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Leukotriene B_4 Mediates Neutrophil Migration Induced by Heme

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High concentrations of free heme found during hemolytic events or cell damage leads to inflammation, characterized by neutrophil recruitment and production of reactive oxygen species, through mechanisms not yet elucidated. In this study, we provide evidence that heme-induced neutrophilic inflammation depends on endogenous activity of the macrophage-derived lipid mediator leukotriene B_4 (LTB_4). In vivo, heme-induced neutrophil recruitment into the peritoneal cavity of mice was attenuated by pretreatment with 5-lipoxygenase (5-LO) inhibitors and leukotriene B_4 receptor 1 (BLT1) receptor antagonists as well as in 5-LO knockout (5-$LO^{-/-}$) mice. Heme administration in vivo increased peritoneal levels of LTB_4 prior to and during neutrophil recruitment. Evidence that LTB_4 was synthesized by resident macrophages, but not mast cells, included the following: 1) immunolocalization of heme-induced LTB_4 was compartmentalized exclusively within lipid bodies of resident macrophages; 2) an increase in the macrophage population enhanced heme-induced neutrophil migration; 3) depletion of resident mast cells did not affect heme-induced LTB_4 production or neutrophil influx; 4) increased levels of LTB_4 were found in heme-stimulated peritoneal cavities displaying increased macrophage numbers; and 5) in vitro, heme was able to activate directly macrophages to synthesize LTB_4. Our findings uncover a crucial role of LTB_4 in neutrophil migration induced by heme and suggest that beneficial therapeutic outcomes could be achieved by targeting the 5-LO pathway in the treatment of inflammation associated with hemolytic processes. ***The Journal of Immunology*, 2011, 186: 6562–6567.**

Inflammation has emerged as an essential component of pathophysiologic situations in which increased hemolysis can lead to high levels of free heme, such as in malaria (1), sickle cell disease (SCD) (2), the hemolysis, elevated liver enzyme levels, and low platelet count syndrome (3), and regional turbulent blood flow (4). SCD is associated with inflammatory stresses within the microcirculation, such as leukocytosis, elevated levels of inflammatory cytokines, and activation of neutrophils, monocytes, and endothelial cells (5–8). The heme molecule serves as the functional component of a wide variety of crucial proteins and is involved in various cellular processes such as gene transcription/translation, cell differentiation, and proliferation (9–13). Heme is therefore of fundamental importance for life. However, heme is also inherently dangerous, particularly when it escapes from intracellular sites. Free heme has several proinflammatory activities, including induction of cytokines and acute-phase proteins, as well as the ability to induce neutrophil migration and activation (14, 15). Heme oxygenase (HO) is a family of ubiquitous enzymes that catalyze the degradation of heme to bilirubin, producing equimolar amounts of biliverdin, free iron, and carbon monoxide. There are three known HO isoforms: the inducible isoform HO-1 and the constitutive isoforms HO-2 and HO-3. The expression of inducible HO-1 is positively modulated by a number of inflammatory mediators, by oxidative stress, and also by heme itself (16). Neutrophil migration into tissues is the hallmark of numerous acute inflammatory reactions and represents a highly regulated multistep process that is controlled by a variety of inflammatory mediators, including leukotriene (LT) B_4.

LTB_4 is a lipid mediator derived from the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism. 5-LO, in conjunction with 5-LO activating protein, oxygenates arachidonic acid to form LT A_4. Hydrolysis of this intermediate forms LTB_4, a potent leukocyte chemoattractant that also displays leukocyte activating functions (17). Regarding hemolysis-related inflammatory conditions, it has been shown that increased LT concentrations are found in plasma and urine of SCD patients at steady state (18, 19), and that plasma levels of LTB_4 are further increased during vaso-occlusion and acute chest syndrome episodes (20).

In the current study, a potential role of the 5-LO product LTB_4 in heme-induced neutrophil migration was investigated. In addition, we tested the hypothesis that macrophages are the cells involved in generating LTB_4 in response to heme stimulation. Our findings indicate that heme-elicited neutrophil recruitment is mediated by

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Abbreviations used in this article: ADRP, adipose-differentiation-related protein; BLT1, leukotriene B4 receptor 1; EDAC, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide; EIA, enzyme immunoassay; HO, heme oxygenase; 5-LO, 5-lipoxygenase; LT, leukotriene; SCD, sickle cell disease; WT, wild-type.

