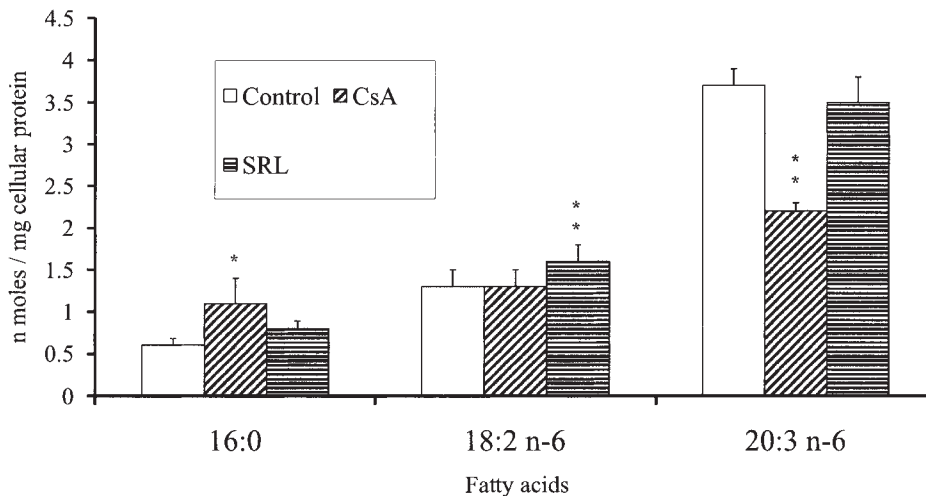


Fig 1. Uptake of radioactivity by HepG₂ cells incubated in the presence of different [1-¹⁴C]fatty acids. Results are expressed as nmoles per mg of cellular protein. Values are the means ± standard errors (n = 7). *P < .05, **P < .01, versus control group. CsA, cyclosporine; SRL, sirolimus.

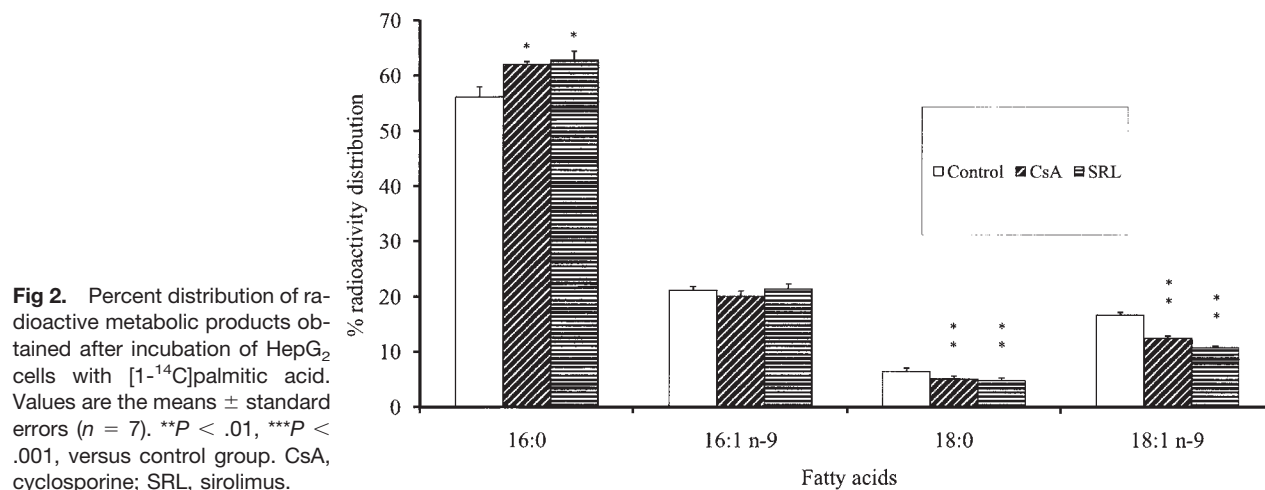


Fig 2. Percent distribution of radioactive metabolic products obtained after incubation of HepG₂ cells with [1-¹⁴C]palmitic acid. Values are the means \pm standard errors ($n = 7$). ** $P < .01$, *** $P < .001$, versus control group. CsA, cyclosporine; SRL, sirolimus.

ing that this human line has an active $\Delta 5$ desaturase, which increased in the presence of both immunosuppressive drugs, a result that was also supported by the increment of the 20:4 n-6/20:3 n-6 ratio: controls = 0.75 ± 0.01 versus CsA = 1.12 ± 0.02 vs SRL = 1.07 ± 0.01 ($P < .01$).

