Published in final edited form as:

J Nutr Biochem. 2018 December ; 62: 28–34. doi:10.1016/j.jnutbio.2018.08.003.

Myeloperoxidase Deficiency Attenuates Systemic and Dietary Iron-induced Adverse Effects

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Abstract

Iron deficiency is routinely treated with oral or systemic iron supplements, which is highly reactive and could induce oxidative stress via augmenting the activity of proinflammatory enzyme, myeloperoxidase (MPO). To investigate the extent to which MPO is involved in iron-induced toxicity, acute (24h) iron toxicity was induced by intraperitoneal administration of FeSO₄ (25 mg/kg body weight) to MPO deficient (*Mpo*KO) mice and their WT littermates. Acute iron-toxicity was also assessed in WT mice pretreated with a MPO inhibitor, 4-aminobenzoic acid hydrazide (ABAH). Systemic iron administration upregulated circulating MPO, neutrophil elastase and elevated systemic inflammatory and organ damage markers in WT mice. However, genetic deletion of MPO or its inhibition significantly reduced iron-induced organ damage and systemic inflammatory responses. In contrast to the acute model, 8 weeks of 2% carbonyl iron diet feeding to WT mice did not change the levels of circulating MPO and neutrophil elastase but promoted their accumulation in the liver. Even though both *Mpo*KO and WT mice displayed similar levels of diet-induced hyperferremia, *Mpo*KO mice showed significantly reduced inflammatory response and oxidative stress than the WT mice. In addition, WT bone marrow-derived neutrophils (BMDN) generated more reactive oxygen species than MPO deficient BMDN upon iron

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Author contribution:

X.X. designed and performed the experiments, analyzed the data and wrote the paper. P.S. and B.S.Y. performed experiments and involved in discussion. J.A.H. and V.S. participated in histologic analysis. M.V-K. designed and directed the project, and co-wrote the paper.

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Conflict of interest

The authors have declared that no conflict of interest exists.

stimulation. Altogether, genetic deficiency or pharmacologic inhibition of MPO substantially attenuated acute and chronic iron-induced toxicity. Our results suggest that targeting MPO during iron supplementation is a promising approach to reduce iron-induced toxicity/side effects in vulnerable population.

Keywords

Neutrophils; MPO; Reactive oxygen species; Acute phase proteins; Proinflammatory cytokines; iron overload

1 Introduction

Iron deficiency is one of the most prevalent micronutrient deficiency throughout the globe. In clinical practice, oral or intravenous iron supplementation are routinely used to treat iron-deficient anemia not only in individuals with iron deficiency, but also in pregnant women and inflammatory bowel disease patients [1, 2]. Paradoxically, iron as a redox active metal ion also participates in Fenton reaction and generates notorious hydroxyl free radicals (OH $\cdot$), which can cause damage to virtually every biomolecule in the cell. Acute iron poisoning may result in mild diarrhea, vomiting to often life-threatening conditions in individuals who take iron supplements [3, 4]. Besides dietary iron overload, genetic iron overload is also common in hereditary hemochromatosis patients, with an incidence of 1 in 300 non-Hispanic white in the United States [5], who are defective in regulating iron absorption and usually suffer from clinical complications, such as liver and heart diseases [6, 7]. Transfusional iron overload is an unavoidable side effect of frequent blood transfusions in sickle cell anemia and thalassemia major patients [8]. The excess iron accumulated in vital organs and other tissues can increase the proinflammatory responses and may eventually result in multiorgan failure [8].

Myeloperoxidase (MPO) is the most abundant neutrophilic proinflammatory heme enzyme, which requires iron for bioactivity; it is stored in the azurophilic granules, accounting for nearly 5.0% of the cellular dry mass [9]. MPO plays a critical role as an antimicrobial agent that can be either released into phagolysosome or secreted out of neutrophils. Its antimicrobial activity is primarily due to its capability to catalyze halide (Cl $^-$, Br $^-$, and I $^-$) and pseudo-halide (SCN $^-$) ions in the presence of H $_2$ O $_2$ to generate their respective potent prooxidant, i.e., hypohalous acid, which mediates microbial killing. As such, MPO level is routinely measured as a direct correlation for the extent of neutrophil infiltration [10]. Although the generation of oxidants by MPO is an appropriate immunologic response to curtail perturbing pathogens, but its inappropriate release/activation can cause substantial collateral damage to host tissues. Besides producing cytotoxic oxidants, MPO facilitates neutrophil recruitment and activation [11, 12], which promotes release of elastase [13, 14] and which can further aggravate tissue damage. Furthermore, MPO is also an endogenous source for free iron that can participate in Fenton reaction causing oxidative stress [15]. Based on accumulated data, we hypothesized that MPO may play a key role in driving the adverse effects induced by excess systemic and dietary iron supplementation.