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macrophage-derived LTB_4, whose intracellular synthesis is compartmentalized within cytoplasmic lipid bodies.

Materials and Methods

Animals

C57BL/6, SV129, or 5-LO–deficient (129-Alox5^{tm1Fun}; 5-LO$^{-/-}$) mice weighing 20–22 g were used. 5-LO$^{-/-}$ and strain-matched wild-type (WT) mice were bred in the unit for transgenic animals at Bio-Rio (Federal University of Rio de Janeiro, Rio de Janeiro, Brazil) from breeders obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were obtained from Instituto Oswaldo Cruz (Rio de Janeiro, Brazil). The animals were housed in temperature-controlled rooms and received water and food ad libitum until used. All experiments were conducted in accordance with National Institutes of Health guidelines on the welfare of experimental animals.

Materials

Heme was purchased from Porphyrin Products (Logan, UT) and LTB_4 from Cayman Chemical (Ann Arbor, MI). Zileuton [*N*-(1-benzo[*b*]thien-2-ylethyl)-*N*-hydroxyurea] was obtained from Ono Pharmaceutical (Osaka, Japan). AA861 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). LY 292476 was obtained from Eli Lilly (Indianapolis, IN). CP 105,696 was a gift from Dr. Henry Showell (Pfizer Laboratories, Groton, CT). Compound 48/80 and thioglycolate were purchased from Sigma (St. Louis, MO), as well as all other reagents used.

Heme preparation

For in vitro neutrophil chemotaxis experiments, heme stock solutions (5 mM) were made in DMSO and diluted in RPMI 1640 medium or saline immediately before use. All procedures were performed in the dark to avoid generation of free radicals.

Neutrophil migration

Heme was injected i.p. in 0.5 ml sterile saline, and control animals received 0.5 ml saline alone. Four hours after challenge, the animals were sacrificed, and the peritoneal cells were harvested by injecting 3 ml PBS containing 0.1% heparin. Total counts were performed in a Neubauer chamber, and differential cell counts (200 cells) were enumerated on HEMA 3-stained cytocentrifuge (cytospin 3; Shandon) slides. The results are presented as number of neutrophils per cavity.

Measurement of LTB_4

LTB_4 levels were determined by enzyme immunoassay (EIA) according to the manufacturer's instructions (Cayman Chemical). Mouse peritoneal macrophages were harvested with PBS 0.1% heparin, enumerated, and cultured in 24-well plates for 1 h at 37°C in an atmosphere of air with 5% CO_2. The plates were then washed three times with RPMI 1640 to remove the nonadherent cells, and the adherent population was incubated for 30 min at 37°C in fresh medium (control) or in medium containing heme (3, 10, or 30 nmol well^{-1}). Subsequently, the supernatants were discarded, and after three further washes the cells were incubated for 6 h with 0.3 ml RPMI 1640 alone. After this incubation period, the supernatants were recovered and stored at −70°C for LTB_4 determination. Cell-free peritoneal lavage fluids obtained after i.p. challenge with heme (50 nmol in 0.5 ml saline) or saline alone (control) were also collected and frozen (−70°C) until LTB_4 determination.

Peritoneal macrophage population enhancement

Thioglycolate (3% w/v, 1 ml i.p.) was injected in a group of animals, and 3 d later the peritoneal cells were collected, enumerated, and differential cell count performed and compared with that in the control group (treated with 1 ml saline). At day 3, heme (50 nmol) was injected into saline- or thioglycolate-treated mice and neutrophil migration evaluated 4 h later.

Depletion of the peritoneal mast cell

Mice were chronically treated with compound 48/80 for 4 d (0.6 mg kg^{-1}, twice a day for 3 d; and 1.2 mg kg^{-1}, twice a day on the 4th day; i.p.). On the 5th day, the peritoneal cells were harvested by lavage, and the number of mast cells was assessed. The counts obtained were compared with those obtained from the control group (saline treated). At day 5, heme (50 nmol) was then injected into control- and compound 48/80-treated mice, and after 4 h the neutrophil migration was evaluated.

