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In vivo sex differences in leukotriene biosynthesis in zymosan-induced peritonitis

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ABSTRACT

Leukotrienes (LTs) are 5-lipoxygenase (5-LO) metabolites which are implicated in sex-dependent inflammatory diseases (asthma, autoimmune diseases, *etc.*). We have recently reported sex differences in LT biosynthesis in *in vitro* models such as human whole blood, neutrophils and monocytes, due to down-regulation of 5-LO product formation by androgens. Here we present evidences for sex differences in LT synthesis and related inflammatory reactions in an *in vivo* model of inflammation (mouse zymosan-induced peritonitis). On the cellular level, differential 5-LO subcellular compartmentalization in peritoneal macrophages (PM) from male and female mice might be the basis for these differences. Sex differences in vascular permeability and neutrophil recruitment (cell number and myeloperoxidase activity) into peritoneal cavity were evident upon intraperitoneal zymosan injection, with more prominent responses in female mice. This was accompanied by higher levels of LTC_4 and LTB_4 in peritoneal exudates of female compared to male mice. Interestingly, LT peritoneal levels in orchidectomized mice were higher than in sham male mice. In accordance with the *in vivo* results, LT formation in stimulated PM from female mice was higher than in male PM, accompanied by alterations in 5-LO subcellular localization. The increased formation of LTC_4 in incubations of PM from orchidectomized mice confirms a role of sex hormones. In conclusion, sex differences observed in LT biosynthesis during peritonitis *in vivo* may be related, at least in part, to a variant 5-LO localization in PM from male and female mice.

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Introduction

Leukotrienes (LTs) are biologically active lipid mediators with key roles in inflammation [1], including regulation of vascular permeability and of leukocyte extravasation and migration [2,3]. LTs derive from arachidonic acid (AA) through the action of 5-lipoxygenase (5-LO), which converts AA to LTA_4, representing the first step in the synthesis of all LTs [4]. In fact, LTA_4 can be hydrolyzed to the chemotactic agent LTB_4 by LTA_4 hydrolase or conjugated with reduced glutathione by LTC_4 synthase to form LTC_4. LTC_4 is then metabolized by sequential proteolytic hydrolysis to LTD_4 and LTE_4, which are together known as cysteinyl-LTs (cys-LTs) with vascular actions. LT biosynthesis is regulated by different mechanisms and among them post-translational modifications of 5-LO, including phosphorylation at serine residues, Ca^{2+} binding, interactions with certain phospholipids and glycerides, and interactions with 5-LO-activating protein and coactosin-like protein [4]. Also, LT synthesis is strongly influenced by the intracellular localization of 5-LO and the amount of trafficable enzyme. Thus, 5-LO is a soluble protein in the cytosol or nucleoplasm of resting cells, and translocates to perinuclear membranes when intracellular Ca^{2+} levels increase or/and after its phosphorylation by mitogen-activated protein kinase (MAPK) [3,5–7].

LTs are implicated in a range of inflammatory diseases, often presenting sex-related differences in the incidence and/or the

Abbreviations: 5-LO, 5-lipoxygenase; AA, arachidonic acid; cys-LTs, cysteinyl-leukotrienes; DMEM, Dulbecco's modified Eagle's medium; EIA, enzyme immunoassay; ERK, extracellular signal-regulated kinases; LTs, leukotrienes; MAPKs, mitogen-activated protein kinases; MPO, myeloperoxidase; ORCH, orchidectomized; PM, peritoneal macrophages; PMSF, phenylmethylsulfonyl, fluoride; PG, prostaglandin.

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course, such as asthma, allergic rhinitis, and autoimmune diseases [8–10]. Interestingly, the sex has emerged as a fundamental variable for the LT pathway and we have recently reported sex differences in LT biosynthesis in *in vitro* models such as human whole blood, neutrophils [11] and monocytes [12]. Thus, male sex hormones repressed LT biosynthesis in human neutrophils by affecting 5-LO localization *via* differential activation of extracellular signal-regulated kinase (ERK) [11]. Also, sex-related differences in LT biosynthesis were observed in human monocytes where, *in vitro*, testosterone caused a repression of phospholipase D, resulting in impaired 5-LO product biosynthesis due to lack of activating diacylglyceride [12]. Moreover, data from animal models showing sex-specific attenuation of atheroma formation in dual 12-/15- and 5-LO knock-out mice [13] and exclusive protection of female mice from platelet-activating factor-induced shock after knocking out of the LTB_4 receptor 1 [14] suggested a major contribution of LTs in the pathophysiology of females. Nevertheless no *in vivo* data about sex-dependent LT biosynthesis are available.

Here, we investigated whether the sex influences the production of LTs *in vivo*, using zymosan-induced peritonitis as experimental model. This model displays all classical signs of acute inflammation, including increased vascular permeability, edema, leukocyte influx and release of inflammatory mediators. We show for the first time, that LT production is sex-biased *in vivo* and significantly higher in the peritoneum of female mice challenged with zymosan. The data indicate that this bias can be traced back to sex differences in the LT-synthetic capacities of peritoneal macrophages (PM) and are accompanied by sex-specific 5-LO subcellular localization.

