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Blunting the response to endotoxin in healthy subjects: effects of various doses of intravenous fish oil

THESE

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par

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Rapport de synthèse

Enjeux et contexte

La recherche de cette dernière décennie sur les acides gras n-3 PUFA contenus dans l'huile de poisson a montré que ceux-ci, et particulièrement l'EPA et le DHA, avaient des propriétés anti-inflammatoires et anti arythmiques puissantes, potentiellement utiles chez les septiques et « cardiaques ». Les mécanismes sous-jacents sont nombreux, incluant l'incorporation des acides gras dans les membranes de phopholipides, la réduction de la production de médiateurs pro-inflammatoires (prostaglandines, leukotrienes, thromboxane), l'augmentation de la production de résolvines et protectines dérivées du DHA, et la régulation de voies de signalisation cellulaire. Cependant, les doses de n-3 PUFA utilisées dans les études cliniques et chez le sujet sain avant le travail de Yann-Karim Pittet étaient nettement supérieures aux doses nutritionnelles de l'ordre de 5-8 g/j par voie orale ou 1 g/kg par voie intraveineuse. De plus, la voie entérale avait la réputation de nécessiter plusieurs jours à semaines de traitement avant d'aboutir à une incorporation d'acides gras membranaire suffisante pour avoir un impact clinique; quant au temps minimal requis pour obtenir cet effet par voie IV, il était inconnu.
Depuis, le développement d'émulsions lipidiques intraveineuses destinées à la nutrition parentérale a permis d'imaginer l'administration de prétraitements IV rapides. Pour les étudier, notre laboratoire a développé un modèle d'endotoxine (LPS d'E.Coli) qui mime les réponses physiologique, endocrinienne et biologique du sepsis chez le sujet sain, utilisant des doses de 2 ng/kg IV. Les réponses sont totalement réversibles en 8 heures.
Dans le but de réduire à la fois la dose de lipides et le temps de perfusion, ce travail a étudié l'influence de 3 doses dégressives de n-3 PUFA sur les réponses à l'endotoxine, et sur l'incorporation membranaire de ces acides gras.

Méthodes

Etude prospective chez 3 groupes consécutifs de sujets sains soumis à un challenge d'endotoxine.
Intervention : perfusions d'huile de poisson (0.5 et 0.2 g/kg de n-3 PUFA, Omegaven® 10%) ou placebo, administrées en 3 heures ou en 1 heure, soit le jour avant ou le jour-même du test d'endotoxine.
Mesures : variables physiologiques (T°, fc, tension artérielle, calorimétrie indirecte)
Laboratoire – prises de sang à T0, 60, 120 et 360 min après l'injection de LPS: TNF-α, hs-CRP, hormones de stress, composition en acides gras des membranes plaquettaires.

Statistiques

Les résultats ont été rapportés en moyennes et écarts types. Des aires sous la courbe (AUC) ont été calculées avec la méthode des parallélépipèdes pour toutes les variables déterminées de manière répétée. L'effet du temps a été exploré par des two-way ANOVA pour mesures répétées. Les comparaisons post-hoc ont été réalisées avec des tests de Dunnett's ou de Scheffe. Les modifications de composition membranaires ainsi que les AUC ont été analysées par des tests non-paramétriques (Kruskal–Wallis).

Résultats

Après LPS, la température, les concentrations d'ACTH et TNF-α ont augmenté dans les 3 groupes. Ces réponse ont été significativement atténuées ($p<0.0001$) par l'huile de poisson comparé à ce que nous avions observé dans le groupe contrôle de Pluess et al (ICM 2007). Les concentrations les plus faibles d'ACTH, de TNF-α, et les AUC les plus basses des températures, ont été observées après une dose unique de 0.2 g/kg de n-3 PUFA administrée 1 heure avant le LPS. Par contre, l'incorporation membranaire d'EPA est dose-dépendante.

Conclusions

Sachant que la réponse à l'endotoxine est reproductible, cette étude montre que 3 doses différentes d'huile de poisson atténuent de manière différente cette réponse. La perfusion de 0.2 g/kg administrée juste avant l'endotoxine s'est avérée la plus efficace à atténuer la réponse fébrile, les cytokines et les hormones de stress, suggérant une capture de l'endotoxine par l'émulsion lipidique qui se surajoute aux effets systémiques et membranaires.

