European Journal of Immunology

Myeloperoxidase accumulates at the neutrophil surface and enhances cell metabolism and oxidant release during pregnancy

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Pregnancy is a unique immunological state. Pregnancy neutrophils differ from those of non-pregnant women as they cannot be fully activated for oxidant production, but yet have higher levels of unstimulated oxidant production. Although reduced activation is due to decreased hexose monophosphate shunt activity, the mechanism enhancing basal oxidant levels is unknown. We hypothesize that myeloperoxidase (MPO) trafficking affects the basal oxidant release by maternal neutrophils. Immunofluorescence microscopy has demonstrated MPO at the surface of pregnancy neutrophils, whereas non-pregnancy cells do not exhibit surface MPO. Adherent pregnancy neutrophils were characterized by high-amplitude metabolic oscillations, which were blocked by MPO inactivation. Conversely, metabolic oscillatory amplitudes of control neutrophils were heightened by incubation with PMA or exogenous MPO. Importantly, MPO decoration of cell surfaces and high-amplitude metabolic oscillations were observed for neutrophils from pregnant but not from non-pregnant mice. However, cells from pregnant MPO knockout mice did not exhibit MPO expression or high-amplitude metabolic oscillations. Unstimulated neutrophils from pregnant women were found to release reactive oxygen metabolites (ROM) and reactive nitrogen intermediates (RNI), but cells from non-pregnant women did not. MPO inhibition returned ROM and RNI formation to non-pregnant levels. Hence, MPO trafficking influences metabolic activity and oxidant production in pregnancy.

Received 16/8/05
Revised 31/1/06
Accepted 22/3/06

[DOI 10.1002/eji.200535391]

Key words: Metabolism · Myeloperoxidase · NADPH oxidase · Neutrophil activation · Pregnancy

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Abbreviations: **DAF-2DA:** diaminofluorescein-2 diacetate · **fMLP:** *N*-formyl-Met-Leu-Phe · **G-6-P:** glucose-6-phosphate · **G-6-PDase:** G-6-P dehydrogenase · **HMS:** hexose monophosphate shunt · **HQ:** hydroxyquinone · **H_2TMRos:** dihydrotetramethylrosamine · **MPO:** myeloperoxidase · **pHBAH:** p-hydroxybenzoic acid hydrazide · **RNI:** reactive nitrogen intermediates · **ROM:** reactive oxygen metabolite · **SHA:** salicylhydroxamic acid · **TMRos:** tetramethylrosamine

Introduction

Pregnancy is characterized by poorly understood changes in a mother's host defense strategies, to permit survival of the fetal semi-allograft. One key cell type contributing to innate and adaptive host defense is neutrophils. Previous studies have suggested that certain neutrophil functions, such as the release of reactive oxygen metabolites (ROM) and reactive nitrogen intermediates (RNI), are depressed during pregnancy [1–8]. Emerging studies show that pregnancy is associated with a systemic inflammatory response and

that pregnancy neutrophils exhibit markers of activation [9–11]. Our recent work has resolved part of this apparent discrepancy by showing that unstimulated, but adherent, pregnancy neutrophils exhibit intermediate levels of ROM production that are "suppressed" in comparison to fully activated (*e.g.* IFN-γ + LPS) non-pregnancy neutrophils but "activated" in comparison to unstimulated neutrophils from non-pregnant women [12–14]. For example, unstimulated normal pregnancy neutrophils produce ROM *in vitro* at the same rate as LPS-treated non-pregnancy neutrophils [12]; clearly, a heightened state of neutrophil activity. Nonetheless, several molecules known to stimulate the hexose monophosphate shunt (HMS) [*e.g. N*-formyl-Met-Leu-Phe (fMLP) and LPS] are unable to increase ROM release from pregnancy neutrophils, and co-incubation of fMLP or LPS with neutrophil-priming agents (PMA or IFN-γ) has no effect on pregnancy neutrophils. The multiple states of activation of non-pregnancy neutrophils and the one state found for pregnancy cells suggest that at least two broad pathways of neutrophil activation exist.

Our recent studies have outlined one pathway contributing to reduced levels of oxidant release by stimulated neutrophils from pregnant women in comparison to fully activated cells from non-pregnant women. ROM and RNI production require NADPH [Eqs. (1) and (2)]. Superoxide is produced by the NADPH oxidase according to:

(1) $1/2\ NADPH + O_2 \rightarrow 1/2\ NADP^+ + 1/2\ H^+ + O_2^-$

RNI production begins with the synthesis of NO:

(2) L-arginine + $NADPH + H^+ + O_2 \rightarrow N^G$-hydroxy-L-arginine + $NADP^+ + H_2O$ and

$2\ N^G$-hydroxy-L-arginine + $NADPH + H^+ + O_2 \rightarrow$ 2 L-citrulline + $NADP^+ + 2\ H_2O + 2\ NO$

Superoxide and NO may then yield additional downstream ROM and RNI. A key source of NADPH is the HMS. Glucose transport, which is required to drive the HMS [15, 16], is accelerated during activation [15, 17]. Glucose-6-phosphate (G-6-P), produced by hexokinase, is metabolized by the HMS and glycolysis. The first step of the HMS is mediated by G-6-P dehydrogenase (G-6-PDase), which converts G-6-P into 6-phosphogluconolactone and NADPH. 6-Phosphogluconolactone dehydrogenase converts 6-phosphogluconolactone into ribose-5-phosphate and NADPH. We have discovered that the intracellular trafficking of an HMS enzyme complex regulates the HMS and ROM production [12–14]. In non-pregnant women, HMS enzymes are found at the cell periphery where G-6-P is produced by hexokinase and is readily available to the HMS. However, in pregnant women, HMS enzymes undergo retrograde transport on microtubules to the cell's microtubule organizing center [12–14]. In this location there is less G-6-P available to the HMS, as much of it is metabolized by glycolytic enzymes at the cell periphery. Although these findings explain the reduction in ROM levels found in stimulated pregnancy neutrophils, they do not account for the higher basal levels of ROM release found for unstimulated adherent pregnancy neutrophils [12].

