Nutritional lipid emulsions modulate cellular signaling and activation of human neutrophils

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Abstract Although numerous studies suggest that nutritional lipids modulate human immune responses, the mechanism behind this observation remains unclear. On the basis of the hypothesis that lipids might affect cellular signaling we evaluated the effects of various lipid emulsions on two major pathways involved in neutrophil activation: second messenger (Ca^{2+}) mobilization and protein kinase C (PKC) activation. Activation by opsonized yeast particles (serum-treated zymosan; STZ) increased cytosolic [Ca^{2+}] ([Ca^{2+}]$_i$) in neutrophils, with an initial slow rise that turned into a fast phase until a plateau was reached. The PKC activator 4-α-phorbol 12-myristate 13-acetate (PMA) markedly increased the initial STZ-induced [Ca^{2+}]$_i$ rise. This PMA effect was mimicked by emulsions containing medium-chain triglycerides (MT), but not by long-chain triglycerides (LT) or structured lipids (SL). However, like PMA, all emulsions decreased the STZ-induced [Ca^{2+}]$_i$ plateau and all activated purified PKC, suggesting that only MT emulsions activate PKC in the context of the intact cell. MT, like PMA, evoked a leftward shift of the dose-response curve for the STZ-induced [Ca^{2+}]$_i$ rise, indicating PKC-dependent sensitization of neutrophils for stimulation by STZ. This study is the first to show that nutritional lipids distinctively modulate cellular signaling and stimulation of neutrophils through effects on calcium mobilization and PKC activation: *i*) MT emulsions sensitize neutrophils for STZ in a PKC-dependent manner, and *ii*) MT, LT, and SL emulsions all reduce the stimulatory effect of STZ in a nonspecific manner. —Wanten, G., S. van Emst-de Vries, T. Naber, and P. Willems. **Nutritional lipid emulsions modulate cellular signaling and activation of human neutrophils. *J. Lipid Res.* 2001.** 42: **428–436.**

Supplementary key words immune response • lipids • nutrition • emulsion • calcium signaling • protein kinase C

There is growing evidence that dietary constituents influence the function of the human immune system and clinical studies have suggested that lipids in particular may affect our defenses against invading microorganisms (1–3). However, the mechanism behind the presumed immune-modulating properties of lipids remains unclear; several studies have yielded equivocal results (4–13). Most investigations have been conducted with intravenous (parenteral) nutrition formulations. This treatment modality, in which lipids are administered as a sterile water-soluble emulsion, free of endotoxins, offers a model that allows a convenient evaluation of lipid effects on the immune system. The most widely used (and investigated) lipid emulsions contain long-chain triglycerides (LT). Data regarding more recently developed emulsions, such as physical mixtures of long- and medium-chain triglycerides (LT/MT), or synthetic structured lipids (SL), with long-chain and medium-chain fatty acids attached to one glycerol molecule, are limited or lacking (14–16). The latter also applies for a new clinically applied vitamin E (α-tocopherol)-enriched LT/MT lipid emulsion (LT/MT-E).

We reported distinct effects of lipids on several effector functions (oxygen radical production, adhesion, degranulation, migration) of (quantitatively) the most important leukocyte, the neutrophil granulocyte (17–19). It seems that MT emulsions, in a clinically relevant concentration range (up to 10 mM), contrary to LT and SL, directly activate neutrophils in a concentration-dependent manner. Because cell activation involves signal transduction pathways, we postulated that the observed effects of lipids might be mediated through effects on cellular signaling. In the present study we therefore evaluated various lipids for their effects on two major signaling pathways known to be involved in neutrophil activation: second messenger (Ca^{2+}) mobilization and protein kinase C (PKC) activation. Besides the membrane-permeable receptor-independent PKC-activating 4-α-phorbol 12-myristate 13-acetate (PMA), a phorbol ester, two membrane receptor-mediated neutrophil activators were used: opsonized yeast particles (serum-

Abbreviations: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LT, long-chain triglycerides; LT/MT, mixture of long- and medium-chain triglycerides; LT/MT-E, vitamin E (α-tocopherol)-enriched LT/MT emulsion; MT, medium-chain triglycerides; PKC, protein kinase C; PMA, 4-α-phorbol 12-myristate 13-acetate; SL, synthetic structured lipids; STZ, serum-treated zymosan.

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treated zymosan; STZ) as a model for opsonized pathogens and the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) as a soluble stimulus.

MATERIALS AND METHODS

Blood samples were drawn from healthy volunteers and processed as described. Serum triglyceride content of these samples was always in the normal range (0.55–1.56 mM), as determined colorimetrically on a Hitachi (Tokyo, Japan) 747 analyzer. The study was approved by the Human Ethics Committee of the University Medical Center Nijmegen, the Netherlands, and subjects gave their informed consent.