EicosaCell for immunodetection of intracellular, newly formed LTB_4 within peritoneal leukocytes

LTB_4 immunodetection at its subcellular sites of synthesis within leukocytes was performed as previously described (21). In brief, leukocytes were recovered from peritoneal cavities 2 h after heme or sterile saline injection by washing the cavity with 500 μl HBSS and immediately mixing with 500 μl water-soluble 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC in HBSS; 0.5% final concentration with cells) (Sigma), used to cross-link eicosanoid carboxyl groups to amines in adjacent proteins. After 15 min incubation at 37°C with EDAC to promote cell fixation and permeabilization, peritoneal leukocytes were then washed with HBSS, cytospun onto glass slides, and blocked with HBSS containing 1% BSA for 30 min. The cells were then incubated overnight with anti-LTB_4 Ab (Cayman Chemical) or irrelevant IgG overnight and the anti–adipose-differentiation-related protein) (anti-ADRP; 1:300 final dilution) to distinguish cytoplasmic lipid bodies within leukocytes. The cells were washed with HBSS for 10 min (three times) and incubated with Alexa 488-labeled anti-rabbit IgG (1:1000 final dilution) plus Alexa 546-labeled anti-guinea pig (1:1000 final dilution) secondary Abs for 1 h.

The specificity of the LTB_4 immunolabeling was demonstrated by 1) lack of immunofluorescence within leukocytes recovered from heme-injected animals that were incubated with irrelevant IgG (data not shown); 2) lack of LTB_4 immunolabeling within resident leukocytes recovered from saline-injected animals that were incubated with anti-LTB_4 Ab; and 3) lack of LTB_4 immunolabeling within resident leukocytes recovered from heme-injected animals that were treated with the 5-LO inhibitor zileuton (60 μg cavity^{-1}, i.p.) 1 h before peritoneal cell recovery with 500 μl HBSS containing 25 μM zileuton and then incubated with anti-LTB_4 Ab.

The images were obtained using an Olympus BX51 fluorescence microscope equipped with a Plan Apo ×100 1.4 Ph3 objective and an Olympus 72 digital camera (Olympus Optical, Japan) in conjunction with Cell^F Imaging Software for Life Science Microscopy (Olympus Life Science Europa, Germany). The images were edited using Adobe Photoshop 5.5 software (Adobe Systems, San Jose, CA).

Cell culture

To determine whether peritoneal macrophages release neutrophil chemotactic activity after heme stimulation, peritoneal cells were harvested and cultured in 24-well plates for 1 h at 37°C in an atmosphere of air with 5% CO_2. The plates were then washed three times with RPMI 1640 to remove the nonadherent cells. The adherent cells were incubated for 30 min at 37°C in fresh medium (control) or in medium containing heme (30 nmol well^{-1}) in the presence or not of 5-LO inhibitor (AA861; 10 μM). Subsequently, the supernatants were discarded, and after three further washes the cells were incubated for 6 h with medium alone or medium containing AA861. At the end of the incubation period, the supernatants were collected, centrifuged to remove cells, filtered through an 0.22-μm membrane, and then injected i.p. in mice pretreated or not with CP 105,696 (3 mg kg^{-1}), an LTB_4 receptor 1 (BLT1) receptor antagonist. After 4 h, neutrophil migration was evaluated.

Statistical analysis

Data shown are mean ± SEM and are representative of at least two separate experiments. Statistical analysis of means was determined by ANOVA with Bonferroni *t* test for unpaired values or Student *t* test, as appropriate and as indicated in the figure legends of this article. Statistical significance was set at $p < 0.05$.

Results

Heme-induced in vivo neutrophil migration depends on newly synthesized LTB_4 acting on BLT1 receptors

It has been previously shown that the intrathoracic administration of heme induces a dose-dependent neutrophil accumulation in rat pleural cavities (15). However, the mechanisms by which heme induced neutrophil recruitment were not characterized. Because LTB_4 is a well-characterized chemoattractant of neutrophils (22, 23) and has been implicated in neutrophil migration induced by several inflammatory mediators, such as TNF-α (24), IL-1β (25), IL-18 (26), MIP-1α (27), and MIP-2 (28), we hypothesized LTB_4 to be a mediator of heme-induced neutrophil migration. Such involvement of LTB_4 was confirmed by using two completely distinct experimental approaches. First, WT or 5-LO$^{-/-}$ mice were injected i.p. with heme (50 nmol) or saline, and neutrophil accumulation in the peritoneal cavity was evaluated 4 h later. As expected, heme administration was effective in triggering significant neutrophil recruitment to the peritoneal cavity in WT mice when compared with saline-injected mice (control). However, heme-induced neutrophil migration was clearly reduced in 5-LO$^{-/-}$ mice compared with that in heme-injected WT mice (Fig. 1*A*). Additionally, the migration induced by heme injection was also significantly inhibited in mice (WT, C57BL/6 mice) pretreated with the 5-LO inhibitors zileuton (Fig. 1*B*) and AA861 (not shown).