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Abstract *Objective:* To test the dose response effect of infused fish oil (FO) rich in n-3 PUFAs on the inflammatory response to endotoxin (LPS) and on membrane incorporation of fatty acids in healthy subjects. *Design:* Prospective, sequential investigation comparing three different FO doses. *Subjects:* Three groups of male subjects aged 26.8 ± 3.2 years (BMI 22.5 ± 2.1). *Intervention:* One of three FO doses (Omegaven®10%) as a slow infusion before LPS: 0.5 g/kg 1 day before LPS, 0.2 g/kg 1 day before, or 0.2 g/kg 2 h before. *Measurements and results:* Temperature, hemodynamic variables, indirect calorimetry and blood samples (TNF-α, stress hormones) were collected. After LPS temperature, ACTH and TNF-α concentrations increased in the three groups: the responses were significantly blunted ($p < 0.0001$) compared with the control group of the Pluess et al. trial. Cortisol was unchanged. Lowest plasma ACTH, TNF-α and temperature AUC values were observed after a single 0.2 g/kg dose of FO. EPA incorporation into platelet membranes was dose-dependent. *Conclusions:* Having previously shown that the response to LPS was reproducible, this study shows that three FO doses blunted it to various degrees. The 0.2 g/kg perfusion immediately before LPS was the most efficient in blunting the responses, suggesting LPS capture in addition to the systemic and membrane effects.

Keywords Inflammation · Sepsis · PUFA · Downregulation

Introduction

The interest in polyunsaturated fatty acid (PUFA) supplementation has generated a large body of research during the last decade. Among PUFA, the n-3 PUFAs eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA), produced by plankton and incorporated by fish, are the most biologically active. Fish oil (FO) consumption increases the proportion of EPA and DHA in the cell membrane phospholipids, particularly in the monocytes and inflammatory cells. They modulate the inflammatory processes by various mechanisms, including downregulation of proinflammatory eicosanoids and cytokines, adhesion molecules, as well as genes involved in inflammation, by modifying phospholipid membrane composition in the immune cells [1, 2]. In addition, the n-3 PUFAs modulate key metabolic pathways, platelet functions and the excitability of cardiomyocytes. Oral FO has been shown to exert beneficial effects in patients with inflammatory diseases and elevated cardiovascular risk [1]. While oral administration requires time to achieve these beneficial effects [3], data in volunteers show that the intravenous administration achieves biological effects in a few hours [4]. The development of intravenous lipid emulsions containing FO enables exploring such properties in acute conditions.

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In a previous trial we showed that the intravenous administration of two doses of 0.5 g/kg of FO in healthy subjects resulted in a rapid incorporation of EPA and DHA into the platelet membrane phospholipids [4]. This was associated with a significant downregulation of the neuroendocrine responses to endotoxin (LPS), as well as of some components of the inflammatory response. In the previous study, the 0.5 g/kg FO dose required a prolonged infusion time (6 h). We hypothesized that smaller doses of FO could exert similar effects with the advantage of a reduction of infusion time and of total fat dose. Indeed in acute medical settings, the two latter characteristics might be an advantage when a rapid effect is required or the fat tolerance is low (e.g., acute coronary conditions).

The present study aimed at comparing the effect of various doses of intravenous FO in healthy subjects by consecutive trials with three gradually decreasing FO doses on membrane incorporation of n-3 PUFAs, as well as on the physiological response to LPS.

Subjects and methods

After approval by the ethics committee of Lausanne's University School of Medicine, and with written informed consent, healthy male subjects were enrolled in three consecutive trials. A complete history (including a questionnaire on the frequency of fish intake) and physical examination were performed before the study; a 12-lead electrocardiogram was recorded. The inclusion criteria were the following: age 18–35 years, being in good health and taking no medications. Exclusion criteria were smoking >10 cigarettes/day, alcohol consumption >1 glass wine or 1 beer/day, and a sea fish intake > twice weekly, or EPA/DHA supplementation.

Experimental design and treatment

Three consecutive studies were carried out testing various regimens of intravenous FO in subjects submitted to the same endotoxin challenge, using decreasing doses. The trial used the same design as our previous randomized trial comparing two doses of 0.5 g/kg FO versus placebo [4]:

- Day −1, 0.5 g/kg group: one 0.5 g/kg infusion of FO in 6 h, 24 h before the investigation day ($n = 8$).
- Day −1, 0.2 g/kg group: one 0.2 g/kg infusion of FO in 3 h, 24 h before the investigation day ($n = 6$).
- Day 0, 0.2 g/kg group: one 0.2 g/kg infusion of FO in 3 h, 2 h before the investigation ($n = 7$).