Our study demonstrates that genetic deletion of MPO offers protection against dietary and systemic iron-induced toxicity in mice. Additionally, mice treated with MPO-specific inhibitor, 4-aminobenzoic acid hydrazide (ABAH), better withstand the systemic iron overload-induced adverse effects than their vehicle-treated counterparts. In conclusion, our results indicate that neutrophil-restricted MPO plays a key role in iron-induced toxicity and that targeting MPO may be a viable strategy to mitigate the side effects associated with iron supplementation.

2. Materials and Methods

2.1 Materials

Duoset mouse MPO, neutrophil elastase (ELA2), lipocalin 2 (Lcn2), serum amyloid A (SAA), interleukin (IL)-6, IL-1 β , and CXCL1 (KC) ELISA kits were obtained from R&D Systems. Aspartate transaminase (AST) and alanine transaminase (ALT) kits were purchased from Randox Laboratories. Ferrous sulfate heptahydrate was obtained from Alfa Aesar. 4-aminobenzoic acid hydrazide (ABAH) was procured from Sigma. qScript cDNA synthesis kit and SYBR® Green mix was acquired from Quanta Biosciences. All other fine chemicals used in present study were reagent grade and procured from Sigma.

2.2 Mice

MPO-deficient (*Mpo*KO) mice on C57BL/6 background were procured from Jackson Laboratory (Bar Harbor, ME) and bred with C57BL/6 wild type (WT) mice. The resulting off springs were crossed to generate homozygous *Mpo*KO mice and their WT littermates. These mice were bred in-house in the animal facility at The Pennsylvania State University. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at The Pennsylvania State University.

2.3 Systemic iron administration

Eight weeks old male *Mpo*KO mice and WT littermates (n=5–6) were intraperitoneally administered with ferrous sulfate (FeSO₄·7H₂O) in sterile saline (25 mg/kg body weight of Fe²⁺) and control mice were given saline alone. Reconstitution of FeSO₄ in PBS results in precipitation, therefore saline was used. In a separate experiment, female WT mice (n=4–5) were pretreated with MPO inhibitor, 4-aminobenzoic acid hydrazide (ABAH, 40 mg/kg), or vehicle 1h before and 12h after iron administration. Body weights were measured before and 24h after iron injection. Mice were bled retro-orbitally and hemolysis-free serum collected for the quantification of organ damage and inflammatory markers.

2.4 Carbonyl iron enriched diet

Seven weeks old male *Mpo*KO mice and their WT littermates (n=4–5) were either fed on a lab chow (TestDiet 5001; 241g protein/kg, 50g fat/kg, 487g carbohydrate/kg, 52g crude fiber/kg and 240 ppm iron) or lab chow fortified with 2% carbonyl iron (*TestDiet* 1816708–201; 20,000 ppm iron) formulated by *TestDiet* (St. Louis, MO) for 8 weeks. Mice were monitored for body weights weekly. At termination of the experiment, mice were euthanized via CO₂ asphyxiation. Hemolysis-free serum and liver tissue were collected and stored at –80°C until further analysis.

2.5 Enzyme-linked immunosorbent assay (ELISA)

Liver samples were homogenized in RIPA buffer with protease inhibitor to make 100 mg/ml suspension and centrifuged (4°C, 10,000g for 10 min). Clear supernatants were collected and diluted for MPO, ELA2, Lcn2, IL-6 and IL-1 β levels measurement. MPO, ELA2, Lcn2, SAA and KC levels were measured in serum samples. All samples were diluted and analyzed using DuoSet ELISA kits according to manufacturer instructions.

2.6 Assay of serum transaminases

Serum aspartate transaminase (AST) and alanine transaminase (ALT) were measured using kits from Randox (Crumlin, UK) according to the manufacturer's instructions.

2.7 Measurement of serum total iron and liver non-heme iron

Total iron was measured in serum samples as described in [16]. Briefly, serum samples were deproteinized to liberate any protein-bound iron. After centrifuging, the supernatants were collected and mixed with chromogen solution and the optical density of the chromogen was measured at 562 nm. Hepatic iron concentration was measured by the non-heme iron assay protocol as described by Torrance and Bothwell [17]. Both serum total iron and hepatic iron concentration were determined using a standard curve generated using the iron AA standard.

