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Original Article

Parenteral medium-chain triglyceride-induced neutrophil activation is not mediated by a Pertussis Toxin sensitive receptor☆

Michelle W.J. Versleijen [a], Joantine C.J. van Esterik [a], Hennie M.J. Roelofs [a], Sjenet E. van Emst-de Vries [b], Peter H.G.M. Willems [b], Geert J.A. Wanten [a,*]

[a] Department of Gastroenterology and Hepatology, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB, Nijmegen, the Netherlands
[b] Department of Biochemistry, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands

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SUMMARY

Background & aims: Lipid-induced immune modulation might contribute to the increased infection rate that is observed in patients using parenteral nutrition. We previously showed that emulsions containing medium-chain triglycerides (LCT/MCTs or pure MCTs), but not pure long-chain triglycerides (LCTs), impair neutrophil functions, modulate cell-signaling and induce neutrophil activation *in vitro*. It has recently been shown that medium-chain fatty acids are ligands for GPR84, a pertussis toxin (PT)-sensitive G-protein-coupled receptor (GPCR). This finding urged us to investigate whether MCT-induced neutrophil activation is mediated by PT-sensitive GPCRs.

Methods: Neutrophils isolated from blood of healthy volunteers were pre-incubated with PT (0.5–1 μg/mL, 1.5 h) and analyzed for the effect of this pre-incubation on LCT/MCT (2.5 mmol/L)-dependent modulation of serum-treated zymosan (STZ)-induced intracellular Ca^{2+} mobilization and on LCT/MCT (5 mmol/L)-induced expression of cell surface adhesion (CD11b) and degranulation (CD66b) markers and oxygen radical (ROS) production.

Results: PT did not inhibit the effects of LCT/MCT on the STZ-induced increase in cytosolic free Ca^{2+} concentration. LCT/MCT increased ROS production to 146% of unstimulated cells. However, pre-incubation with PT did not inhibit the LCT/MCT-induced ROS production. Furthermore, the LCT/MCT-induced increase in CD11b and CD66b expression (196% and 235% of unstimulated cells, respectively) was not inhibited by pre-incubation with PT.

Conclusion: LCT/MCT-induced neutrophil activation does not involve the action of a PT-sensitive G-protein-coupled receptor.

1. Introduction

A major problem in patients on Total Parenteral Nutrition (TPN) remains the occurrence of infectious complications. Immune-modulating properties of nutritional components, mainly lipids, may play a role herein.[1] In accordance with others,[2,3] we previously reported that *in vitro* (pre-) incubation with parenteral lipid emulsions containing medium-chain triglycerides, such as mixed long- and medium-chain triglycerides (LCT/MCTs) or pure MCT emulsions, in contrast to pure LCT emulsions, induces neutrophil activation and neutrophil function impairment.[4] For example, we could show that MCTs, in this experimental setting, induce the production of reactive oxygen species (ROS),[5] sensitize neutrophils for opsonized yeast particles (serum-treated zymosan (STZ))-induced intracellular calcium signaling[6] and induce an increment in the expression of relevant adhesion and degranulation markers (CD11b, CD66b, CD63 and CD62L) on neutrophils and monocytes.[7] Furthermore, we demonstrated that MCTs inhibit the migration of neutrophils[8] and decrease their capacity to kill *Candida albicans*.[9] The latter might be explained by exhaustion of the neutrophil cellular metabolism due to LCT/MCT-induced continuous, and therefore probably inadequate, cell activation.

The exact mechanism by which MCTs exert these effects is still not elucidated. However, several G-protein-coupled receptors (GPCRs) have recently been identified as receptors for free fatty acids on leukocytes and might, therefore, play a role in the

Nonstandard abbreviations: STZ, serum-treated zymosan; PT, pertussis toxin; GPCR, G-protein-coupled receptor; ROS, reactive oxygen species; MCFFAs, medium-chain free fatty acids; LCFFAs, long-chain free fatty acids; SCFFAs, short-chain free fatty acids; CT, cholera toxin; PAF, platelet activating factor.

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* Corresponding author. Tel.: +31 24 3614760; fax: +31 24 3540103.
E-mail address: g.wanten@mdl.umcn.nl (G.J.A. Wanten).

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lipid-induced immune modulation. For example, the GPR40, which is expressed in monocytes, mediates the effects of medium-chain free fatty acids (MCFFAs) and/or long-chain free fatty acids (LCFFAs),[10,11] whereas the GPR41 and GPR43, which are expressed in neutrophils, have short-chain free fatty acids (SCFFAs) as their ligands.[10,12,13] In a recent study, the GPR84 was de-orphanized and shown to have MCFFAs as ligands and to be abundantly expressed in monocytes and neutrophils.[14] This GPCR might, therefore, be involved in MCT-induced neutrophil activation.

The G-proteins involved in GPCR signaling are heterotrimeric proteins that bind guanine-nucleotides. They constitute a large and diverse family and are involved in many physiological processes. G-proteins are classified into the $G_{i/o}$, G_s, G_q and G_{12} families, each of which interacts with its own effector molecules to modulate the production of intracellular second messengers. Effector molecules include adenylyl cyclase, cGMP-phosphodiesterase, phospholipase C-β (PLC-β) and ion-channels.[15] Members of the G_i family are pertussis toxin (PT)-sensitive; ADP ribosylation of their α_i-subunit by this toxin prevents coupling of the ligand-bound receptor to the G_i-protein and consequent exchange of GDP for GTP on this α_i-subunit. Because the GTP-bound α_i-subunit inhibits adenylyl cyclase, its PT-induced ADP ribosylation prevents the receptor-mediated inactivation of this enzyme. In addition, PT can inhibit G_i-protein-induced PLC-β activation since Gβγ-dimers, that are released from the heterotrimeric G_i-proteins, have been shown to activate PLC-β2 in granulocytes.[16] Members of the G_s, G_q and G_{12} family are PT-resistant but the α_s-subunit is ADP-ribosylated by Cholera toxin (CT). PT and CT have been shown to be useful in indentifying the involvement of heterotrimeric G-proteins in signaling systems. MCFFA-induced activation of the GPR84 has been shown to be PT-sensitive.[14]

The present study was conducted to evaluate the involvement of a PT-sensitive GPCR, such as the GPR84, in the immune-modulating effects of MCT-based parenteral lipid emulsions.