A purified FO solution was used in all groups (Omegaven®10%, provided by Fresenius Kabi AG, Bad Homburg, Germany): 100 ml contain 10 g of FO, providing 1.25–2.82 g of EPA; 1.44–3.09 g of DHA and 15–29.6 mg of D,L-α-tocopherol as antioxidant.

Infusion protocol

On the investigation day, subjects came at 8 a.m. to the metabolic laboratory after an overnight fast. They were requested to void, and the urine was discarded. Thereafter, they remained recumbent for the next 8 h. A venous catheter was inserted into a vein of the left forearm for LPS injection and labeled glucose infusion, and another catheter was placed into a wrist vein of the right forearm for blood sampling to avoid contamination of blood samples by the infusion. The right hand was kept in a thermo-stabilized box, heated at 56°C to achieve partial arterialization of venous blood.

Intervention

A dose of 2 ng/kg of endotoxin from *E. Coli* (USP, Rockville, MD, batch no. 2, as a 200 ng/ml solution in NaCl 0.9%) was given as an intravenous bolus at time T0 (injection time: 1 min).

Vital functions, including heart rate, respiratory rate, rectal temperature (Hellige, Servomed), non-invasive arterial blood pressure (Critikon, Dinamap), cardiac output [thoracic bioimpedance (NCCOM3 cardiodynamic monitor, BoMed, Irvine, CA] and oxygen saturation (Ohmeda, Biox 3740 Pulse oximeter), were monitored. Subjective symptoms such as muscle pain and headache, nausea and vomiting were recorded.

Blood collection and analysis

Blood was drawn twice before the endotoxin challenge: before the first infusion of FO and before the challenge. Blood was drawn in tubes containing EDTA and plasma immediately separated by low speed centrifugation (228 GIGA for 10 min at 4°C). The plasma supernatant was further centrifuged for 20 min at 1428 GIGA. Platelets were then "washed" twice and centrifuged in a TRIS solution. Thereafter, storage was done in formol at −80°C until analysis. For fatty acid (FA) determination in platelet membranes, the lipid components, triglycerides, phospholipids and cholesteryl esters were first separated by two-dimensional thin layer chromatography. FAs in phospholipids were then analyzed by gas chromatography (Agilent, GC system, 6890 series; 6890A) [5]. During the LPS challenge blood was sampled in all subjects every 30–60 min over the 8-h protocol to determine hormone levels (cortisol, ACTH, catecholamines), inflammatory markers (TNF-α, hsCRP), glucose, lactate, free FA and triglyceride plasma levels. Blood samples were collected

into heparinized tubes and centrifuged (3,000/min for 10 min at 4°C) to separate plasma. Plasma was stored at −20 or −80°C depending on the matrix until analysis. Cortisol (kit from Diagnostic Products Corporation, Los Angeles, CA) was determined by radioimmunoassay. ACTH was determined by chemiluminescence assay (Nichols Institute Diagnostics, San Juan Capistrano, CA) (reference range 10–60 ng/l). Plasma catecholamines were determined by HPLC with electrochemical detection.

ELISA in streptavidin-coated microtiter plates (Roche, Mannheim, Germany) was used for in vitro determination of TNF-α. High sensitivity C-reactive protein (hsCRP) was measured by turbidimetry on a Hitachi 917(Roche) and determined only at T0 and at the end of the trial.

Plasma glucose and lactate concentrations were measured enzymatically using a Yellow Spring Instrument YSI 2300 STAT PLUS (Yellow Spring, OH). Plasma free FA concentrations were measured with a colorimetric method using a kit from Wako (Neuss, Germany). Enzymatic determination of triglycerides in plasma was performed with a TG PAP 150 kit (BioMérieux, Lyon, France).

Indirect calorimetry was performed continuously during the whole study (Datex, Deltatrac2, Helsinki, Finland). Respiratory gases were collected with a canopy to determine resting energy expenditure (REE) and net substrate oxidation. REE was calculated using the equation of Livesey and Elia [6].

