



Lipid Pattern in Kidney-Transplanted Rats Without Immunosuppressive Therapy

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ABNORMALITIES IN lipid metabolism are frequently observed in patients with end-stage renal disease and following renal transplantation. Dislipidemia has been recognized as one of the risk factors for atherosclerosis. It may not only contribute to increase morbidity and mortality in this population, but it can also influence the development and progression of graft dysfunction and its loss.¹ Additional support for the role of lipids in progressive renal injury can be obtained from studies in which the administration of 3-hydroxy-3-methyl glutaryl coenzyme A reductase inhibitors (statins), a lipid-lowering treatment, decreased the glomerular damage.² The mechanism by which lipids may increase glomerular injury includes an interaction between macrophages, alterations in vascular and mesangial functions, changes in the production of mediator substances, and alteration in membrane fluidity.³⁻⁵ Few data dealing with the potential role of the renal transplant lipid abnormalities in the progression of renal damage and chronic rejection are available.³ The purpose of the present study was to determine the lipid profile of different tissues in kidney-transplanted rats, in the absence of immunosuppressive therapy. The behavior of the lipid pattern under these experimental conditions was studied to establish possible treatment strategies.

MATERIALS AND METHODS

Animals

Fourteen male outbred Wistar rats weighing 280 to 350 g served as donors and recipients for seven kidney transplants. The rats were housed in a climate-controlled room on a 12-hour light-dark cycle, with food (standard laboratory rat chow) and tap water ad libitum. They were fasted overnight prior to sample collection, surgery procedure, and sacrifice. This study was conducted according to the guidelines for the use and care of small laboratory animals in transplantation research.⁶

Transplantation

Both donors and recipients were anesthetized with intraperitoneal administration of atropine (0.01 mg/kg), diazepam (0.05 mg/kg), and ketamine (75 mg/kg). In short, the donor's left kidney was perfused in situ with 3 mL cold (4°C), heparinized, lactated Ringer's solution via the infrarenal aorta, until it was macroscopically bloodless. The kidney was then removed including the renal artery attached to a segment of the aorta; the renal vein was cut in its origin. It was then stored in the same solution to be used as the graft (transplanted kidney: TK).

The donor's right kidney (DK) (after removing the renal capsule), the liver, and the testis (free of tunica albuginea) were removed, weighed, and homogenized in cold (4°C) homogenizing solution (HS) (1:3 wt/vol). The HS contained 0.25 mol/L sucrose, 62 mmol/L phosphate buffer (pH 7.0), 0.15 mol/L KCL, 5 mmol/L MgCl₂, and 100 μmol/L EDTA.

Before transplantation the right recipient kidney (EK) was excised and homogenized in HS as described above. The graft was transplanted into the abdominal cavity. Both the aorta and inferior cava vein from the recipient were sutured by an end-to-side anastomoses to the donor's aorta and renal vein respectively, with 9-0 nonabsorbable monofilament nylon suture. The average total anastomotic time was 33 ± 3 minutes. End-to-end ureter anastomosis was performed using 10-0 nonabsorbable monofilament nylon suture. The left native kidney of the recipient (native kidney: NK) was not removed at the time of transplantation to study the microsome lipid pattern at the time of sacrifice. The average total ischemic time was 78 ± 28 minutes.

On the first transplantation day the rats were fed ad libitum and treated with buprinorphine (0.04 mg/kg) as analgesic therapy every 12 hours until the third postoperative day. The animals were sacrificed on the seventh postoperative day.

Isolation of Kidney, Liver, and Testis Microsomes and Erythrocyte Membranes

One week after surgery rats were killed by decapitation. Blood was drained off and collected into test tubes containing an anticoagulant EDTA solution (Wiener Lab, Rosario, Argentina). Liver, kidney, and testis were excised, weighed, and homogenized as indicated before. The microsomal fraction was separated by differential centrifugation at 105,000 g as described elsewhere.⁷

Whole blood was centrifuged, the plasma was immediately separated, and the packed red blood cells were washed four times, at 4°C, with a buffered solution containing NaCl (140 mmol/L), KCl (5 mmol/L), NaHSO₄ (1 mmol/L), Tris buffer (10 mmol/L), pH 7.4. After agitation they were kept at 4°C for 10 minutes, and centrifuged at 16,000 g for 15 minutes. This last procedure was done

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Table 1. Fatty Acid Composition of Total Lipids of Kidney Microsomes

