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Involvement of p38 MAPK in haemozoin-dependent MMP-9 enhancement in human monocytes

RUNNING TITLE: P38 MAPK AND MMP-9 IN HAEMOZOIN-FED MONOCYTES

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Abstract

The lipid moiety of natural haemozoin (nHZ, malarial pigment) was previously shown to enhance expression and release of human monocyte matrix metalloproteinase-9 (MMP-9), and a major role for its lipoperoxidation product 15-hydroxy-eicosatetraenoic acid (15-HETE) was proposed. Here the underlying mechanisms were investigated, focusing on the involvement of p38 mitogen-activated protein kinase (MAPK). Results showed that either natural nHZ or 15-HETE promoted p38 MAPK late phosphorylation without affecting basal protein levels, whereas lipid-free synthetic (s)HZ and delipidized (d)HZ did not. SB230580, a synthetic inhibitor of p38 MAPK, abrogated nHZ- and 15-HETE-increased mRNA/protein expression of proMMP-9, as well as nHZ- and 15-HETE-enhanced release of active MMP-9. These data suggest that 15-HETE, a major component of the lipid moiety of nHZ, upregulates MMP-9 expression and release in human monocytes through activation of p38 MAPK pathway. The present work provides new evidence on mechanisms underlying MMP-9 dysregulation in malaria, which might be helpful to design new specific drugs for adjuvant therapy in complicated malaria.

Key words

haemozoin (HZ); 15-hydroxy-eicosatetraenoic acid (15-HETE); matrix metalloproteinase-9 (MMP-9); p38 mitogen-activated protein kinase (P38 MAPK); human monocyte; *falciparum* malaria.

Introduction

Matrix metalloproteinase-9 (MMP-9) is an extracellularly acting Zn^{2+}-dependent endopeptidase subject to complex regulation at the level of transcription, translation and protein activation, as it is released extracellularly in a latent pro-form with the enzymatic site covered by a propeptide that has to be cleaved off to reveal the activity (Van den Steen PE et al 2002). MMP-9 can process a large kaleidoscope of substrates, including proteins of the extracellular matrix, precursors of pro-inflammatory molecules (cytokines and chemokines), growth factors, adhesion and junction molecules, and haemostatic factors: as a consequence of cleavage by MMP-9, these molecules undergo different fates, including shedding, activity modulation, or degradation (Cauwe B et al 2007). This makes MMP-9 a pivotal molecule in either physiological processes, such as developmental tissue morphogenesis and wound healing (Kähäri VM and Saarialho-Kere U 1997), or pathological, including cancer (Ito Y and Nagase H 2002) and neuro-inflammation (Rosenberg GA 2002).

In the recent years, growing evidence on the involvement of MMP-9 in malaria - the infectious disease caused by parasites of the genus *Plasmodium* - has emerged from either *in vivo* or *in vitro* studies, and a possible role as marker of disease severity, as well as candidate target for adjuvant therapy has been proposed (Prato M and Giribaldi G 2011; Geurts N et al 2012; Prato M and Giribaldi G 2012; Piña-Vázquez C et al. 2012). This appears extremely important, as the most part of deaths due to malaria (655.000 in 2010, among more than 200 million clinical cases (WHO 2011)) occur after insurgence of dramatic complications such as cerebral malaria, pulmonary oedema, acute renal failure and severe anaemia (Medana IM and Turner GD 2006; Mohan A et al 2008; Buffet PA et al 2011; Das BS 2008). Unfortunately, mechanisms underlying the clinical switch towards complicated malaria are not fully understood. Basically, it is characterized by the binding of *Plasmodium*-infected red blood cells (IRBCs) to the microvascular endothelium (cytoadherence) and to non-infected erythrocytes (rosetting), thereby generating a reduction in the blood flow, and resulting in tissue hypoxia and necrosis (Dondorp AM et al 2004).

Besides, after phagocytosis of circulating IRBCs and free natural haemozoin (nHZ, malaria pigment), a parasite waste product from haemoglobin digestion (Egan TJ 2008), human monocytes undergo a serious functional impairment, coupled with an exacerbated pro-inflammatory response and not accompanied by cell death (Prato M and Giribaldi G 2011). nHZ has a scaffold structure composed by the ferric haem and the lipid moiety, which contains large amounts of mostly esterified monohydroxy derivatives (OH-PUFAs: hydoxy-octadecadienoic acids, HODEs; and hydoxy-eicosatetraenoic acids, HETEs), the stable end products of peroxidation of polyenoic fatty acids, generated through non-enzymatic haem-catalysis (Schwarzer E et al 2003). The lipid moiety of nHZ has been related to several impaired functions of human monocytes (Prato M and Giribaldi G 2011). In particular, in human monocytes 15-HETE was previously associated with nHZ-dependent enhancement of expression and release of MMP-9 and several MMP-9-related pro-inflammatory molecules, including IL-1β, TNF-α, IL-8/CXCL8, and ENA-78/CXCL5 (Prato M et al 2008, Prato M et al 2010a, Giribaldi G et al 2010). The mechanisms underlying nHZ-dependent upregulation of MMP-9 are not entirely known, although convincing evidence on the involvement of NF-kappaB pathway was recently reported (Prato M et al 2010b; Dell'Agli M et al. 2010). Nonetheless, nHZ-enhanced MMP-9 expression and release could also depend on several other complementary routes. In particular, mitogen-associated protein kinases (MAPKs), which were previously related to MMP-9 enhancement in human monocytes (Lai WC et al, 2003; Heidinger M et al 2006; Nguyen J et al 2006), might be involved.

