Leukotriene B4 inhibits neutrophil apoptosis via NADPH oxidase activity: Redox control of NF-κB pathway and mitochondrial stability

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ARTICLE INFO

ABSTRACT

Leukotriene B4, an arachidonic acid-derived lipid mediator, is a known proinflammatory agent that has a direct effect upon neutrophil physiology, inducing reactive oxygen species generation by the NADPH oxidase complex and impairing neutrophil spontaneous apoptosis, which in turn may corroborate to the onset of chronic inflammation. Despite those facts, a direct link between inhibition of neutrophil spontaneous apoptosis and NADPH oxidase activation by leukotriene B4 has not been addressed so far. In this study, we aim to elucidate the putative role of NADPH oxidase-derived reactive oxygen species in leukotriene B4-induced anti-apoptotic effect. Our results indicate that NADPH oxidase-derived reactive oxygen species are critical to leukotriene B4 pro-survival effect on neutrophils. This effect also relies on redox modulation of nuclear factor kappaB signaling pathway. We have also observed that LTB4-induced Bad degradation and mitochondrial stability require NADPH oxidase activity. All together, our results strongly suggest that LTB4-induced anti-apoptotic effect in neutrophils occurs in a reactive oxygen species-dependent manner. We do believe that a better knowledge of the molecular mechanisms underlying neutrophil spontaneous apoptosis may contribute to the development of more successful strategies to control chronic inflammatory conditions such as rheumatoid arthritis.

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1. Introduction

Neutrophils are considered the first line of defense and they bear a highly specialized killing machinery [1]. Among the several cytotoxic mechanisms displayed by these terminally differentiated leukocytes, the most aggressive ones are the release of pre-formed intracellular granules stored in the cytoplasm and the generation of reactive oxygen and nitrogen species [2]. Among all the ROS-generating systems, the NADPH oxidase (NADPHox) is the most specialized one, being absolutely essential to an appropriate host defense [3,4].

This multi-enzymatic complex, which is constituted by membrane and cytosolic subunits, can be activated by both particulated and soluble stimuli which are able to induce the translocation of the cytosolic subunits to the membrane-bound ones [5–7]. Once assembled, this complex can transfer electrons from cytosolic NADPH to the molecular oxygen present in the extracellular or phagosomal environment, generating superoxide ($O_2^{\bullet-}$) and, subsequently, $O_2^{\bullet-}$–derived species [3,8].

A growing body of evidence has suggested that beside their microbicidal and toxic properties, ROS can also act as signal transduction mediators in several biological systems [8]. However, ROS apparently exhibit a dual role in cell physiology, which implies that the amount of ROS produced is a critical parameter for the cell outcome [9]. High concentrations of ROS generally induce cell death either by necrosis or apoptosis [10–13]. The latter is particularly observed in a specialized type of cell death, known as phagocytosis-induced cell death (PICD), in which NADPHox-derived ROS contributes to neutrophil death during phagocytosis of opsonized pathogens [14]. Conversely, subtoxic levels of ROS may account for the activation of pro-survival pathways, leading to inhibition of neutrophil spontaneous apoptosis [15–18].

Several cytokines and inflammatory mediators can alter cellular redox status, inducing paradoxical ROS-mediated effects. It is well established that proinflammatory agents not only can prolong neutrophil responsiveness but also can extend its lifespan through regulation of anti-apoptotic routes, therefore delaying neutrophil spontaneous apoptosis [19,20]. This phenomenon is highly desirable during the onset of an acute inflammatory response, as these cells will remain exerting their defense functions for a greater period of time. On the other hand, excessively prolonged neutrophil survival can threaten tissue homeostasis due to the release of highly histotoxic products, including ROS [21,22]. This inappropriate extension of neutrophil lifespan is accompanied by impaired clearance of apoptotic bodies and consequent inhibition of resolution of inflammation [23,24].

Leukotriene B4 (LTB4), a lipid mediator derived from arachidonic acid via 5-lipoxygenase pathway, is produced primarily by inflammatory cells, being considered a potent neutrophil activator [25,26]. Among its effects, LTB4 promotes changes in calcium influx, cell polarization, chemotaxis and degranulation, as well as generation of NADPHox-derived ROS [27–29]. In many cell types, LTB4 can also promote nuclear factor kappaB (NF-κB) activation. NF-κB is a redox-sensitive transcription factor usually confined to the cytoplasm of resting cells, as these molecules are associated to chaperones called inhibitor of κB (IκB). However, a number of stimuli trigger the activation of IκB kinases (IKK), which phosphorylate IκB, leading to the release of NF-κB (which in turn translocate to the nucleus) and signals IκB degradation by the proteasome 26S [30].

NF-κB induces the transcription of various proinflammatory molecules such as cytokines, growth factors, and adhesion molecules [31,32]. NF-κB is often related to cell survival, since it controls genes encoding pro-survival proteins, particularly anti-apoptotic members of the Bcl-2 family such as Bcl-2, Bcl-xL, A1, and Mcl-1 [33]. Bcl-2 proteins, which can also be proapoptotic (Bad, Bim, Bik, Bax, Bak among others), are considered both "gatekeepers" and "gate crashers", as they control mitochondrial outer membrane integrity and therefore mitochondrial transmembrane potential ($\Delta\Psi_m$, an early apoptosis indicator), a critical checkpoint of apoptosis [34].

It has been previously shown that LTB4 delays neutrophil spontaneous apoptosis [29,35]. Pétrin and collaborators [36] have reported that LTB4-stimulation activates signaling pathways classically involved in cell survival, such as PI3K and ERK, as well as enhanced Mcl-1 expression. However, the correlation between LTB4-induced delay of neutrophil apoptosis and the generation of ROS evoked by this eicosanoid remained unknown.

This study aims to elucidate the putative role of NADPHox-derived ROS in LTB4-induced anti-apoptotic effect. Adopting a pharmacological approach, based in the use of two structurally and functionally unrelated compounds, diphenyleneiodonium (DPI, a flavoprotein inhibitor extensively used as NADPHox negative modulator) and apocynin (an inhibitor of p47^{phox} cytosolic subunit translocation), we assessed NADPHox involvement in LTB4 anti-apoptotic effect on human peripheral blood neutrophils and the putative role of redox-dependent NF-κB activation on these effects. In order to clarify the underlying molecular mechanisms controlling LTB4 anti-apoptotic effect, the impact of NADPHox and NF-κB systems on the expression of the proapoptotic protein Bad as well as on $\Delta\Psi_m$, of LTB4-stimulated neutrophils was also investigated.

2. Material and methods

2.1. Reagents

DPI, apocynin, pyrrolidine dithiocarbamate (PDTC), Trolox™, bovine serum albumin (BSA), phenylmethylsulphonyl fluoride (PMSF), phorbol myristate acetate (PMA), DNAse, RNAse, polyethylenoglycol-superoxide dismutase (peg-SOD), BAY 11-7082 and soybean trypsin inhibitor (SBTI) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Dulbecco's modified eagle's medium (DMEM) was acquired from GIBCO-BRL (Carlsbad, CA, USA). Polyvinylidene difluoride membrane (PVDF) was obtained from Amersham (Piscataway, NJ, USA). Ficoll-Paque™ was purchased from GE Healthcare. All antibodies utilized were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence system (ECL) was obtained from Pierce Biotechnology (Rockford, IL, USA). JC-1 and annexin V were purchased from Molecular Probes (Eugene, OR, USA). LTB4 originated from Biomol (Plymouth Meeting, PA, USA) and was kindly donated by Dr. Peters-Golden (University of Michigan Health System, Ann Arbor, MI, USA). 12-HHT and LY 255283 were purchased from Cayman (Ann Arbor, MI, USA). CP-105,696 was donated by Dr. H. Showell (Pfizer, Groton, CT, USA).