Materials

Myelin basic protein (MBP), aprotinin, leupeptin, trypsin inhibitor, fMLP, and PMA were purchased from Sigma (St. Louis, MO). Staurosporine and calpain inhibitor were obtained from Boehringer (Mannheim, Gemany); phenylmethylsulfonyl fluoride (PMSF) was from Serva (Heidelberg, Germany); [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol) was from Amersham (Buckinghamshire, UK); U73122 and U73343 were from Upjohn (Kalamazoo, MI); phosphatidylserine (PS) was from Lipid Products (Nutfield Nurseries, Surrey, UK); PMA was from LC Services (Woburn, MA); human serum albumin (HSA) was from Behring (Westwood, MA); and Fura-2/AM from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade. PMA was stored as a 10^{-3} M stock in fMLP (DMSO) at −20°C. STZ was prepared as described (20). fMLP was stored as a 10^{-3} M stock in DMSO at −20°C. Hanks' buffered saline solution (HBSS) was from Life Technologies (Paisley, Scotland). Phosphate-buffered saline (PBS) contained 163.9 mM Na^+, 140.3 mM Cl^-, 10.9 mM HPO_4^{2-}, and 1.8 mM $H_2PO_4^-$ (pH 7.4). Isotonic lysis solution contained 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4). Incubation medium contained HBSS supplemented with 0.5% (w/v) HSA. Percoll (ρ 1.129 g/ml at 20°C) was from Pharmacia Biotech (Uppsala, Sweden). LT emulsion (Intralipid; 20%, w/v) was from Pharmacia & Upjohn (Stockholm, Sweden). LT/MT and α-tocopherol-enriched (170 ± 40 mg/l) LT/MT emulsions (Lipofundin; 20%, w/v) were from B. Braun Melsungen (Melsungen, Germany). SL emulsion was from Pharmacia & Upjohn Parenterals (Stockholm, Sweden). A pure 20% MT (w/v) emulsion was provided by Pharmacia & Upjohn (Clayton, NC). For lipid emulsion characteristics see **Table 1**. Blood samples were collected in 10-ml Monoject tubes (Sherwood Medical, Ballymoney, N. Ireland) with 143 USP units of lithium heparin.

Cell isolation

Neutrophils were purified from blood anticoagulated with lithium heparin (21). The blood, diluted 1:1 with PBS with 0.4% (w/v) trisodium citrate (pH 7.4), was placed on Percoll (ρ 1.076 g/ml) and centrifuged (700 *g*, 18 min, 25°C). The pellet was suspended in ice-cold lysis solution for 10 min. After centrifugation (5 min, 400 *g*, 4°C), the remaining erythrocytes were lysed in fresh lysis solution for another 5 min. The cells were then washed and resuspended in medium to a final concentration of 2×10^6 cells/ml and kept at room temperature. Cytospin preparations were >97% pure and >99% viable as determined by May-Grünwald/Giemsa and trypan blue staining.

Fluorescence measurements in suspensions of human neutrophils

Neutrophils (2×10^6 cells/ml) were loaded with 5 μM Fura-2/AM for 30 min at 37°C. Excess Fura-2/AM was removed by washing the cells twice with HBSS-0.5% (w/v) HSA. Next, neutrophils were transferred to a cuvette placed in a Shimadzu (Kyoto, Japan) RF-5301 spectrofluorophotometer equipped with a magnetic stirrer and a thermostatted cuvette holder. The fluorescence emission ratio at 490 nm was monitored as a measure for the average cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) after excitation at 340 and 380 nm.

TABLE 1. Composition and characteristics of lipid emulsions

	LT	LT/MT-E	SL	MT
Fractionated soybean oil (g/l)	200	100	0	0
Medium-chain triglycerides (g/l)	0	100	0	200
Fatty acid [% (w/w) of total]				
Caproic acid ($C_{6:0}$)	—	0.5	0.1	⩽2%
Caprylic acid ($C_{8:0}$)	—	28.5	23.4	50–63%
Capric acid ($C_{10:0}$)	—	20	10.4	36–47%
Lauric acid ($C_{12:0}$)	—	1	0.2	⩽2%
Palmitic acid ($C_{16:0}$)	9	6.5	7.5	—
Stearic acid ($C_{18:0}$)	5	2	3.2	—
Oleic acid ($C_{18:1}$)	25	11	16.2	—
Linoleic acid ($C_{18:2}$)	55	26	33.3	—
Linolenic acid ($C_{18:3}$)	8	4	4.2	—
Arachidonic acid ($C_{20:4}$)	1	0.5	—	—
Structured triacylglycerols (g/l)	0	0	200	0
Mean molecular triglyceride weight	865	634	683	505
Fractionated egg phospholipids (g/l)	12	12	12	12
Glycerol (g/l)	22.5	25	22.5	20
Tocopherol (mg/l)				
α	8.8[a] 16.9[b]	50[a] (200) 21.8[b]	NIA[a]	NA[a]
β	NIA[a] 4.3[b]	NIA[a] NIA[b]	NIA[a]	NA[a]
δ	43.1[a] 119.8[b]	NIA[a] 53.9[b]	NIA[a]	NA[a]
γ	84.4[a] 44.3[b]	NIA[a] 27.9[b]	NIA[a]	NA[a]
pH	8.0	8.0	6.5–8.5	8.0

Composition and characteristics are according to [a] manufacturer or [b] literature (33).
Abbreviations: NA, none added; NIA, no information available.

PKC activity measurements

PKC activity was determined as described previously (22). The reaction mixture (200 μl) contained 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.1), 0.1 mM PMSF, 2 μM leupeptin, aprotinin (0.05 μg/ml), 0.1 μM calpain inhibitor, trypsin inhibitor (0.02 mg/ml), 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (specific activity, 0.05 Ci/mmol), 50 μg/ml MBP, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.2 mM EDTA, and 1.39 mM $CaCl_2$ (approximately 300 μM free Ca^{2+}). Where indicated, the reaction mixture contained in addition PS (100 μg/ml) and/or PMA (50 nM) and/or lipid emulsion (2.5 mM). The reaction mixture was preincubated for 2 min at 30°C and the reaction was started by addition of 20 μl of enzyme solution. The enzyme solution consisted of a cytosolic fraction of Chinese hamster ovary (CHO) cells transiently overexpressing PKCα. After 30 min, the reaction was stopped by addition of 5 ml of ice-cold 5% (w/v) trichloroacetic acid-0.1 M H_3PO_4 and the acid-precipitable material was collected on a Schleicher & Schuell (Keene, NH) AE-95 membrane filter (pore size 0.8 μm). The filters were washed three times with 5 ml of ice-cold 5% (w/v) trichloroactic acid-0.1 M H_3PO_4, added to 4 ml of Opti-fluor (Packard, Meriden, CT), and counted by liquid scintillation analysis. In each experiment, the level of MBP phosphorylation obtained with Ca^{2+}, PS, and PMA was set at 100%, to which all other values were related.

