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Changes in plasma free fatty acid concentrations in rheumatoid arthritis patients during fasting and their effects upon T-lymphocyte proliferation

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Abstract

Objective. To measure whether changes in the concentrations of circulating free fatty acids (FFAs) after a 7 day fast in rheumatoid arthritis (RA) patients would inhibit *in vitro* T-lymphocyte proliferation.

Methods. The concentration and composition of plasma FFAs were measured in nine RA patients at the conclusion of a 7 day fast. A FFA mixture was made up based on these findings (20% linoleic, 43% oleic, 10% stearic, 27% palmitic acid). Mitogen-induced lymphocyte proliferative responses were measured after co-culture of peripheral blood mononuclear cells (PBMC) from healthy individuals in the presence of increasing concentrations of this FFA mixture (from 0 to 2000 μM) and in the presence of FFA mixtures where the relative proportions of fatty acids varied.

Results. Both the concentration of the FFA mixture and the ratio between the unsaturated and saturated fatty acids significantly influenced *in vitro* lymphocyte proliferation ($P < 0.0001$). Unexpectedly, the highest concentrations of the FFA mixture increased lymphocyte proliferation. At equimolar concentrations (600 μM), manipulating the amounts of oleic and linoleic fatty acids relative to stearic and palmitic fatty acids had a potent inhibitory effect upon lymphocyte proliferation.

Conclusion. Fasting-associated increases in total plasma FFA concentrations do not inhibit, but rather enhance, *in vitro* lymphocyte proliferation. An inhibitory effect could only be achieved by manipulating the balance between the unsaturated and saturated fatty acids.

KEY WORDS: Rheumatoid arthritis, Fasting, Fatty acids, Lymphocyte proliferation.

There has been considerable interest in recent years regarding the immunomodulatory effects of polyunsaturated fatty acids and their therapeutic potential as anti-inflammatory agents [1]. Both clinical [2, 3] and *in vitro* studies [4–6] have established that long-chain n-3 and n-6 fatty acids inhibit T-lymphocyte function.

Whilst it is known that long-chain n-3 and n-6 fatty acids have immunomodulatory effects, it is not known whether changes in the total concentration of circulating free fatty acids (FFAs) influence immune responses. Substantial increases in the total concentrations of circulating FFAs occur under certain environmental and metabolic conditions, such as prolonged fasting [7] and insulin resistance, and may be of pathological significance [8].

For unknown reasons, fasting has significant beneficial effects upon both clinical and laboratory variables of disease activity in rheumatoid arthritis (RA) [9, 10]. We hypothesized that fasting-induced changes in the concentrations of serum FFAs might inhibit lymphocyte function and could contribute to the observed anti-inflammatory effects.

We have measured the concentration of plasma FFAs in nine RA patients before and after a 7 day fast. Lymphocyte proliferative responses after mitogenic stimulation with anti-CD3 were measured in the presence of increasing concentrations of a FFA mixture, the composition of which was based on our *in vivo* findings. As it has been suggested that the clinical improvements in RA patients during fasting may be related to changes in the fatty acid composition of cell membranes [10],

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we also measured the cellular fatty acid composition of peripheral blood mononuclear cells (PBMC) pre- and post-fasting.

Patients and methods

Study design and patients

Briefly, nine RA patients (eight female and one male) underwent a 7 day supervised subtotal fast [<50 g carbohydrate/day, total energy <900 kJ (215 kcal)/day]. All patients had active disease, as defined by the presence of three of the four following criteria: >3 swollen joints (28 joints), >6 tender joints (28 joints), duration of morning stiffness >45 min, erythrocyte sedimentation rate (ESR) >28 mm in the first hour. All patients had radiological erosions of grade 3 or below, and a body mass index (BMI) between 22 and 28 kg/m^2, and did not have other serious medical conditions which would preclude fasting. Median age and disease duration were 51 yr (range 31–65) and 2.9 yr (range 0.2–12), respectively. Four patients were taking second-line drugs, one was taking corticosteroids (5 mg/day), seven were taking non-steroidal anti-inflammatory drugs (NSAIDs) and three were taking painkillers.

Sample collection and storage

Pre- and post-fasting blood samples, drawn between 10.00 and 11.30 a.m., were obtained. Ethylenediaminetetraacetic acid (EDTA)–whole blood was collected and placed on ice. Plasma was separated and frozen at -70°C for subsequent analysis of FFA composition and concentrations. PBMC were isolated from the RA patients pre- and post-fasting, and were frozen at -70°C until subsequent analysis of total cellular fatty acid composition.