2.2. Human neutrophil isolation and culture

Neutrophils were isolated from EDTA (0.5%)-treated peripheral venous blood of healthy donors by density gradient centrifugation over Ficoll-Paque™ followed by dextran sedimentation as previously described [37]. Residual erythrocytes were removed by hypotonic lysis by addition of 0.2% sterile saline and solution concentration is reconstituted to 0.9% after addition of equal volume 1.6% sterile saline. Isolated neutrophils were incubated in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin and 100 mg/mL of streptomycin or Hank's balanced salt solution (HBSS), at 37 °C in a humidified atmosphere containing 5% CO$_2$. In all experimental conditions, more than 98% of the cells were viable, according to trypan blue dye exclusion assay.

2.3. ROS production assays

2.3.1. Chemiluminescence assay

Neutrophils (6×10^5 cells/well, final volume of 200 μL) were resuspended in HBSS and placed in a white 96 well plate. Cells were loaded with lucigenin (250 μM) or luminol (500 μM) and pretreated or not with apocynin (10 μM), DPI (10 μM) or polyethylenoglycol-superoxide dismutase (peg-SOD, 200 U/mL) for 15 min at 37 °C in a 5% CO$_2$ incubator. Cells were stimulated or not with LTB4 (30–300 nM) or PMA (30 nM), a known NADPHox activator (positive control). Non-stimulated cells were considered the control group. Chemiluminescence was measured in each well for 20 s throughout 60 min (lucigenin assay) or 10 min (luminol assay) using a VICTOR3 1420 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA).

2.3.2. Cytochrome c reduction assay

Neutrophils (1×10^6) were suspended in HBSS containing cytochrome c (500 μg/mL) and catalase (11 μg/mL). After apocynin (10 μM) pretreatment for 15 min in the indicated groups, cells were stimulated or not with LTB4 (30–300 nM) or PMA (30 nM) in the presence or the absence of SOD (200 U/mL, internal control) for 30 min at 37 °C and 5% CO$_2$ atmosphere. Cells were centrifuged, supernatants were collected and absorbance ($\lambda = 550$ nm) was measured in multilabel plate reader Envision 2104 (PerkinElmer, Waltham, MA, USA). The molar coefficient was calculated as: $\varepsilon_{550} = 21 \times 10^3$ M$^{-1} \times$ cm^{-1}.

2.4. Apoptosis detection

2.4.1. Annexin V-FITC binding

Annexin V is a serum protein that selectively interacts with phosphatidylserine residues, which are exposed on the outer leaflet of the plasma membrane when cells undergo apoptosis [16].

In order to estimate the content of annexin V-positive cells, fresh neutrophils (control group) or neutrophils incubated or not with LTB4 (30–300 nM) for 20 h at 37 °C in a 5% CO$_2$ atmosphere were washed with binding buffer (10 mM HEPES (pH = 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$ and 1.8 mM CaCl$_2$) and resuspended

in 100 μL binding buffer containing annexin V conjugated to FITC (1:500 dilution). After a 20 min-incubation at room temperature in the dark, 400 μL of binding buffer was added and the samples were immediately analyzed in a FACScalibur™ flow cytometer (Becton–Dickinson).

2.4.2. Morphology

After pre-incubation or not with DPI (10 μM), apocynin, (10 μM), PDTC (100 nM), BAY 11-7082 (10 μM), CP-105,696 (1 μM), peg-SOD (200 U/mL), Trolox™ (50 μM) or LY 255283 (0.3 μM) for 15 min, neutrophils were stimulated with LTB_4 (300 nM) or 12-HHT (15–300 nM) for 20 h at 37 °C in a 5% CO_2 incubator. Cells were cytocentrifuged, stained with Diff-Quik™ and counted under an Olympus BX41 optical microscopy (×1000) to determine the proportion of cells showing apoptotic morphological features. Cells were considered apoptotic when displaying pyknotic nuclei and cell volume shrinkage. At least 400 cells were counted per slide.

2.5. Cell extract preparation

For whole cell extracts, neutrophils (5×10^6 cells) were resuspended in lysis buffer (50 mM HEPES, pH 6.4, 1 mM $MgCl_2$, 10 mM EDTA, 1% Triton X-100, 1 μg/mL DNase, 0.5 μg/mL RNase) containing the following protease inhibitors: 1 mM PMSF, 1 mM benzamidine, 1 μM leupeptin, and 1 μM soybean trypsin inhibitor (Sigma-Aldrich). Total protein content was determined by Bradford method [38].

For nuclear extracts, neutrophils (5×10^6 cells) were lysed in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF), and after a 15-min incubation in ice, Nonidet P-40 was added to a final concentration of 0.5% (v/v). Nuclei were collected by centrifugation ($1810 \times g$; 5 min at 4 °C). The nuclear pellet was suspended in ice-cold buffer C (20 mM HEPES (pH = 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 μg/mL pepstatin, 1 μg/mL leupeptin, and 20% (v/v) glycerol) and incubated for 30 min. Nuclear proteins were collected in the supernatant after centrifugation ($12,000 \times g$; 10 min at 4 °C).

2.6. Western Blot analysis

Whole cell as well as nuclear extracts were performed as previously described [22]. Briefly, lysates were denatured in sample buffer containing SDS and 2-mercaptoethanol and heated in boiling water for 3 min. Whole cell extracts (30 μg of protein) or nuclear extracts (25 μL) were submitted to a 12% or 15% SDS-PAGE and proteins were transferred to PVDF membranes. Rainbow molecular weight markers (Amersham Pharmacia Biotech) were run in parallel to estimate molecular weights. Membranes were blocked with tween-PBS containing 2% BSA for 30 min and incubated for approximately 15 h with anti-Bad (1:500), anti-α-tubulin (1:1000), anti-IκB-α (1:500), anti-phosphorylated IκB-α (1:500), anti-NF-κB p65 subunit (1:1000) or anti-Histone H3 (1:1000). After three washes (10 min each) with tween-PBS at room temperature, PVDF membranes were incubated with anti-rabbit or anti-mouse IgG antibodies conjugated to biotin (1:10,000) for 1 h and then incubated with streptavidin conjugated to peroxidase (1:10,000, Caltag Laboratories, Burlingame, CA). Immunoreactive proteins were visualized by ECL system. Protein bands were quantified by optical densitometry by Scion Image software (Scion Co., MD). Results are expressed as the band densitometry mean ± SEM of at least three independent experiments.

2.7. Flow cytometry assessment of mitochondrial transmembrane potential

The mitochondrial stability was measured by the use of the cationic dye JC-1, which incorporates to the mitochondrial intermembrane space. The monomer (green) can polymerize forming clusters known as J-aggregates (red) in a transmembrane potential-dependent manner

[39]. Therefore, viable, non-apoptotic cells exhibit a pronounced reddish fluorescence of mitochondria that can be detectable by flow analysis (in the FL-2 channel). Conversely, apoptotic process results in loss of $\Delta\Psi_m$ and a subsequent decrease of the reddish fluorescence shifting to an increase in green fluoresce (as seen in FL-1 channel, [40]).