DISCUSSION

The study of regulatory factors that modulate $\Delta 9$, $\Delta 6$, and $\Delta 5$ desaturases system is essential to understand PUFA biosynthesis, which strongly depends on these enzymes. It is well known that immunosuppressive drugs adversely influence serum lipids. This study focused on the mechanism that influences the FA profile in many membrane tissues as we have observed in previous studies dealing with kidney transplanted rats.^{4,20} The liver plays an essential role in FA biosynthesis. Hepatocyte cell lines derived from human tumors have been used as an alternative model for studies on human hepatic lipid metabolism.^{21,22} The advantage of this system is the possibility to investigate the enzymatic activities involved in FA biosynthesis (elongases and/or de-

saturases) in a chemically defined medium, avoiding many complex biological factors always present in the intact mammalian organism. The high content of oleic acid present in the FA composition of total lipids and the low levels of linoleic acid and PUFAs are the most frequently abnormalities in lipids of neoplasm.²³ The different FA composition observed between control and immunosuppressive-treated cells may be explained to be a consequence of an increment in the $\Delta 6$ and $\Delta 5$ desaturase activity evidenced through the enhancement of 22:4 n-6, 22:5 n-6, and 22:5 n-3. In order to investigate the cellular uptake of the FA precursors added to the medium and their conversion into the corresponding higher homologs, cells were treated with specifically labeled FA.

Delta-9 desaturase is critical in the biosynthesis of neutral lipids: triglycerides, cholesterol esters, wax esters, and 1-alkyl-2,3-diacylglycerol.²⁴ Oleic acid is the preferred substrate for acyl-CoA cholesterol acyltransferase and diacylglycerol acyltransferase, the enzymes responsible for cholesterol esters and triglyceride synthesis, respectively. The decrease observed in the conversion index indicated that

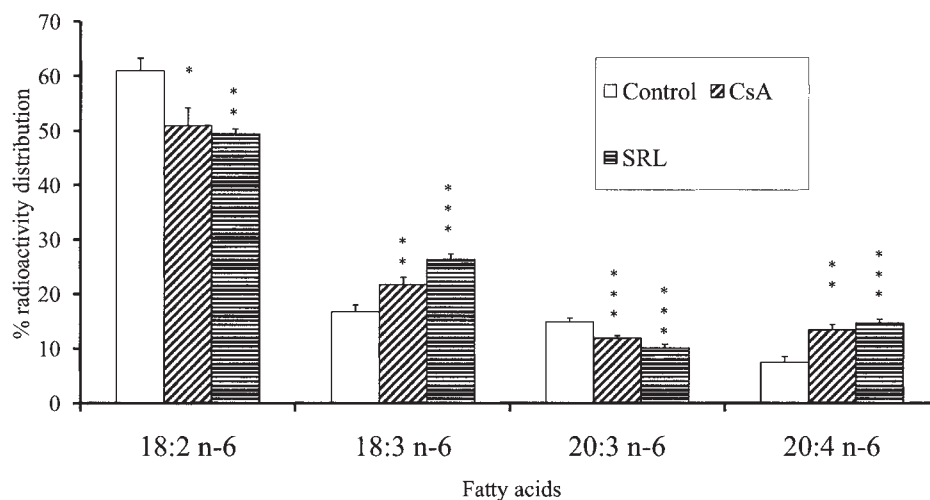


Fig 3. Percent distribution of radioactive metabolic products obtained after incubation of HepG₂ cells with [1-¹⁴C]linoleic acid. Values are the means \pm standard errors ($n = 7$). ** $P < .01$, *** $P < .001$, versus control group. CsA, cyclosporine; SRL, sirolimus.