In a recent work, we showed that phagocytosis of nHZ promoted early phosphorylation of p38 MAPK in human adherent monocytes, an event directly related to monocyte degranulation and early lysozyme release (Polimeni M et al 2012a). However, no data on long-term activation of p38 MAPK pathway were available so far, and the role of p38 MAPK in nHZ-enhanced expression and release of human monocyte MMP-9 was not known yet. Thus, the present work aimed at

investigating the long-term effects of the different components of nHZ (haem and lipid moiety) on p38 MAPK in human adherent monocytes, along with the possible involvement of p38 MAPK in nHZ- and 15-HETE-dependent upregulation of MMP-9 expression and release.

Materials and Methods

Materials

Unless otherwise stated, reagents were obtained from Sigma-Aldrich, St. Louis, MO. Sterile plastics were from Costar, Cambridge, UK; Panserin 601 monocyte medium was from PAN Biotech, Aidenbach, Germany; Percoll was from Pharmacia, Uppsala, Sweden; Diff-Quik parasite stain was from Baxter Dade AG, Dudingen, Switzerland; TRIzol, M-MLV, oligo-dT, sense and anti-sense primers and Platinum Taq DNA Polymerase were from Invitrogen, Carlsbad, CA; DNA-free kit was from Ambion, Austin, TX; Beacon Designer 2.1 software was from Premier Biosoft International, Palo Alto, CA; dNTPs were from Applied Biosystem, Foster City, CA; 15-HETE were from Cayman, Ann Arbor, MI; p38 MAPK inhibitor SB230580 was from Cell Signaling Technology, Danvers, MA; anti-human MMP-9, p38 MAPK, and phospho-p38 MAPK monoclonal antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA; ECL Kit and HRP-conjugated anti-mouse secondary antibodies were from GE-Healthcare, Milan, Italy; Geldoc computerized densitometer and electrophoresis reagents were from Bio-Rad Laboratories, Hercules, CA.

Culturing of P. falciparum, isolation of nHZ, and preparation of dHZ and sHZ

P. falciparum parasites (Palo Alto strain, mycoplasma-free) were kept in culture as described (Prato M et al 2005). After centrifugation at 5,000 x *g* on a discontinuous Percoll-mannitol density gradient, nHZ was collected from the 0–40% interphase, washed five times with 10 mM HEPES (pH 8.0) containing 10 mM mannitol at 4°C and once with PBS, and stored at 20% (v/v) in PBS at –20°C or immediately used for opsonization and phagocytosis. dHZ and sHZ were prepared as previously reported (Prato M et al 2011).

Preparation and handling of monocytes

Human monocytes were separated by Ficoll centrifugation from freshly collected buffy coats discarded from blood donations by healthy adult donors of both sexes provided by the local blood bank (AVIS, Associazione Volontari Italiani Sangue, Torino, Italy) (Prato M et al 2010c). Separated lympho-monocytes were resuspended in RPMI 1640 medium and plated on wells of 6-well plates. Each well received 2 ml of cell suspension containing $15x10^6$ cells in RPMI 1640. The plates were incubated in a humidified CO_2/air-incubator at 37°C overnight. The day after, non-adherent cells were removed by 3 washes with RPMI 1640, and adherent cells were reincubated at 37°C in RPMI 1640. Shortly before starting phagocytosis, wells were washed with RPMI 1640 and Panserin medium added (2 ml/well).

Pre-selection of NF-κB-quiescent monocytes by flow cytometry and Real Time RT-PCR

Before starting experiments, a pre-selection of cell populations was taken as a precautionary measure, as previously described (Prato M et al 2010b). First, cell cultures isolated through Ficoll separation were analyzed by flow cytometry: only cell populations showing at least 70% monocytes were used for following experiments. Second, in order to avoid the use of NF-κB pre-activated monocytes, cells were analyzed by Real Time RT-PCR: in each cell preparation a cell aliquot was stimulated or not with LPS (1 μg/ml) for 4 h, and TNFα RNA production was measured in lysates by Real Time RT-PCR. GAPDH was used as house-keeping gene. Only unstimulated monocyte

populations ("NF-κB-quiescent" cells) showing at least a 3-PCR-cycles gap of cDNA amplification between controls and LPS-stimulated cells were used for following experiments.

Phagocytosis of nHZ, sHZ, and dHZ and treatments with 15-HETE and SB203580.

To each well of a 6-well plate with approximately $2x10^6$ adherent monocytes, nHZ, dHZ, or sHZ (120 nmoles HZ haem, an amount comparable to 50 µl trophozoites on haem content basis) were added. All phagocytic meals were opsonized with fresh autologous serum. Alternatively 1 µM 15-HETE was added. The plates were incubated in a humidified CO_2/air-incubator at 37°C for 2 h. After the end of the phagocytic period, cells were checked by optical microscopy: as an average, HZ-containing monocytes were 25-35% of total cells, a percentage compatible with *in vivo* levels measured in patients with severe malaria showing high parasitaemia (Were T et al 2009). Therefore, cells were washed and incubated in Panserin 601 medium for 15 h or 24 h in the presence/absence of p38 MAPK inhibitor SB230580. Therefore, cell supernatants were collected, and cells were lysed for mRNA and protein extraction.

mRNA extraction from cell lysates

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO_2/air-incubator at 37°C for 15 h. Total cellular RNA from $2x10^6$ cells was isolated from monocytes by TRIzol, according to the manufacturer's instructions, and eluted in 20 µl diethyl pyrocarbonate water. To remove any contaminating DNA, RNA was treated with Ambion's DNA-free kit. After extraction, the concentration of RNA was determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer using quartz cuvettes.

Isolation of cytosolic protein fractions in cell lysates.

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO_2/air-incubator at 37°C for 24 h. Thereafter cells were mechanically scraped in PBS and washed, then resuspended in lysis buffer (15 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 µg/ml aprotinin, 2 µg/ml leupeptin, 0.1% NP-40, pH 7.6). Cell suspensions were then incubated for 15 min on ice with occasional vortexing, and centrifuged for 30 s to pellet nuclei. Supernatants with cytosolic proteins were collected for following experiments.