Isolated neutrophils (5×10^6/mL) were stimulated with LTB_4 (0–300 nM) after being pretreated or not with apocynin (10 μM) or PDTC (100 nM) for 15 min. Cells were incubated for 20 h at 37 °C in a 5% CO_2 atmosphere. JC-1 dye (10 μg/mL) was then added to each group and after a 30-min incubation at 37 °C in 5% CO_2 atmosphere, labeled cell suspensions were submitted to FACScalibur analysis. FL-1 (515–545 nm) and FL-2 (564–606 nm) channels were assessed. Results expressed are mean ± SEM of FL-2/FL-1 ratio.

2.8. Statistical analysis

Statistical significance was evaluated by Analysis of Variance (ANOVA) followed by Bonferroni comparison test.

3. Results

3.1. Role of NADPHox-derived ROS and NF-κB activation in LTB_4-delayed neutrophil apoptosis

The LTB_4-dependent ROS generation by human neutrophils was evaluated by two different chemiluminogenic probes, lucigenin (selective to extracellular $O_2^{\bullet-}$ generation) and luminol (broad-spectrum, cell permeable probe). As shown in Fig. 1, LTB_4 evoked a transient and potent ROS generation, in a concentration dependent manner, peaking in very early time points (≤1 min; Fig. 1A–B). In order to estimate the ROS accumulation promoted by LTB_4, area under curve was calculated, revealing that only higher concentrations of LTB_4 (100–300 nM) promoted a significant increase in ROS production when compared to non-stimulated cells (Fig. 1C–D). The observed ROS generation was significantly inhibited when cells were pretreated with the NADPHox inhibitors apocynin (10 μM) and DPI (10 μM), strongly suggesting that these ROS are derived from NADPHox (Fig. 1E). Similar results were also observed using the cytochrome *c* reduction assay, a classical ROS generation measurement method (Fig. 1F).

It has been reported that LTB_4 delays neutrophil spontaneous apoptosis in vitro [29,35,36]. Coincidently, this effect was observed in a concentration range in which this eicosanoid is able to promote ROS generation. Accordingly, we observed that LTB_4 was able to inhibit neutrophil spontaneous apoptosis at the same concentration range (Fig. 2A). Moreover, LTB_4-induced inhibition of neutrophil spontaneous apoptosis seems to require NADPHox activity, since LTB_4 pro-survival effect was abrogated by DPI (Fig. 2B–C) and apocynin (Fig. 2D). The ROS scavenger Trolox™ and the cell-permeable antioxidant enzyme peg-SOD (which dismutate superoxide anion) were also able to revert LTB_4-anti-apoptotic effect (Fig 2E). Confirming LTB_4 receptors' involvement, pretreatment of neutrophils with BLT_1 or BLT_2 antagonists, CP-105,696 and LY 255283 respectively, reverted LTB_4 anti-apoptotic effect (Fig. 2 F). Interestingly, NF-κB inhibition by PDTC and BAY 11-7082, also abrogated LTB_4 anti-apoptotic effect (Fig. 2D).

3.2. LTB_4-induced ROS generation impact on NF-κB activation

NF-κB is a transcription factor whose activation is triggered by a number of proinflammatory agents, leading to cell activation, cytokine synthesis and extension of neutrophil lifespan [33]. LTB_4 promoted NF-κB p65 subunit accumulation in nuclear extracts, a parameter of activation of this signaling route. This effect was abrogated by NADPHox inhibition (Fig. 3A). LTB_4 (300 nM) was also able to strongly induce both IκB-α protein degradation and phosphorylation, critical steps to NF-κB activation. NADPHox-derived ROS also plays an essential role in this event, since apocynin pretreatment
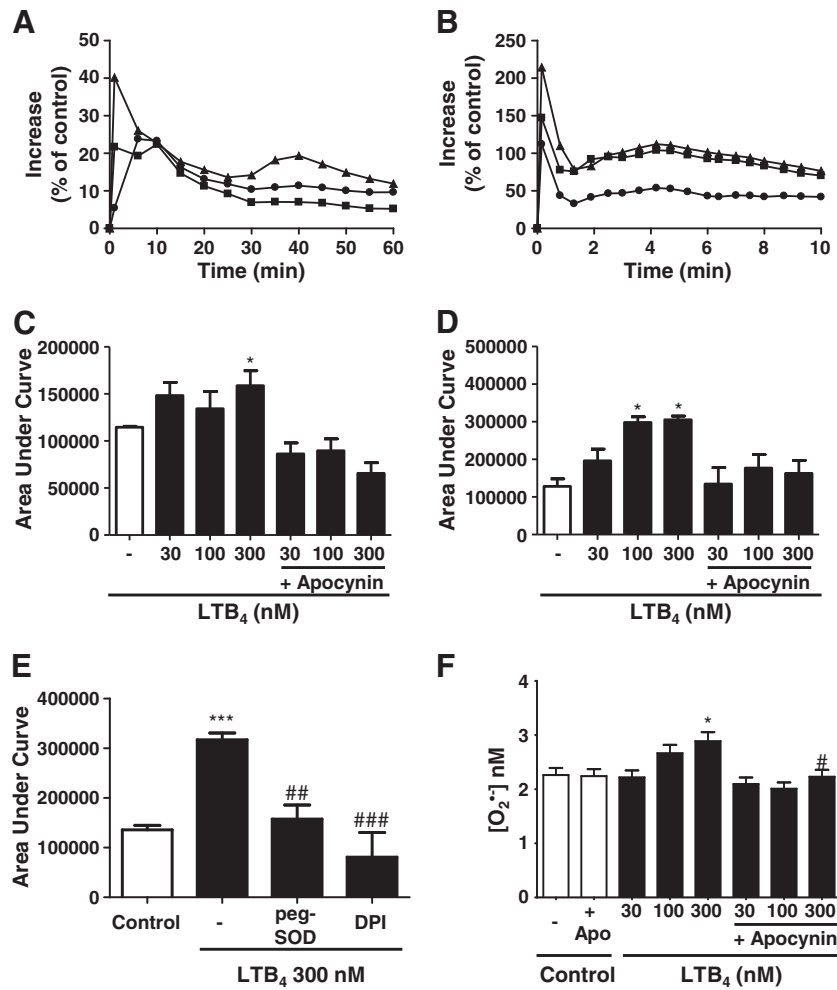
Fig. 1. LTB$_4$ induction of neutrophil ROS production. (A) Neutrophils (6×10^5 cells/well) were placed in a white flat-bottom 96-well plate and stimulated or not with 30 nM (circles), 100 nM (squares) and 300 nM (triangles) of LTB$_4$ in the presence of lucigenin. Data are expressed as percentage of increase when compared to the control group. (B) Same experiment was performed using luminol as chemiluminogenic probe. Data shown are representative of three to six independent experiments. (C–E) Cells were pre-treated or not with apocynin (10 μM), DPI (10 μM) or peg-SOD (200 U/mL) and then stimulated with LTB$_4$ (30–300 nM). ROS accumulation was evaluated calculating the area under curve using lucigenin for 60 min (C), or luminol for 10 min (D, E). (F) Cells (1×10^6 cells/mL) were incubated with cytochrome *c*-supplemented medium in the absence or in the presence of apocynin (10 μM) for 15 min, and stimulated or not with LTB$_4$ (30–300 nM) after 30 min, cytochrome *c* reduction was assessed as detailed in the Material and methods section. Results are mean ± SEM of three to six independent experiments. The asterisks indicate a significant difference when compared to the control (*, $p < 0.05$; ***, $p < 0.001$). #, ## and ### indicate that the treatments significantly reverted LTB$_4$ effects ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively).

inhibited LTB$_4$-induced IκB-α degradation and phosphorylation (Fig. 3B–C), suggesting that LTB$_4$-induced ROS lead to activation of IKKs, resulting in IκB-α phosphorylation and consequent degradation, and therefore NF-κB activation.

