Vol. 40, No. 4, November 1996 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 833-842

THE EFFECT OF N-3 PUFA RICH DIET UPON MACROPHAGE AND LYMPHOCYTE METABOLISM AND FUNCTION.

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Received July 16, 1996
Received after revision September 13, 1996

Summary

A large body of evidence from experimental studies has documented that n-3 fatty acids can modify a variety of cell functions and disease states. As lymphocytes and macrophages are important cells for the development of the inflammatory and non-inflammatory immune response and are known to utilize high rates of glucose and glutamine, we have evaluated the effect of n-3 PUFA rich diet on the metabolisation of glucose and glutamine these cells, as well as the effect of one such diet upon the proliferative response of lymphocytes and the phagocytic capacity and hydrogen peroxide production by macrophages. The diet provoked an increase in the flux of glucose through the Krebs cycle in macrophages as well as a reduction in G6PDh and glutaminase activity in these cells. Lymphocytes from n-3 PUFA rich diet-fed rats showed a reduction in glucose and glutamine decarboxylation. Taken together the data show that, at least in part, the functional changes observed in macrophages and lymphocytes from n-3 PUFA-rich diet fed rats are related to the effect of this diet upon glucose and glutamine metabolism, leading to immunosupression

Key words: macrophage, lymphocyte, n-3 PUFA, glucose, glutamine

Introduction

Dietary n-3 polyunsaturated fatty acids are essential for the ensurance of normal health in mammals. A large body of evidence from experimental studies has documented that n-3 fatty acids can modify a variety of cell functions and disease states (1, 2), having a protective effect in inflammatory and autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and psoriasis (3). It has been proposed that such benefits may be related to their selective inhibitory effects on the cyclooxigenase and lipoxygenase pathways (4). However, there is still some controversy, since the inhibitory effect of unsaturated fatty acids has also been shown to be independent of eicosanoid synthesis (5, 6) and lipid peroxidation (7). The effect of this type of fatty acid

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1039-9712/96/040833-10$05.00/0

could, however, be related to changes in the production of the different cytokines involved in the inflammatory and immunological responses. Various studies have shown that mononuclear cells from n-3 fatty acids fed rats produce less IL-1(interleukin-1) and TNF (tumour necrosis factor) (8) when compared with controls. Such results are in contrast with those from Chang and coworkers (4) who showed an increase in the mRNA expression of TNF-alfa in macrophages treated with LPS (lipopolysaccharide).

The present study was undertaken to explore at the metabolic level the mechanism by which fish oil rich diet induces changes in the immune response. Despite the fact that macrophages are fully differentiated cells, while lymphocytes, on the other hand, are highly proliferative cells, both cell types show the same metabolic pattern, utilizing glucose and glutamine at high rates (9, 10). These substrates allow them to proliferate (lymphocytes) and produce a large amount of proteins and lipids. The ability to proliferate, for lymphocytes and to produce and secrete large amounts of substances, for macrophages and lymphocytes, are strictly linked to the proper development of the immune inflammatory and non-inflammatory reaction, as well as to the effective control of tumour growth and wound healing. Since the ability to proliferate and mantain a secretory pattern is essential for those cells to mantain their function, and that it has been previously shown that polyunsaturated and saturated fatty acid rich diets affect metabolic and functional parameters both in macrophages and in lymphocytes (10, 11), we have presently evaluated the effect of n-3 PUFA rich diet on the metabolisation of glucose and glutamine in lymphocytes and macrophages, as well as the effect of one such diet upon the proliferative response of lymphocytes and the phagocytic capacity and hydrogen peroxide production by macrophages.

Material and Methods

Animals and diets - Male Wistar rats weighing 180 g (about 2 months of age) were obtained from the Biomedical Sciences Institute - São Paulo. The rats were maintained at 23°C under a cycle of 12h light:12 h darkness, receiving water *ad libitum*. Animals were fed either a control diet - Purine commercial chow, to which casein was added, providing a 20% final protein content and 3% of fat content (p/s 2.9) or a n-3 PUFA-rich diet, prepared by adding 20% fish oil (P/S 9.7). The n-3 PUFA-rich diet was prepared twice a week and kept at 4°C to avoid oxidation.