Measurement of MMP-9 mRNA levels in cell lysates by Real Time RT-PCR

6 µg of RNA were reverse transcribed into single-stranded cDNA using M-MLV (200 U/µl final concentration) and oligo-dT (25 µg/µl final concentration). Real Time RT-PCR analysis was performed with the iCycler Instrument and the iCycler iQ Real Time Detection System Software version 3.0 (Biorad, Hercules, CA) as previously described (Giribaldi G et al 2011). MMP-9 (GenBank accession no. NM_004994) primers (forward: 5'-CCT GGA GAC CTG AGA ACC AAT C-3'; reverse: 5'-CTC TGC CAC CCG AGT GTA AC-3') were obtained from Invitrogen. Oligonucleotide sequences were identified using Beacon Designer Software package (PREMIER Biosoft International, Palo Alto, CA) and designed to be intron-spanning allowing the differentiation between cDNA and DNA-derived PCR products. As housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used; primer sequences were from the Bio-Rad library (forward: 5'-GAA GGT GAA GGT CGG AGT-3'; reverse: 5'-CAT GGG TGG AAT CAT ATT GGA A-3'). For each 25 µl PCR reaction mix: 1 µl cDNA (corresponding to 10^5 cells); 1.0 µl sense primer (10 µM); 1.0 µl anti-sense primer (10 µM); 0.5 µl dNTP (10 mM); 1.5 µl $MgCl_2$ (50 mM); 1.25 U Platinum Taq DNA Polymerase; 2.5 µl Buffer (10x); 1.7 µl SYBR Green

(stock 1:10,000); and 14.55 µl PCR-grade water were mixed together. DNA polymerase was pre-activated for 2 min at 94°C and the amplification was performed by 50 cycles (MMP-9) or 35 cycles (GAPDH) with denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. Relative quantification for MMP-9, expressed as –fold variation over untreated control cells, was calculated with the efficiency-corrected quantification model (Pfaffl MW 2001) after determination of the difference between C_T of the given gene A (MMP-9) and that of the calibrator gene B (GAPDH). C_T values are means of triplicate measurements. To validate the use of the method, serial dilutions of cDNA from monocytes, stimulated for 15 h by 20 ng/ml rhTNFα, were tested. Analyzed transcripts exhibited high linearity amplification plots ($r>0.98$) and similar PCR efficiency (99.7% for MMP-9 and 92.2% for GAPDH), confirming that the expression of each of these genes can be directly compared to one another. The specificity of PCRs was confirmed by melt curve analysis. The melting temperatures for each amplification product were 85.8°C for MMP-9 and 86.5°C for GAPDH.

Measurement of p38 MAPK, phospho-p38 MAPK, and MMP-9 protein levels in cell lysates by Western blotting

12 µg cytosolic protein/sample were added to the loading buffer. The samples were loaded on 8% and 12% polyacrylamide gels under denaturing and reducing conditions, with addition of Laemmli buffer, blotted on a polyvinylidene difluoride membrane, and probed with anti-human MMP-9, p38 MAPK, phospho-p38 MAPK, and actin monoclonal antibodies at 1/1000 final dilution. Bands were visualized by enhanced chemiluminescence. Densitometric analysis of the bands was performed using a computerized densitometer. Every blot was re-used for multiple times by stripping and re-staining. Firstly, all blots were analyzed for actin protein levels (housekeeping gene, data not shown), in order to verify that equal protein amounts were present in each lane. Therefore, all blots were stripped, and grouped in two separate batches. The first batch was re-used twice, to study both p38 MAPK and P-p38 MAPK proteins; the second one was re-used once, for MMP-9 protein studies.

Measurement of active MMP-9 protein levels in cell supernatants by gelatin zymography

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO_2/air-incubator at 37°C for 24 h. Thereafter the levels of active MMP-9 were evaluated by gelatin zymography in the cell supernatants as indicated (Mitola S et al 2003). Briefly, 15 µl cell supernatants/lane were loaded on 8% polyacrylamide gels containing 0.1% gelatin under non-denaturing and non-reducing conditions. Following electrophoresis, gels were washed at room temperature for 2 h in milliQ water containing 2.5% (v/v) Triton-X100 and incubated for 18 h at 37°C in a collagenase buffer containing (mM): NaCl, 200; Tris, 50; $CaCl_2$, 10; and 0.018% (v/v) Brij 35, pH 7.5, with or without 5 mM EDTA to exclude aspecific bands. At the end of the incubation, the gels were stained for 15 min with Coomassie blue (0.5% Coomassie blue in methanol/acetic acid/water at a ratio of 30:10:60). The gels were destained in milliQ water. Densitometric analysis of the bands, reflecting the total levels of active MMP-9, was performed using a computerized densitometer.

Statistical analysis

For each set of experiments, data are shown as mean values + SEM (Real Time RT-PCR studies and densitometric analyses) or one representative image (western blotting or gelatin zymography studies) of three independent experiments. All data were analyzed by Student's *t*-test (equal variances).

Results

The lipid moiety of nHZ promotes long-term phosphorylation of p38 MAPK in human adherent monocytes: role of 15-HETE.

nHZ was recently shown to promote p38 MAPK early (2 h after the end of phagocytosis) phosphorylation in human adherent monocytes (Polimeni M et al 2012a). Here nHZ long-term (24 h) effects were evaluated, and a differential analysis with various components of nHZ moieties was performed. Cells were left unfed or fed for 2h with nHZ, dHZ or sHZ; alternatively, cells were treated for 2 h with 1 μM 15-HETE, a major nHZ lipoperoxidation product (Schwarzer E et al 2003). After washings, cells were incubated for 24 additional h. Therefore, p38 MAPK protein expression and phosphorylation in cell lysates were evaluated by Western blotting and subsequent densitometry. Results are shown in Figure 1. Phosphorylation of p38 MAPK was not observed in untreated monocytes, whereas it was induced by nHZ; 15-HETE mimicked nHZ effects, whereas lipid-free sHZ and sHZ did not (panel 1A). None of treatments did affect the basal levels of p38 MAPK protein (panel 1B). As expected, p38 MAPK phosphorylation was not detected in nHZ-fed or 15-HETE-treated monocytes incubated with 10 μM SB230580, a synthetic inhibitor of p38 MAPK pathway (data not shown).