3.3. LTB$_4$-induced ROS production on the modulation of proapoptotic members of Bcl-2 family and mitochondrial stability

The apoptotic process is critically regulated by Bcl-2 family proteins, since the complex interaction between its members can cause the preservation or the dissipation of $\Delta\Psi_m$, being the latter a phenomenon closely related to the onset of apoptosis [41]. It had already been described that LTB$_4$ induces Mcl-1 accumulation in human neutrophils [36]. We observed that LTB$_4$ (300 nM) rapidly induced degradation of Bad, a Bcl-2 proapoptotic member, in a NADPHox-derived ROS dependent manner (Fig. 4).

LTB$_4$ (100–300 nM) is able to maintain the FL-2/FL-1 ratio similar to fresh, non-apoptotic neutrophils, thus preventing $\Delta\Psi_m$ dissipation (Fig. 5A). LTB$_4$-induced $\Delta\Psi_m$ maintenance relies on NADPHox-derived

ROS signaling and NF-κB activation, once both apocynin and PDTC abolished LTB$_4$ protective effect (Fig. 5B).

4. Discussion

Our data strongly indicate the importance of ROS signaling, particularly those derived from NADPHox complex, to the LTB$_4$ antiapoptotic effect in human neutrophils in vitro. LTB$_4$ is a potent chemoattractant, exerting its effects at nanomolar concentrations, eliciting adherence and aggregation of leukocytes to the blood vessels [42,43]. Moreover, LTB$_4$ stimulation also induces neutrophil degranulation, leading to increased release of lysosomal enzymes as well as NADPHox enzymatic complex activation which causes subsequent ROS production. Interestingly, the LTB$_4$ secretagogue effect is exerted at higher concentrations when compared to those required for neutrophil chemotaxis [27,44–46].

In our work, we have observed LTB$_4$-induced ROS generation using the chemiluminogenic probes lucigenin and luminol for its sensitivity and accuracy. In addition, these assays allow continuous
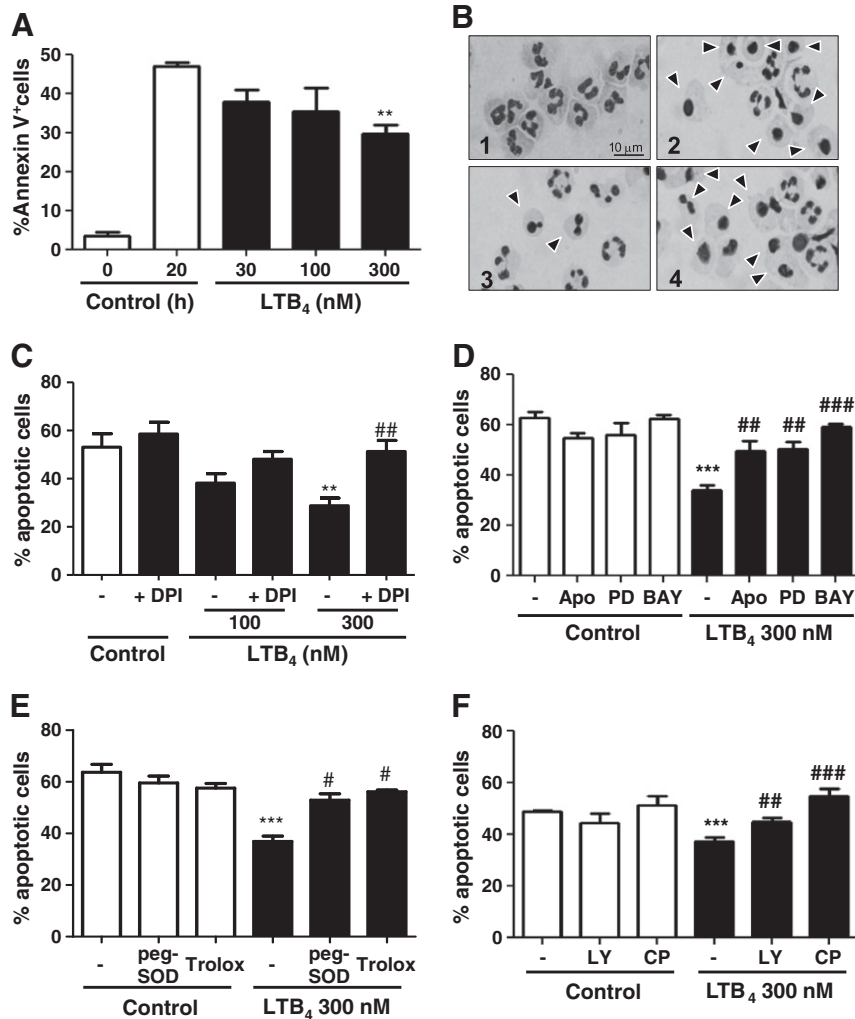
Fig. 2. Role of NADPHox and NF-κB activities on LTB4 anti-apoptotic effect. (A) Annexin V binding to fresh (control 0 h) or aged neutrophils (control 20 h) incubated in the absence or presence of LTB4 (30–300 nM) was assessed by flow cytometry. (B) Morphological analysis of neutrophils: (1) medium alone 0 h; (2) medium alone 20 h; (3) LTB4 300 nM 20 h; and (4) DPI (10 μM) + LTB4 300 nM 20 h. Arrowheads indicate apoptotic neutrophils. (C) Neutrophils (5×10^6/mL) were pre-treated or not with DPI (10 μM), in the absence or in the presence of different concentrations of LTB4 (30–300 nM) for 20 h in vitro. Following pre-incubation with (D) apocynin, (10 μM), PDTC (100 nM), BAY 11-7082 (10 μM) or (E) peg-SOD (200 U/mL), ROS scavenger Trolox™ (50 μM) or (F) LY 255283 (0.3 μM) and CP 105,696 (1 μM), neutrophils were stimulated or not with LTB4 (300 nM). After 20 h, cells were cytocentrifuged and the number of apoptotic cells was determined by morphological analysis. Data shown are the results (mean ± SEM) of three to seven independent experiments. The asterisks indicate a significant difference when compared to the control 20 h (**, $p<0.01$; ***, $p<0.001$). #, ## and ### indicate that the treatments significantly reverted LTB4 effects ($p<0.05$, $p<0.01$ and $p<0.001$, respectively).

monitoring of ROS production in contrast to other fixed-time, end point assays. They also make it possible to discriminate which ROS are being generated [47,48]. Lucigenin is mostly selective for $O_2^{•-}$ released at the extracellular milieu while luminol detects a group of reactive species derived from the myeloperoxidase (MPO)–hydrogen peroxide (H_2O_2) system, which in turn, requires $O_2^{•-}$ as substrate. Among the reactive species detected by luminol are H_2O_2, hydroxyl radical, hypochlorite and peroxynitrite [49].