A variety of bioactive products generated via the 5-LO pathway are candidates for mediating this neutrophil recruitment. Fig. 1*C* implicates heme-driven LTB$_4$ as a mediator of neutrophil migration via activation of BLT1 receptors, as in mice pretreated with two structurally unrelated BLT1 receptor antagonists, CP 105,696 and LY 292476 (Fig. 1*C*), heme-induced peritoneal neutrophilia was significantly reduced.

In vivo heme-elicited LTB$_4$ synthesis takes place within newly assembled lipid bodies of resident peritoneal macrophages

In agreement with a role for endogenous LTB$_4$, in vivo stimulation with heme elicited rapid synthesis/release of LTB$_4$, which preceded heme-induced neutrophil migration. As shown in Fig. 2, peritoneal lavage fluid recovered 2 h after heme injection contained higher levels of LTB$_4$ than after saline injection.

The cellular source of LTB$_4$ synthesized during heme-driven inflammation was investigated by the direct intracellular immunofluorescent localization of newly formed LTB$_4$ within mouse peritoneal leukocytes recovered 2 h after heme stimulation. By using the EicosaCell—a methodology that cross-links and immunolabels LTB$_4$ at its sites of synthesis—resident macrophages were identified as the cell population responsible for LTB$_4$ production during heme-induced inflammatory reaction (Fig. 3, *top panel*). As illustrated in the images of immunolabeled ADRP (Fig. 3) and enumerated in osmium-stained cells (data not shown), besides LTB$_4$ synthesis, heme administration was also able to trigger rapid (2 h) assembly of new lipid bodies within resident peritoneal macrophages. The parallel between increased numbers

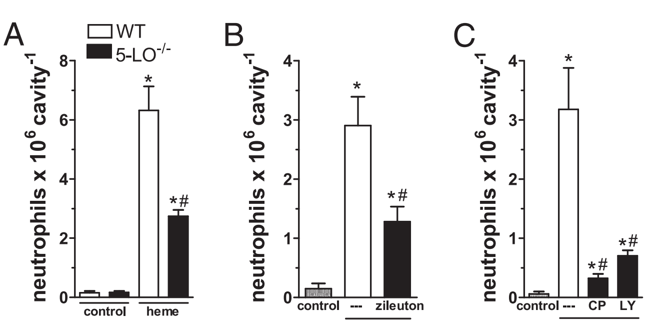

FIGURE 1. Heme-induced neutrophil migration in vivo depends on LTB$_4$/BLT1. *A*, WT (sv129) or 5-LO$^{-/-}$ mice were injected i.p. with 50 nmol heme in 0.5 ml saline or saline alone (control). *B*, C57BL/6 mice were treated with saline (—; i.v.; 20 min before) or zileuton (3 mg kg^{-1}; i.v.; 20 min before) and then challenged with heme (50 nmol in 0.5 ml saline) or saline alone (control). *C*, C57BL/6 mice were injected with saline (—; s.c.; 30 min before) or CP 105,696 (CP; 3 mg kg^{-1}; s.c.; 30 min before) or LY 292476 (LY; 2 mg kg^{-1}; s.c.; 30 min before) and administered heme (50 nmol) or saline (control). Mice were sacrificed 4 h later, peritoneal cavity cells harvested, and neutrophil migration was determined. Data are presented as the mean ± SEM. The results are of one experiment representative of three independent experiments performed with six mice per group. *$p < 0.05$ (compared with respective control), $^{\#}p < 0.05$ (compared with heme group in WT mice).