To account for the higher basal levels of oxidant production in unstimulated adherent pregnancy neutrophils, we evaluated other pathways relevant to ROM management. MPO can influence metabolic reactions as well as the amount and kind of oxidants produced [18, 19]. MPO is a highly cationic heme protein of 144 kDa found in neutrophils and monocytes. In neutrophils, it constitutes 5% of the total cellular protein and is found primarily in azurophilic granules. The functional significance of MPO is usually considered to be in the formation of oxidants, such as hyperchlorous acid, and the oxidation of (pseudo)halides, aromatic amino acids, indoles, xenobiotics, and others. It also contributes to bactericidal and tumoricidal functions [18, 20–22]. The reaction mechanism of MPO, the peroxidase cycle, is a highly nonlinear dynamic process that can be best understood using computational biology [23–26]. Peroxidases, including human MPO, in the presence of their substrates and modifiers, are capable of sustained autonomous oscillations [23–27], which may help protect this highly reactive enzyme from itself [25]. This oscillatory system resembles the Belousov–Zhabotinsky reaction where four mutually dependent chemical reactions couple to form temporal oscillations in the concentrations of products [28]. Other intracellular chemical concentration oscillations, such as NAD(P)H and superoxide, are well known and can be described by their periods (time between concentration peaks) and amplitudes (differences between low and high concentrations) [29–31]. Recent computational and experimental studies have shown that these pathways combine when chemically coupled to one another to yield high-amplitude oscillations and heightened ROM release [26]. From a cellular standpoint, chemical coupling of MPO and NADPH oxidase is possible when they are deposited in the same compartment, such as a phagosome or the plasma membrane, or when a molecule such as melatonin is used to "short-circuit" the insulation provided by their residence in separate membrane-bound compartments. Hence, neutrophils may regulate the extent of their activation by altering MPO enzyme trafficking, just as they can regulate the HMS by translocation of enzyme complexes.

As previous studies indicated that the amplitude of metabolic oscillations was enhanced during pregnancy [12, 13], we tested the hypothesis that MPO influences metabolic oscillations and the basal levels of ROM production observed during pregnancy. We show that MPO accumulates at the cell surface of neutrophils from pregnant women, but not in cells from non-pregnant

women, which may account for certain functional differences.

Results

MPO accumulates at the surface of neutrophils from pregnant women

As MPO is an important enzyme associated with ROM production and cell metabolism [26], we hypothesized that MPO trafficking is changed during pregnancy. To test this hypothesis, we performed direct immunofluorescence microscopy experiments of neutrophils from non-pregnant and pregnant women using anti-MPO Ab. When neutrophils were fixed with paraformaldehyde, bright cellular labeling was observed (Fig. 1A, B) for cells from both non-pregnant and pregnant women. Quantitatively, cells from non-pregnant and pregnant women displayed indistinguishable fluorescence intensity levels of ~10 000 counts/s per cell (Fig. 2). However, as MPO constitutes 5% of the neutrophil protein, it would be difficult to distinguish intracellular MPO from an MPO fraction associated with the cell surface. To avoid permeabilization of the plasma membrane, we examined live cells using anti-MPO Ab. Fig. 1C shows that neutrophils from non-pregnant women were only dimly labeled by the anti-MPO Ab (~2000 counts/s/cell; Fig. 2). However, neutrophils from pregnant women were labeled brightly with anti-MPO Ab (Fig. 1D). This distribution of the fluorescence indicates that the label was associated with the cell surface. This was further confirmed using z-scanning