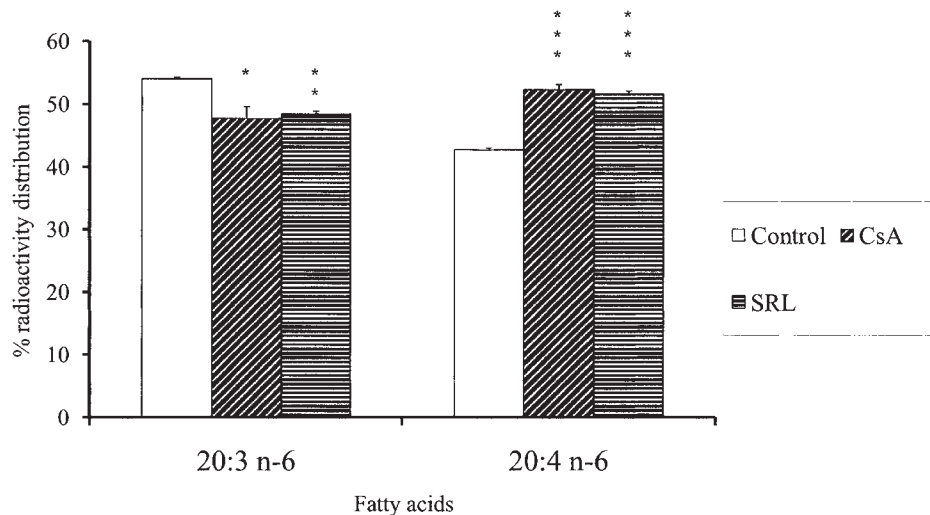


Fig 4. Percent distribution of radioactive metabolic products obtained after incubation of HepG₂ cells with [1-¹⁴C]eicosa-8,11,14-trienoic acid. Values are the means \pm standard errors ($n = 7$). ** $P < .01$, *** $P < .001$, versus control group. CsA, cyclosporine; SRL, sirolimus.

both immunosuppressive drugs were responsible for the diminution of $\Delta 9$ desaturase activity.

The metabolic conversion of PUFAs, such as 18:2 n-6 and 20:3 n-6, in neoplastic cells and their modulation are of particular interest since they are the precursors of arachidonic (n-6), eicosapentaenoic, and docosahexaenoic acids (n-3), which are related to cell proliferation and induction of apoptosis.²⁵ Recently, there has been increasing interest in the effects of various drugs on the FA composition of many tissues and their relationship to the immune system.^{4,6} Kew et al⁶ reported that 20:5 n-3 was the long chain (n-3) PUFA with the strongest modulating effects upon lymphocyte proliferation. In this regard, we observed that immunosuppressive-treated cells showed an increment in PUFAs as evidenced by the conversion index consequent to the enhancement of $\Delta 6$ and $\Delta 5$ desaturase activities.

De Alaniz and Marra^{26,27} studied the influence of steroid hormones and FA desaturases, concluding that glucocorticoids modulate the microsomal desaturase activities in different ways: increasing $\Delta 9$ and decreasing $\Delta 6$ and $\Delta 5$ desaturase activities. This mechanism blocks the generation of eicosanoids by cells leading to dramatic anti-inflammatory effects. However, glucocorticoids also provoke an adverse serum and membrane lipid pattern in contrast to CsA and SRL, which were demonstrated to exert a beneficial increase in $\Delta 6$ and $\Delta 5$ desaturase activities on membrane lipids. These results support current knowledge concerning the abolition of steroid treatment after solid organ transplantation.

In conclusion, CsA and SRL modulate the biosynthesis of unsaturated FA decreasing $\Delta 9$ desaturase and increasing $\Delta 5$ and $\Delta 6$ desaturase enzymes. However, the mechanism of action is different for each type of immunosuppressant. Further experiments will be necessary to investigate whether this is a genomic or nongenomic mechanism of immunosuppressive drugs.

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