Statistical analysis data are presented as mean ± SD. Areas under the curve (AUC) calculated using the trapezoidal method were used to compare the overall effect of FO between groups. The effect of time was explored by means of two-factor repeated measures ANOVA and n-3 PUFA dose (group = dose, group × time = intervention). Post hoc comparisons were carried out by Dunnett's (effect of time versus baseline in each group) or Scheffe's test (between group comparisons at the same time point). Membrane incorporation changes and AUC were analyzed by non-parametric tests (Kruskal–Wallis). The statistical package was: JMP® Version 5.1., SAS Institute Inc., Cary, NC. Significance was considered at $p \leq 0.05$.

Results

The 23 subjects were aged 26.8 ± 3.2 years with a mean body mass index (BMI) of 22.5 ± 2.1 kg/m^2. Demographic variables were similar between the groups.

Platelet membrane incorporation

EPA incorporation in platelet phospholipids was observed in all FO groups and was significantly higher in the group receiving the largest FO dose (day 1, 0.5 g/kg) compared with the two other groups ($p < 0.018$) (Table 1). DHA was also incorporated in platelet membranes, but the changes were not significant.

Systemic effects of LPS

Significant increases in heart rate (+52% $p < 0.0001$) and cardiac output (+40%, $p < 0.0001$) peaking at T240 after LPS were observed in all subjects, while systolic, diastolic, mean blood pressure and SpO_2 did not change significantly. REE variability was substantial and did not reach steady state in the various parts of the study precluding statistical analysis because of marked shivering (data not shown). Body temperature increased significantly in all groups after LPS (Fig. 1). All subjects complained of transient headache, nausea and muscle pain. They were all discharged home symptom free, with no medication.

Metabolism, cytokines and hormones

There were modest changes in plasma glucose and lactate concentrations along the study from baseline, but these were not significant.

LPS induced significant increases in plasma TNF-α, norepinephrine, epinephrine and ACTH levels in all groups (Table 2). Figure 2 shows that FO attenuated several aspects of this response, with significant blunting

Table 1 EPA and DHA in platelet phospholipids before and after FO infusion (T0)

Group	EPA (% molar)			DHA (% molar)		
	Before FO	T0	Change	Before FO	T0	Change
Day −1, 0.5 g/kg	0.41 ± 0.29	1.2 ± 0.15$^{\#}$	+0.79$^{\#\#}$	2.23 ± 0.40	2.61 ± 0.37	+0.38
Day −1, 0.2 g/kg	0.32 ± 0.12	0.68 ± 0.16**	+0.36	1.97 ± 0.33	2.13 ± 0.55	+0.16
Day 0, 0.2 g/kg	0.27 ± 0.25	0.67 ± 0.28*	+0.40	2.49 ± 0.38	2.90 ± 0.61	+0.41

The incorporations of the previous trial delivering 2 × 0.5 g/kg FO were higher (EPA +1.4, DHA +0.8% molar) [4]

Statistically significant within-group changes by two-way ANOVA are indicated as * $p = 0.05$, ** $p = 0.01$, $^{\#}$ $p < 0.001$. Difference between groups $^{\#\#}$ $p = 0.018$

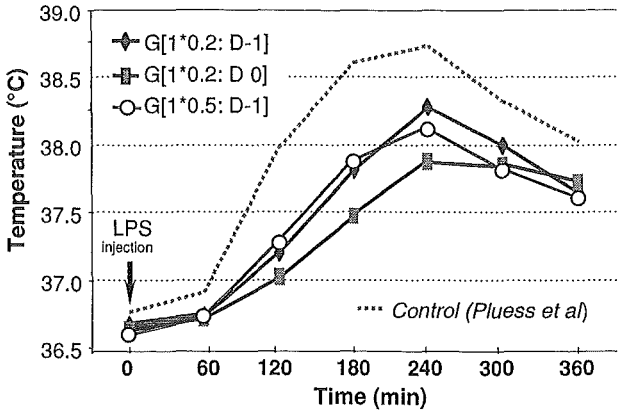

Fig. 1 Temperature over time in the three groups after LPS injection: the increase was significantly blunted in all FO groups ($p = 0.0002$) compared with the control group (*dotted line*) of Pluess et al. [4]

of the overall response of plasma TNF-α by AUC ($p < 0.0001$) in the day −1, 0.2 g/kg and day-0, 0.2 g/kg groups compared to the day −1, 0.5 g/kg group.