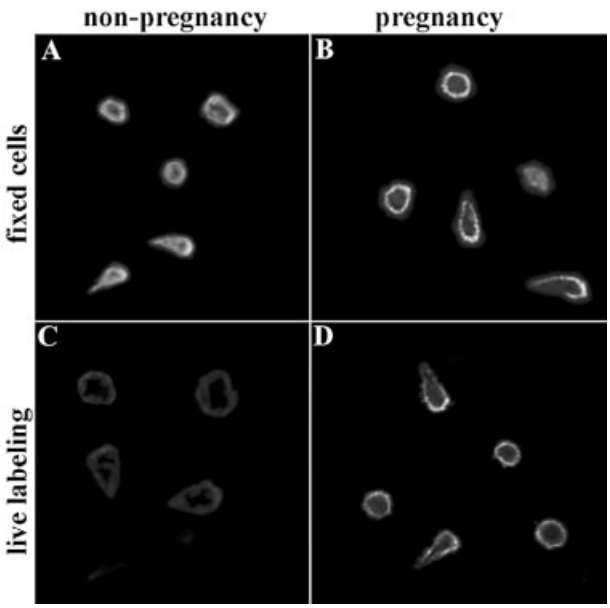

Figure 1. Direct immunofluorescence microscopy using anti-MPO-FITC Ab-stained neutrophils from non-pregnant (A, C) and pregnant (B, D) women. Cells were attached to coverslips, but were otherwise unstimulated. When cells were fixed using 3.7% paraformaldehyde, neutrophils from both non-pregnant and pregnant women were brightly stained with anti-MPO Ab (A, B). As conventional fixation methods may permeabilize the plasma membrane, these results include fluorescence from both intracellular granules and plasma membrane-associated MPO. To avoid cell permeabilization, live cells were examined. When live cells were evaluated, cells from non-pregnant women were stained at very low levels with anti-MPO Ab (C), whereas neutrophils from pregnant women were stained brightly (D). Magnification ×610; n = 30.

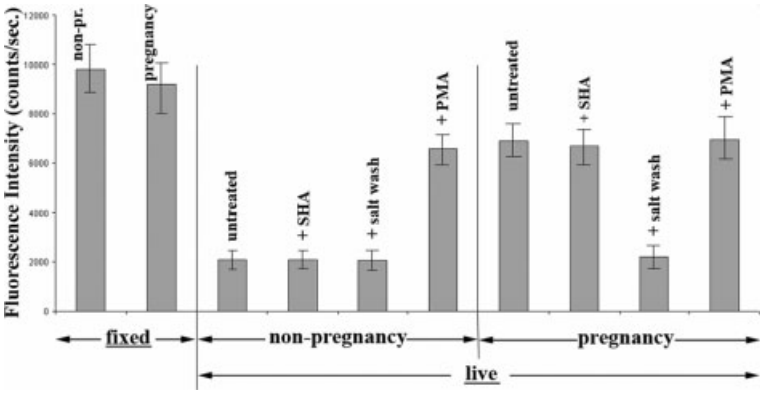

Figure 2. Quantitative analyses of the fluorescence intensity of neutrophils from non-pregnant and pregnant women after MPO staining are shown. The vertical axis gives the fluorescence intensity in counts/s. Each value represents the mean + SD. All cells prepared by fixation with paraformaldehyde show bright staining. When living neutrophils from non-pregnant women were evaluated, their fluorescence intensity level was low. Although this intensity was not affected by the MPO inhibitor SHA or a brief salt wash, it was increased by incubation with PMA. Pregnancy neutrophils displayed high levels of fluorescence that were not affected by SHA, but were dramatically reduced by the salt wash procedure. PMA, however, did not measurably change this fluorescence intensity level. Untreated and SHA-treated cells from non-pregnant women had significantly reduced fluorescence intensities in comparison to neutrophils from pregnant women (p <0.001); other comparisons between samples from pregnant and non-pregnant women were not significant. n = 12.

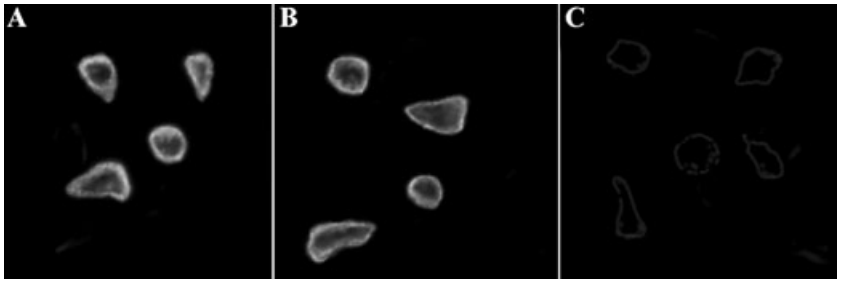

Figure 3. Immunofluorescence microscopy of MPO surface labeling of unfixed cells. Pregnancy neutrophils were evaluated without treatment (A), after exposure to SHA (B) and after exposure to a hypertonic salt wash (C). Magnification ×840; $n = 10$.

and image deconvolution software (data not shown). This corresponded to an average fluorescence intensity of ~6000 counts/s/cell (Fig. 2). The level of MPO surface expression during pregnancy is similar to the amount of surface MPO found for cells from non-pregnant women after incubation with PMA (Fig. 2). Therefore, the cell surface displays a considerable fraction of the total cell-associated MPO during pregnancy.

MPO surface expression was evaluated during conditions expected to modify its activity or association with the cell surface. Untreated pregnancy neutrophils express surface MPO (Fig. 3A), as described above. Salicylhydroxamic acid (SHA) is a well-known inhibitor of MPO [32], which blocks enzyme activity. It did not affect MPO staining of cells from pregnant mothers (Fig. 2, 3B) or non-pregnant women (Fig. 2). MPO possesses a high positive charge and thereby may electrostatically bind to cells. To test this idea, control cells or neutrophils from pregnant women were washed with a hypertonic (400 mM) NaCl solution for 40 s while adherent to microscope slides. Following this treatment, surface MPO was stained as described above. The staining of pregnancy neutrophils was dramatically decreased by this protocol (Fig. 3C). Quantitatively, the staining was reduced to levels consistent with cells from non-pregnant women (Fig. 2). However, the salt wash had no effect on cells from non-pregnant women (Fig. 2).