Our results showed that LTB4 (300 nM) induced ROS production in neutrophils in vitro, which depends on NADPHox activity, by both chemiluminescence methods. Nevertheless, LTB4 (100 nM) ROS production was detected in the luminol assay, an event also modulated by apocynin pretreatment, whereas we did not detect ROS production incited by this concentration using lucigenin assay. As luminol chemiluminescence depends on MPO involvement, it is possible that LTB4 stimulation provokes mobilization of granular NADPHox followed by plasma membrane NADPHox activation [50].

LTB4 inhibition of neutrophil apoptosis seems to require NADPHox-derived ROS, since two different NADPHox inhibitors prevented this prosurvival effect. DPI is a flavoprotein inhibitor

widely used to prevent NADPHox activity [51,52], while apocynin seems to have a more selective effect upon p47^phox assembly and consequent NADPHox activation [53,54]. Some recent works have identified other effects related to apocynin treatment, including non-specific NADPHox inhibition and oxidative stress induction, but only in much higher concentrations [55–57].

We have also observed that peg-SOD and Trolox™ pretreatments also abrogated the LTB4-induced anti-apoptotic effect. These data are in agreement with the concept that ROS signaling mediated by proinflammatory stimuli can trigger prosurvival pathways, supporting ROS ambiguity in cell life/death balance [1,58].

LTB4 biological effects are exerted through two receptors: the widely-studied high affinity receptor BLT1 and the low affinity receptor BLT2. The high concentration range in which LTB4 induces ROS generation has led to the proposition that this effect was modulated by LTB4 binding to BLT2 [59,60]. However, our study suggests that the two receptors are involved in LTB4 anti-apoptotic effect. BLT1 had been related to neutrophil survival [61] whereas BLT2 role in cell survival was only observed in other cell types [62,63]. In fact, we described that BLT2 activation by its agonist 12-HHT [64] can
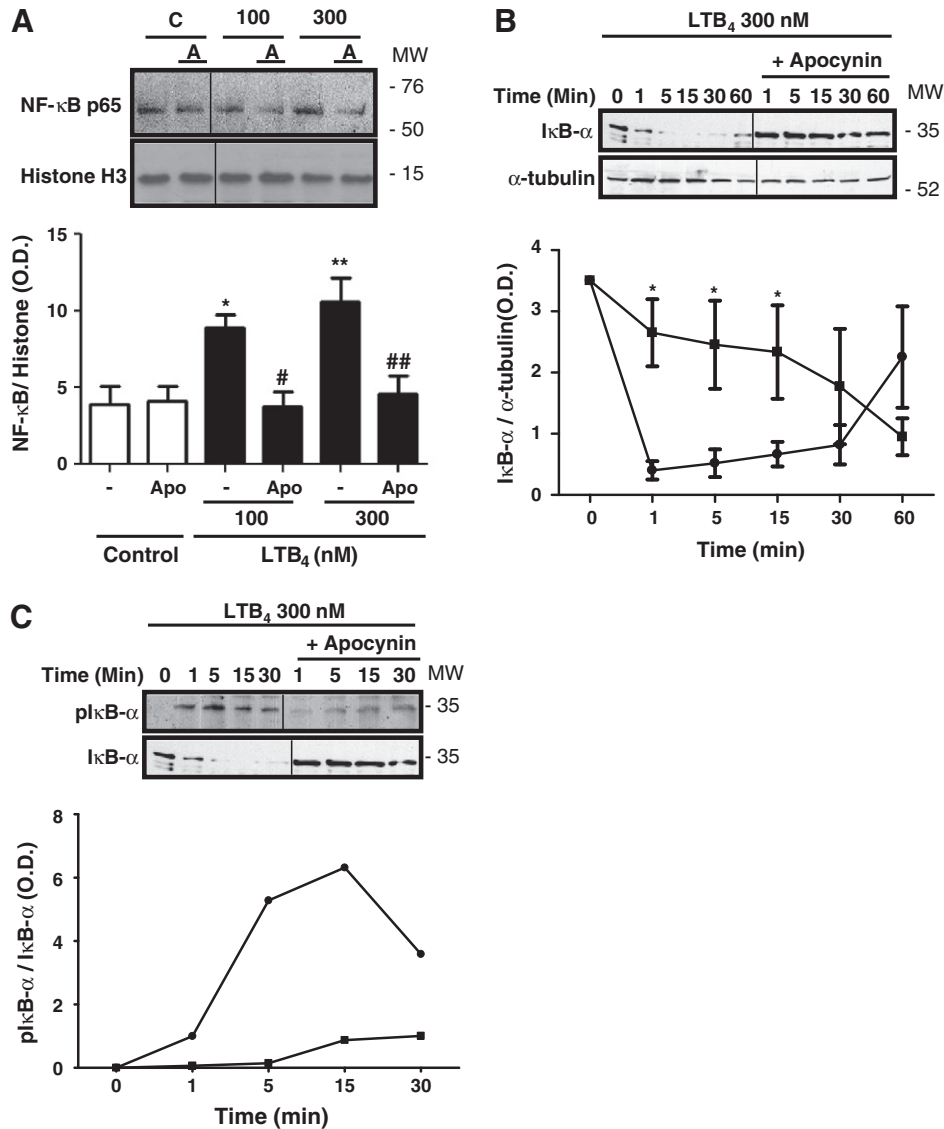
Fig. 3. LTB$_4$ modulation of NF-κB pathway dynamics — involvement of NADPHox-derived ROS. (A) Neutrophils (5×10^6/mL) were incubated for 15 min in the absence or in the presence of apocynin (10 μM) before LTB$_4$ stimulation (100 and 300 nM) for 1 h. The nuclear extracts were obtained and submitted to Western blot. Data are shown as mean ± SEM of five independent experiments. The asterisks indicate a significant difference (*, $p < 0.05$; and ** $p < 0.01$) when compared to the control (non-stimulated cells). # and ##, indicate reversal of LTB$_4$ effect ($p < 0.05$ and $p < 0.01$. respectively). Whole cell extracts of neutrophils (5×10^6/mL) pre-treated (squares) or not (circles) with apocynin (10 μM) and stimulated by LTB$_4$ 300 nM for different times were submitted to electrophoresis and total IκB-α (B) or phosphorylated IκB-α expression (C) was accessed by Western blot. In (B) α-tubulin protein expression was used as equal loading and data are shown as mean ± SEM of five independent experiments. The asterisk indicates a significant difference ($p < 0.05$) between apocynin-treated and non-treated groups. Data shown in (C) are representative of three similar experiments.

delay neutrophil apoptosis, although in a less extensive manner than LTB$_4$ (Supplementary Fig. 1). Redundant roles for BLT receptors [65,66] inclusive a possible heterodimerization mechanism [25], had been already observed and further experiments are necessary to investigate this hypothesis.