Involvement of p38 MAPK in nHZ-enhanced MMP-9 expression and release from human adherent monocytes.

nHZ was previously shown to enhance MMP-9 mRNA/protein expression and protein release from human adherent monocytes (Prato M et al 2005). Here the involvement of p38 MAPK pathway in such an enhancement was investigated. Cells were left unfed or fed for 2h with nHZ and then incubated for 15 h (mRNA studies) or 24 h (protein studies) in the presence/absence of 10 μM SB230580. Thereafter, cell lysates and supernatants were collected. MMP-9 mRNA expression was studied by Real Time RT-PCR in 15 h cell lysates; proMMP-9 protein expression was evaluated by Western blotting and subsequent densitometry in 24 h cell lysates; and active MMP-9 protein release was analyzed by gelatin zymography and subsequent densitometry in 24 h cell supernatants. Results are shown in Figure 2 (panel A: mRNA expression; panels B-C: protein expression; panels D-E: protein release). As expected, all evaluated parameters of MMP-9 were enhanced by nHZ. Such a nHZ-dependent enhancement of MMP-9 was fully abrogated by p38 MAPK inhibitor at all levels. SB230580 had no effects on unfed cells.

Involvement of p38 MAPK in nHZ-enhanced MMP-9 expression and release from human adherent monocytes.

15-HETE was previously shown to be responsible for nHZ-dependent enhancement of MMP-9 mRNA/protein expression and protein release from human adherent monocytes (Prato M et al 2008). Here the role of p38 MAPK in achieving 15-HETE effects was investigated. Cells were left unfed or fed for 2h with 15-HETE and then incubated for 15 h (mRNA studies) or 24 h (protein studies) in the presence/absence of 10 μM SB230580. Thereafter, cell lysates and supernatants were collected. MMP-9 mRNA expression was studied by Real Time RT-PCR in 15 h cell lysates; proMMP-9 protein expression was evaluated by Western blotting and subsequent densitometry in 24 h cell lysates; and active MMP-9 protein release was analyzed by gelatin zymography and subsequent densitometry in 24 h cell supernatants. Results are shown in Figure 3 (panel A: mRNA expression; panels B-C: protein expression; panels D-E: protein release). As expected, all evaluated parameters of MMP-9 were enhanced by 15-HETE. Such a 15-HETE-dependent enhancement of MMP-9 was fully abrogated by p38 MAPK inhibitor at all levels. SB230580 had no effects on cells not treated with 15-HETE.

Discussion

Proteases originating from either *Plasmodium* parasites or human/murine hosts play critical functions in malaria pathophysiology (Piña-Vázquez C et al. 2012; Khadjavi A et al. 2010). *Plasmodium* proteases are involved in parasite invasion, migration, and development within human tissues. Cysteine protease activity is critical for hepatocyte invasion by sporozoites, liver stage development, host cell survival and merozoite liberation (Coppi A et al 2005; Coppi A et al 2007; Sturm A et al 2006). Moreover, *Plasmodium* utilizes aspartic proteases (plasmepsins), cysteine proteases (falcipains), and serine proteases (subtilases) for hydrolysis of haemoglobin, rupture of erythrocytes by mature schizonts, and subsequent invasion of other erythrocytes by free merozoites (Spaccapelo R et al. 2010; Rosenthal PJ 2004; Withers-Martinez C et al 2004;).

On the other hand, in the recent years growing interest in looking for some host proteases as possible mediators of clinical switch from uncomplicated to severe malaria has emerged, paying a major attention to MMPs (Szklarczyk A et al 2007; Dejonckheere E et al. 2011; Prato M and Giribaldi F 2011; Geurts N et al 2012; Piña-Vázquez C et al. 2012). Few years ago, Van den Steen and his group published a huge amount of data on murine MMP regulation in different organs during uncomplicated malaria and CM (Van den Steen PE et al. 2006). Interestingly, in this study the expression and activation of monocyte (CD11b+) MMP-9 was enhanced in the brains of mice infected with different strains of *P. berghei*, and such an increase was significantly higher in mice with CM. Moreover, in the brains of human patients with fatal CM, accumulations of MMP-1 (Deininger MH et al 2003) and urokinase-type plasminogen activator receptor (uPAR) (Fauser S et al. 2000) were observed in Durck granulomas, along with a lack of anti-angiogenic endostatin/collagenXVIII in ring haemorrhage areas (Deininger MH et al. 2002), suggesting that the pro-MMP-9 proteolytic machinery is activated in areas of intense parasite sequestration and vascular damage. Consistently, a microarray analysis performed on blood as a whole from children with severe malaria showed an activation of the human MMP-9 gene by *P. Falciparum* (Griffiths MJ et al. 2005). On the contrary, sera of patients with uncomplicated or severe malaria displayed higher levels of MMP-8 but not of MMP-9 (Armah HB et al. 2007; Dietmann A et al. 2008); however, it has been complained that serum might not be an ideal source for reliable data on MMP levels, since their release from blood cells during sampling process might give a highly non-specific background result, interfering with the determination of true concentrations of circulating MMPs (Jung K 2008).