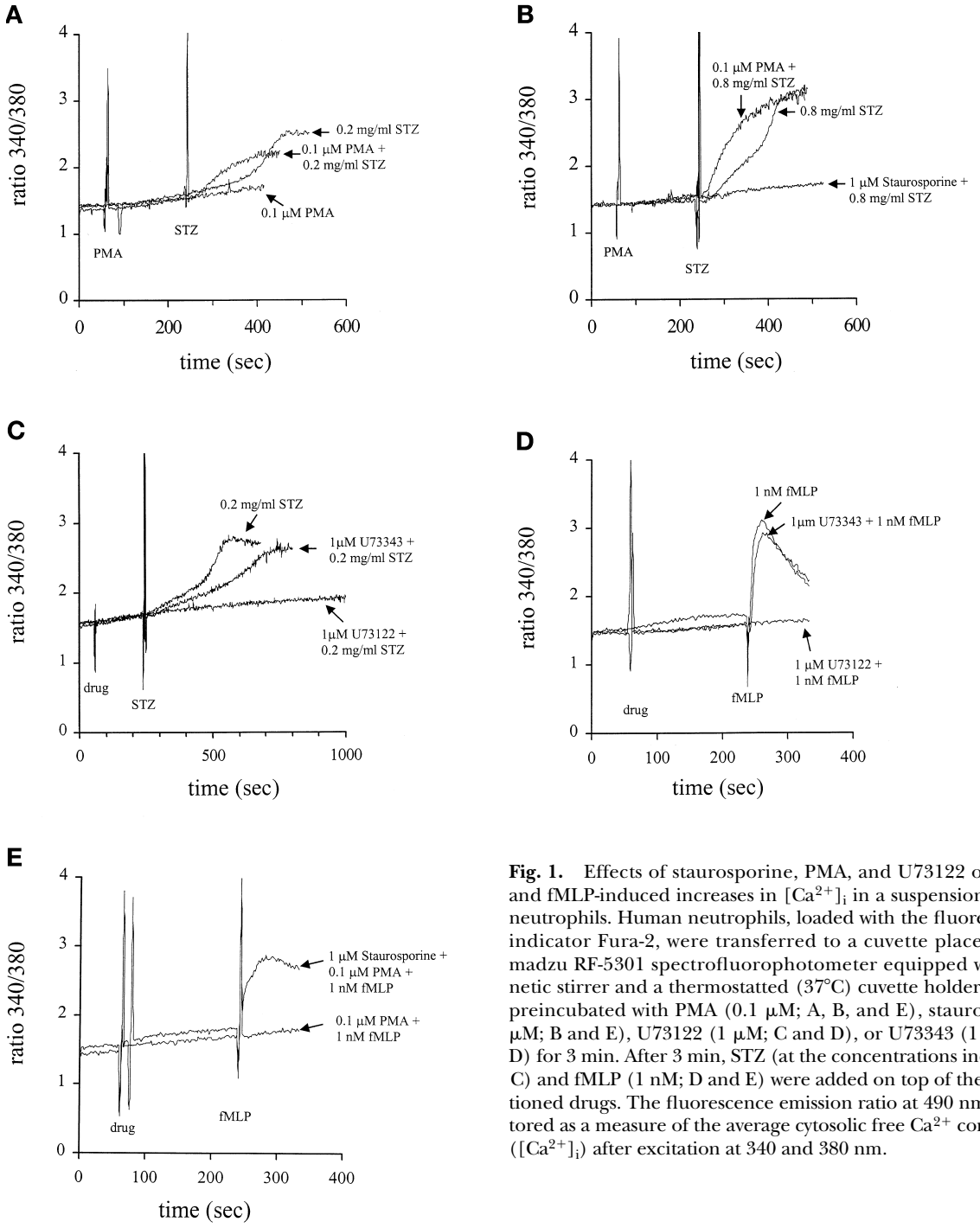

Fig. 1. Effects of staurosporine, PMA, and U73122 on the STZ- and fMLP-induced increases in $[Ca^{2+}]_i$ in a suspension of human neutrophils. Human neutrophils, loaded with the fluorescent Ca^{2+} indicator Fura-2, were transferred to a cuvette placed in a Shimadzu RF-5301 spectrofluorophotometer equipped with a magnetic stirrer and a thermostatted (37°C) cuvette holder. Cells were preincubated with PMA (0.1 μM; A, B, and E), staurosporine (1 μM; B and E), U73122 (1 μM; C and D), or U73343 (1 μM; C and D) for 3 min. After 3 min, STZ (at the concentrations indicated; A–C) and fMLP (1 nM; D and E) were added on top of the aforementioned drugs. The fluorescence emission ratio at 490 nm was monitored as a measure of the average cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) after excitation at 340 and 380 nm.

Statistical analysis

In all experiments, the data are expressed as means ± SEM. Overall statistical significance was determined by analysis of variance. In the case of significance ($P < 0.05$) individual groups were compared by contrast analysis according to Scheffé. *P* values of less than 0.05 were considered significant.

RESULTS

Effects of staurosporine, PMA, and U73122 on the STZ- and fMLP-induced increases in $[Ca^{2+}]_i$ in a neutrophil suspension

STZ induced a typical biphasic increase in the fluorescence emission ratio in a suspension of Fura-2-loaded neutrophils (**Fig. 1A–C**). This increase, which reflects the increase in average cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), consisted of a first (slow) phase that gradually turned into a second (fast) phase until a plateau $[Ca^{2+}]_i$ was reached that was maintained for prolonged periods of time. Increasing the STZ concentration from 0.2 to 0.8 mg/ml markedly increased both the initial rate of $[Ca^{2+}]_i$ rise and the final plateau $[Ca^{2+}]_i$ without changing, however, the biphasic nature of the STZ response.