High-amplitude metabolic oscillations of pregnancy neutrophils accompany MPO accumulation at the cell surface

Previous studies have shown that (1) neutrophil metabolism oscillates in time, (2) these oscillations correlate with ROM production [29–31], and (3) oscillations increase in amplitude during pregnancy [12–14]. As MPO promotes heightened NAD(P)H amplitudes in neutrophils during melatonin treatment [26], we tested the hypothesis that MPO accumulation at the cell surface participates in pregnancy-associated metabolic changes. To evaluate cell metabolism, NAD(P)H autofluorescence was monitored by quantitative microfluorometry. We first measured the percentage of neutrophils staining positively for MPO and the percentage of cells exhibiting high-amplitude metabolic oscillations. For cells from both non-pregnant and pregnant women, the percentages of cells exhibiting MPO staining and high-amplitude oscillations paralleled one another: Cells from non-pregnant women had very low percentages of both parameters (1 ± 1 and $2 \pm 1\%$) whereas cell from pregnant women expressed very high levels (74 ± 4 and $72 \pm 4\%$). To provide further evidence that metabolic dynamics is affected by MPO during pregnancy, we evaluated the effect of MPO inhibitors. Polarized neutrophils from non-pregnant individuals exhibit NAD(P)H oscillations with a period of ~20 s (Fig. 4A) [29–31]. Cells were treated with a panel of MPO inhibitors, SHA, p-hydroxybenzoic acid hydrazide (pHBAH), hydroxyquinone (HQ) and cyanide, to minimize the impact of non-specific properties of any one compound [32, 33]. For example, neutrophils were treated with 50 mM SHA for 20 min at 37°C followed by experimentation. Although SHA had no effect on untreated control cells, it blocked high-amplitude metabolic oscillations of pregnancy neutrophils (Fig. 4). This effect, however, was not specific for SHA, as pHBAH, HQ, and cyanide had a similar effect (Fig. 4). Moreover, this result could not be explained by the drugs causing MPO to dissociate from the cell membrane, as MPO staining remains after treatment with MPO inhibitors (*e.g.* Fig. 3). This suggests that MPO participates in forming the high-amplitude metabolic oscillations of pregnancy.

As illustrated by Fig. 3C, MPO can be removed from pregnancy neutrophil surfaces by using a brief wash at high salt concentration. Although this wash protocol has no effect upon the metabolic oscillations of control cells, it reduced the amplitudes of metabolic oscillations of pregnancy neutrophils (Fig. 4). Quantitative data are shown in Table 1. This finding is consistent with a role of MPO in cell metabolism. We next sought to transform the oscillatory metabolic phenotype of cells from non-pregnant individuals into one resembling pregnant women. We confirmed the role of MPO in high-amplitude metabolic oscillations using a reconstitution approach. We added purified MPO (10 mg/mL) to

Table 1. Quantitative analysis of metabolic oscillations

	Amplitude ($\times 10^4$ counts/s)		Period (s)	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
Untreated	2.2 ± 0.11	10.1 ± 0.5[a)]	22.5 ± 0.7	23.0 ± 0.6
SHA	2.3 ± 0.13	2.3 ± 0.2	23.5 ± 0.7	21.8 ± 1.2
pHBAH	2.3 ± 0.11	2.3 ± 0.2	22.3 ± 1.1	22.5 ± 1.3
Cyanide	2.3 ± 0.1	2.4 ± 0.2	23.2 ± 0.9	23.1 ± 1.4
HQ	2.3 ± 0.12	2.3 ± 0.2	22.9 ± 0.9	22.2 ± 1.2
NaCl wash	2.3 ± 0.13	2.4 ± 0.3	22.5 ± 0.9	22.1 ± 1.0
MPO	10.0 ± 0.3	10.1 ± 0.4	22.9 ± 1.3	23.0 ± 0.9

[a)] Does not include a minor population of low-amplitude cells.