In vitro, a tight relationship between malarial pigment and MMP-9 has been well established by several authors through consistent studies. Geurts et al. demonstrated that sHZ binds the haemopexin domain of MMP-9 thereby priming the activation of the zymogen by other MMPs, such as MMP-3 (Geurts et al. 2008). Moreover, either nHZ or nHZ-containing IRBCs were shown to promote MMP-9 expression and release in several cells, including human monocytes, rat macrophages and human endothelial cells (Prato M and Giribaldi G 2011; Schrimpe A et al. 2009; D'Alessandro S et al 2012).

Among these cells, human monocytes are those studied more intensively for nHZ-dependent enhancement of nHZ, and to date several soluble mediators, biological effects, and signalling pathways have been partially identified. Phagocytosis of nHZ by human monocytes promotes expression and secretion of a large number of pro-inflammatory molecules, including IL-1β, TNFα and MIP-1α/CCL3 (Giribaldi G et al 2010). In a series of previous studies, these three molecules were shown to be required for nHZ-dependent enhancement of MMP-9 expression and release through a double approach with neutralizing antibodies and mimicking recombinant molecules (Prato M et al 2005; Prato M et al 2008; Giribaldi G et al 2011). Similarly, IL-1β, TNFα and MIP-1α/CCL3 also mediate nHZ-induced expression and secretion of lysozyme (Polimeni M et al 2012a) and TIMP-1 (Polimeni M et al 2012b). Chemical inhibition of MMP-9 in nHZ-fed

monocytes abrogated TNFα shedding from its membrane-bound precursor, thereby suggesting that nHZ triggers an auto-enhancing loop between TNFα and MMP-9; additionally, MMP-9 inhibition reduced extracellular matrix invasion by monocytes, indicating that nHZ promotes a pro-invasive phenotype on human monocytes (Prato M et al 2005).

Mechanisms underlying nHZ-dependent upregulation of MMP-9 have been partially explored, and a role for NF-κB transcription system has been proposed (Prato M et al 2010; Dell'Agli M et al 2010). However, the possible involvement of complementary pathways along with NF-κB should not be neglected. Indeed, in human monocytes MMP-9 gene expression can be also induced through several other routes, such as mitogen-associated protein kinases (MAPKs) (Lai WC et al, 2003; Heidinger M et al 2006; Nguyen J et al 2006). Interestingly, several *in vitro* and *in vivo* studies from animal or human malaria models have proposed the nHZ-dependent activation of MAPKs routes. In murine macrophages or monocytes, nHZ induced activation of p38 MAPK (Cambos M et al 2010) and ERK1/2 (Jaramillo et al 2003; Jaramillo et al 2005; Griffith JW et al 2009), but not JNK-2/STAT-1 (Jaramillo et al 2003, Jaramillo et al 2005) pathways. No data describing the effects of nHZ on ERK1/2 and JNK-2/STAT-1 pathways in human monocytes are available so far. However, in a recent work nHZ was shown to early activate p38 MAPK signaling by inducing p38 MAPK phosphorylation without altering basal protein levels 2 h after the end of phagocytosis (Polimeni M et al 2012a).

In the present work we show that p38 MAPK phosphorylation occurs 24 h after phagocytosis of nHZ, suggesting that nHZ promotes also a long-term activation of this signal transduction pathway. Moreover, SB230580, a chemical inhibitor of p38 MAPK phosphorylation, contrasted the enhancing effects of nHZ on MMP-9 mRNA/protein expression and release of the active zymogen. The present data indicate that the activation of p38 MAPK pathway is required for MMP-9 enhancement in nHZ-laden human monocytes. Besides, the involvement of p38 MAPK has been also proposed for nHZ-dependent upregulation of expression and secretion of lysozyme (Polimeni M et al 2012a), TIMP-1 and related pro-inflammatory molecules IL-1β, TNFα and MIP-1α/CCL3 (Polimeni M et al 2012b).

The finding that nHZ promotes long-term phosphorylation of p38 MAPK paves the way to possibly clarify another currently unsolved problem regarding the mechanisms underlying nHZ-dependent upregulation of MMP-9. Indeed, it was previously demonstrated that among differential components of nHZ (ferric haem and lipid moiety), OH-PUFAs are responsible for enhancing MMP-9 expression and release, and 15-HETE plays a major role (Prato M et al 2008). In particular, 15-HETE was shown to enhance production of MMP-9 and related pro-inflammatory molecules IL-1β and TNFα by activating NF-κB pathway (Prato M et al 2010). However, HETEs and parent compounds HODEs can bind peroxisome proliferator-activated receptor-γ (Huang JT et al 1999); thus, they could suppress the NF-κB system (Cabrero A et al 2002), in apparent conflict with current evidence. Nevertheless, literature data also reported that HETEs may activate the NF-κB system by alternative mechanisms. Short-term activation of NF-κB by HETEs requires activation of protein kinase C (Sharma *et al.*, 2005, Wyke *et al.*, 2005, Chen *et al.*, 2008), which in turn activates IKK (Smith *et al.*, 2004), the kinase responsible for I-κBalpha phosphorylation and degradation (Verma *et al.*, 1997) and subsequent activation of NF-κB. Consistently, transient activation of protein kinase C by HZ has been reported (Schwarzer *et al.*, 1993), which may explain short-term NF-κB activation (Prato M et al 2010, Polimeni M et al 2012a). On the other hand, long-term activation of NF-κB pathway by HETEs was suggested to involve MAPK cascades, including ERK and p38 MAPK (Di Mari JF et al 2007; Ishizuka T et al 2007; Cheng J et al 2010). The p38 MAPK cascade was also proposed for HETEs-dependent modulation of MMP-9 (Nguyen *et al.*, 2006). Here we show that the lipid moiety of nHZ promotes long-term activation of p38MAPK pathway. Indeed, lipid-free sHZ and dHZ did not reproduce nHZ effects on p38 MAPK phosphorylation, whereas 15-HETE mimicked nHZ effects by inducing long-term phosphorylation of p38MAPK without affecting basal protein levels. Moreover, activation of p38 MAPK was mandatory for 15-HETE-dependent upregulation of MMP-9 at all investigated levels: mRNA expression, protein

expression, and secretion of the active zymogen. Thus, long-term 15-HETE-dependent activation of NF-κB transcription system in nHZ-fed human monocytes could be likely explained by concurrent 15-HETE-dependent activation of p38 MAPK pathway.