The tracings depicted in Fig. 1A and B show that pretreatment with the PKC activator PMA (0.1 μM) markedly potentiated the first (slow) phase of the STZ-induced $[Ca^{2+}]_i$ rise, while abolishing, however, the second (fast) phase of this response. In addition, PMA significantly lowered the plateau $[Ca^{2+}]_i$ obtained with the lower (0.2 mg/ml), but not the higher (0.8 mg/ml), STZ concentration. PMA alone did not change the fluorescence emission ratio, indicating that activation of PKC in itself had no effect on $[Ca^{2+}]_i$ (Fig. 1A). Conversely, the potent inhibitor of protein kinase activity, staurosporine (1 μM), abolished the STZ-induced increase in $[Ca^{2+}]_i$ (Fig. 1B).

Pretreatment with the phospholipase C inhibitor U73122 (1 μM) abolished the STZ-induced increase in $[Ca^{2+}]_i$ (Fig. 1C). In contrast, U73343 (1 μM), which is an inactive analog of U73122, did not prevent STZ-induced Ca^{2+} mobilization. However, it should be noted that U73343 decreased the rate of $[Ca^{2+}]_i$ rise both during the first (slow) phase and the second (fast) phase of the STZ response.

Figure 1D shows that the peptide hormone fMLP (1 nM) evoked an immediate and rapid increase in $[Ca^{2+}]_i$. The effect of fMLP was transient by nature in that $[Ca^{2+}]_i$, after having reached its peak, more gradually returned to lower levels. In addition, Fig. 1D shows that the fMLP-induced increase in $[Ca^{2+}]_{i,av}$ was abolished by U73122 (1 μM). In contrast, U73343 (1 μM) did not interfere with the fMLP-induced $[Ca^{2+}]_i$ response. Pretreatment with PMA (0.1 μM) abolished the fMLP-induced increase in $[Ca^{2+}]_i$ (Fig. 1E). This inhibitory action of PMA was completely reversed by staurosporine (1 μM). Importantly, none of the effects of PMA was observed after pretreatment of the neutrophils with the inactive phorbol ester, PMA (data not shown).

Effects of various lipid emulsions on the STZ- and fMLP-induced increases in $[Ca^{2+}]_i$ in a neutrophil suspension

Figure 2A shows that LT/MT (2.5 mM) completely mimicked the effects of PMA in that it increased the first (slow) phase, abolished the second (fast) phase, and lowered the sustained plateau of the increase in $[Ca^{2+}]_i$ in response to a suboptimal concentration of STZ (0.2 mg/ml). Conversely, LT (2.5 mM) did not affect the biphasic response to STZ with the understanding, however, that it lowered the plateau $[Ca^{2+}]_i$ (Fig. 2B).

The effects of LT/MT were also observed with LT/MT enriched with α-tocopherol (LT/MT-E) and MT (all 2.5 mM). But, whereas PMA (0.1 μM) evoked a 5-fold increase in the initial rate of the STZ-induced increase in $[Ca^{2+}]_i$, LT/MT, LT/MT-E, and MT increased this initial rate only by a factor of 2 to 3 (**Fig. 3A**). Similar to LT/MT and LT (see Fig. 2), all other lipid emulsions significantly

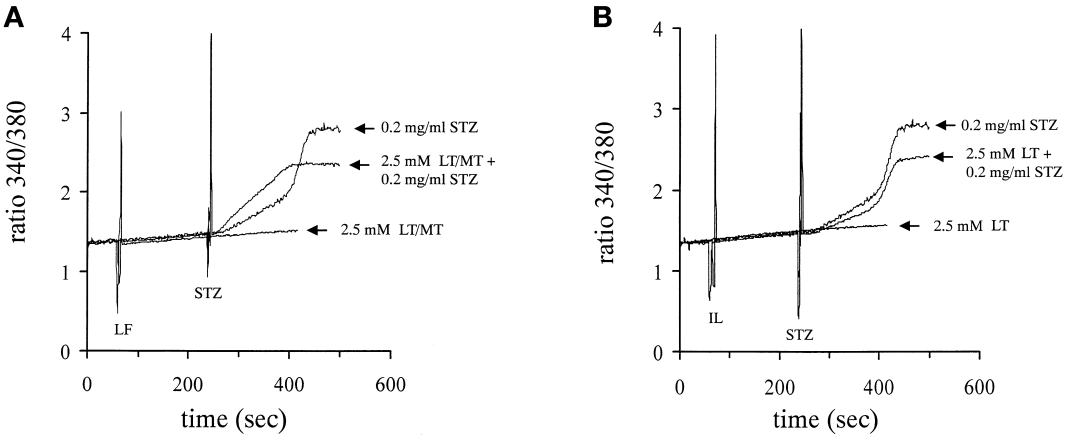

Fig. 2. Effects of the nutritional lipid emulsions LT/MT and LT on the STZ-induced increase in $[Ca^{2+}]_i$ in a suspension of human neutrophils. Fura-2-loaded neutrophils were preincubated with either LT/MT (2.5 mM; A) or LT (2.5 mM; B) for 3 min and subsequently stimulated with STZ (0.2 mg/ml). Details on $[Ca^{2+}]_i$ measurement are given in the legend to Fig. 1.