NF-κB activation is also critically involved in LTB$_4$-evoked pro-survival signaling, as supported by our observations that pretreatment with both PDTC and BAY 11-7082, a highly selective NF-κB inhibitor, prevented LTB$_4$ anti-apoptotic effect. It has been reported previously that lower LTB$_4$ (<30 nM) was only able to trigger a mild NF-κB activation [31]. Furthermore, it seems that the 100–300 nM concentration range is critical to induce ROS-dependent p65 subunit translocation to the nucleus as well as to induce a prompt redox-sensitive IκB-α phosphorylation and degradation. We hope these results corroborate to strengthen ROS-mediated modulation of NF-κB as a key mechanism involved in immunity and inflammation [18,67,68].

Our data are in agreement with work by Pétrin and collaborators [36], which shows that sub-micromolar concentrations of LTB4

promote inhibition of neutrophil spontaneous apoptosis in a PI3K- and MAPK-dependent manner, involving induction of Mcl-1 expression. Noteworthy, both pathways are redox-sensitive signaling routes that converge to NF-κB activation, that corroborates to our hypothesis.

LTB$_4$ stimulation alters the expression of Bcl-2 family proteins, which are devoted to the control of apoptosis at the mitochondrial level [36,69]. Resting neutrophils, as found in circulation under normal conditions, express low levels of anti-apoptotic Bcl-2 family proteins, contrasting with extremely high expression of proapoptotic ones. Proinflammatory/anti-apoptotic factors invert this scenario: they promote a dramatic decrease in proapoptotic Bcl-2 proteins availability (both inducing proteolysis and heterodimerization with 14-3-3 proteins [70]), while inducing the expression of anti-apoptotic members [71,72]. As expected, LTB$_4$ promotes rapid Bad degradation in a NADPHox activity-dependent fashion, which is in agreement with reports describing modulation of Bcl-2 family expression by ROS [18,73]. This transient, but dramatic decrease in Bad cellular content may prevent the insertion of proapoptotic Bcl-2 members (Bax, Bak) in the
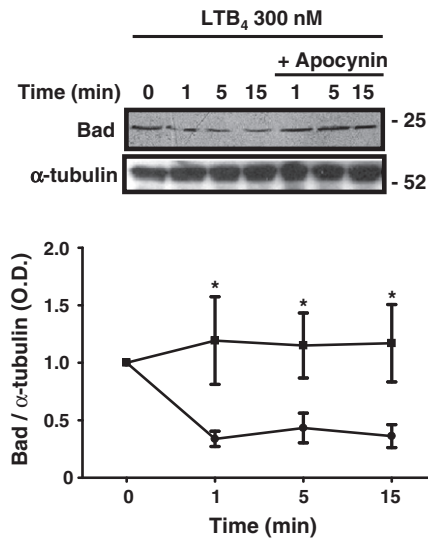
Fig. 4. LTB$_4$ modulation of Bad protein levels. Neutrophils (5×10^6/mL) were pre-treated (squares) or not (circles) with apocynin (10 μM) and then stimulated with LTB$_4$ (300 nM) for 0–15 min. Cells were then harvested and whole cell extracts were submitted to Western Blot procedure to access Bad expression. α-Tubulin protein expression was used as load control. Data shown are expressed as mean ± SEM of three independent experiments. Asterisks indicate a significant difference ($p < 0.05$) between apocynin-treated and non-treated groups.

mitochondrial outer membrane, resulting in $\Delta\Psi_m$ maintenance, thus avoiding the release of proapoptotic factors confined to the mitochondrial intermembrane space to the cytosol. We demonstrated in this study that NADPHox-derived ROS are critical to $\Delta\Psi_m$ preservation mediated by LTB$_4$. NF-κB activity was also essential to LTB$_4$-induced mitochondrial stability, probably inducing expression of anti-apoptotic Bcl-2 members, such as Mcl-1 [36].
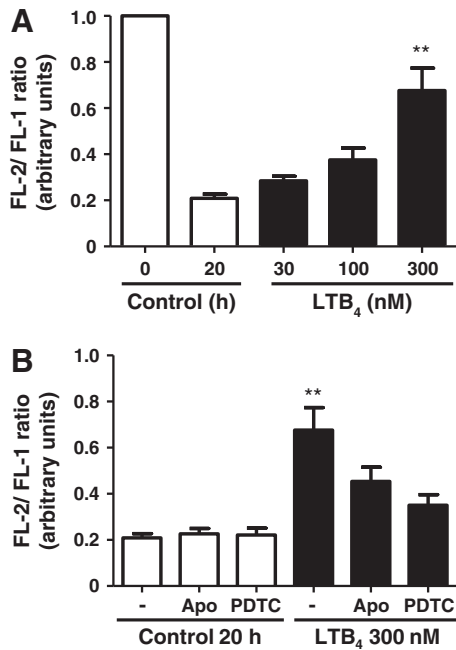

Fig. 5. Role of NADPHox and NF-κB activities on LTB$_4$-evoked $\Delta\Psi_m$ maintenance. Neutrophils (5×10^6/mL) were incubated for 20 h in the absence or in the presence of (A) increasing concentrations of LTB$_4$ (30–300 nM); or (B) LTB$_4$ 300 nM with or without apocynin (10 μM) or PDTC (100 nM). Cells were then stained with JC-1 dye and analyzed by flow cytometry. Results displayed as the ratio between reddish (FL-2) and greenish (FL-1) JC-1-emitted fluorescence. Data shown are the results (mean ± SEM) of three independent experiments, with the asterisks (**) indicating a significant difference ($p < 0.01$) when compared to the control (20 h) group.

5. Conclusions

In summary, we demonstrate that LTB$_4$, at concentrations found in inflammatory sites, evokes NADPHox activation and subsequent ROS production, leading to neutrophil survival. This effect relies on redox-sensitive NF-κB activation as well as Bad degradation, maintaining mitochondrial stability. As LTB$_4$-induced anti-apoptotic effect is involved in many inflammatory conditions, elucidating the molecular events responsible for its signaling could help to design more rational and selective therapies, particularly those that aim to control chronic inflammatory disorders.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.07.012.