For proliferation assays, PBMC were isolated from healthy controls and were resuspended in RPMI 1640 containing 10% fetal calf serum and 0.6 mM bovine serum albumin (BSA; essentially fatty acid free; Sigma Chemical Co., St Louis, MO, USA) at a concentration of 2×10^6 cells/ml. More than 96% of the cells were viable as assessed by the trypan blue exclusion test.

Fatty acid analysis

FFAs from EDTA–plasma and total cellular lipids were extracted and measured by gas–liquid chromatography (GLC) as fatty acid methyl esters. After extraction of plasma [11], the FFAs were incubated with 1 ml methanolic HCl (3 M) for 15 min at 37°C. The mixtures were neutralized by 2 ml $NaHCO_3$ (0.7 M) and extracted twice with 2 ml *n*-hexane. After evaporation of the solvent, the lipids were redissolved in *n*-hexane, and an aliquot was injected into the chromatograph (Shimadzu, GC-14A), equipped with a polar capillary column (Supelco SPE 2560, length 100 m). The oven temperature was programmed to rise from 135 to 193°C at 5–6°C/min. Identification of individual methyl esters was performed by comparisons with authentic standard mixtures. Heptadecanoic acid (C17:0) was used as an internal standard for quantification of unesterified fatty acids.

After extraction of PBMC, the residual lipids were redissolved in 1 ml benzene and methylated overnight in 2 ml methanolic HCl (3 M) and 200 ml 2,2-dimethoxypropane. The mixtures were neutralized by 4 ml $NaHCO_3$ (0.7 M) and the procedure for analysis of FFAs was followed as above.

Lymphocyte proliferation assay

Fifty microlitres (1×10^5 cells) of the cell suspension were added to each well of a round-bottom 96 well microtitre plate (Falcon Plastics). A FFA mixture (final concentration 2666 μM) based on the average percentage composition of FFAs *in vivo* was made up. The mixture consisted of 20% linoleic acid, 43% oleic acid, 10% stearic acid and 27% palmitic acid (all Sigma Chemical Co., St Louis, MO, USA) and contained 0.6 mM BSA (equivalent to *in vivo* albumin concentration). Various amounts of the fatty acid mixture (from 0 to 150 μl) were added to the cell suspension as required and the volume in each well was brought up to 200 μl by adding RPMI 1640 containing 0.6 mM BSA.

For additional experiments, two fatty acid mixtures were made: an unsaturated mixture containing equimolar concentrations of oleic acid and linoleic acid, and a saturated mixture containing equimolar concentrations of palmitic and stearic acid. BSA was added at a final concentration of 0.6 mM. Cell cultures were incubated in 20% O_2/5% CO_2 at 37°C. After 24 h, an anti-CD3 (OKT3D) monoclonal antibody (MAb) was added, and the mixtures were incubated for a further 72 h under identical conditions.

Viability was assessed by the trypan blue exclusion test; 1 mCi methyl [^{3}H]thymidine was added for the final 18 h before harvesting and [^{3}H]thymidine incorporation was measured by liquid scintillation. All experiments were carried out in triplicate and results were expressed as counts per minute (c.p.m.).

Statistical analyses

Within-group differences at fixed time points were tested by Wilcoxon's signed rank test. To test whether different concentrations or compositions of fatty acids influenced lymphocyte proliferation *in vitro*, we used analysis of variance. A randomized complete block model using triplicate values as a blocking factor was used. Fisher's protected least square difference was used to identify which combinations of the unsaturated fatty acid mixture and the saturated fatty acid mixture differed significantly from the representative *in vivo* combination. Two-sided P values of <0.05 were considered as significant. The analyses were carried out using STATVIEW 4.1 and Super ANOVA 1.11 (both Abacus Concepts, Berkeley, CA, USA).

Results

Concentration and composition of plasma FFAs during fasting

There were significant increases in total FFAs, palmitic acid, stearic acid, oleic acid (all $P < 0.01$), myristic acid,

palmitoleic and linoleic acid (18:2 n-6) after fasting (all $P < 0.05$). With the exception of oleic acid, the amount of each fatty acid as a percentage of the total concentration of FFAs did not change. In PBMC, we found no significant changes in the amounts of fatty acids as a percentage of total cellular lipids (Table 1).

Effects of fasting-induced changes in FFA concentrations upon lymphocyte proliferation in vitro

The concentration of the FFA mixture had a significant effect upon lymphocyte proliferation ($P < 0.001$) with proliferative responses increasing with higher concentrations of fatty acids (Fig. 1a). Linoleic acid alone (240 μM) was used to confirm that our culture conditions could replicate the previously reported inhibitory effect of linoleic acid upon lymphocyte proliferation [6].