2. Subjects and materials

2.1. Subjects

Appropriate informed consent from all participating volunteers was obtained and the human experimentation guidelines of the author's institution were followed in the conduct of this study. After overnight fasting, blood samples drawn from 7 healthy volunteers, none of whom was on medication, were collected in 10 mL Monoject tubes with 170 IU of lithium heparin (Beliver Industrial Estate, Plymouth PL6 7BP, UK) and processed as described.

2.2. Materials

Reagents were from Sigma Chemicals (St Louis, MO, USA), unless stated otherwise. Hank's balanced salt solution (HBSS) was purchased from Life Technologies (Paisley, Scotland, UK). Percoll (ρ 1.130 g/mL at 20 °C) was obtained from GE Healthcare Biosciences AB (Uppsala, Sweden) and phosphate buffered saline (PBS) was produced by B. Braun Melsungen AG, Melsungen Germany. Isotonic lysis solution (pH 7.4) contained 155 mmol/L NH_4CL and 10 mmol/L $KHCO_3$. Neutrophil medium consisted of HBSS supplemented with 0.5% (wt/vol) bovine serum albumin (BSA). *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was stored as a 0.04 mmol/L stock in DMSO at −20 °C and Luminol as a 10 mmol/L stock in DMSO at room temperature (RT). PT was stored as a 100 μg/mL stock in aquadest at 4 °C. Serum-treated zymosan (STZ) (10 mg/mL stock) was prepared as described previously.[17] Fura-2/AM was from Molecular Probes Inc. (Eugene, OR, USA). LCT/MCT emulsion (Lipofundin, 20% w/v) was from B. Braun Melsungen AG (Melsungen, Germany). All antibodies used for the measurement of leukocyte membrane surface antigen expression were purchased from Beckman Coulter (Miami, FL, USA).

3. Methods

3.1. Neutrophil isolation

Neutrophils were purified from blood anti-coagulated with lithium heparin. The blood, diluted 1:1 with PBS, was placed on Percoll (ρ 1.076 g/mL) and centrifuged (700 × g, 20 min, RT). The granulocyte-containing pellet was suspended in 50 mL ice-cold lysis solution and lysis of erythrocytes was performed on ice for 10–15 min. After centrifugation (400 × g, 5 min, RT), remaining erythrocytes were lysed on ice in fresh lysis solution for another 5 min. The granulocytes were then washed twice, suspended to 8×10^6 cells/mL in neutrophil medium and kept at RT. This method of neutrophil isolation yields >97% pure and >99% viable cell samples as determined by May-Grünwald/Giemsa and trypan blue staining.[5]

3.2. PT (pre-) incubation

Neutrophils (8×10^6 cells/mL in neutrophil medium) were incubated with PT (0.5 or 1.0 μg/mL) for 1.5 h by gentle head-over-head turning at 37 °C. Then, the cells were washed (5 min, 400 × g, RT) and resuspended in neutrophil medium to the desired final concentration. The efficacy of PT treatment was tested by determining its effect on the fMLP (0.3 nmol/L)-induced increase in cytosolic free Ca^{2+} concentration, shown to be abolished by this toxin in isolated neutrophils.[18,19]

3.3. STZ-induced cytosolic calcium signaling

After PT incubation and washing, neutrophils (4×10^6 cells/mL) were loaded with 5 μmol/L Fura-2/AM for 15 min at 37 °C. Excess Fura-2/AM was removed by washing the neutrophils first with neutrophil medium and second with HBSS. Subsequently, the neutrophils were transferred to a cuvette placed in a Shimadzu RF-5301 spectrofluorophotometer equipped with a magnetic stirrer and a thermostated cuvette holder (37 °C). In the cuvette, cells were pre-incubated with LCT/MCT, in a clinically relevant concentration of 2.5 mmol/L, for 3 min and thereafter stimulated with STZ. The change in fura-2 fluorescence emission ratio at 490 nm was monitored as a measure of the average of the individual changes in cytosolic free Ca^{2+} concentration of all cells in the suspension after excitation at 340 and 380 nm.[6,20] All measurements were performed in duplicate.

3.4. Oxygen radical production

Oxygen radical production was evaluated by means of luminol-enhanced chemiluminescence, as measured in an automated LB96V Microlumat Plus Luminometer (EG & G Berthold, Bad Wildberg, Germany).[21] Briefly, after PT pre-incubation, 200 μL of neutrophil suspension (1×10^6 cells/mL) was added to a 96-well microplate; either with no stimulus or with LCT/MCT emulsion, in a clinically relevant concentration of 5 mmol/L, and the reaction was started by the addition of 20 μL of luminol (stock 1:10 diluted in neutrophil medium). Each measurement was performed in fivefold and chemiluminescence was monitored every 145 s at 37 °C for 1 h. Luminescence was expressed as relative light units per second (RLU/sec). The area under the curve (AUC), representing the overall oxygen radical production during 1 h, was calculated. Data were analyzed with Winglow software (EG & G Berthold, Bad Wildberg, Germany).