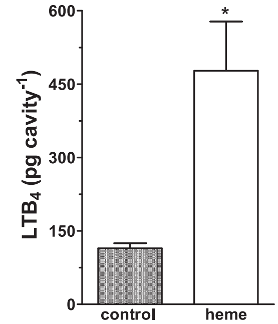

FIGURE 2. Heme-induced LTB$_4$ production in vivo. Concentration of LTB$_4$ in the peritoneal lavage fluids obtained 2 h after i.p. challenge with heme (50 nmol in 0.5 ml saline) or saline alone (control) was determined by EIA. Results are mean ± SEM; one experiment representative of three separate experiments performed with six mice per group. *$p < 0.05$ (compared with control group by Student *t* test).

of lipid bodies and levels of secreted LTB$_4$ observed in heme-induced peritoneal macrophages appeared to reflect a functional correlation, as lipid body localization of newly formed LTB$_4$ within macrophages was ascertained by colocalization with ADRP (Fig. 3). Supporting the specificity of LTB$_4$ immunostaining within cytoplasmic lipid bodies of heme-stimulated peritoneal macrophages, virtually no immunofluorescent staining for LTB$_4$ was localized within peritoneal macrophages of saline-injected mice (Fig. 3, *inset panels*) or 5-LO inhibitor-treated heme-stimulated mice (Fig. 3, *bottom panels*). Therefore, the presence of heme within the peritoneal compartment induces activation of resident macrophages with rapid formation of distinctive lipid bodies endowed with the enzymatic machinery necessary for LTB$_4$ synthesis.

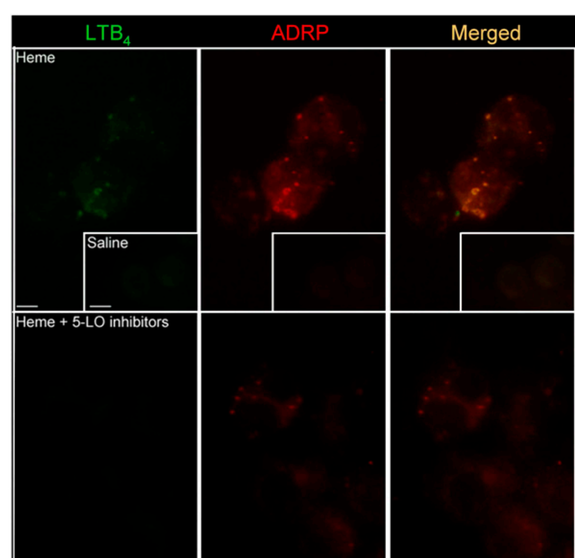

FIGURE 3. Heme elicits LTB$_4$ synthesis within cytoplasmic lipid bodies of peritoneal macrophages. EicosaCell analysis of LTB$_4$ synthesis was performed 2 h after heme administration. An anti-LTB$_4$ field was merged with an identical anti-ADRP field of fluorescent images of macrophages recovered from mice stimulated with saline (*inset panels*), heme (*top panels*), or heme plus zileuton treatment (*bottom panels*). Images show LTB$_4$ immunoreactive lipid bodies (as identified by anti-ADRP) of heme-stimulated peritoneal macrophages. Image is representative of three separate experiments with three mice per group. Scale bars, 5 μm.