Effects of varying the ratio between an unsaturated and a saturated fatty acid mixture at equimolar concentrations upon lymphocyte proliferation in vitro

In contrast to the dose-dependent inhibitory effect of individual fatty acids upon lymphocyte proliferation [12], we noted that increasing concentrations of fatty acids as a part of a mixture had the opposite effect. We therefore investigated how varying the ratio between an unsaturated fatty acid mixture (oleic acid and linoleic acids at 1/1 ratio) and a saturated fatty acid mixture (palmitic and stearic acids at 1/1 ratio) influenced lymphocyte proliferation at equimolar concentrations (600 μM).

Altering the ratio between the unsaturated fatty acid mixture and the saturated fatty acid mixture significantly influenced lymphocyte proliferation ($P < 0.001$) (Fig. 1b). The post hoc analysis revealed that as compared to 400 μM unsaturates + 200 μM saturates together (representative of the *in vivo* ratio), 600 μM of the unsaturates alone had a significant inhibitory effect (68% reduction; $P < 0.001$). Substitution of only 50 μM of the unsaturates with 50 μM of the saturates reversed this inhibitory effect. Incubation of the cells in the presence of saturates only also had a significant inhibitory effect as compared to the *in vivo* ratio (65% reduction; $P < 0.001$). Substitution of 50 μM of the saturated mixture with 50 μM of the unsaturated mixture (50 μM unsaturated + 550 μM saturated) had a less potent but still significant inhibitory effect as compared to the *in vivo* ratio (44% reduction; $P < 0.001$).

TABLE 1. Effects of fasting upon plasma FFA concentrations and PBMC fatty acid composition (mean ± S.E.M.; $n = 9$)

	Before fasting	After fasting
Plasma (μM) (mean percentage of total FFAs shown in parentheses)		
Total FFAs	534 ± 50	1254 ± 184**
Myristic acid (14:0)	6 ± 2 (1%)	17 ± 5* (1%)
Palmitic acid (16:0)	149 ± 14 (28%)	341 ± 43** (27%)
Palmitoleic acid (16:1 n-7)	19 ± 4 (4%)	52 ± 11* (4%)
Stearic acid (18:0)	69 ± 5 (13%)	134 ± 16** (11%)
cis-Vaccenic acid (18:1 n-7)	8 ± 4 (1%)	25 ± 10 (2%)
Oleic acid (18:1 n-9)	185 ± 22 (35%)	476 ± 74** (38%**)
Linoleic acid (18:2 n-6)	96 ± 12 (18%)	209 ± 43* (17%)
PBMC cellular membranes (expressed as % of total cellular lipids)		
Myristic acid (14:0)	5.3 ± 2.7	3.7 ± 2.1
Palmitic acid (16:0)	28.4 ± 2.5	30.2 ± 2
Palmitoleic acid (16:1 n-7)	1.6 ± 0.7	1.6 ± 0.7
Stearic acid (18:0)	42.0 ± 5.7	41.7 ± 3
Oleic acid (18:1 n-9)	8.3 ± 3.2	9.3 ± 2.5
Linoleic acid (18:2 n-6)	4.0 ± 2.6	2.9 ± 0.7
Arachidonic acid (20:4 n-6)	9.8 ± 4.4	10 ± 2.8

Significantly different from pre-fasting: *$P < 0.05$; **$P < 0.01$.