References

[1] M.A. Arruda, C. Barja-Fidalgo, NADPH oxidase activity: in the crossroad of neutrophil life and death, Front. Biosci. 14 (2009) 4546–4556.
[2] M.T. Quinn, M.C. Ammons, F.R. Deleo, The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction, Clin. Sci. (Lond.) 111 (2006) 1–20.
[3] S.J. Chanock, J. El Benna, R.M. Smith, B.M. Babior, The respiratory burst oxidase, J. Biol. Chem. 269 (1994) 24519–24522.
[4] C. Kim, M.C. Dinauer, Rac2 is an essential regulator of neutrophils nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways, J. Immunol. 166 (2001) 1223–1232.
[5] G.Y.N. Iyer, M.F. Islam, J.H. Quastel, Biochemical aspects of phagocytosis, Nature 192 (1961) 535–540.
[6] J.D. Lambeth, G. Cheng, R.S. Arnold, W.A. Edens, Novel homologs of gp91phox, Trends Biochem. Sci. 25 (2000) 459–461.
[7] B.M. Babior, J.D. Lambeth, W. Nauseef, The neutrophil NADPH oxidase, Arch. Biochem. Biophys. 397 (2002) 342–344.
[8] W. Dröge, Free radicals in the physiological control of cell function, Physiol. Rev. 82 (2002) 47–95.
[9] K.R. Martin, J.C Barret, Reactive oxygen species as double-edged swords in cellular processes: low-dose cell signaling versus high-dose toxicity, Hum. Exp. Toxicol. 21 (2002) 71–75.
[10] M. Higuchi, T. Honda, R.J. Proske, E.T.H. Yeh, Regulation of reactive oxygen species-induced apoptosis and necrosis by caspase 3-like proteases, Oncogene 17 (1998) 2753–2760.
[11] E. Rollet-Labelle, M.J. Grange, C. Elbim, C. Marquetty, M.A. Gougerot-Pocidalo, C. Pasquier, Hydroxyl radical as a potential intracellular mediator of polymorphonuclear neutrophils apoptosis, Free Radic. Biol. Med. 24 (1998) 563–572.
[12] J. Chandra, A. Samali, S. Orrenius, Triggering and modulation of apoptosis by oxidative stress, Free Radic. Biol. Med. 29 (2000) 323–333.
[13] B. Zhang, J. Hirahashi, X. Cullere, T.N. Mayadas, Elucidation of molecular events leading to neutrophil apoptosis following phagocytosis: cross-talk between caspase 8, reactive oxygen species, and MAPK/ERK activation, J. Biol. Chem. 278 (2003) 28443–28454.
[14] T.N. Mayadas, X. Cullere, Neutrophil beta2 integrins: moderators of life or death decisions, Trends Immunol. 26 (2005) 388–395.
[15] E.M. Conner, M.B. Grisham, Inflammation, free radicals and antioxidants, Nutrition 12 (1996) 274–277.
[16] B. Fadeel, A. Ahlin, J.I. Henter, S. Orrenius, M.B. Hampton, Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species, Blood 92 (1998) 4808–4818.
[17] K. Asehnoune, D. Strassheim, S. Mitra, J.Y. Kim, E. Abraham, Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kappa B, J. Immunol. 172 (2004) 2522–2529.
[18] M.A. Arruda, P. Barcellos-de-Souza, A.L. Sampaio, A.G. Rossi, A.V. Graça-Souza, C. Barja-Fidalgo, NADPH oxidase-derived ROS: key modulators of heme-induced mitochondrial stability in human neutrophils, Exp. Cell Res. 312 (2006) 3939–3948.

[19] F. Colotta, F. Re, N. Polentarutti, S. Sozzani, A. Mantovani, Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products, Blood 80 (1992) 2012–2020.

[20] C. Haslett, A. Lee, J.S. Savill, L. Meagher, M.K. Whyte, Apoptosis (programmed cell death) and functional changes in aging neutrophils. Modulation by inflammatory mediators, Chest 99 (3 Suppl.) (1991) 6S.

[21] J.B. Klein, M.J. Rane, J.A. Scherzer, P.Y. Coxon, R. Kettritz, J.M. Mathiesen, A. Buridi, K.R. McLeish, Granulocyte–macrophage colony stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways, J. Immunol. 164 (2000) 4286–4291.

[22] M.A. Arruda, A.G. Rossi, M.S. de Freitas, C. Barja-Fidalgo, A.V. Graça-Souza, Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-kappaB, J. Immunol. 173 (2004) 2023–2030.

[23] C.N. Serhan, J. Savill, Resolution of inflammation: the beginning programs the end, Nat. Immunol. 6 (2005) 1191–1197.

[24] G. Bannenberg, M. Arita, C.N. Serhan, Endogenous receptor agonists: resolving inflammation, ScientificWorldJournal 7 (2007) 1440–1462.

[25] K.A. Lundeen, B. Sun, L. Karlsson, A.M. Fourie, Leukotriene B4 receptors BLT1 and BLT2: expression and function in human and murine mast cells, J. Immunol. 177 (2006) 3439–3447.

[26] T.G. Brock, M. Peters-Golden, Activation and regulation of cellular eicosanoid biosynthesis, ScientificWorldJournal 7 (2007) 1273–1284.

[27] M.P. Wymann, V. von Tscharner, D.A. Deranleau, M. Baggiolini, The onset of the respiratory burst in human neutrophils. Real-time studies of H_2O_2 formation reveal a rapid agonist-induced transduction process, J. Biol. Chem. 262 (1987) 12048–12053.

[28] W.R. Henderson, The role of leukotrienes in inflammation, Ann. Intern. Med. 121 (1994) 684–697.

[29] M.J. Hébert, T. Takano, H. Holthöfer, H.R. Brady, Sequential morphologic events during apoptosis of human neutrophils. Modulation by lipoxygenase-derived eicosanoids, J. Immunol. 157 (1996) 3105–3115.

[30] L.F. Chen, W.C. Greene, Shaping the nuclear action of NF-κB, Nat. Rev. Mol. Cell Biol. 5 (2004) 392–401.

[31] P.P. McDonald, A. Bald, M.A. Cassatella, Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils, Blood 89 (1997) 3421–3433.

[32] M. Karin, Y. Ben-Neriah, Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity, Annu. Rev. Immunol. 18 (2000) 621–663.

[33] S. François, J. El Benna, P.M. Dang, E. Pedruzzi, M.A. Gougerot-Pocidalo, C. Elbim, Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF-kappaB signaling pathways, leading to increased levels of Mcl-1, A1 and phosphorylated Bad, J. Immunol. 80 (2005) 554–561.

[34] H. Clevers, At the crossroads of inflammation and cancer, Cell 118 (2004) 671–674.

[35] J. Murray, C. Ward, J.T. O'Flaherty, I. Dransfield, C. Haslett, E.R. Chilvers, A.G. Rossi, Role of leukotrienes in the regulation of human granulocyte behaviour: dissociation between agonist-induced activation and retardation of apoptosis, Br. J. Pharmacol. 139 (2003) 388–398.

[36] D. Pétrin, S. Turcotte, A.K. Gilbert, M. Rola-Pleszczynski, J. Stankova, The anti-apoptotic effect of leukotriene B4 in neutrophils: a role for phosphatidylinositol 3-kinase, extracellular signal-regulated kinase and Mcl-1, Cell. Signal. 18 (2006) 479–487.

[37] A. Boyum, Isolation of lymphocytes, granulocytes and macrophages, Scand. J. Immunol. 5 (1976) 9–15.

[38] M. Bradford, A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.

[39] G. Fossati, D.A. Moulding, D.G. Spiller, R.J. Moots, M.R. White, S.W. Edwards, The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis, J. Immunol. 170 (2003) 1964–1972.

[40] M. Ankarcrona, J.M. Dypbukt, E. Bonfoco, B. Zhivotovsky, S. Orrenius, S.A. Lipton, P. Nicotera, Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function, Neuron 15 (1995) 961–973.

[41] H. Harada, S. Grant, Apoptosis regulators, Rev. Clin. Exp. Hematol. 7 (2003) 117–138.

[42] A.W. Ford-Hutchinson, M.A. Bray, M.V. Doig, M.E. Shipley, M.J. Smith, Leukotriene B₄, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes, Nature 286 (1980) 264–265.

[43] R.A. Lewis, K.F. Austen, R.J. Soberman, Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases, N. Engl. J. Med. 323 (1990) 645–655.