Materials and methods

Materials

Enzyme immunoassay (EIA) kits were from Cayman Chemical Company (Aurogene, Rome, Italy). [^3H-PGE_2] was from PerkinElmer Life Sciences (Milan, Italy). All other reagents and compounds were obtained from Sigma–Aldrich (Milan, Italy).

Animals

Male and female CD-1 mice (8–9 weeks old, Charles River, Calco, Italy) were housed in a controlled environment (21 ± 2 °C) and provided with standard rodent chow and water. All animals were allowed to acclimate for four days prior to experiments and were subjected to 12 h light – 12 h dark schedule. Experiments were conducted during the light phase.

To investigate the impact of sex hormones, male mice were orchidectomized (ORCH group) and allowed to recover for 5 weeks. This time period ensures that sex hormones have been metabolized and are no longer in the blood, and it is sufficient to allow the turnover of immune cells generated under the influence of this reproductive hormone [15,16]. In particular, orchidectomies were performed through scrotal incision. Sham groups were performed through the similar procedures, except that the gonads were not removed. Plasma testosterone was measured with a commercially available EIA (Cayman Chemical Company). The experimental protocols were approved by the Animal Care Committee of the University of Naples Federico II, in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116/92) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986).

Zymosan-induced peritonitis

Peritonitis was induced by i.p. injection (0.5 mL) of zymosan (2 mg mL^{-1} in saline, boiled and washed; Sigma, Milan, Italy). At selected time points after zymosan injection (0, 15, 30, 60, 120, 180 and 240 min), mice were euthanized and peritoneal exudates were collected using 2 mL of PBS. The cells were counted using a light microscope in a Burker's chamber after vital trypan blue staining. Exudates were centrifuged at 20,000 × *g* for 20 min and supernatants and cell pellets were collected and frozen at −80 °C for measurements of eicosanoids and myeloperoxidase (MPO) activity, respectively. The peritoneal exudate levels of LTC_4 and LTB_4 were measured by EIA kits according to manufacturer's instructions (Cayman Chemical; Aurogene, Rome, Italy) and expressed as ng mL^{-1}.

Vascular permeability

Evans blue dye (40 mg kg^{-1}, 0.3 mL; Sigma, Milan, Italy) was injected into the tail vain followed by an *i.p.* injection of zymosan (0.5 mL; 2 mg mL^{-1}). After 0, 15, 30, 60, 120, 180 and 240 min, mice were euthanized and peritoneal exudates were collected using PBS (0.1 mL g^{-1} of body weight). After centrifugation, supernatants (diluted 1:3) were analyzed for Evans Blue bound to plasma albumin by reading at 610 nm in a plate reader (MultiskanGo, Thermo Scientific).

MPO assay

The use of MPO as an index of neutrophil infiltration is well documented [17]. Cell pellets were disrupted by sonication in 2 mL PBS (50 mM, pH 6) with 0.5% hexadecyltrimethylammonium bromide, freeze-thawed three times and centrifuged (32,000 × *g*, 20 min) to collect supernatants which were used in MPO assay, performed as described by Bradley et al. [17]. Briefly, 20 μl samples were added to a 96 well plate and the reaction was initiated by the addition of 0.2 mL of assay buffer containing 0.167 mg mL^{-1} of *o*-dianisidine and 0.0005% hydrogen peroxide. The rate of change of absorbance was monitored in kinetic mode by a plate reader (IMarkmicroplate Reader, Bio-Rad, Segrate, Milan, Italy). Levels of MPO in samples were determined from the calibration curve using human neutrophil MPO as the reference standard. The levels of MPO were expressed as U mL^{-1}.

Peritoneal macrophages

Resident PM were obtained by lavage of the peritoneal cavity of mice with 7 mL of cold Dulbecco's modified Eagle's medium (DMEM) with heparin (5 U mL^{-1}). PM were then centrifuged at 500 × *g* 4 °C for 5 min, resuspended at 1×10^7 cells mL^{-1} and 0.5 mL of cell suspension were added to a 15 mL conical polypropylene tube for each experimental condition. Cells were then incubated at 37 °C with ionophore A23187 (4.5 mL, final concentration 2.5 μM, 15 min), zymosan (4.5 mL, final concentration 13 particles per cell, 30 min) or vehicle (0.25% dimethyl sulfoxide in DMEM for A23187 and DMEM for zymosan). EDTA at a final concentration of 4 mM was added at the end of incubation time to A23187-stimulated cells. All samples were then centrifuged at 500 × *g* at 4 °C for 5 min. Supernatants were collected and frozen at −80 °C for measurements of LTC_4 and prostaglandin $(PG)E_2$ by EIA (Cayman Chemical Company; Aurogene, Rome, Italy) and radioimmunoassay (PerkinElmer Life Sciences, Milan, Italy; Sigma, Milan, Italy) respectively. The cell pellets were washed by resuspending in ice-cold PBS, centrifuged and collected and frozen at −80 °C for 5-LO evaluation.

Cell disruption and fractionation

Total cell lysates were prepared by lysis for 15 min at 4 °C with lysis buffer (20 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), 15 μg mL^{-1} soybean trypsin inhibitor, 3 μg mL^{-1} pepstatin A, 2 μg mL^{-1} leupeptin, 1% Nonidet P-40, 20% glycerol,

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