Taken altogether, the present data suggest that in human adherent monocytes the lipid moiety of nHZ promotes long-term phosphorylation of p38 MAPK, and 15-HETE may play a major role. As a consequence of nHZ- and 15-HETE-dependent activation of p38 MAPK pathway, MMP-9 expression and release is promoted. Present evidence might be helpful in order to find effective adjuvant therapies for complicated malaria; broad spectrum drugs, such as MMP or kinase inhibitors, as well as drugs specifically targeting MMP-9 or upstream signaling molecules, (i.e. phospho-p38 MAPK) could be good candidate molecules.

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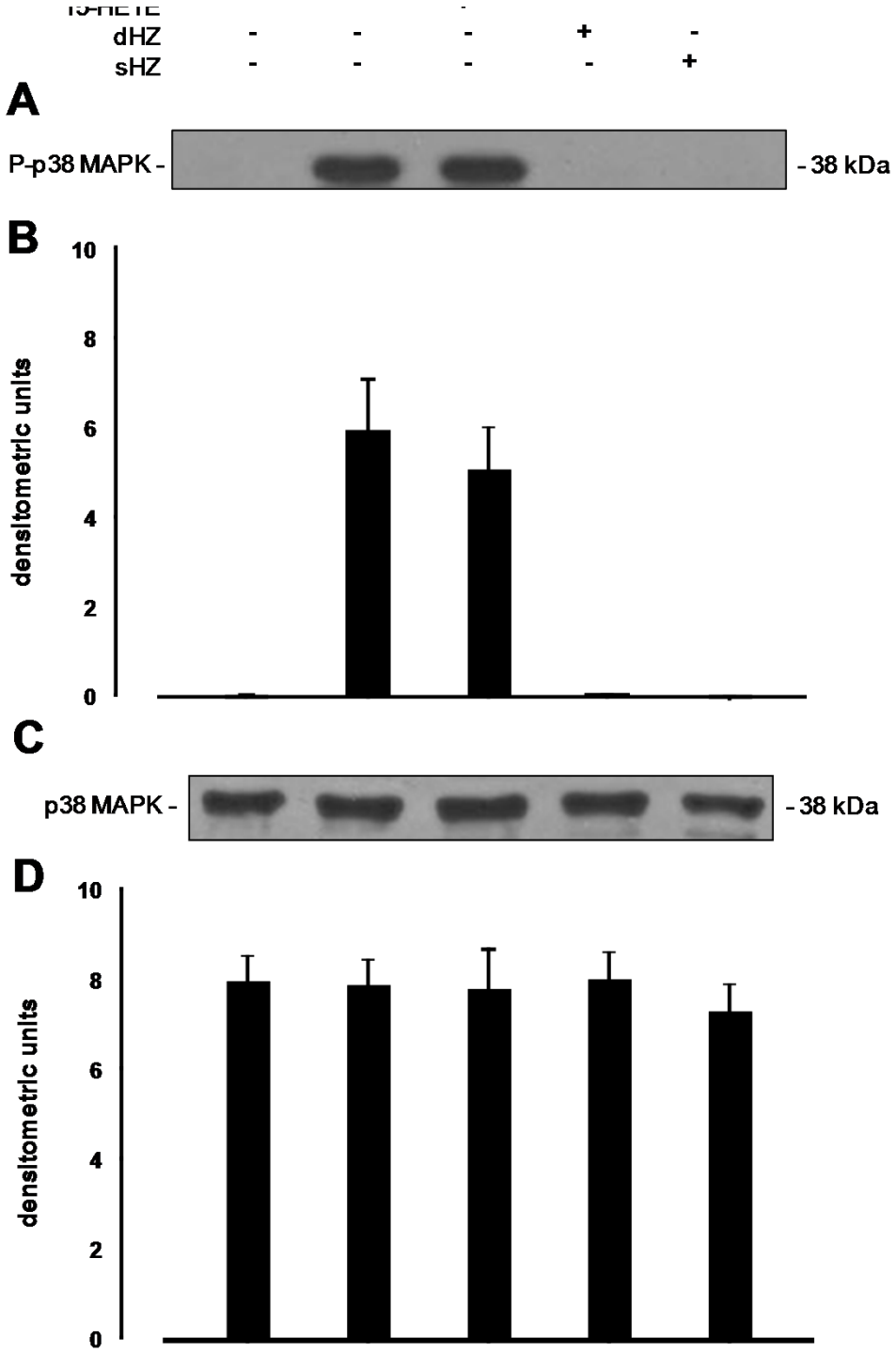
Figure legends

Figure 1.
Effects of lipid and ferric moieties of nHZ on p38 MAPK expression and phosphorylation in human adherent monocytes. Cells were left unfed/untreated (control cells), fed with nHZ, dHZ or sHZ, or treated with 1 μM 15-HETE for 2h; after washings, cells were incubated for 24 h and lysed. Then p38 MAPK protein phosphorylation (panels A-B) and expression (panels C-D) were evaluated by Western blotting and subsequent densitometry. Results are shown as representative blots (A, C) or means+SEM (B, D) of three independent experiments. Densitometric data were also evaluated for significance by Student's t-test. Panel B. nHZ-fed vs control cells: $p<0.01$; 15-HETE-treated vs control cells: $p<0.01$; all other comparisons: no significant differences. Panel D. no significant differences.