The AUC of ACTH and the peak levels after LPS were similarly lower in the day −1, 0.2 g/kg and day-0, 0.2 g/kg groups compared to the day −1, 0.5 g/kg group (Fig. 2). Plasma norepinephrine did not differ among the three groups (Table 1). The increase in plasma epinephrine AUC was blunted significantly only in the day −1, 0.2 g/kg group. Baseline hsCRP concentrations were modestly higher in the day −1, 0.5 g/kg group (ns: $p = 0.14$) and increased significantly only in the day −1, 0.2 g/kg group (mg/l), remaining a trend in both other groups.

Discussion

In the present study, we tested the effects of three different decreasing regimens of intravenous FO on the cell membrane phospholipid composition and on the physiological response to LPS. The data were further compared with those of our previous study, which was carried out with the same design and which included a control group [4]. The aim was to reduce both infusion time and the fat load, while keeping the blunting effects of FO observed in the first trial. We found that one or two 0.2-g/kg doses of FO, administered within the 24 h before LPS challenge, significantly influenced both membrane EPA content and important aspects of the response to LPS.

Table 2 Evolution of the plasma TNF-α, hormone concentrations and hsCRP after the LPS challenge

	T0	T60	T120	T240
TNF-α (<30 pg/ml)				
Day −1, 0.5 g/kg ($n = 8$)	9.4 ± 7.3	129.5 ± 69.8	383.6 ± 155.5	91.0 ± 20.0
Day −1, 0.2 g/kg ($n = 6$)	6.9 ± 5.7	28.0 ± 8.8**	111.4 ± 25.7*	46.4 ± 9.9*
Day 0, 0.2 g/kg ($n = 7$)	21.2 ± 4.1	36.7 ± 6.5**	121.2 ± 48.8*	62.0 ± 15.0
	T0	T120	T240	T360
Norepinephrine (reference range 0.47–2.94 nM/l)				
Day −1, 0.5 g/kg	2.57 ± 0.84	3.86 ± 1.14	1.75 ± 0.59	1.44 ± 0.39
Day −1, 0.2 g/kg	2.13 ± 0.37	4.02 ± 1.45	2.01 ± 0.32	1.61 ± 0.29
Day 0, 0.2 g/kg	1.97 ± 0.21	1.93 ± 0.32	1.91 ± 0.41	1.46 ± 0.15
Epinephrine (reference range 0.02–0.44 nM/l)				
Day −1, 0.5 g/kg	0.35 ± 0.08	0.34 ± 0.02	0.36 ± 0.07	0.31 ± 0.05
Day −1, 0.2 g/kg	0.14 ± 0.02	0.20 ± 0.04*	0.21 ± 0.03*	0.18 ± 0.03
Day 0, 0.2 g/kg	0.19 ± 0.03	0.24 ± 0.05	0.31 ± 0.09	0.20 ± 0.05
ACTH (reference range 10–60 ng/l)				
Day −1, 0.5 g/kg	30.9 ± 7.3	129.8 ± 43.9	109.4 ± 40.1	20.4 ± 3.9
Day −1, 0.2 g/kg	6.2 ± 1.3***	22.7 ± 3.9***	21.8 ± 6.5**	3.0 ± 0.0**
Day 0, 0.2 g/kg	3.6 ± 0.6***	16.9 ± 8.4***	33.4 ± 21.3**	6.3 ± 2.3**
Cortisol (reference range 170–630 ng/l)				
Day −1, 0.5 g/kg	226 ± 51	411 ± 123	458 ± 158	204 ± 96
Day −1, 0.2 g/kg	297 ± 55	420 ± 95	490 ± 125	215 ± 80
Day 0, 0.2 g/kg	195 ± 81	392 ± 145	515 ± 161	320 ± 235
hsCRP (reference range <0.11 mg/l)				
Day −1, 0.5 g/kg	1.52 ± 2.83	–	–	2.81 ± 3.53
Day −1, 0.2 g/kg	0.37 ± 0.12	–	–	0.57 ± 0.16*
Day 0, 0.2 g/kg	0.74 ± 0.35	–	–	0.81 ± 0.34

Statistics: All the hormone concentration changes over time were highly significant by two-way ANOVA, whereas the hsCRP change was significant only in the day −1, 0.5 g/kg group. Post hoc tests between groups, * $p = 0.05$, ** $p = 0.01$, *** $p < 0.001$