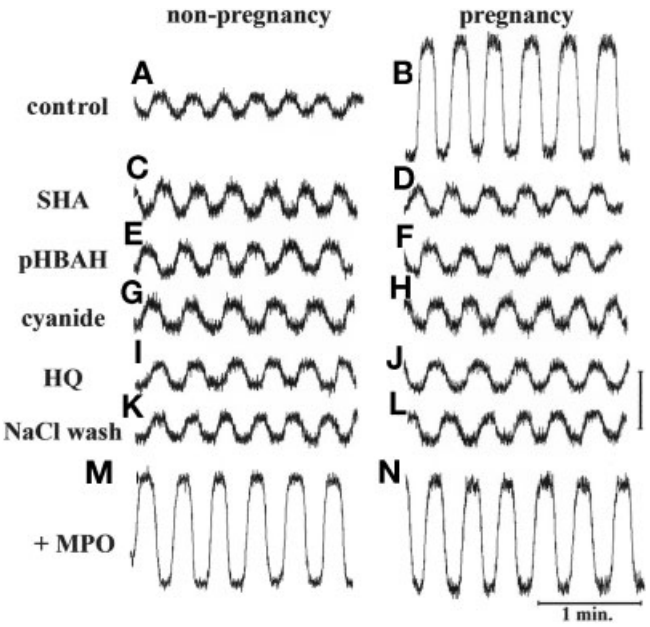

Figure 4. Representative NAD(P)H oscillations in living adherent neutrophils from non-pregnant (left-hand side) and pregnant (right-hand side) women. The amplitudes of NAD(P)H oscillations are higher in pregnancy neutrophils than in those from non-pregnant women (A, B). The inhibitors SHA, pHBAH, cyanide, and HQ had no effect on cells from non-pregnant women (C, E, G, I). However, the high-amplitude oscillations of pregnancy neutrophils were reduced to the level found for cells from non-pregnant women (D, F, H, J). Although a brief hypertonic salt wash had no effect on the metabolic dynamics of cells from non-pregnant women, it dramatically reduced the NAD(P)H oscillatory amplitude of pregnancy cells (K, L). As shown in Fig. 2 and 3, this protocol removes MPO from the cell surface. If exogenous MPO is added to cell samples, it dramatically increased the oscillatory behavior of cells from non-pregnant women, but had no effect upon cells from pregnant women (M, N). See Fig. 2 for quantitative results. Vertical bar = 5 × 10^4 counts; horizontal bar = 10 s; n = 12.

untreated neutrophils. As anticipated, the amplitude of the metabolic oscillations increased dramatically (Fig. 4). Furthermore, these MPO-exposed cells stained positively for MPO using direct immunofluorescence as described above (data not shown). Therefore, our evidence supports the idea that MPO accumulation at the cell surface contributes to altered metabolic dynamics of pregnancy neutrophils.

As the above experiments focused upon either cell labeling or NAD(P)H autofluorescence, we reconfirmed the link between these two variables by performing simultaneous microscopic assays. Neutrophils from non-pregnant and pregnant women were labeled with anti-MPO Ab and then analyzed by microfluorometry. Cells from non-pregnant women displayed minimal MPO labeling and low-amplitude oscillations (Fig. 5). As previously reported [12], about 70% of the neutrophils from normal pregnant women display high-amplitude oscillations. When checked on a cell-by-cell basis, only the MPO-positive cells in the pregnancy population exhibited high-amplitude metabolic oscillations (Fig. 5). This further confirms the link between MPO surface association and metabolic changes in neutrophils.

MPO knockout mice do not exhibit MPO accumulation at the cell surface

To provide unambiguous genetic evidence to support differences in MPO trafficking during pregnancy, we examined the surface MPO staining of neutrophils from pregnant and non-pregnant wild-type and MPO knockout mice. Non-pregnant female mice from both wild-type and MPO knockout mice did not display surface MPO staining (Fig. 6A, B, E, F). As expected, unstimulated neutrophils from pregnant wild-type mice displayed surface MPO staining (Fig. 6C, D). However, neutrophils from pregnant MPO knockout mice did not exhibit MPO staining (Fig. 6G, H). Hence,

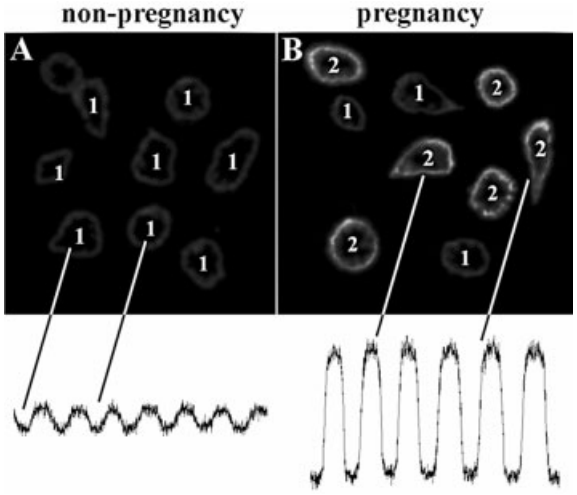

Figure 5. Combined visualization of MPO staining and NAD(P)H oscillations for the same viable cells. Living cells from non-pregnant (A) and pregnant (B) women were stained with anti-MPO. Cells from non-pregnant women were negative for both MPO staining and high-amplitude NAD(P)H oscillations. As mentioned in Fig. 4, approximately 70% of the pregnancy neutrophils display MPO staining and high-amplitude metabolic oscillations. Cells staining positively for MPO also displayed high-amplitude NAD(P)H oscillations (B). By staining and analyzing the same cells, we showed for the population of cells a one-to-one correspondence between the surface expression of MPO and high-amplitude metabolic oscillations. $n = 10$.

MPO trafficking in murine cells resembles that of human cells.

Using this genetic model, we studied the metabolic phenotype and unstimulated oxidant production of neutrophils from knockout and control mice. Cells from non-pregnant mice demonstrated conventional metabolic oscillations and minimal levels of ROM production under all conditions tested (Fig. 7). Neutrophils from pregnant C57BL/6 mice display metabolic oscillations of enhanced amplitude, which could be blocked by incubation with SHA or using a salt washing procedure, as described above. The ROM production rate was also measured using a single-cell assay, as described [30, 31]. ROM production was enhanced in neutrophils from normal mice, but not MPO knockout mice (Fig. 7). However, conditions that inactivate MPO or remove it from the surface, SHA or salt wash treatments, respectively, blocked the enhanced basal rate of ROM release associated with murine pregnancy. This provides another line of evidence supporting a role for MPO in regulating the basal rates of ROM production in pregnancy.