Fatty acid	DK	EK	TK	NK
16:0	19.5 ± 0.4	19.5 ± 0.3	20.0 ± 0.9	20.5 ± 0.4
16:1	0.6 ± 0.05	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.04
18:0	20.5 ± 0.4	20.7 ± 0.3	20.3 ± 0.8	20.2 ± 0.4
18:1 n-9	7.6 ± 0.1	7.2 ± 0.3	9.0 ± 0.5	8.0 ± 0.5
18:1 n-7	1.5 ± 0.06	1.2 ± 0.1	1.6 ± 0.1	1.7 ± 0.1
18:2 n-6	14.8 ± 0.4	14.4 ± 0.2	14.4 ± 1.1	13.5 ± 0.7
20:3 n-6	1.2 ± 0.07	1.1 ± 0.1	1.0 ± 0.06	0.9 ± 0.1
20:4 n-6	30.7 ± 0.4 ^a	31.6 ± 0.2 ^b	26.9 ± 1.2 ^a	27.7 ± 1.2 ^b
22:4 n-6	0.8 ± 0.03 ^c	0.7 ± 0.1 ^{a,b}	1.9 ± 0.3 ^{a,c}	2.6 ± 0.5 ^b
22:5 n-6	0.2 ± 0.01 ^a	0.3 ± 0.07 ^b	0.7 ± 0.1 ^a	0.5 ± 0.04 ^b
22:5 n-3	0.4 ± 0.01 ^{a,c}	0.4 ± 0.02 ^d	0.6 ± 0.06 ^{a,b}	0.7 ± 0.1 ^d
22:6 n-3	2.2 ± 0.02 ^a	2.3 ± 0.6 ^b	3.0 ± 0.3 ^a	3.0 ± 0.2 ^b

Remaining donor's kidney (DK); excised recipient's kidney (EK); transplanted kidney (TK); recipient's native kidney (NK).

Fatty acids are identified by: number of carbon atoms in the chain is given first, value following the colon represents number or double bonds (0 means saturated fatty acid); number following n-indicates the position of the last double bond counting the double bond from the terminal methyl group.

Results are the mean of seven determinations ± 1 SEM expressed as % of total fatty acids.

Values with the same superscript letter are significantly different $P < 0.05$.

twice, leaving a substantial hemoglobin-free pellet of erythrocyte membranes, which was resuspended in a small amount of supernatant and stored (-70°C) until assayed.

Lipid Extraction and Analysis

Lipids from erythrocyte membranes and liver, kidney, and testis microsomes were extracted with chloroform-methanol (2:1 vol/vol). Fatty acids were converted to their corresponding methyl esters, then they were analyzed using a Hewlett-Packard Model 5840-A gas-liquid chromatograph, equipped with a flame-ionization detector. An aliquot from the organic phase of kidney microsomes extraction was separated to determine phospholipid and neutral lipid content through flame ionization detector (FID) of an Iatroscan apparatus model TH 10. Lipids were separated on previously activated chromarods type S-III, under a double-development system. The first mobile phase was hexane-benzene (70:30 vol/vol), whereas the second one was benzene-chloroform-formic acid (70:25:2 vol/vol/vol). Lipidic species were quantified by comparison with known amounts of pure standards run under the same conditions. The signals from the FID were registered on a Hewlett-Packard model HP-3396 A integrator.

Fluorescence Anisotropy Measurements

Steady-state fluorescence anisotropy (r_3) was measured in erythrocyte and microsomal membranes in an SLM 4800 C spectrofluorometer as previously described by Garda and Brenner.⁸ The probe used was 1,6-diphenyl-1,3,5 hexatriene (DPH). Excitation wavelength was 360 nm and the emitted light was passed through a sharp cutoff filter (Schott KV 389).

Light scattering of blanks represented less than 5% and fluorescence values were corrected accordingly. The phospholipid:probe ratio was maintained at more than 200:1 (mol:mol) to minimize possible probe-probe interactions.

Chemical Determinations

To measure the plasma cholesterol and triglyceride levels, blood was collected by puncture of the tail vein 1 week before surgery and at the time of sacrifice. Both variables were assayed by commercial enzymatic methods (Sera-Pak Bayer Corporation, USA). One week before transplantation and on day 4 and 7 after surgery, blood

was obtained by the same procedure to determine creatinine and urea plasma levels (Bio Systems, S. A. Barcelona, España, and Wiener Lab, Rosario, Argentina, enzymatic methods, respectively).

Histopathological Analysis

Light microscopic examination was performed on kidney graft removed on the seventh postoperative day. Biopsies were fixed with Bouin's solution and embedded in paraffin. Five $5\text{-}\mu\text{m}$ sections were stained with haematoxylin-eosin and periodic acid-Schiff standard procedures.