[44] C.N. Serhan, A. Radin, J.E. Smolen, H. Korchak, B. Samuelsson, G. Weissmann, Leukotriene B4 is a complete secretagogue in human neutrophils: a kinetic analysis, Biochem. Biophys. Res. Commun. 107 (1982) 1006–1012.

[45] A.G. Rossi, D.E. MacIntyre, C.J. Jones, R.M. McMillan, Stimulation of human polymorphonuclear leukocytes by leukotriene B4 and platelet-activating factor: an ultrastructural and pharmacological study, J. Leukoc. Biol. 53 (1993) 117–125.

[46] L. Flamand, M.J. Tremblay, P. Borgeat, Leukotriene B4 triggers the in vitro and in vivo release of potent antimicrobial agents, J. Immunol. 178 (2007) 8036–8045.

[47] R.C. Allen, Phagocytic leukocyte oxygenation activities and chemiluminescence: a kinetic approach to analysis, Methods Enzymol. 133 (1986) 449–493.

[48] H. Hasegawa, K. Suzuki, S. Nakaji, K. Sugawara, Analysis and assessment of the capacity of neutrophils to produce reactive oxygen species in a 96-well microplate format using lucigenin- and luminol-dependent chemiluminescence, J. Immunol. Methods 210 (1997) 1–10.

[49] A.J. Kettle, C.C. Winterbourn, Myeloperoxidase: a key regulator of neutrophil oxidant production, Redox Rep. 3 (1998) 3–15.

[50] A. Karlsson, C. Dahlgren, Assembly and activation of the neutrophils NADPH oxidase in granule membranes, Antioxid. Redox Signal. 4 (2002) 49–60.

[51] A.R. Cross, O.T. Jones, The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils. Specific labelling of a component polypeptide of the oxidase, Biochem. J. 237 (1986) 111–116.

[52] D. Sanmun, E. Witasp, S. Jitkaew, Y.Y. Tyurina, V.E. Kagan, A. Ahlin, J. Palmblad, B. Fadeel, Involvement of a functional NADPH oxidase in neutrophils and macrophages during programmed cell clearance: implications for chronic granulomatous disease, Am. J. Physiol. Cell Physiol. 297 (2009) C621–C631.

[53] J. Stolk, T.J. Hiltermann, J.H. Dijkman, A.J. Verhoeven, Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol, Am. J. Respir. Cell Mol. Biol. 11 (1994) 95–102.

[54] V.F. Ximenes, M.P. Kanegae, S.R. Rissato, M.S. Galhiane, The oxidation of apocynin catalyzed by myeloperoxidase: proposal for NADPH oxidase inhibition, Arch. Biochem. Biophys. 457 (2007) 134–141.

[55] M. Vejrazka, R. Mícek, S. Stípek, Apocynin inhibits NADPH oxidase in phagocytes but stimulates ROS production in non-phagocytic cells, Biochim. Biophys. Acta 722 (2005) 143–147.

[56] C. Riganti, C. Costamagna, A. Bosia, D. Ghigo, The NADPH oxidase inhibitor apocynin (acetovanillone) induces oxidative stress, Toxicol. Appl. Pharmacol. 212 (2006) 179–187.

[57] J. Stefanska, R. Pawliczak, Apocynin: molecular aptitudes, Mediators Inflamm. (2008) 106507 (Epub 2008 Dec 2).

[58] J.P. Fruehauf, F.L. Meyskens Jr., Reactive oxygen species: a breath of life or death? Clin. Cancer Res. 13 (2007) 789–794.

[59] C.H. Woo, M.H. Yoo, H.J. You, S.H. Cho, Y.C. Mun, C.M. Seong, J.H. Kim, Transepithelial migration of neutrophils in response to leukotriene B4 is mediated by a reactive oxygen species-extracellular signal-regulated kinase-linked cascade, J. Immunol. 170 (2003) 6273–6279.

[60] C. Kim, J.Y. Kim, J.H. Kim, Cytosolic phospholipase A(2), lipoxygenase metabolites, and reactive oxygen species, BMB Rep. 41 (2008) 555–559.

[61] J. Stankova, S. Turcotte, J. Harris, M. Rola-Pleszczynski, Modulation of leukotriene B4 receptor-1 expression by dexamethasone: potential mechanism for enhanced neutrophil survival, J. Immunol. 168 (2002) 3570–3576.

[62] J.M. Seo, K.J. Cho, E.Y. Kim, M.H. Choi, B.C. Chung, J.H. Kim, Up-regulation of BLT2 is critical for the survival of bladder cancer cells, Exp. Mol. Med. 43 (2011) 129–137.

[63] K.J. Cho, J.M. Seo, J.H. Kim, Bioactive lipoxygenase metabolites stimulation of NADPH oxidases and reactive oxygen species, Mol. Cells 32 (2011) 1–5.

[64] T. Okuno, Y. Iizuka, H. Okazaki, T. Yokomizo, R. Taguchi, T. Shimizu, 12(S)-Hydroxyheptadeca-5Z, 8E, 10E-trienoic acid is a natural ligand for leukotriene B4 receptor 2, J. Exp. Med. 205 (2008) 759–766.

[65] E. Sánchez-Galán, A. Gómez-Hernández, C. Vidal, J.L. Martín-Ventura, L.M. Blanco-Colio, B. Muñoz-García, L. Ortega, J. Egido, J. Tuñón, Leukotriene B4 enhances the activity of nuclear factor-kappaB pathway through BLT1 and BLT2 receptors in atherosclerosis, Cardiovasc. Res. 81 (2009) 216–225.

[66] Y.H. Nam, A. Min, S.H. Kim, Y.A. Lee, K.A. Kim, K.J. Song, M.H. Shin, Leukotriene B(4) receptors BLT1 and BLT2 are involved in interleukin-8 production in human neutrophils induced by Trichomonas vaginalis-derived secretory products, Inflamm. Res. 61 (2012) 97–102.

[67] R. Schreck, P. Rieber, P.A. Baeuerle, Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1, EMBO J. 10 (1991) 2247–2258.

[68] C. Bubici, S. Papa, K. Dean, G. Franzoso, Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance, Oncogene 25 (2006) 6731–6748.

[69] J.F. Ohd, C. Wikström, A. Sjölander, Leukotrienes induce cell-survival signaling in intestinal epithelial cells, Gastroenterology 119 (2000) 1007–1018.

[70] S.R. Datta, A. Katsov, L. Hu, A. Petros, S.W. Fesik, M.B. Yaffe, M.E. Greenberg, 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation, Mol. Cell 6 (2000) 41–51.

[71] P. Weinmann, P. Gaehtgens, B. Walzog, Bcl-Xl- and Bax-alpha mediated regulation of apoptosis of human neutrophils via caspase-3, Blood 93 (1999) 3106–3115.

[72] H.R. Luo, F. Loison, Constitutive neutrophil apoptosis: mechanisms and regulation, Am. J. Hematol. 83 (2008) 288–295.

[73] N. Perskvist, M. Long, O. Stendahl, L. Zheng, Mycobacterium tuberculosis promotes apoptosis in human neutrophils by activating caspase-3 and altering expression of Bax/Bcl-xL via an oxygen-dependent pathway, J. Immunol. 168 (2002) 6358–6365.