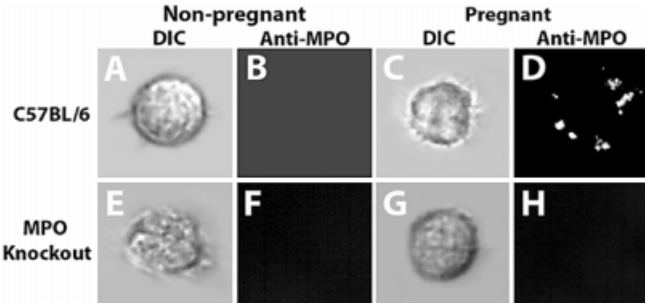

Figure 6. Direct immunofluorescence microscopy using anti-MPO-FITC Ab-staining of neutrophils of non-pregnant (A, B, E, F) and pregnant (C, D, G, H) mice. (A–D) Cells from control C57BL/6 animals; (E–H) cells from MPO knockout mice. Living cells were allowed to attach to coverslips. Cells from non-pregnant C57BL/6 mice did not exhibit MPO staining (B), whereas neutrophils from pregnant C57BL/6 mice did (D). Cells from both non-pregnant (F) and pregnant (H) MPO knockout mice did not display surface MPO staining. Magnification ×950; $n = 3$.

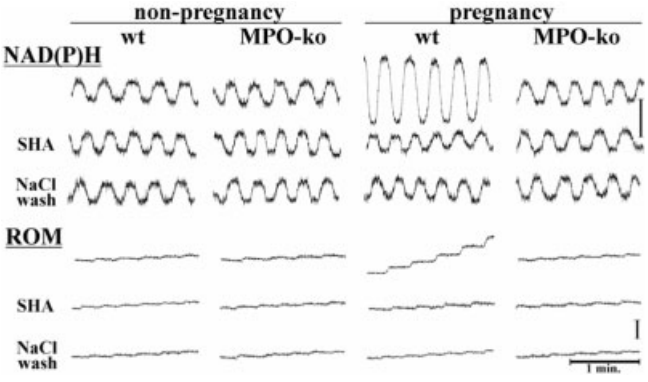

Figure 7. Representative NAD(P)H oscillations and ROM production kinetics for adherent neutrophils from non-pregnant (left-hand side) and pregnant (right-hand side) mice. Cells were unstimulated. Results obtained for wild-type (w.t.) and MPO knockout (MPO-ko) mice are shown at the left- and right-hand sides for these two groups. Enhanced NAD(P)H oscillatory amplitudes and ROM release kinetics are only seen for pregnant wild-type mice; these changes could be reversed by incubation with SHA or by a salt wash protocol. Neutrophils from pregnant MPO knockout mice could not be distinguished from non-pregnant mice. Vertical bars = 10^4 counts; horizontal bar = 1 min; $n = 10$.

MPO influences ROM release from neutrophils of pregnant women

Previous studies have shown that high-amplitude NAD(P)H oscillations may be observed during periods of heightened ROM production [29–31] and that under unstimulated conditions metabolic oscillations and ROM release are higher in adherent polarized neutrophils from pregnant women [12–14]. As MPO decoration of the plasma membrane occurs during pregnancy and is

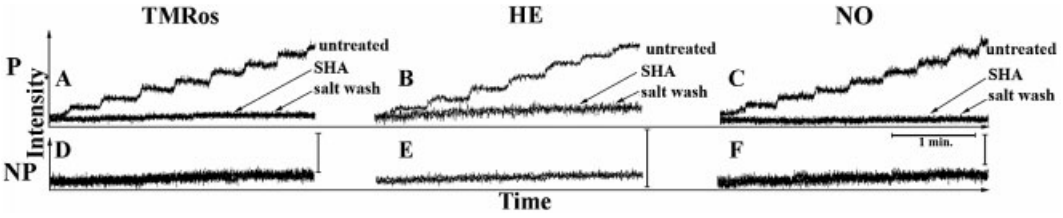

Figure 8. Rates of ROM (A, D), superoxide (B, E) and NO (C, F) release by neutrophils from pregnant (A–C) and non-pregnant subjects (D–F). ROM, superoxide or NO release was measured during cell adherence/migration through a matrix containing H_2TMRos, which is sensitive to several ROM, hydroethidine, which is sensitive to superoxide, or DAF-2DA, respectively. Although untreated pregnancy cells demonstrated oxidant production, they did not do so after exposure to SHA or a salt wash protocol. Cells from non-pregnant women did not release substantial levels of oxidants under these conditions. Vertical bar = 5×10^4 counts; horizontal bar = 10 s; $n = 30$.

Table 2. Quantitative analysis of the rate of oxidant release[a)]

Sample	Treatment	TMRos	HE	NO
Pregnant	Untreated	2.54 ± 0.17	1.73 ± 0.09	3.3 ± 0.34
	SHA	0.39 ± 0.09	0.24 ± 0.04	0.69 ± 0.12
	NaCl wash	0.42 ± 0.09	0.24 ± 0.04	0.74 ± 0.12
Non-pregnant	Untreated	0.4 ± 0.07	0.25 ± 0.04	0.75 ± 0.13
	SHA	0.37 ± 0.12	0.23 ± 0.05	0.71 ± 0.09
	NaCl wash	0.42 ± 0.07	0.24 ± 0.05	0.73 ± 0.08

[a)] The values shown are the rates of change in fluorescence intensity in counts $\times 10^4$/min.

associated with high-amplitude oscillations, we examined if MPO affects the total amount of oxidant release. Fig. 8 and Table 2 show experiments on ROM and NO release from neutrophils migrating in a matrix containing reporters that become fluorescent upon oxidant exposure. Cells from non-pregnant women generate very low levels of ROM and NO under all conditions tested (Fig. 8, Table 2). However, unstimulated adherent cells from pregnant women generate ROM and NO at significant rates (Fig. 8). If this difference is associated with MPO, it should be possible to block it by chemical inhibition of MPO and by using salt washing. This work shows that ROM and NO production by pregnancy neutrophils are dramatically reduced by treatment with SHA or after a brief salt wash. Hence, MPO trafficking appears to regulate the basal levels of ROM and NO production by pregnancy neutrophils.