Statistical Analysis

Results were tested statistically using either the Student's t test compared to the respective control or the one-way analysis of variance (ANOVA) as appropriate.

RESULTS

Fatty Acid Profile of Plasma, Erythrocyte Membranes, and Liver, Testis, and Kidney Microsomal Membranes

The fatty acid composition of kidney microsomal total lipids is shown in Table 1. No changes were seen in the relative percentages of the different fatty acids between the remaining donor's kidney (DK) and the excised recipient's kidney (EK). A significant decrease in arachidonic acid and an increment in fatty acids of n-6 series 22:4 and in fatty acids of n-3 series 22:5 and 22:6 were observed comparing the DK with the graft (TK). Seven days after surgery, the fatty acid profile of microsomes of the NK, left in place during the surgery procedure, was similar to that of TK. The comparison of NK and EK showed the same changes to those described for DK and TK.

Table 2 shows the fatty acid composition of testis microsomes. In the values of the transplanted rats, a significant increase of 18:1n-9 and 22:5 n-6 acids as well as a decrease of stearic and linoleic acids were observed.

The main change observed in the erythrocyte membranes of the transplanted rats was a decrease in linoleic acid, compared to those of non-transplanted animals (mean ±

Table 2. Fatty Acid Composition of Total Lipids of Testis Microsomes in Donors and Transplanted Rats

Fatty Acid	Donors Rats	Transplanted Rats
16:0	28.6 ± 0.7	31.1 ± 1.0
16:1	1.3 ± 0.3	1.0 ± 0.1
18:0	10.9 ± 0.6	8.6 ± 0.3*
18:1 n-9	10.3 ± 0.3	11.6 ± 0.2*
18:1 n-7	1.6 ± 0.03	1.5 ± 0.05
18:2	9.4 ± 0.1	6.0 ± 0.3*
20:3	1.1 ± 0.05	1.4 ± 0.03
20:4	16.7 ± 0.4	16.2 ± 0.5
22:4	2.1 ± 0.2	1.8 ± 0.2
22:5 n-6	16.5 ± 0.7	18.9 ± 0.4*
22:5 n-3	Tr	0.2 ± 0.03
22:6	1.5 ± 0.3	1.2 ± 0.1

Fatty acids are identified by: number of carbon atoms in the chain is given first, value following the colon represents number or double bonds (0 means saturated fatty acid); number following n-indicates the position of the last double bond counting the double bond from the terminal methyl group.

Results are the mean of seven determinations ± 1 SEM expressed as % of total fatty acids.

*Indicates significant differences between donors rats and transplanted rats values, $P < .01$.

SEM were 9.9 ± 0.4 vs 11.7 ± 0.1 , $P < .01$). No changes were observed in the fatty acid pattern of plasma and liver microsomal membranes of both groups of rats (data not shown).

Steady-State Fluorescence Anisotropy of DPH

The mean steady-state fluorescence polarization values of DPH measured in erythrocyte membranes from transplanted rats at the time of sacrifice were not different from those obtained in the same membranes of the nontransplanted animals; therefore the results were not shown.

Relative Percentages of the Lipid Species in Kidney Microsomal Membranes

The amount of the different lipid species in kidney microsomal membranes is shown in Table 3. No differences were observed comparing the DK, the graft 7 days after surgery, and the EK. A significant decrease in the free fatty acids was observed between EK and NK at the time of sacrifice.

Plasma Cholesterol and Triglycerides Levels

Cholesterol and triglyceride levels in the transplanted rats 7 days after surgery were compared to those of nontransplanted animals. A significant increase in cholesterol levels

after the transplantation was noted (mean ± SEM were 260 ± 30 mg/dL vs 390 ± 20 mg/dL, $P < .01$). Plasma triglyceride levels were similar in both groups of rats (mean ± SEM were 530 ± 40 mg/dL vs 570 ± 50 mg/dL).

Plasma Urea and Creatinine Levels

After 4 days of transplantation, a significant increment was observed in both parameters compared to the basal measurements obtained before the transplant procedure (mean ± SEM were: urea 54 ± 3.44 mg/dL vs 40 ± 4.0 mg/dL, $P < .01$, and creatinine 2.50 ± 0.20 mg/dL vs 1.05 ± 0.6 mg/dL, $P < .01$). On day 7 both values showed a decrease but they still remained statistically significant compared to the basal (mean ± SEM were: urea 48.47 ± 2.61 mg/dL vs 40 ± 4.0 mg/dL, $P < .01$, and creatinine 2.35 ± 0.21 mg/dL vs 1.05 ± 0.6 mg/dL, $P < .01$).