2.8 Liver histology and iron staining

Liver samples were fixed in 10% neutral buffered formalin and then processed for paraffin embedding. Serial paraffin sections (5.0 μ m) were stained with hematoxylin and eosin (H&E). To visualize iron, sections were stained by iron stain kit (Richard-Allan Scientific, MI) according to the manufacturer's instructions.

2.9 Quantitative RT-PCR

Mouse liver were collected in *RNA Later* (Sigma) and stored in -80°C until analysis. Total mRNA was extracted by using Trizol reagent (Sigma) and cDNA was synthesized from mRNA (0.8 μ g) for qRT-PCR using SYBR green (Quanta) according to manufacturer's protocol. The following primers were used to assess gene expression: *Hamp* (hepcidin) 5'-AGAAAGCAGGGCAGACATTG-3' and 5'-CACTGGGAATTGTTACAGCATT-3' [18]; *36B4* 5'-TCCAGGCTTTGGGCATCA-3' and 5'-CTTTATTTCAGCTGCACATCACTCAGA-3' [19]. *36B4* was used to normalize relative mRNA expression by using Ct (2^{-Ct}) method. Fold change was determined by comparison to the untreated control group.

2.10 Lipid peroxidation

Lipid peroxidation in liver was measured by estimating thiobarbituric acid reactive substances as malondialdehyde (MDA) as described in [20].

2.11 Bone marrow-derived neutrophils (BMDN) generation and measurement of reactive oxygen species (ROS)

BMDN were isolated from female WT and *Mpo*KO mice (6–8 weeks old) using the Histopaque gradient method [21][21][21]. This method yielded >95% pure and >99% viable

Ly6G⁺ neutrophils as analyzed by flow cytometry. Isolated BMDN (2×10^5 cells per well) were incubated with or without 25 μM Fe²⁺ or Fe³⁺ as indicated for 3 h at 37°C and 5% CO₂. Cells were washed with PBS, stained with 5.0 μM CellROX Deep Red Reagent (Molecular Probes) for 30 min at 37°C in the dark, and washed twice with PBS. Fluorescence was measured by flow cytometry (Accuri C6; BD Biosciences) and analyzed using BD Accuri C6 software (BD Biosciences). Intracellular ROS was expressed as the fold change of mean fluorescence intensity (MFI) normalized to the controls.

2.12 Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical significance between two groups was analyzed using unpaired, two-tailed t-test. Data from more than two groups was compared using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests to compare the mean of each column with the mean of every other column. $p < 0.05$ was considered as statistically significant. All statistical analyses were performed with the GraphPad Prism 6.0 program (GraphPad Inc, La Jolla, CA).

3. Results:

3.1 Genetic deletion of MPO alleviates iron-induced acute toxicity in mice

The redox metal ion, iron is proinflammatory and is known to induce acute systemic inflammatory response [22]. However, the effective mediators for such inflammatory response is less clear. To investigate the role of prooxidative metalloenzyme MPO in systemic iron administration induced acute inflammatory response, we analyzed several parameters of adverse effects of iron at 24h post-administration in MPO deficient (*Mpo*KO) mice and their WT littermates. As shown in the (Figure 1A), WT mice lost more body weight compared to *Mpo*KO mice, yet, did not reach statistical significance. WT mice challenged with iron displayed 44% increase in serum MPO (Figure 1B), which was not detectable (N.D.) in *Mpo*KO mice confirming the specificity of ELISA and genotype of the mice. Organ damage markers AST and ALT levels were highly upregulated in WT mice, whereas such increase was not observed in *Mpo*KO mice (Figures 1C-D). Neutrophil elastase (ELA2), which is specifically secreted by neutrophils, was markedly increased in WT mice (Figure 1E), indicating more neutrophil oxidative burst occur upon iron challenge. Lipocalin 2 (Lcn2), [aka; neutrophil gelatinase-associated lipocalin (NGAL) in human] and serum amyloid A (SAA), which are general inflammatory markers, were elevated in both WT and *Mpo*KO mice (Figures 1F-G). However, compared to *Mpo*KO mice, the systemic increase of these markers were 1.5 and 1.7 folds higher for Lcn2 and SAA levels, respectively, in WT mice (Figures 1F-G). In addition, WT mice challenged with iron also showed significant upregulation of chemokine KC, a neutrophil chemoattractant [23], than *Mpo*KO mice (Figure 1H).