Membrane incorporation of FO

Fatty acids are incorporated into cell membrane phospholipids and play key roles in the physico-chemical and functional characteristics of the cell membranes. This in turn modifies important cell properties, including regulation of the inflammatory response by modulating ecosanoid synthesis and down-regulating cell signaling and gene expression. The dietary FAs determine in part the composition of membrane phospholipids [7]. Several important membrane characteristics are influenced by their fatty acid composition, as shown by experiments analyzing the effects of various FAs in murine macrophage cultures [8]; incorporation of saturated FA into macrophage membranes decreases their ability to phagocytize and to adherence to surfaces, while the phagocytic rates increase with an increasing degree of unsaturation. This is caused by the changes in membrane structure and fluidity, the latter being increased by n-3 and reduced by n-6 PUFAs [9] compared with saturated fatty acids [8], influencing multiple pathways [10].

In this study, we observed that a single intravenous infusion of FO significantly increased EPA content in cell membranes. We observed a direct relation between the dose of FO and the platelet membrane incorporation: the lowest incorporations were observed in the two groups receiving 0.2 g/kg, while the highest uptake of EPA (two times higher) was found in the group receiving the largest FO dose (day −1, 0.5 g/kg; $p < 0.0001$). Furthermore, the EPA incorporation was higher in our previous study using two doses of 0.5 g/kg FO, being 3.5 higher than in the groups receiving the lowest dose of the actual study. Such a finding suggests a direct dose effect.

The n-3 PUFA incorporation after oral FO supplements is a rather slow process. In healthy subjects receiving 6–9 g FO/day for 12 months, the incorporation half-life of EPA in serum cholesteryl esters was calculated to be 4.8 days, while a plateau was reached after 4–8 weeks [7]. Recent data, including ours, show that the intravenous route strongly accelerates the incorporation into human cell membranes [4, 11]. In rats the bolus administration of a novel MCT-FO solution achieves incorporation of n-3 PUFAs into cell membranes after only 60 min [12]. The data of the present study confirm these preliminary data and report combined pharmacological and biological data.

Modulation of the physiological response to LPS

Several nutrients have been shown to modulate acute inflammation, such as FO, antioxidant micronutrients and glutamine. A large body of literature shows that FO downregulates the inflammatory processes by complex mechanisms including a decreased production of proinflammatory eicosanoid mediators (prostaglandins, leukotrienes, thromboxanes), increased production of DHA-derived resolvin D1 and protectin D1, which attenuate neutrophil migration and attenuate NF-κB activation and tissue damage in several disease models [13], regulation of cell signaling of pathways involved in immune responses and regulation of gene expression involved in inflammation (transcription factors PPARs and SREBPs) [9].

Experimental studies show that FO may influence the response to LPS: FO increases survival in guinea pigs submitted to lethal doses of LPS [14], improves splanchnic and vital organ hemodynamics, and reduces bacterial translocation [15]. This is also observed in critically ill patients with acute inflammatory conditions such as ARDS sepsis and surgery receiving FO with parenteral nutrition [13].

In two previous studies in healthy subjects, FO was found to reduce fever, as well as the neuro-endocrine and inflammatory responses to LPS after 2–3 week of oral supplementation, respectively [16], or much faster with two large intravenous 0.5 g/kg FO doses [4]. In the present study, lower doses or even a single dose achieved similar changes. Interestingly, the blunting of the LPS effects differed with the dose and the timing of administration, and was not directly proportional to the total FO dose. Fever was attenuated in all groups compared to the previous trial's control group (Fig. 1). TNF-α response was significantly blunted only in the two groups with the lowest FO dose. The hypothalamo-pituitary adrenal responses were blunted in all groups compared to the control group of the previous trial [4]. Blunting of the ACTH response was stronger in the 0.2 g/kg FO groups compared to the 0.5 g/kg group ($p = 0.0019$). In the low FO doses groups, the responses were not as clear cut for the sympato-adrenal responses (epinephrine and norepinephrine).

Dose effect

The study design, with consecutive trials, allowed describing the relationship between different doses of FO and biological responses. A direct effect of increasing doses of FO was observed with membrane EPA incorporation, completing the results of our previous study [4], suggesting a mass effect. Table 1 mentions the previous trial's higher incorporation after the double 0.5-g/kg dose. Such a dose pattern was not observed with DHA due to substantial inter-individual variability and the limited power of the study.