Chemicals - All chemicals and enzymes were obtained from Boehringer Mannheim GmbH, Lewes, East Sussex, U.K. and Sigma, St. Louis, MO, USA except for [U-^{14}C]-glucose, [U-^{14}C]-glutamine and [2-^{14}C]-thymidine from Amersham, UK. All measurements were performed using a Gilford (Model Response) spectrophotometer.

Peritoneal cell preparation - Cells present in the intraperitoneal cavity of the rats were collected using 6 ml phosphate-buffered saline (PBS) and considered as resident macrophages (unstimulated). Cell viability was confirmed by Trypan blue exclusion (>95%). In all experiments, at least 92% of the peritoneal exudate cells were macrophages as determined by differential counting.

Lymphocyte preparation - The cells were obtained as previously described (9) from the mesenteric lymph nodes and spleen pressed against a steel screen. Peripheral blood lymphocytes were obtained by the centrifugation of the blood against a gradient of Percoll, density 1.077. The cell suspension was then filtered (Whatman 105 cat n° 2105841 filter, UK) and centrifuged at 1500 rpm for 15 min at 4°C. The pellet was resuspended in the extraction buffer, which was specific for each enzyme, as described in the "Enzyme assays" section, or platted for culture procedures. The population of cells obtained consisted of T and B lymphocytes and the total contamination with macrophages was lower than 1% (9).

Incubation procedure - Macrophages and lymphocytes were incubated (1.0×10^6 cells/ flask) at 37°C in Krebs-Ringer medium with 2% fat-free bovine serum albumin and in the presence of glucose (5mM). After incubation, the cells were disrupted by the addition of 0.2 ml of 25% perchloric acid. Protein was removed by centrifugation and the supernatant was neutralized with 20µl of a 40% KOH solution and a tris-(hydroxymethyl) aminomethane/KOH (0.5 to 2.0 M) solution for the measurement of the metabolites.

Metabolite measurements - Neutralized samples of the incubation medium were analyzed for the measurement of lactate (12) and glucose (13).

The $^{14}CO_2$ produced from [U-^{14}C]-glucose and [U-^{14}C]-glutamine was collected as previously described (14). Macrophages and lymphocytes were incubated for 1 hour in the presence of one of the radiolabelled substrates in a sheltered erlenmeyer (25ml), containing one compartment for cell incubation and another for CO_2 collection. After this period of time the cells were killed using 200µl of a perchloric acid solution (25%). The labelled CO_2 was collected for one hour in a solution of phenylethylamine:methanol (1:1) and the radioactivity counted, using Bray's scintillation cocktail, in a Beckman-LS 5000TD liquid scintillator (Beckman Instruments, Fullerton, CA, USA).

Macrophage phagocytosis - Macrophages were incubated with 10 ml PBS containing opsonized zymosan for 30 min at 37°C so that phagocytosis could be quantified by counting (in a counting chamber) the percentage of cells that had phagocytosed more than four particles of zymosan.

Hydrogen peroxide production - The production of H_2O_2 was measured by a modification of the method described by Pick and Mizel (15). The cells were incubated in siliconized flasks (25 ml), in 1 ml PBS, in the presence of glucose (5 mM), under an atmosphere of 5% CO_2/95% air at 37°C and in the presence of phorbol-myristate-acetate (PMA) (10 ng/ml) when indicated. After one hour incubation, a solution of phenol red and horseradish-peroxidase (HRPO) was added to the medium to quantify the hydrogen peroxide content. After 10 min the reaction was stopped with 100µL of 1 N NaOH and the amount of hydrogen peroxide formed was measured spectrophotometrically at 620 nm.

Enzyme assays - Enzyme activities were measured as previously described (16, 17). The extraction medium for hexokinase (EC 2.7.1.1) contained 25 mmol Tris-HCl/l, 1 mmol EDTA/l and 30 mmol β-mercaptoethanol/l at pH 7.4 and that for glutaminase (EC 3.5.1.2) contained 150 mmol potassium phosphate/l, 1 mmol EDTA/l and 50 mmol Tris-HCl/l at pH 8.6. The extraction medium for citrate synthase (EC 4.1.3.7) contained 50 mmol Tris-HCl/l and 1 mmol EDTA/l; the final pH was 7.4. To all enzyme assays, 0.05% (v/v) Triton X-100 was added to complete the extraction of the enzymes. The final volume of the assay mixtures in all cases was 1.0 ml. Citrate synthase was assayed by following the rate of change in absorbance at 412 nm, and the remainder of the enzymes was assayed by following the rate of change in absorbance at 340 nm. All spectrophotometric measurements were performed at 25°C, except for glutaminase which was determined at 37°C. Preliminary experiments established that extraction and assay procedures were such as to produce maximum enzyme activities (18) for all enzymes studied.