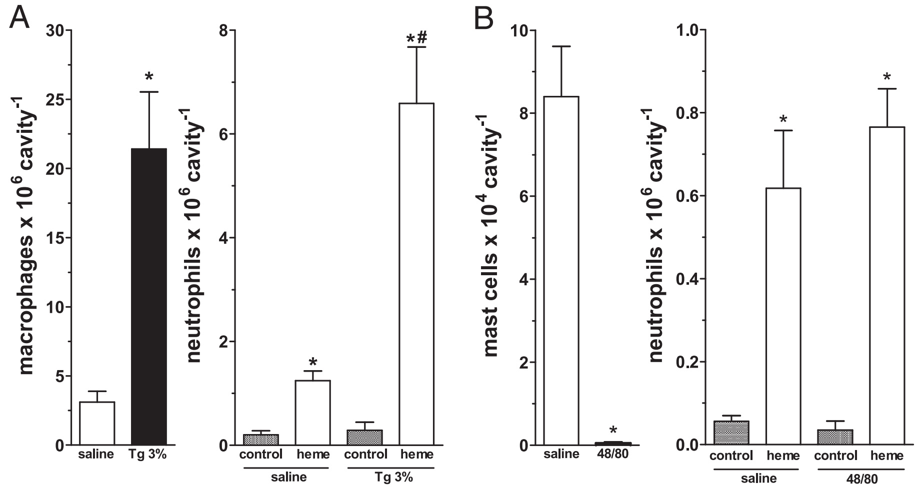

FIGURE 4. Macrophages, but not mast cells, participate in heme-induced neutrophil migration. *A*, Macrophage population in saline-pretreated and in thioglycolate (Tg)-pretreated (Tg 3%) groups was determined as described in *Materials and Methods*. Saline alone (0.5 ml; control) or heme (50 nmol in 0.5 ml saline) was injected in saline- and thioglycolate-pretreated groups. Four hours later mice were killed, peritoneal cavity cells harvested, and neutrophil migration was determined. *B*, Mast cell population in saline-pretreated and in compound 48/80 (48/80)-pretreated animals was estimated as described. Saline alone (0.5 ml; control) or heme (50 nmol in 0.5 ml saline) was injected in saline-pretreated and in compound 48/80-pretreated mice. Neutrophil recruitment was evaluated 4 h later. Data are presented as the mean ± SEM. The results are of one experiment representative of two independent experiments performed with five to six mice per group. $^*p < 0.05$ (compared with respective control), $^\#p < 0.05$ (compared with heme group in saline-pretreated mice by Student *t* test).

Macrophages, but not mast cells, participate in heme-induced neutrophil migration by generating LTB_4

Besides macrophages, resident peritoneal mast cells could represent an additional cellular source of LTB_4 involved in heme-induced neutrophil migration. To assess the differential contributions of these two cell populations in heme-induced peritoneal neutrophilia, the macrophage population was enhanced by thioglycolate administration, and functional mast cells were depleted by chronic treatment with the mast cell degranulator compound 48/80. As observed in Fig. 4*A* (*left panel*), thioglycolate injection resulted in an increase in the peritoneal macrophage population after 72 h, as expected. The administration of heme in the peritoneal cavity of mice 72 h after pretreatment with thioglycolate caused a marked enhancement of neutrophil migration (Fig. 4*A*, *right panel*). In contrast, depletion of functional peritoneal mast cells by chronic 48/80 treatment (Fig. 4*B*, *left panel*) or by distilled water injection (data not shown) did not abrogate heme-induced neutrophil accumulation (Fig. 4*B*, *right panel*). Supporting the conclusion that macrophages play a central role in heme-induced neutrophil migration by producing LTB_4, we demonstrated that challenging thioglycolate-treated macrophage-enriched mice with heme resulted in an enhanced LTB_4 production compared with that observed in saline-treated animals injected with heme (Fig. 5*A*). Furthermore, mast cell depletion with compound 48/80 had no effect on LTB_4 production in vivo after saline or heme administration into the peritoneal cavity (Fig. 5*B*), reinforcing that mast cells are not involved in heme-induced neutrophil migration. Moreover, the same observation was also noted in H_2O-treated mice (data not shown). Together, these data suggest that resident macrophages, but not mast cells, control heme-evoked LTB_4-mediated neutrophil influx.

Heme-induced LTB_4 production in vitro

To evaluate whether heme is able to trigger LTB_4 synthesis within macrophages by a direct effect on these cells, we stimulated purified mouse peritoneal macrophages with heme in vitro. As shown in Fig. 6, peritoneal macrophages synthesize/release LTB_4 in a dose-dependent manner in response to in vitro heme stimulation, indicating that free heme directly triggers macrophage activation characterized by LTB_4 synthesizing activity.

Supernatant chemotactic activity from heme-stimulated macrophages is due to the presence of LTB_4

As can be observed in Fig. 7, the instillation of supernatants obtained from heme-stimulated macrophages induced neutrophil recruitment into the peritoneal cavity of naive mice, which mimicked the neutrophil migration induced by heme administration itself. In contrast, the i.p. injection of supernatants recovered from nonstimulated macrophages did not induce neutrophil accumulation. To evaluate if heme-stimulated macrophage supernatant chemotactic activity was a consequence of the presence of