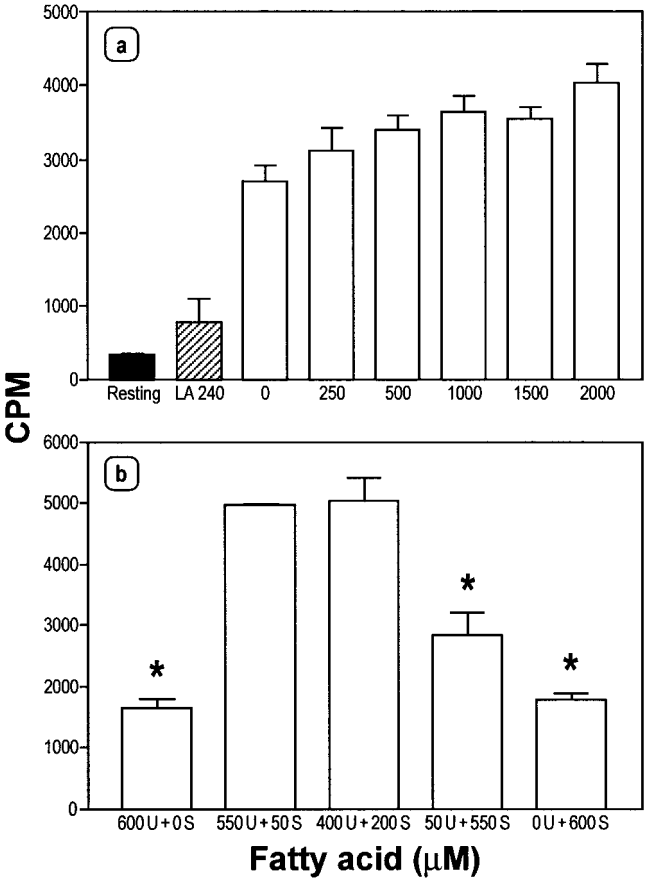

FIG. 1. (a) Mean (± S.E.M.) lymphocyte proliferative responses either with or without (black bar) mitogenic stimulation for 72 h in the presence of different concentrations of a fatty acid mixture containing palmitic (27%), stearic (10%), oleic (43%) and linoleic (20%) acids in proportions equivalent to *in vivo* findings. LA, linoleic acid (18:2 n-6). BSA was held constant at 0.6 mM. FFA concentration had a significant effect upon lymphocyte proliferation ($P < 0.001$). (b) Mean (± S.E.M.) lymphocyte proliferative responses after mitogenic stimulation for 72 h in the presence of different combinations of an unsaturated fatty acid mixture (U) and a saturated fatty acid mixture (S). The ratio between saturated and unsaturated fatty acids had a significant effect upon lymphocyte proliferation ($P < 0.001$). *Significantly different by post hoc test from 400 μM U + 200 μM S ($P < 0.001$). Data were obtained from 3–4 independent experiments using PBMC isolated from 3–4 healthy subjects.

Cell viability

There were no significant differences in cell viability related either to the type or concentration of fatty acids

used in culture. The mean cell viability in the cell cultures activated with anti-CD3 was 84% (range 79–88%). Mean cell viability in PBMC isolated from wells containing resting cells was 94%.

Discussion

The 2- to 3-fold increase in the concentration of plasma FFAs at the end of the 7 day fast is in accordance with earlier studies in healthy subjects [13], as are our findings that the predominant circulating FFAs both under normal conditions and during prolonged fasting are palmitic, myristic, oleic and linoleic acid [14]. However, in contrast to Hafström *et al.* [10], who found a significant increase in the percentage of arachidonic acid in serum from RA patients after fasting, we could not find any significant changes in the proportions of any plasma fatty acids. Neither did we observe any changes in the relative amount of individual fatty acids in PBMC as a percentage of total cellular lipids.

That *in vitro* lymphocyte proliferation increased with increasing concentrations of the FFA mixture does not support the idea that fasting-induced increases in circulating FFAs inhibit lymphocyte proliferation. The concentrations of FFAs we selected for our *in vitro* experiments approximated the observed changes *in vivo*, where the lowest concentrations of FFAs pre-fasting and the highest concentration post-fasting were 230 and 2400 μM, respectively. Thus, in contrast to the dose-dependent inhibitory effect upon lymphocyte proliferation which occurs with individual fatty acids [15], the opposite occurs when the FFAs are presented as a mixture of saturated and unsaturated fatty acids. Possible explanations for this effect may be that increased concentrations of FFAs provide a greater energy source for proliferating lymphocytes [16]. The increasing FFA/albumin ratio (from 0.37 at the 250 μM concentration to 3.33 at the 2000 μM concentration) may also be of significance.

To achieve an inhibitory effect upon lymphocyte proliferation *in vitro* using the predominant circulating FFAs, it was necessary to alter the balance of fatty acids towards either an excess of unsaturated or saturated fatty acids. Similar findings have been reported in concanavalin A-stimulated rat lymph node lymphocytes [12]. The inhibitory effects we observed may occur secondary to changes in membrane fluidity induced by an excess of either unsaturated fatty acids or saturated fatty acids. The fluidity and flexibility of the cell membrane play a central role in both receptor expression and protein movement in the membrane [17], both of which are intimately involved in T-cell activation.

The practical implications of these observations to the *in vivo* situation are unclear. Although circulating FFAs are ultimately determined by diet, it is unlikely that the ratio of unsaturates to saturates required for T-cell inhibition to occur *in vitro* could be achieved *in vivo*. However, it has been reported that a very low intake of saturated fats is beneficial in multiple sclerosis, where, like in RA, CD4+ lymphocytes are thought to play a pathogenic role [18]. Whether such a diet could have immunosuppressive effects *in vivo* and could thus be of benefit in the treatment of RA should be investigated.

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