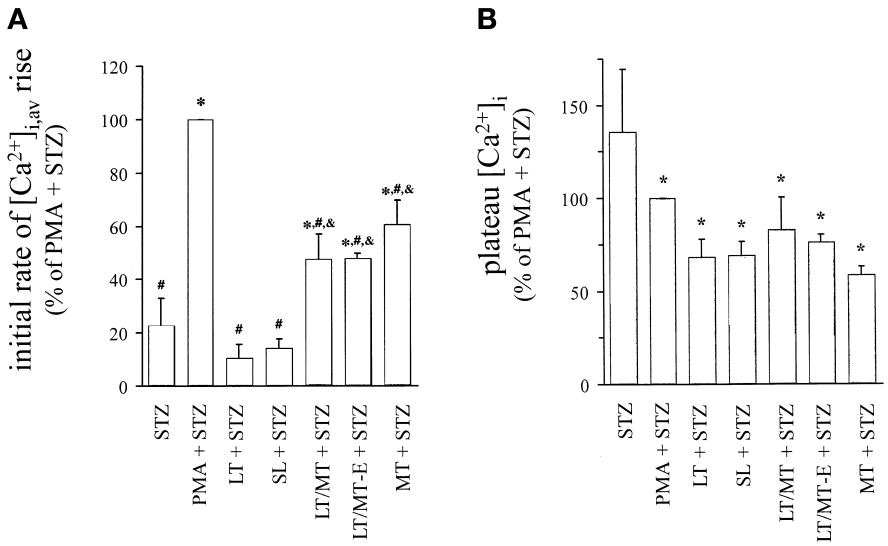

Fig. 3. Effects of PMA and the various lipid emulsions on the initial rate of $[Ca^{2+}]_i$ rise and the $[Ca^{2+}]_i$ plateau after stimulation with STZ. Fura-2-loaded neutrophils were preincubated with either PMA (0.1 μM) or a 2.5 mM concentration of the indicated lipid emulsion for 3 min. Thereafter, the cells were stimulated with STZ (0.2 mg/ml) and the increase in $[Ca^{2+}]_i$ was monitored as described in the legend to Fig. 1. A: The initial rate of $[Ca^{2+}]_i$ rise obtained with PMA-treated cells is set at 100%, to which all other values are related. B: The value for the $[Ca^{2+}]_i$ plateau obtained with PMA-treated cells is set at 100%, to which all other values are related. The data represent means + SEM of at least three measurements. * Significantly different from STZ alone ($P < 0.05$); # significantly different from PMA + STZ ($P < 0.05$); & significantly different from both LT + STZ and SL + STZ ($P < 0.05$).

lowered the STZ-induced $[Ca^{2+}]_i$ plateau (Fig. 3B). Importantly, none of the lipid emulsions alone had an effect on $[Ca^{2+}]_i$ (see also Fig. 2).

The potentiating effect of LT/MT on the first (slow) phase of the STZ-induced increase in $[Ca^{2+}]_i$ was dose dependent and a more than 3-fold increase was obtained with a lipid concentration of 5 mM (**Fig. 4**). Unfortunately, lipid concentrations beyond 5 mM caused technical problems, making it impossible to assess whether LT/MT was as efficacious as PMA. Figure 4 also shows that even at a maximal concentration of 5 mM, LT did not affect the STZ-induced increase in $[Ca^{2+}]_i$.

The rate at which $[Ca^{2+}]_i$ increased during the first (slow) phase of the STZ-induced increase in $[Ca^{2+}]_i$ clearly depended on the STZ concentration (**Fig. 5A**). At concentrations below 0.13 mg/ml, STZ did not affect $[Ca^{2+}]_i$, while the maximal effect on the initial rate of $[Ca^{2+}]_i$ increase was obtained at a concentration of 0.6 mg/ml. Both PMA (0.1 μM) and LT/MT (2.5 mM) sensitized the human neutrophils for stimulation by STZ as indicated by a shift to the left of the dose-response curve for the effect of STZ on the initial rate of $[Ca^{2+}]_i$ increase. Moreover, both PMA and LT/MT potentiated the initial rate of $[Ca^{2+}]_i$ increase at all STZ concentrations tested, thereby abolishing the second (fast) phase (see Figs. 1A and 2A). Figure 5B shows also that the $[Ca^{2+}]_i$ plateau depended on the STZ concentration and that a maximum was reached at 0.4 mg/ml. Both PMA and LT/MT lowered the STZ-induced plateau with the understanding, however, that the inhibitory effect of PMA, unlike that of LT/MT, was reversed at higher STZ concentrations.

The peak increase in $[Ca^{2+}]_i$ obtained with fMLP was a function of the fMLP concentration (**Fig. 6**). The peptide hormone did not affect $[Ca^{2+}]_i$ at concentrations at or below 0.1 nM, whereas its maximum effect was observed at a concentration of 0.1 μM. PMA (0.1 μM) desensitized

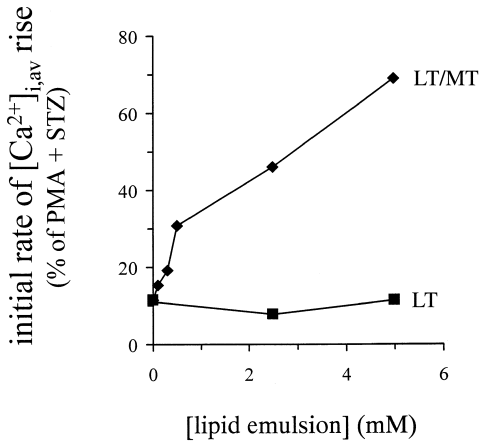

Fig. 4. Dose dependence of the effects of LT/MT and LT emulsions on the initial rate of $[Ca^{2+}]_i$ rise in response to STZ stimulation. Fura-2-loaded cells were preincubated in the presence of either 2.5 mM LT/MT or 2.5 mM LT for 3 min and subsequently stimulated with STZ (0.2 mg/ml). The initial rate of $[Ca^{2+}]_i$ rise after stimulation with STZ was monitored as described in the legend to Fig. 1, and is expressed as a percentage of the value obtained with cells preincubated with PMA (0.1 μM). The figure represents a single experiment.