Incorporation of [2-^{14}C]-thymidine into cultured lymphocytes. - Lymphocytes were cultured in RPMI-1640 medium for 24 h at 37°C in an artificially humidified atmosphere of 5% CO_2 in air at sterile conditions. Cultures were performed in a LAB-LINE Microprocessor CO_2 incubator (LAB LINE, USA) in a 96-well plates (Corning, NY, USA), 1x 10^5 cells per well (total volume, 200 µl). After 24h in culture, more than 98% of lymphocytes were viable, as measured by trypan blue dye exclusion. After 48h, cells were pulsed with 20 µl of 0.02 µCi [2-^{14}C]-thymidine (sp. act. 56.0 mCi/nmol), diluted in sterile PBS yelding final concentration of 1 µg/ml. Cells were then maintained under this conditions for an additional 15h and automatically harvested by using a multiple cell harvester and filter papers cat. n° 11731 (Skatron Combi, Sufolk, UK). The paper discs containing labelled cells were counted in 5 mL of Bray's scintillation cocktail, in a Beckman-LS 5000TD liquid scintillator.

Expression of the results - The enzyme activities are expressed as nmol substrate utilized/ min per mg of protein. The rates of glucose consumption, lactate formation and of decarboxylation of [U-^{14}C]-glucose and [U-^{14}C]-glutamine are presented as nmol/h per mg of protein

Statistical analysis - Results are expressed as mean ± SEM. The paired "t" test and ANOVA were employed and differences were considered significant at $p<0.05$.

Results

Peritoneal macrophages obtained from n-3 PUFA rich diet fed rats showed a reduced phagocytic capacity (46%) and H_2O_2 production (61%) (Table 1). The diet did not alter the maximal activity of hexokinase (HK) and citrate synthase (CS) but reduced those of glucose-6-phosphate dehydrogenase - G6PDh- (48%) and glutaminase - GLUTase- (52%) (Table 2). These changes were accompanied by a decrease in lactate production (25%) and glutamine decarboxylation (53%), as illustrated in Table 3. Glucose consumption and its metabolisation through the Krebs cycle, however, were not affected by the n-3 PUFA rich diet (Table 3).

Table 1. Number of cells present in the peritoneal cavity, percentage of cells showing phagocytosis and hydrogen peroxide production of macrophages from the control and n-3 PUFA rich diet-fed animals. The results are presented as mean ± SEM of 7 experiments. * $p<0.05$ for comparison with the control group.

	Control	n-3
Number of cells ($x10^6$)	1.07 ± 0.2	0.98 ± 0.1
% phagocytosis	44.3 ± 2.1	23.6 ± 1.6*
Hydrogen peroxide	3.02 ± 0.12	1.16 ± 0.11*

Table 2. Maximal activities of hexokinase (HK), citrate synthase (CS), glucose-6-phosphate dehydrogenase (G6PDh) and glutaminase (GLUTase) from macrophages obtained from control and n-3 PUFA rich diet fed animals. The results are expressed as mean ± SEM from 7 experiments (nmol/min/mg protein). * $p<0.05$ for comparison with the control group

	control	n-3
HK	248.84 ± 22.09	271.16 ± 27.55
CS	46.79 ± 2.82	40.49 ± 2.99
G6PDh	8.85 ± 0.11	4.58 ± 0.26*
GLUTase	221.35 ± 10.12	107.94 ± 9.08*

The proliferative response of lymphocytes obtained from the mesenteric lymph nodes (LMLN), peripheral blood (PBL) and spleen (SL) from n-3 PUFA rich diet-fed rats were also evaluated. This diet reduced the proliferation of the lymphocytes obtained from the three different sites - 57.7% for PBL, 34.7% for SL and 27.9% for LMLN (Table 4). The same effect was observed when the cells were stimulated with concanavalin-A (Con-A), a mitogen for T cells or LPS, a mitogen for B-cells (19) (Table 4).