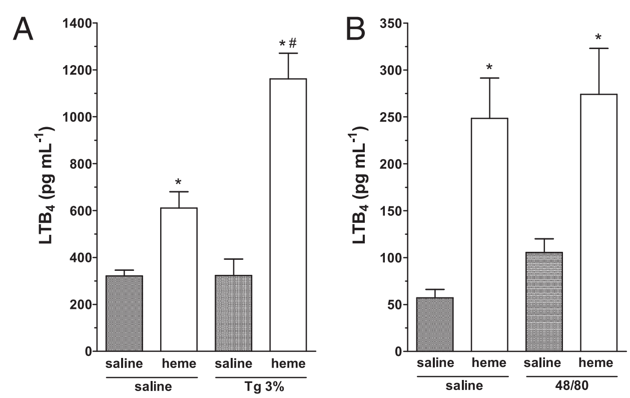

FIGURE 5. Overproduction of LTB_4 induced by heme challenge in thioglycolate-treated mice. Macrophage population was enhanced in thioglycolate (Tg)-pretreated groups (Tg 3%) (*A*) and mast cell population was depleted in compound 48/80 (48/80)-pretreated groups (*B*) as described in *Materials and Methods*. Saline (0.5 ml; control) or heme (50 nmol in 0.5 ml saline) was injected in saline-, thioglycolate-, and compound 48/80-pretreated groups. LTB_4 concentration in the peritoneal lavage fluids obtained 4 h after i.p. challenge was determined by EIA. Results are mean ± SEM. The results are of one experiment representative of three independent experiments performed with six mice per group. $^*p < 0.05$ (compared with respective control), $^\#p < 0.05$ (compared with heme group in saline-pretreated mice by Student *t* test).

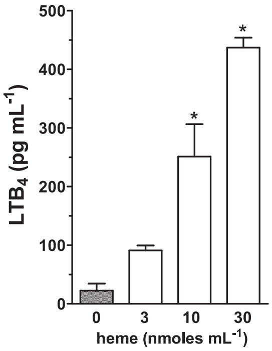

FIGURE 6. Heme-induced LTB$_4$ production in vitro. Peritoneal macrophages were cultured and stimulated with heme at the indicated doses as described in *Materials and Methods*. LTB$_4$ concentration in the supernatants collected was measured by EIA. Results are mean ± SEM. One experiment representative of two separate experiments performed in quadruplicate for each sample. *$p < 0.05$ (compared with control group).

LTB$_4$, we first evaluated the effect of a 5-LO inhibitor on its release. As shown in Fig. 7*A*, macrophage treatment with AA861 (5-LO inhibitor) was able to inhibit the release of the neutrophil chemotactic factor by heme-stimulated macrophages. Furthermore, injection of heme-stimulated macrophage supernatant failed to elicit neutrophil migration in mice pretreated with a BLT1 receptor antagonist (CP 105,696; Fig. 7*B*). Together, these data point to an essential role for macrophage-derived LTB$_4$ acting via the high-affinity receptor, BLT1, to induce neutrophil migration.

Discussion

Tissue injury after hemolytic episodes is associated with intense neutrophil accumulation. In pathological states such as SCD, high levels of free heme up to 20 μM have been observed (29). Many studies have demonstrated that neutrophil activation is present in patients with SCD (30–34). In this regard, it has been previously shown that heme causes neutrophil migration in vivo and in vitro

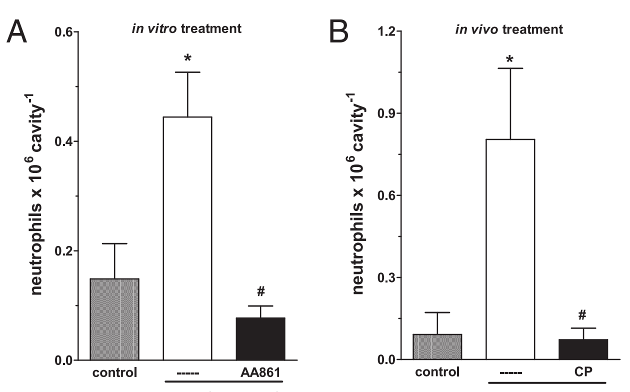

FIGURE 7. Supernatant chemotactic activity from heme-stimulated macrophages is due to the presence of LTB$_4$. *A*, Neutrophil migration was induced in naive animals by the i.p. administration of 1 ml supernatant obtained from peritoneal macrophages incubated in medium (control) or in medium containing heme (30 nmol well^{-1}) in the presence or not of 5-LO inhibitor (AA861, 10 μM). *B*, Neutrophil migration was induced by the administration of 1 ml heme-stimulated peritoneal macrophage supernatant in naive or in CP 105,696 (CP; 3 mg kg^{-1}; s.c.; 30 min before challenge)-treated mice. Neutrophil migration was quantified 4 h after the supernatant injections, and the values are presented as the mean ± SEM. The results are of one experiment representative of two independent experiments performed with five to six mice per group. *$p < 0.05$ (compared with control supernatant), $^{\#}p < 0.05$ (compared with heme-stimulated supernatant by Student *t* test).

as well as reactive oxygen species formation (15), but the mechanisms involved in this activation phenomenon are not fully understood. Data presented in this study demonstrate that heme-induced neutrophil migration and activation are LTB$_4$ dependent. In addition, the results also point to a central role for resident macrophages as the major LTB$_4$ source.