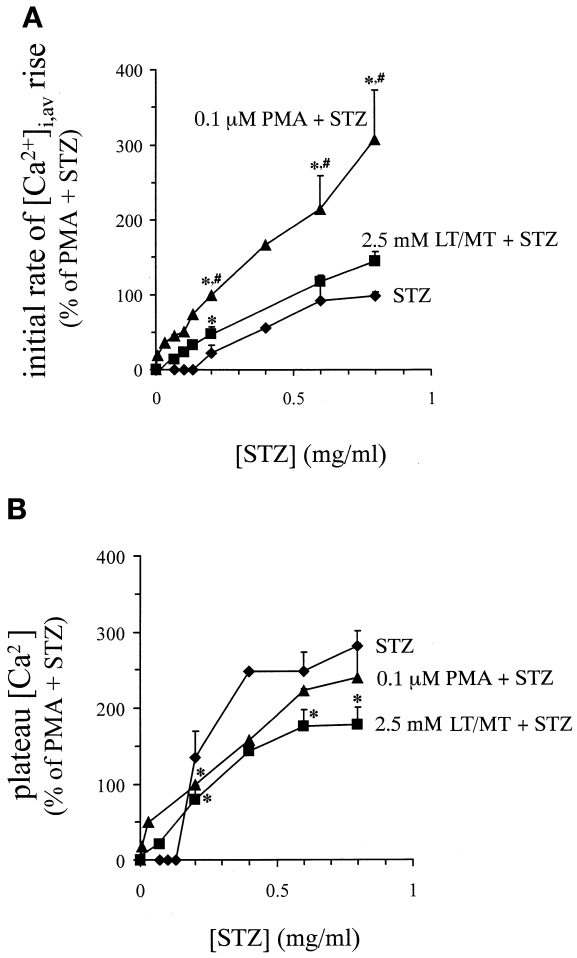

Fig. 5. Effects of PMA and LT/MT on the dose-response curve for the STZ-induced initial rate of $[Ca^{2+}]_i$ rise in human neutrophils. Fura-2-loaded neutrophils, preincubated with either PMA (0.1 μM) or LT/MT (2.5 mM) for 3 min, were stimulated with the indicated concentration of STZ. The increase in $[Ca^{2+}]_i$ was monitored as described in the legend to Fig. 1. A: The initial rate of $[Ca^{2+}]_i$ rise obtained with PMA-treated cells is set at 100%, to which all other values are related. B: The value for the $[Ca^{2+}]_i$ plateau obtained with PMA-treated cells is set at 100%, to which all other values are related. Where indicated by error bars, the data represent means ± SEM of at least three measurements. * Significantly different from STZ alone ($P < 0.05$); # significantly different from LT/MT + STZ ($P < 0.05$).

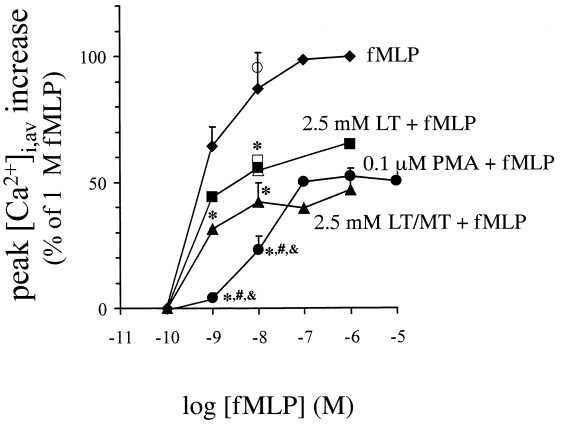

Fig. 6. Effects of PMA, LT/MT, and LT on the dose-response curve for the fMLP-induced peak increase in $[Ca^{2+}]_i$ in human neutrophils. Neutrophils loaded with Fura-2 were preincubated in the presence of PMA (0.1 μM), LT/MT (2.5 mM), or LT (2.5 mM) and in the absence (closed symbols) or presence (open symbols) of staurosporine (1 μM) for 3 min, after which the cells were stimulated with the indicated concentration of fMLP. $[Ca^{2+}]_i$ was monitored as described in the legend to Fig. 1. The peak increase in $[Ca^{2+}]_i$ obtained with 1 μM fMLP is set at 100%, to which all other values are related. Where indicated by error bars, the data represent means + SEM of at least three measurements. * Significantly different from fMLP alone ($P < 0.05$); # significantly different from LT + fMLP ($P < 0.05$); & significantly different from LT/MT + fMLP ($P < 0.05$).

the neutrophils for fMLP, as was indicated by a shift to the right of the dose-response curve for the fMLP-induced peak increase in $[Ca^{2+}]_i$. Moreover, PMA halved the maximal effect of fMLP.

LT/MT markedly lowered the maximal effect of fMLP on the peak increase in $[Ca^{2+}]_i$ without causing, however, a significant shift to the right of the dose-response curve for the effect of the hormone. Unexpectedly, the same result was obtained with LT. This latter finding urged us to investigate the involvement of PKC. Pretreatment of the cells with the PKC inhibitor staurosporine (0.1 μM) abolished the inhibitory effect of PMA, while only slightly reducing the inhibitory effect of LT/MT (Fig. 6, open symbols). The inhibitor did not interfere with the inhibitory action of LT. These findings indicate that the primary effect of lipid emulsions on fMLP-induced Ca^{2+} mobilization occurs in a PKC-independent manner.