Lymphocytes from n-3 PUFA fed rats did not show any changes in the maximal activity of HK and G6PDh (data not shown) but presented a decreased activity of GLUTase (of 36, 27 and 35% for PBL and SL and for LMLN, respectively) and CS (30% for the three types) (Table 5). These enzymatic changes are related to a reduced flux of glucose and glutamine through the Krebs cycle (Table 6). In these cells, however, there were no changes in glucose consumption, neither in lactate production (Table 6).

Discussion

It is well known that n-3 PUFA alleviate certain inflammatory and disease states and affect immune function (3), as shown by various epidemiological studies with human subjects (20) and in animal models (21). The presence of these fatty acids in the diet can modify some of the functions of lymphocytes and macrophages, such as the production and secretion of cytokines, proliferative response and prostaglandin production (3, 20). However, little is known about the effect of this diet upon the metabolism of glucose and glutamine in these cell types. Changes in the capacity of these cells in metabolising those substrates can impair cell function, since both represent essential metabolites for lymphocytes and macrophages (9, 10).

Table 3. Consumption of glucose and lactate production and [U-^{14}C]-glucose and [U-^{14}C]-glutamine decarboxylation from macrophages obtained from control and n-3 PUFA rich diet-fed rats. The results are presented as mean ± SEM of 7 experiments (nmol/h/mg protein). * $p<0.05$ for comparison with the control group

	Control	n-3
Glucose consumption	154.3 ± 9.8	148.6 ± 8.4
Lactate production	274.2 ± 12.7	204.5 ± 10.3*
Glucose decarboxylation	16.4 ± 1.2	18.3 ± 1.1
Glutamine decarboxylation	9.3 ± 0.5	4.3 ± 0.2*

Table 4. Effect of dietary n-3 PUFA on lymphocyte proliferation. Lymphocytes from the spleen (SL), mesenteric lymph nodes (MLN) and from the peripheral blood were cultured in the presence of autologous serum, the T-cell mitogen, ConA and the B-cell mitogen, LPS. [2-^{14}C]-Thymidine incorporation was measured over the final 18h of a 66h culture period, and is expressed as the percentage of the incorporation into cells obtained from rats fed control diets (1594.2 ± 106.3, 3357.1 ± 189.3 and 2678.9 ± 153.2 for no addition, ConA and LPS, respectively). * $p<0.05$ for comparison with the control group.

	No addition	ConA	LPS
Blood lymphocyte	42.3 ± 3.5	54.3 ± 2.1	73.1 ± 2.7
Spleen lymphocyte	65.3 ± 1.9	68.2 ± 0.8	79.3 ± 4.1
MLN lymphocyte	72.1 ± 2.3	69.5 ± 2.7	83.1 ± 4.5

Table 5. Effect of n-3 PUFA rich diet on the maximal activity of glutaminase (GLUTase) and citrate synthase (CS) in lymphocytes from rat peripheral blood, spleen and mesenteric lymph nodes. The results are presented as mean ± SEM (nmol/min/mg protein) from 7 experiments. *p<0.05 for comparison with the results presented for the control diet group.

	Control	n-3
GLUTase		
Blood lymphocyte	97.1 ± 4.0	61.2 ± 1.5*
Spleen lymphocyte	63.6 ± 5.1	46.9 ± 2.3*
MLN lymphocyte	68.2 ± 3.1	44.1 ± 2.9*
CS		
Blood lymphocyte	131.4 ± 9.3	91.2 ± 4.3*
Spleen lymphocyte	148.5 ± 3.7	102.3 ± 5.6*
MLN lymphocyte	156.3 ± 4.1	112.6 ± 7.3*

Table 6. Consumption of glucose and lactate production and [U-^{14}C]-glucose and [U-^{14}C]-glutamine decarboxylation from lymphocytes from the peripheral blood, spleen and mesenteric lymph nodes obtained from control and n-3 PUFA rich diet-fed rats. The results are presented as mean ± SEM of 7 experiments (nmol/h/mg protein)