3.2 Pharmacological inhibition of MPO activity alleviates iron-induced acute toxicity

Next, we investigated whether specific pharmacologic inhibition of MPO can recapitulate our findings with *Mpo*KO mice. Therefore, we pretreated WT mice with 4-aminobenzoic acid hydrazide (ABAH), which is an irreversible and specific inhibitor of MPO [24]. After 24h of iron administration, the loss of body weight was substantially reduced in mice treated

with ABAH (Figure 2A). Control WT mice treated with ABAH alone did not show increase in serum proinflammatory cytokines or acute phase proteins (data not shown). In ferrous iron injected mice, ABAH treatment did not reduce MPO levels in the serum (Figure 2B). However, ABAH treatment reduced the iron-induced elevation of serum AST (Figure 2C), ALT (Figure 2D), ELA2 (Figure 2E), Lcn2 (Figure 2F), SAA (Figure 2G) and KC (Figure 2H) levels, indicating that iron-induced adverse effects are probably mediated downstream of MPO activity.

3.3 MPO deficiency protects against the adverse effects of excess dietary iron

Next, we asked whether MPO deficient mice were also protected against chronic adverse effects of dietary iron overload. We maintained WT and *Mpo*KO mice on 2% carbonyl iron diet and monitored for 8 weeks. WT mice fed on high iron diet gained a lesser body weight (albeit differences were not significant) compared to other groups throughout the feeding period (Figure 3A). As anticipated, high iron diet increased serum total iron (Figure 3B) and liver non-heme iron (Figure 3C) in both WT and *Mpo*KO mice. Furthermore, iron staining also reflects iron accumulation in the liver of high iron diet-fed WT and *Mpo*KO mice (Figure 3E). Correlated to liver non-heme iron estimation results, *Mpo*KO liver displayed noticeably more iron (Figures 3C, E). Liver is not only a major organ for iron storage, but also plays a critical role in iron homeostasis. Therefore, we analyzed dietary iron induced hepatic gene expression of *hepcidin* (*Hamp*) in both WT and *Mpo*KO mice; however, there was no significant difference (Figure 3D), indicating the iron status are comparable between WT and *Mpo*KO high iron-fed mice. Unlike the systemic iron challenge, dietary excess iron did not elevate serum MPO (Figure 4A) and ELA2 (Figure 4B) levels. Even though no significant differences were observed in serum AST, ALT and SAA levels (S. Figure 1A-C), serum Lcn2 and KC levels were significantly higher in hyperferremic WT mice, but such increase was not displayed in *Mpo*KO mice (Figures 4C-D), suggesting that MPO also plays key role in dietary iron-induced chronic adverse effects.

Since liver is the primary organ for iron accumulation, we next analyzed liver pathology associated with iron overload. No obvious liver injury/damage was observed by H&E staining (S. Figure 1D). Excess dietary iron fed WT mice displayed elevated hepatic MPO levels (Figure 4E) that correlated with significant increase in ELA2 (Figure 4F) and Lcn2 (Figure 4G), representing the higher neutrophil activation in WT liver. Pro-inflammatory cytokines IL-6 (Figure 4H) and IL-1 β (Figure 4I) only increased in WT mice but not in *Mpo*KO mice fed with high iron diet. Interestingly, malondialdehyde (MDA), an end product of lipid peroxidation, was 2-fold higher in *Mpo*KO mice at basal levels compared to WT mice (Figure 4J), which could be due to activation of compensatory pathways in the absence of MPO. As anticipated, iron overload markedly increased hepatic MDA levels in WT mice, however, the excess iron accumulation did not further increase MDA levels in *Mpo*KO mice (Figure 4J), demonstrating that excess iron induced more oxidative stress in WT mice than *Mpo*KO mice.

3.4 MPO deficient BMDN generates less reactive oxygen species (ROS) upon iron stimulation

MPO is one of the major generator of hypochlorous acid, a potent oxidizing agent that could further increase ROS generation, which promotes oxidative stress. To investigate the extent to which MPO participates in iron-induced ROS generation, we stimulated BMDN with ferrous and ferric iron *ex vivo*. Even though both ferrous and ferric forms of iron are physiologically functional, the reduced form has higher pro-oxidative property. WT BMDN were able to upregulate five-fold higher ROS in response to ferrous iron than unstimulated BMDN, whereas such response was reduced by 50% in MPO deficient BMDN (Figures 5A-B). Similarly, ferric iron was able to induce ROS in WT BMDN (albeit 50% lesser ROS than ferrous), but MPO deficient BMDN failed to induce ROS in response to oxidized form of iron (Figures 5A-B).