Effect of PMA and various lipid emulsions on the PKC activity in a cytosolic extract

Figure 7 shows that optimal PKC activity in a cytosolic extract was obtained in the presence of Ca^{2+}, PS, and PMA (Fig. 7). In the presence of Ca^{2+} alone, MBP phosphorylation was only 7% of maximal and addition of PMA increased the level of MBP phosphorylation by a factor of 2. None of the lipid emulsions changed the phosphorylation level obtained with Ca^{2+} alone or with Ca^{2+} and PMA. Addition of PS increased the level of MBP phosphorylation to approximately 70% of maximal and a further increase to 100% was achieved when in addition PMA was added to the reaction mixture. This latter effect of PMA was mimicked by all lipid emulsions (2.5 mM) tested, with the understanding that the increase obtained with lipid emulsion was even higher than that obtained with PMA. The combination of PMA and lipid emulsion did not further increase the level of MBP phosphorylation. Importantly, these findings indicate that all lipid emulsions mimic the PMA effect and not the PS effect.

DISCUSSION

Clinical studies suggest that the administration of lipids in parenteral nutrition regimens may cause phagocyte

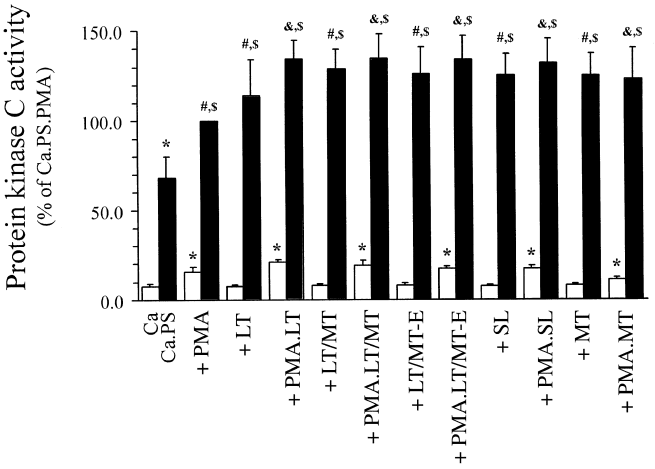

Fig. 7. Effect of the various nutritional lipid emulsions on PKC activity. A cytosolic fraction of CHO cells transiently overexpressing PKCα was used to assess the effects of the various lipid emulsions on PKC activity. PKC activity was measured at an ambient free Ca^{2+} concentration of 0.3 mM and a temperature of 37°C and is defined as the PMA- and PS-dependent increase in MBP phosphorylation. In each experiment, the level of MBP phosphorylation obtained with PMA (50 nM) and PS (100 μg/ml) is set at 100%, to which all other values are related. Where indicated, lipid emulsions were added at a concentration of 2.5 mM. Measurements were performed in the presence of either Ca^{2+} (open bars) or Ca^{2+} and PS (closed bars). The data represent means + SEM of three measurements. * Significantly different from Ca^{2+} or Ca^{2+} + corresponding lipid emulsion alone ($P < 0.05$); # significantly different from Ca^{2+} + PMA or Ca^{2+} + corresponding lipid emulsion ($P < 0.05$); & significantly different from Ca^{2+} + PMA + corresponding lipid emulsion ($P < 0.05$); $ significantly different from Ca^{2+} + PS ($P < 0.05$).

dysfunction, resulting in infectious complications such as bacteremia, pneumonia, and wound abscesses (1, 3). The present study provides evidence that nutritional lipid emulsions influence responses of the human immune system by modulation of cellular signal transduction, as represented by second-messenger (Ca^{2+}) mobilization and PKC activation. Importantly, the nature of the response seems to depend on the structure of the lipid that is administered. Emulsions containing medium-chain triglycerides are capable of activating PKC in neutrophil granulocytes and this activation sensitizes the neutrophils for stimulation by opsonized particles (STZ). This conclusion is based on the observation that MT, in contrast to LT and SL, mimicked the stimulatory effect of the PKC-activator PMA on the initial (slow) rate of the STZ-induced increase in $[Ca^{2+}]_i$, thereby shifting the dose-response curve for the effect of STZ to the left. Importantly, these effects were observed at a clinically relevant triglyceride concentration of 2.5 mM. Because staurosporine was found to abolish the STZ-induced increase in $[Ca^{2+}]_{i,av}$ it could not be used to provide additional evidence of the involvement of PKC in the mechanism of action of the MT-containing emulsions. However, at this point it should be noted that staurosporine is not a specific PKC inhibitor and may inhibit other types of kinases as well.

In agreement with the idea that MT-containing emulsions affect STZ-induced Ca^{2+} mobilization through PKC, these emulsions were found to activate partially purified PKC in a PMA-like fashion. Intriguingly, however, the LT and SL emulsions also displayed this activity. The finding that the latter emulsions did not mimic the effect of PMA on the STZ-induced $[Ca^{2+}]_i$ rise then suggests that they do not activate PKC in the context of the intact cell.

In cell physiological terms, the experimental data obtained with the PKC activator PMA unveil an interesting and new priming mechanism involving PKC. PKC activation sensitizes the neutrophil to phagocytosis and killing of opsonized pathogens (represented by STZ particles) and, in the mean time, desensitizes the neutrophil from being distracted from this activity by chemoattractants such as the chemotactic peptide fMLP. As far as the nutritional lipid emulsions are concerned, those that contain MT mimic the PKC-mediated sensitizing effect of PMA, whereas all lipid emulsions decrease the maximal effect of STZ and fMLP in a PKC-independent manner. The importance of these lipid effects is evident, regarding the immune-compromised state of the patient category receiving parenteral nutrition and their increased susceptibility to infectious complications.