The effect of FO on the response to LPS is complex. The effect on fever was not influenced by the dose: the blunting appears similar to that previously observed (Fig. 1). Surprisingly, the suppressive effect on TNF-α

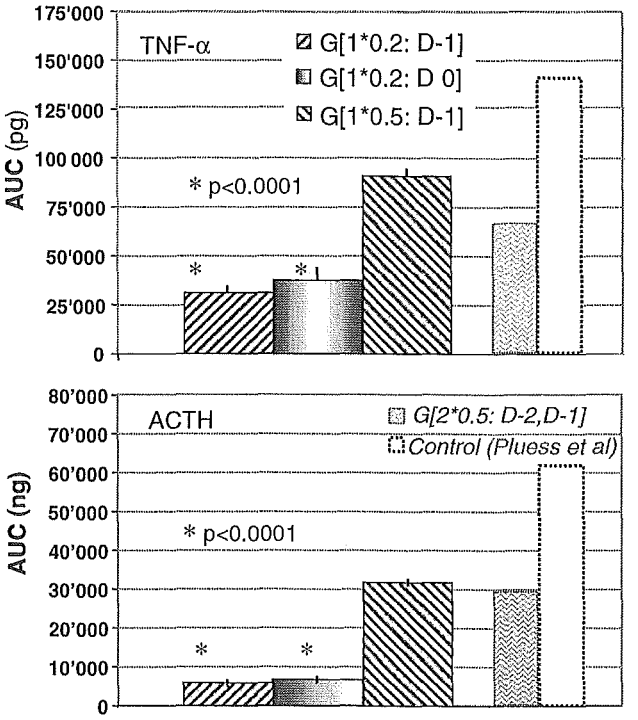

Fig. 2 Areas under the curve (AUC) of TNF-α and ACTH after LPS infusion in the three groups: the strongest blunting was observed with the two lowest FO doses. The data from the previous trial are indicated in *dotted bars* (Pluess et al. [4])

and ACTH was significantly more pronounced in the two low-dose groups (0.2 g/kg) compared with the single or double 0.5-g/kg doses of the previous trial (Fig. 2). We have no clear explanation for this observation: timing, dose of FO, membrane incorporation and genetic factors seem to be the main causative factors. Fat emulsion composition is another possible cause. Various types of phospholipids, including phospholipids contained in any parenteral nutrition fat emulsion, strongly inhibit LPS-induced cytokine release by complex mechanisms [17], including the direct binding of LPS. Even if the present protocol, in absence of data, does not allow to reach conclusions about direct LPS inhibition by fat emulsion, such a mechanism is likely in the subjects receiving FO immediately before LPS (day 0, 0.2 g/kg), as suggested by the strongest blunting of LPS response in this group. On the other hand, we have no clear explanation for the large ACTH and TNF-α blunting observed in the day −1, 0.2 g/kg group in which FO was infused 24 h before the LPS challenge.

Limitations

The study has limitations. The number of subjects per group was low, reducing the power of the statistical analysis. The biological effects of FO have substantial inter-individual variability with a potential for outliers related to factors including genetics: this might be amplified by the small numbers, although the data do not show real outliers.

The limited power probably explains why the DHA membrane incorporation was not significant, contrasting with that of EPA, which had greater changes with a lower variability. AUCs were calculated for every variable, which is an appropriate method for a kinetic study with the advantage of reducing the negative impact of the small numbers with repeated determinations. We determined membrane FA composition only in platelets and not in other immune cells responsible for the inflammatory response. A recent study performed in healthy volunteers receiving intravenous FO showed similar incorporation dynamics in platelets, monocytes and erythrocytes [11], although with different rates of incorporation. This suggests that monocyte membranes in our healthy volunteers were also influenced by the FO infusion. Further, we did not assess thromboxanes and leukotrienes, important targets of FO administration, which limits the interpretation of our results.

Finally, to limit LPS administration in numerous control groups, a highly disagreeable procedure that incurs laboratory costs, we proceeded by consecutive trials. Such a design is commonly used in pharmacological studies to reduce the number of experiments. This trial was not designed to determine the lowest dose of intravenous FO achieving a blunting effect, only one that could be used in clinical settings.

Conclusion

Altogether the results show that a small dose of parenteral FO administered just before a LPS challenge produces a significant EPA membrane incorporation already 6 h after the injection and attenuates the physiological response to LPS. This was also observed with a single 0.2-g/kg dose. These results open important perspectives in pre-conditioning of patients before interventions known to elicit strong inflammatory responses.

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