Heme-induced neutrophil migration into the peritoneal cavity of mice depends on the LTB$_4$/BLT1 axis, as pretreatment of the animals with 5-LO inhibitors (zileuton and AA861) or BLT1 receptor antagonists (CP 105,696 and LY 292476) inhibited neutrophil accumulation. Further confirmation was obtained with the use of 5-LO$^{-/-}$ mice, in which neutrophil recruitment in response to heme challenge was also attenuated. Both macrophages and neutrophils are recognized to have a robust capacity for LTB$_4$ synthesis, and generation of this activating lipid was triggered by heme in both cell types. Data in the literature support a role for LTs in SCD pathophysiology, in which urinary and plasma levels have been proposed as potential biomarkers (19, 35). To assess the involvement of LTB$_4$ in heme-induced neutrophil recruitment, we determined its production in vivo. As presented in Fig. 2, LTB$_4$ production after i.p. heme challenge was 3-fold higher than that in the control group. The idea that LTs contribute to SCD pathophysiology is reinforced by the recent finding that 5-LO and 5-LO activating protein, key LT biosynthetic proteins, were significantly increased in PBMCs obtained from patients with SCD compared with controls (36).

Mast cells and macrophages are resident cells implicated in recruiting neutrophils and eosinophils through the release of chemotactic factors (37–39). It has been demonstrated that manipulating the numbers of these cell types alters neutrophil recruitment induced by several inflammatory mediators, such as TNF-α, LTB$_4$, IL-8, and CCL2 (MCP-1) (36, 37, 40). Thus, we evaluated the effects on neutrophil migration induced by heme administration of enhancing the macrophage population and depleting the mast cell population. We found that heme-induced neutrophil migration was dependent on resident macrophages, but not on mast cells. Corroborating the central position of macrophages in neutrophil recruitment induced by heme, we noted a marked enhancement of heme-induced LTB$_4$ production when the macrophage population was increased. Ex vivo peritoneal macrophage stimulation with heme also induced LTB$_4$ production in a dose-response manner, confirming the in vivo observations. Neutrophil chemotactic activity was also noted in supernatants from heme-stimulated macrophages. Although our data implicate LTB$_4$ as a major mediator of heme-induced neutrophil migration, they do not rule out the possible participation of other mediators, such as cytokines and chemokines. In fact, it was demonstrated that murine macrophages release CXC chemokine KC and TNF-α when stimulated with heme (41). Nevertheless several inflammatory cytokines (TNF-α, IL-1β, IL-18, MIP-1α, and MIP-2) induce neutrophil migration in vivo by indirect mechanisms, dependent on 5-LO/LTB$_4$ pathway, demonstrating a close cross-talk between cytokines/chemokines and LTB$_4$, a fact not very often appreciated. The possible interplay between LTB$_4$ and other mediators in inflammatory responses to heme requires further investigation.

It is increasingly recognized that lipid bodies (or lipid droplets) are specialized sites involved in compartmentalization and amplification of eicosanoid synthesis. Lipid bodies are virtually absent in most resting non-adipocytic cells, but increased numbers of these organelles have been described in inflammatory and cancer cells both in experimental models and in clinical conditions (42, 43).

To investigate the site of LTB$_4$ production within macrophages, heme was injected, and 2 h later the cells were harvested, and the intracellular newly formed LTB_4 was immunodetected by the EicosaCell technique. LTB_4 was detected in a clear punctate pattern in the cytoplasm, near but separate from the nucleus, fully consistent in size and form with macrophage lipid bodies. Heme injection also induced lipid body formation (genesis), observed in Fig. 3 and also in osmium-stained cells (data not shown), which was absent or much less apparent in cells recovered from saline-injected mice. The relevance of lipid bodies as a site of LTB_4 formation in human neutrophils stimulated with heme remains to be determined.

To conclude, our data allow us to speculate that the inhibition of the synthesis or the actions of LTB_4 at BLT1 could be beneficial as a strategy to dampen inflammatory responses during pathological circumstances in which free heme is observed.

Disclosures

The authors have no financial conflicts of interest.

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