Histopathology

The histopathological study of the graft from all the animals studied showed the following changes:

Interstitium: Either mild or moderate stromal edema. Severe mixed (mononuclear-neutrophilic) cellular infiltration.

Tubules: Mild inflammation reaction with some tubular focal necrosis.

Glomeruli: Either mild or moderate focal necrosis. Vascular congestion.

Blood vessels: mild endothelial inflammatory reaction with thickening of the intima.

DISCUSSION

Dyslipidemia is one of the first metabolic dysfunction observed in patients with end-stage renal disease, and it has been incriminated as a risk factor for atherosclerotic vascular disease.⁹ Plasma triglycerides, but not cholesterol, are increased in most patients with advanced renal failure.^{10,11} These lipid abnormalities persist even after renal transplantation and coexist with high levels of plasma cholesterol in 50% of the patients 3 months after transplantation.¹² Data obtained from literature show that this phenomenon is mainly attributed to the immunosuppressive therapies.^{2,13-15} In the present study, where immunosuppressive drugs were not included in the therapy, a hypercholesterolemia was observed after transplantation, together with modifications in lipid fatty acid profile of kidney and testis microsomes, and also in erythrocyte membranes.

Table 3. Relative Percentages of the Lipid Species in Kidney Microsomal Membranes

	DK	EK	TK	NK
Triglycerides	3.2 ± 0.4	3.3 ± 0.5	4.3 ± 0.2	3.6 ± 0.3
Free fatty acids	5.0 ± 0.3	5.9 ± 0.5 ^a	4.8 ± 0.2	4.4 ± 0.4 ^a
Cholesterol	24.2 ± 0.7	28.5 ± 2.0	26.0 ± 0.9	23.5 ± 1.5
Phospholipids	67.6 ± 1.2	62.3 ± 2.1	64.9 ± 1.0	67.8 ± 1.7

Remaining donor's kidney (DK); excised recipient's kidney (EK); transplanted kidney (TK); recipient's native kidney (NK).

Results are the mean of seven determinations ± 1 SEM expressed as relative % of total lipid species.

Values with the same superscript letter are significantly different $P < .05$.

The main change observed in the total fatty acid composition of the graft microsomal fraction was a decrease in arachidonic acid partially compensated by an enhancement of the fatty acids of 22 C belonging to n-3 and n-6 series. This effect could be attributed to an increase of eicosanoid synthesis from arachidonic acid as a consequence of the ischemia-reperfusion injury. Alcaraz et al showed a progressive decrease of 6-keto-PGF α /TxB2 ratio in renal cortex with the prolongation of cold ischemia.¹⁶ Nevertheless, this mechanism cannot explain the arachidonic acid decrease observed in the native kidney that did not suffer ischemic injury.

It is well known that lipids are of fundamental importance in the development and functioning of testicular tissues. Lipids from testis contain a high concentration of polyenoic acids, which include docosapentaenoic (22:5 n-6) acid in addition to linoleic and arachidonic acids.¹⁷ The increase in 22:5n-6 acid observed in testis microsomal membranes after transplantation could be the consequence of the depletion of linoleic acid to preserve the function of the tissue. This change cannot be attributed to the ischemic injury, since the blood supply was not interrupted when the kidney was transplanted.¹⁸

The depletion of linolenic acid was also observed in erythrocyte membranes after transplantation. Nevertheless, this change was not so important to modify the rotational mobility of the lipids in the membranes.

Serum urea and creatinine levels are useful markers to monitor the occurrence of early graft rejection. In humans, the serum creatinine level may reach <2.5 mg/dL in ideal circumstances during the first posttransplant week.¹⁹ In the experimental reported here the initial increment of both values could be ascribed either to rejection of the graft or to a compensatory mechanism of the native kidney to depurate the plasma. Then they decline so that the rats tended to achieve stable kidney function within 7 days after surgery.

Related to histopathological studies we can assume that, in general, the changes observed in the graft consisted of features compatible with moderate rejection.

In summary, a remarkable change in the lipid pattern and a moderate rate of the rejection were found 7 days after kidney transplantation in rats maintained without immunosuppressive therapy. We consider that the transplantation

develops a thorough immunologic mechanism that is responsible for the lipid profile changes observed in the transplanted rats, not only in the graft but also in many tissues. The present experimental model could represent a useful tool for testing different therapies used routinely to abolish rejection, to evaluate the safety and efficacy of these treatments, mainly in relation to cardiovascular risk factors.

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