Figure 2.
Involvement of p38 MAPK in nHZ-enhanced MMP-9 mRNA/protein expression and protein release from human adherent monocytes. Cells were left unfed (negative controls) or fed with nHZ (positive controls) for 2 h; after phagocytosis, cells were washed and incubated for 15-24 h alone or with 10 μM p38 MAPK synthetic inhibitor SB230580. Therefore, MMP-9 mRNA expression was measured by Real Time RT-PCR (panel A) in 15 h cell lysates; proMMP-9 protein expression was analyzed by Western blotting (panel B) and subsequent densitometry (panel C) in 24 h cell lysates; and active MMP-9 protein release was evaluated by gelatin zymography (panel D) and subsequent densitometry (panel E) in 24 h cell supernatants. Results are shown as means+SEM (A, C, E) or as representative blot/gel (B, D) of three independent experiments. Real Time RT-PCR and densitometric data were also evaluated for significance by Student's t-test. Panel A. nHZ-fed vs unfed cells: $p<0.005$; nHZ-fed vs SB230580-treated nHZ-fed cells: $p<0.001$; nHZ-fed vs SB230580-treated unfed cells: $p<0.005$; all other comparisons: no significant differences. Panel C. nHZ-fed vs unfed cells: $p<0.0001$; nHZ-fed vs SB230580-treated nHZ-fed cells: $p<0.001$; nHZ-fed vs SB230580-treated unfed cells: $p<0.001$; all other comparisons: no significant differences. Panel E. nHZ-fed vs unfed cells: $p<0.001$; nHZ-fed vs SB230580-treated nHZ-fed cells: $p<0.001$; nHZ-fed vs SB230580-treated unfed cells: $p<0.001$; all other comparisons: no significant differences.

Figure 3.
Involvement of p38 MAPK in 15-HETE-enhanced MMP-9 mRNA/protein expression and protein release from human adherent monocytes. Cells were left untreated (negative controls) or treated with 1 μM 15-HETE (positive controls) for 2 h; after treatment, cells were washed and incubated for 15-24 h alone or with 10 μM p38 MAPK synthetic inhibitor SB230580. Therefore, MMP-9 mRNA expression was measured by Real Time RT-PCR (panel A) in 15 h cell lysates; proMMP-9 protein expression was analyzed by Western blotting (panel B) and subsequent densitometry (panel C) in 24 h cell lysates; and active MMP-9 protein release was evaluated by gelatin zymography (panel D) and subsequent densitometry (panel E) in 24 h cell supernatants. Results are shown as means+SEM (A, C, E) or as representative blot/gel (B, D) of three independent experiments. Real Time RT-PCR and densitometric data were also evaluated for significance by Student's t-test. Panel A. 15-HETE-treated vs untreated cells: $p<0.001$; 15-HETE-treated vs SB230580-/15-HETE-treated cells: $p<0.001$; 15-HETE-treated vs SB230580-treated cells: $p<0.001$; all other comparisons: no significant differences. Panel C. 15-HETE-treated vs untreated cells: $p<0.02$; 15-HETE-treated vs SB230580-/15-HETE-treated cells: $p<0.02$; 15-HETE-treated vs SB230580-treated cells: $p<0.02$; all other comparisons: no significant differences. Panel E. 15-HETE-treated vs untreated cells: $p<0.002$; 15-HETE-treated vs SB230580-/15-HETE-treated cells: $p<0.005$; 15-HETE-treated vs SB230580-treated cells: $p<0.005$; all other comparisons: no significant differences.

Figure 1


15-HETE
dHZ - - - + -
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A
P-p38 MAPK -
- 38 kDa
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densitometric units
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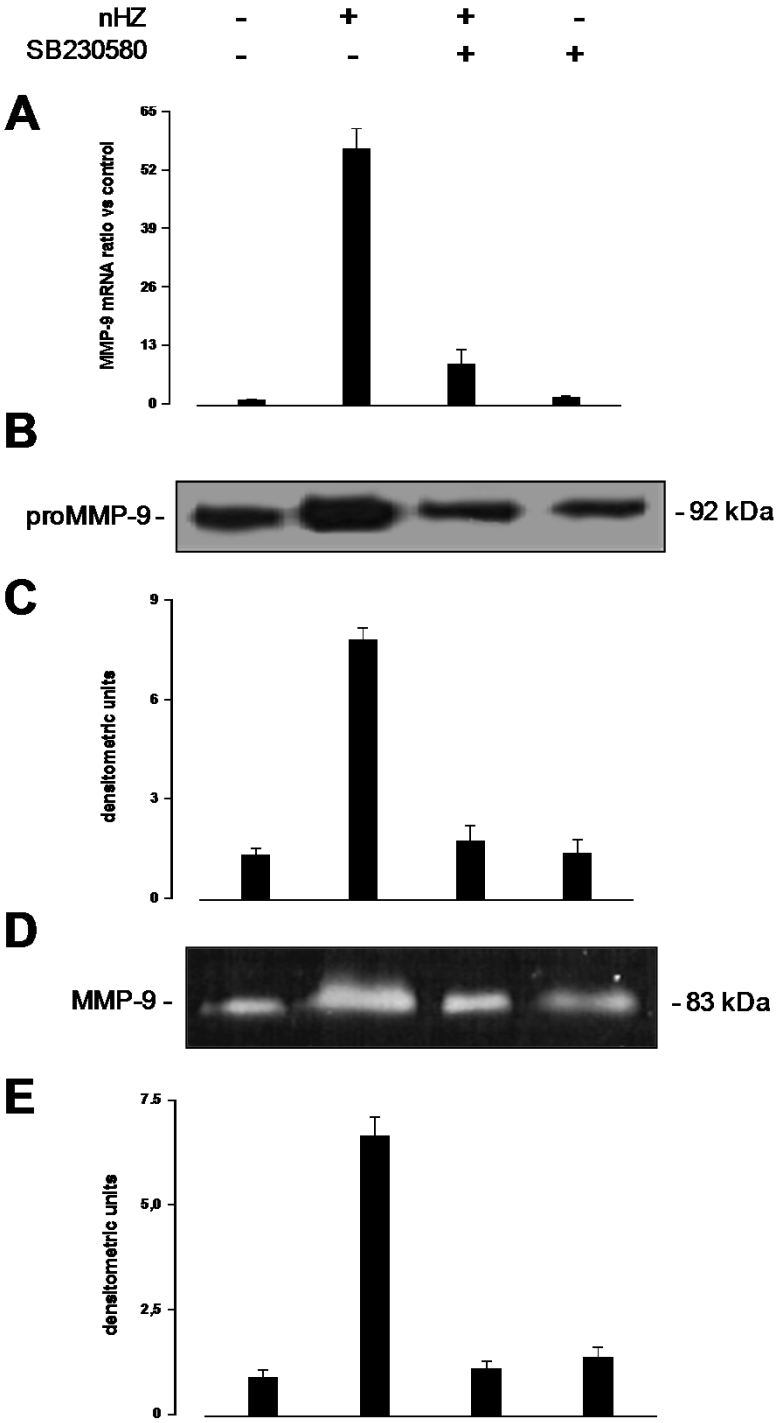
Figure 2