The present finding that only the MT-containing emulsions mimic the effects of the PKC activator, PMA, on the initial phase of the STZ-induced $[Ca^{2+}]_i$ rise is in agreement with previous studies, in which we demonstrated that MT-containing emulsions, but not emulsions that consisted solely of LT or SL, significantly increased oxygen radical production, adhesion, degranulation, and migration of these cells in a PKC-dependent manner (17-19). Here, we provide evidence that these MT-containing emulsions exert their PMA-like actions by directly activating PKC in a PMA-like fashion. Neutrophils express five PKC isoforms, α, βI, βII, δ, and ξ (23), and future research will show which PKC isoforms are involved in the various actions of the MT-containing lipid emulsions on neutrophil function. Importantly, the MT-containing emulsions did not increase $[Ca^{2+}]_i$ by themselves. This indicates that these lipids do not act through an effect on $[Ca^{2+}]_i$ to increase oxygen radical production, adhesion, degranulation, and migration.

The antioxidant effects of lipid-soluble vitamin E, or tocopherol, are considered to be beneficial for both emulsion (stability by prevention of lipid peroxidation) and patient (detoxification of oxygen radicals produced by immune cells). On the other hand, the α-tocopherol isoform has shown well-defined inhibitory effects on the respiratory burst of leukocyes, that is, inhibition of PKC as well as impaired assembly of the NADPH-oxidase (24). However, enrichment of an LT/MT emulsion with α-tocopherol at 170 ± 40 mg/l (see Table 1) did not influence the emulsion effect in our study with regard to PKC activity.

The present work furthermore shows that both an MT-containing emulsion and an emulsion that consisted solely of LT can markedly lower the fMLP-induced increase in $[Ca^{2+}]_i$. Although the same effect was obtained with PMA, the involvement of PKC (or some other staurosporine-inhibitable kinase) in the mechanism of action of these

lipid emulsions was excluded by the fact that staurosporine did not significantly reduce their inhibitory effect, whereas it abolished the inhibition by PMA. Similarly, all lipid emulsions that were tested were found to reduce the STZ-induced $[Ca^{2+}]_i$ plateau. Most probably this also occurs in a PKC-independent fashion. For reasons outlined above, however, we could not use staurosporine to experimentally verify this hypothesis. Taken together, these findings show that all nutritional lipid emulsions, when added at clinically relevant concentrations, can significantly reduce the fMLP-induced peak increase in $[Ca^{2+}]_i$ and the STZ-induced $[Ca^{2+}]_i$ plateau, and that this reduction occurs in a PKC-independent fashion. It remains open to speculation whether this finding represents an "aspecific" lipid effect. Lipid-receptor interactions have been suggested in previous studies regarding an LT emulsion (7).

It is unlikely that the "PMA-like" factor present in the lipid emulsions tested in this study consists of a short-chain carboxylic acids, as these acids have been shown to evoke a rapid increase in inositol 1,4,5-trisphosphate and cytosolic free Ca^{2+} (25). Similarly, phosphatidic acid has been shown to evoke a rapid rise in $[Ca^{2+}]_i$ in human neutrophils (26). Evidence has been provided that polyunsaturated fatty acids, which are present in all emulsions (see Table 1), also can stimulate neutrophil responses such as the oxygen-dependent respiratory burst through activation of phospholipase A_2 (27). It has been demonstrated that neutrophils can secrete phospholipase A_2 (28). In principle, secreted phospholipase A_2 can hydrolyze lipids of the lipid emulsions to produce reactive lipids. These lipids, in turn, may prime the cell for activation by STZ.

STZ evoked a typical biphasic increase in $[Ca^{2+}]_i$ consisting of a first (slow) increase that gradually turned into a second (fast) increase until a plateau was reached. It has been suggested that the first (slow) phase of the STZ-induced $[Ca^{2+}]_i$ rise reflects the inositol 1,4,5-trisphosphate-triggered release of Ca^{2+} from the endoplasmic reticulum (29). Indeed, the present study shows that the phospholipase C inhibitor U73122 abolished both the STZ- and fMLP-induced increase in $[Ca^{2+}]_i$. The present study shows that both PMA and all MT-containing lipid emulsions markedly potentiated the rate of $[Ca^{2+}]_i$ rise during the initial phase of the response to STZ. This finding suggests that PKC activation promotes iC3b receptor signaling, as it has been argued that this receptor, and not FcγR, mediates STZ activation of neutrophils (29). Possible mechanisms for this action of PKC include an increase in *i*) affinity of the receptor for its ligand, *ii*) receptor number, and *iii*) coupling between receptor and G protein. An effect at the level of the inositol 1,4,5-trisphosphate-mediated release of Ca^{2+} from the endoplasmic reticulum is not likely, as PMA decreased rather than increased the fMLP-induced increase in $[Ca^{2+}]_i$. The second (fast) phase of the STZ-induced increase in $[Ca^{2+}]_i$ has been suggested to be brought about by endogenously produced platelet-activating factor (PAF) (29). According to this idea, the present observation that PMA and the MT emulsions abolished this second phase may suggest that PKC inhibits the STZ-triggered production of PAF. Alternatively, however, PKC may accelerate the STZ-induced production of PAF, thus shortening the first (slow) phase. Inhibition of the fMLP-induced increase in $[Ca^{2+}]_i$ after PMA treatment has been reported before (30–32). The same study also mentioned PMA inhibition of the STZ-induced increase in $[Ca^{2+}]_i$. However, these authors did not observe a biphasic increase in $[Ca^{2+}]_i$ and most probably referred to the PMA-induced decrease of the plateau $[Ca^{2+}]_i$ as described in the present study.

In conclusion, the present study provides evidence that nutritional lipids influence human immune responses through modulation of cellular signaling pathways in immune-competent cells. The nature of these effects appears to be dependent on the fatty acid chain length of the lipid: medium-chain triglyceride-containing emulsions sensitize neutrophils for STZ in a PKC-dependent manner and medium-chain, long-chain, and structured lipid emulsions all reduce the stimulatory effect of STZ and fMLP in an aspecific manner.

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