Discussion

Enzyme trafficking contributes to several key events in neutrophil activation. Hexokinase traffics to the cell periphery to accelerate glucose transport to provide G-6-P for the HMS [34]. The HMS, in turn, provides NADPH for the NADPH oxidase, which is also translocated to the plasma membrane during activation [35]. PKC accumulates at the neutrophil periphery during activation to participate in signal transduction [36]. The contents of cytoplasmic granules may also be translocated to the cell surface during activation [37]. Recent studies have found that during pregnancy the respiratory burst is regulated by HMS enzyme trafficking [12]. Enzymes of the shunt, including G-6-PDase, 6-phosphogluconolactone dehydrogenase, transaldolase and perhaps others, form a large supramolecular complex [12–14]. During pregnancy, this complex undergoes retrograde trafficking on microtubules to the microtubule organizing center. As a result, G-6-P, formed at the plasma membrane by hexokinase, is disconnected from the shunt's G-6-PDase, thereby depressing HMS activity, NADPH and ROM production. Although this mechanism explains why pregnancy neutrophils cannot become fully activated for ROM release as cells from non-pregnant women can, it does not account for the higher unstimulated levels of oxidant production found for adherent pregnancy neutrophils [12]. This study reports the discovery of another translocation event – MPO accumulation at neutrophil surfaces – that accounts for metabolic changes and greater basal levels of ROM release during pregnancy.

We have assembled multiple lines of evidence to support the hypothesis that MPO accumulates at the neutrophil surface during pregnancy and thereby alters

a cell's chemical properties. The NAD(P)H oscillatory amplitudes and the ROM/NO production rate change in parallel and are correlated with surface expression of MPO. The pregnancy-associated enhancements of NAD(P)H amplitudes and ROM release were inhibited by each of four MPO inhibitors at doses relevant to MPO inactivation. We also found that MPO could be removed from the cell surface by using a brief washing step with a high-salt solution, which would be expected to remove materials bound to the cell surface by electrostatic forces. This treatment returned NAD(P)H oscillations and ROM release to normal levels; as metabolic dynamics remained intact, cells were not dramatically affected by the protocol. Although pregnant wild-type mice displayed a normal phenotype, neutrophils from MPO knockout mice did not display the enhanced NAD(P)H oscillatory amplitudes or the increase in the basal level of ROM production found for normal adherent pregnancy neutrophils. If MPO delivery to the cell surface was sufficient to mediate these effects, it should be possible to reconstitute this effect by simply adding exogenous MPO to neutrophils from non-pregnant individuals. Indeed, we found this to be the case. Exogenous MPO promoted these changes in leukocyte metabolism and physiology. Based upon recent computational results [26], MPO could be activated by the delivery of both MPO and the NADPH oxidase to the same cell compartment, such as a phagosome or the plasma membrane. The ability of exogenous MPO to reconstitute the effect suggests that delivery to phagosomes is not required to mediate these effects – cell surface delivery is sufficient. Therefore, cell surface MPO accumulation promotes high-amplitude metabolic oscillations and enhances ROM production by adherent cells.

As MPO is stored in intracellular granules of neutrophils [37], it seems possible that degranulation contributes to their delivery to the cell surface. This is consistent with the fact that another highly charged granule protein, α-elastase, also accumulates at the surface of pregnancy neutrophils (unpublished). However, the biological mechanism of pregnancy causing MPO accumulation at the cell surface during pregnancy is not certain. Potential factors that could mediate the effect might be identified based upon their effects on cell metabolism. For example, IL-12 and IFN-γ increase the amplitude of metabolic oscillations. As IL-12 production by monocytes during pregnancy is enhanced [38], it could, for example, act in an autocrine manner to enhance metabolic oscillatory amplitudes *via* MPO trafficking.

There are reports that superoxide release is decreased in neutrophils from pregnant women [1–4], but there are also reports that ROM production is increased in neutrophils from pregnant women [39]. We believe that these claims are not mutually exclusive. We have previously shown that ROM production by adherent pregnancy neutrophils is lower than that of adherent cells from non-pregnant women activated with IFN-γ and fMLP [12]. On the other hand, unstimulated adherent pregnancy neutrophils generate roughly as many ROM as LPS-activated control cells, but far more than unstimulated adherent cells from non-pregnant women [12]. Thus, during pregnancy neutrophils have been primed or "activated" by MPO translocation to the cell surface, but due to the retrograde trafficking of the HMS complex they cannot become fully activated. The enhanced basal level of cell metabolism and oxidant production may offset, in part, the suppression caused by HMS complex trafficking during pregnancy.