nHZ - + + -
SB230580 - - + +
A
MMP-9 mRNA ratio vs control
65
52
39
26
13
0
B
proMMP-9 -
- 92 kDa
C
densitometric units
9
6
3
0
D
MMP-9 -
- 83 kDa
E
densitometric units
7,5
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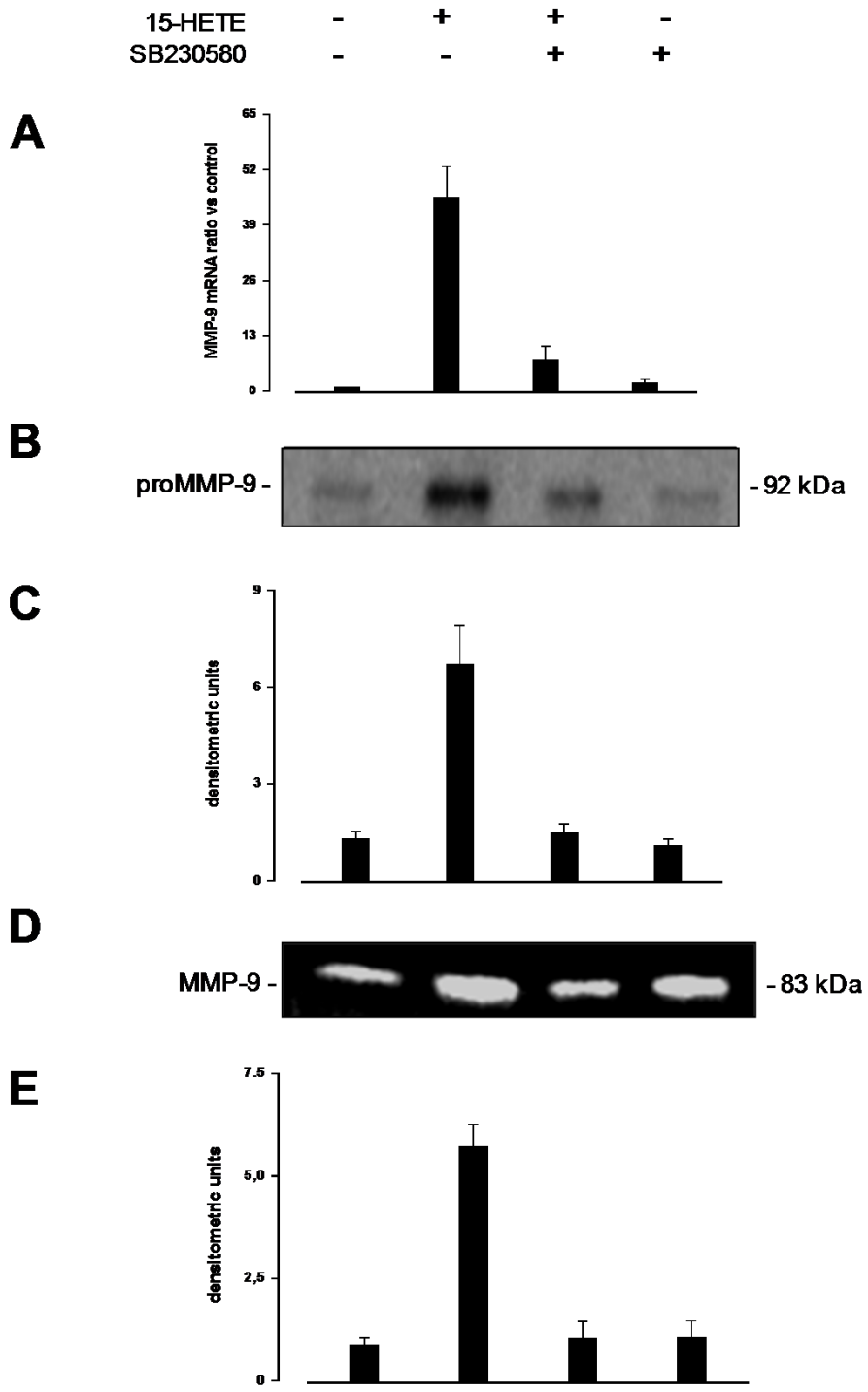
2,5
0

Figure 3


15-HETE - + + -
SB230580 - - + +
A
MMP-9 mRNA ratio vs control
65
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9
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