	Control	n-3
Glucose consumption		
Blood lymphocyte	78.3 ± 1.6	82.1 ± 3.0
Spleen lymphocyte	65.1 ± 2.9	63.5 ± 2.6
MLN lymphocyte	69.2 ± 2.5	67.1 ± 1.1
Lactate production		
Blood lymphocyte	41.9 ± 1.1	37.5 ± 1.2
Spleen lymphocyte	34.6 ± 2.7	36.2 ± 2.4
MLN lymphocyte	37.4 ± 1.3	34.1 ± 2.7
Glucose decarboxylation		
Blood lymphocyte	6.2 ± 0.4	2.5 ± 0.3*
Spleen lymphocyte	3.4 ± 0.2	1.2 ± 0.1*
MLN lymphocyte	2.1 ± 0.1	0.9 ± 0.1*
Glutamine decarboxylation		
Blood lymphocyte	12.3 ± 1.8	4.2 ± 0.3*
Spleen lymphocyte	22.4 ± 0.8	5.9 ± 0.4*
MLN lymphocyte	19.6 ± 1.4	7.8 ± 0.1*

*p<0.05 for comparison with the results presented for the group fed control diet

The n-3 PUFA-rich diet reduced the phagocytic capacity and the production of H_2O_2 by macrophages. A possible mechanism involved in the reduction of H_2O_2 production could be related to changes in the activation of protein kinase C, modulated by the presence of different fatty acids in the diet , directly (6) or indirectly, via altered .polyphosphoinositide turnover (7). A reduction in PKC activity would lead to a reduction in H_2O_2 production, diminishing the activity of the enzyme NADPH-oxidase (22). This lowering effect on PKC activity, however, is still controversial, as shown by the different results concerning TNF synthesis and secretion appearing in the literature, which seem to be modulated by PKC (4, 8). In lymphocytes, however, unsaturated fatty acids inhibit PKC activity (23). It is interesting to note that the diet also provoked a reduction in G6PDh activity, the key enzyme regulating the flux of substrates for NADPH production via reactions of the oxidative segment of the pentose pathway (24). The diet also provoked a marked reduction in the metabolism of glutamine through the Krebs cycle as well as in the maximal activity of GLUTase, a key enzyme for glutaminolysis (25). These metabolic changes, together with the reduction in lactate production by the macrophages and a slightly, but not statiscally different, increase in glucose decarboxylation, suggest that the n-3 PUFA rich diet may facilitate a change from a metabolism which favours glucose utilization via the TCA cycle. These metabolic changes could be altering the secretory pattern and/or ability of the macrophages, contributing, this way, to the anti-inflammatory effect of the n-3 PUFA-rich diet.

The inhibitory effect of a n-3 PUFA rich diet or its components, eicosapentaenoic acid and docosahexaenoic acid, upon lymphocyte proliferation is well established (5, 20, 21), as well as their effect on other aspects of lymphocyte response(3). Little is known, however, about the effect of this type of fatty acids on lymphocyte metabolism of glucose and glutamine.

Lymphocytes obtained from the peripheral blood (PBL), spleen (SL) and mesenteric lymph nodes (LMLN) from rats fed n-3 PUFA rich diets showed a decreased proliferative response. The changes were more pronounced in PBL and more subtle in those from the MLN, indicating that the enviroment interferes in the proliferative response of the cells, as previously demonstrated by Calder (3), when studying the effect of unsaturated and saturated fatty acids on the proliferative response of PBL and SL. This phenomenon was also demonstrated by Ford & Schulzer (26) that showed that lymphocytes obtained from different sources in immunized rats are often functionally distinct from peripheral blood lymphocytes.

Lymphocytes present a high capacity of glucose and glutamine utilisation and the latter is essential for their proliferative capacity (14). N-3 PUFA rich diet induced, in these cells, a reduction in GLUTase and CS activities which were accompanied by a reduction in glucose and glutamine decarboxylation in the three types of lymphocytes. These results suggest that beyond the effects upon cytokine production (3), upon intracellular signalling mechanisms, as PKC activation (23) and upon changes in cell membrane fluidity (20), involved in the control of the proliferative response of these cells, another effect of this diet is the impairment of glutamine utilization and of the flux of substrates through the Krebs cycle, contributing to the diminished proliferative response observed. Taken together the data show that, at least in part, the functional changes observed in macrophages and lymphocytes from n-3 PUFA-rich diet fed rats are related to the effect of this diet upon glucose and glutamine metabolism in these cell types, leading to the generally accepted immunosupression.

Acknowledgements - The authors are grateful for the technical assistance of J.R. Mendonça, G. de Souza and M. Carnelós and to Dr. R Curi for the use of his laboratory facilities.

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