It may seem paradoxical that pregnancy neutrophils, which display a heightened basal ability to produce oxidants, would also display depressed phagocytosis [8]. This may be explained by MPO trafficking. MPO-deficient neutrophils display enhanced phagocytosis [40]. Moreover, addition of exogenous MPO reduced the phagocytic activity of both normal and MPO-deficient neutrophils [40]. Therefore, a reduction in phagocytosis is consistent with surface expression of MPO.

These observations may find application in basic and clinical sciences. First, MPO expression likely constitutes a marker of normal pregnancy. Hence, its expression may be an easy and useful indicator of the status of pregnancy. MPO trafficking may be a fundamental aspect of neutrophil activation in many settings. For example, PMA-mediated cell priming may be explained by the mechanism described above. PMA treatment causes MPO to accumulate at the cell surface (Fig. 2); we suggest that this is the physical event that constitutes what is usually referred to as PMA "priming" of the respiratory burst. When a second signal arrives, such as fMLP, that causes HMS activation or NADPH oxidase transportation to the cell surface, both the shunt and the peroxidase cycle are engaged, resulting in higher amounts of ROM (full activation). Furthermore, the fraction of maternal leukocytes exhibiting high-amplitude oscillations or MPO trafficking may provide a diagnositic tool. For example, preliminary studies suggest that this parameter increases from normal levels of ∼70% to ∼90% in pre-eclampsia, but falls during interuterine fetal death (unpublished).

We believe that MPO trafficking and the peroxidase cycle will be an increasingly important element of leukocyte physiology. For example, we have recently shown that diabetic levels (∼10 to 14 mM) of glucose saturate the glycolytic apparatus, thereby allowing G-6-P to enter the HMS; during pregnancy this causes high levels of unstimulated ROM production due to simultaneous activation of the HMS and peroxidase cycles, perhaps accounting for the heightened rate of

birth defects among certain diabetic mothers [41]. In normal pregnant women, MPO and the constitutively active peroxidase cycle may represent a compromise to decrease the potentially dangerous levels of oxidants that could be produced by a fully activated leukocyte while providing a basal level of host oxidative protection.

Materials and methods

Patients

Peripheral blood samples were obtained from non-pregnant women and pregnant women after written informed consent was provided. Blood collection was IRB approved. Eligible patients were approached at the Detroit Medical Center/ Wayne State University in Detroit, MI. The non-pregnant group consisted of women in the secretory phase of the menstrual cycle who were not taking oral contraceptives and who had no history of acute or chronic inflammatory conditions (such as asthma or recent infections). Women with normal pregnancies had no medical or obstetric complications, and their pregnancies ranged in gestational age from 20 wk to term. All patients were followed through delivery and did not develop complications.

MPO knockout mice

MPO knockout mice were prepared as described [22]. Experiments were IRB approved.

Cell preparation

Neutrophils were isolated from blood samples using Ficoll-Hypaque (Sigma) density gradient centrifugation [12]. Neutrophil viability was >95% as assessed by Trypan blue exclusion. Cells were suspended in HBSS (Life Technologies, Grand Island, NY).

Reagents and antibodies

SHA, pHBAH, cyanide, HQ and PMA were obtained from Sigma Chemical Co. (St. Louis, MO). FITC was obtained from Molecular Probes (Eugene, OR). Rabbit anti-MPO polyclonal Ab was obtained from Chemicon International, Inc. (Temecula, CA). FITC- or TRITC-conjugated Ab were prepared as described [30].

Immunofluorescence staining

Neutrophils were placed on glass coverslips, incubated with 1 μg FITC-conjugated anti-MPO Ab at 4°C for 30 min, then washed again with HBSS at room temperature. In other studies, cells were fixed with 3.7% paraformaldehyde, labeled with anti-MPO Ab, then washed with HBSS.

Fluorescence microscopy

Cells were observed using an Axiovert fluorescence microscope (Carl Zeiss, Inc., New York, NY) with mercury illumination interfaced to a computer using Scion image processing software [33]. A narrow bandpass discriminating filter set (Omega Optical) was used with excitation at 485/22 nm and emission at 530/30 nm for FITC. A long-pass dichroic mirror of 510 nm was used. The fluorescence images were collected with an intensified charge-coupled device camera (Princeton Instruments, Inc., Princeton, NJ).

Detection of NAD(P)H oscillations

NAD(P)H autofluorescence oscillations were detected as described [30, 31]. An iris diaphragm was adjusted to exclude light from neighboring cells. A cooled photomultiplier tube (PMT) held in a model D104 detection system (Photon Tech. Intl., Lawrenceville, NJ) attached to a Zeiss microscope was used [30, 31].

Detection of ROM production

Pericellular release of ROM from single cells was monitored as described [30, 31]. Briefly, adherent neutrophils were surrounded in 1% gelatin containing 100 ng/mL dihydrotetramethylrosamine (H_2TMRos) (Molecular Probes, Eugene, OR). ROM, especially H_2O_2, released by cells entered the gelatin matrix, where they oxidized H_2TMRos to tetramethylrosamine (TMRos), which was detected by fluorescence microscopy. NO production was monitored in the same manner as single-cell production of ROM, with the exception that the gelatin was mixed with 15 μM diaminofluorescein-2 diacetate (DAF-2DA) (Daiichi Kagaku Yakuhin, Tokyo, Japan) in place of H_2TMRos.

Acknowledgements: This work was supported by grants from the NIAID and the National Multiple Sclerosis Society (H.R.P.) and The Division of Intramural Research of NICHD/NIH (R.R.).

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