4. Discussion:

Iron is essential for various biological processes; therefore, several mechanisms are in place to uptake and stringently recycle this metal ion in the body. However, the lack of any physiological mechanism(s) to excrete iron poses a health risk when its dietary intake exceeds the threshold tolerable by the host. When excess iron is not properly contained, it could emerge as a labile iron pool, which serves as a redox engine that readily participate in Fenton reaction, resulting in a vicious cycle of oxidative stress and inflammation. The liver is often the first organ to be affected by iron overdose, given its role as the major repository for iron in the form of ferritin or hemosiderin. In the current study, we document an increase in MPO levels in the liver and the circulation in response to dietary and systemic iron challenge, respectively. These findings led us to envision that such MPO response may be pathological and could be accountable, in part, for the adverse effects of iron when taken in excess. Accordingly, we demonstrate that loss of MPO activity, either due to genetic deficiency or pharmacologic inhibition, substantially protects mice from iron-induced inflammation. Our observation that MPO-deficient neutrophils generate less ROS upon stimulated with iron *ex vivo* further implicates the detrimental role of MPO in this context.

MPO is exclusively expressed by neutrophils and is subsequently stored within their azurophilic granules. When neutrophils get activated, they release MPO to facilitate the generation of the oxidizing agent hypochlorous acid, which lyses microbes by halogenating their membrane components [9]. However, these MPO-derived oxidants are also as harmful to host tissues, causing collateral oxidative damage especially when dispensed under sterile inflammatory conditions [25–28]. The restriction of MPO to neutrophils may be viewed as an evolutionary advantage and adaptive strategy to limit MPO-induced oxidative stress and collateral damage not only during iron overload, but also throughout the course of other inflammatory diseases. The presence of endogenous inhibitor of MPO, ceruloplasmin, whose levels increase during acute and chronic inflammation [29–31] further substantiates the importance to restrict the activity of MPO in both healthy and disease conditions. NADPH oxidase (NOX2), another critical antibacterial enzyme, a major ROS generator, is also expressed in neutrophils; however, they differ in regards to their specific cell-type expression, structure, location, and assembly for enzyme activation. For instance, MPO

display enzyme activity in its soluble form, whereas NOX2 requires proper assembly of cytosolic and membrane subunits for its bioactivity. Unlike MPO, NOX2 is not restricted to neutrophils, but is expressed by other professional phagocytes (e.g. macrophages/monocytes, eosinophils) and dendritic cells [32].

MPO deficiency is the most common inherited disorder of phagocytes and approximately 1 in 4,000 individuals were affected with complete deficiency, but rarely associated with clinical symptoms [33]. In humans, MPO deficiency is known to protect against number of chronic ailments, such as cardiovascular diseases [34, 35], rheumatoid arthritis [36, 37], and MPO levels positively correlate with the severity of multiple sclerosis [38, 39]. Similarly, in many murine models, the deficiency and inactivation of MPO are known to be protect from skin injuries [40, 41] chronic kidney diseases [42], lung inflammation [43, 44], rheumatoid arthritis [45], and coronary artery disease [27]. Aside from the traditional MPO inhibitor ABAH that we used in this study, several other pharmacologic inhibitors (e.g. INV-315, 2-thioxanthines) have emerged [46–48], and have been suggested as potential therapeutic drugs for various inflammation-associated disorders [49]. It could be promising approach to use specific inhibitors of MPO to treat chronic iron overload, i.e., ameliorating iron-induced oxidative stress and inflammatory responses, specifically in individuals susceptible to iron overnutrition. However, one major limitation of prolonged use of MPO inhibitors is that it may compromise the immune system and worsen the risk for opportunistic infections associated with iron overload [50, 51].

Compared to iron deficiency, iron-induced adverse effects due to acute iron poisoning or chronic iron overload draws less attention in public and in clinics, but in fact, this hyperferremic condition also requires equal and immediate medical care. Our study demonstrates that loss of MPO activity moderated iron-induced toxic effects, potentially through ameliorating neutrophil activity and reducing MPO-derived oxidative species generation, which otherwise would aggravate unwanted oxidative stress and prolong the inflammatory responses. We cannot rule out the possibility that excess iron-induced ROS could also negatively influence mitochondrial respiration and membrane potential [52]. Even though our study did not capture iron-induced global adverse responses, but substantially reduced systemic and tissue damage markers in MPO deficient mice may reflect such responses and highlights the key role of MPO in aggravating adverse effects. Collectively, our study highlights that MPO may be a promising therapeutic target for alleviating iron-induced toxicity in vulnerable population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

funding disclosure

This work was supported by grants from the National Institutes of Health (NIH) R01 (DK097865) to M.V-K.

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Figure 1. MPO deficiency protects against iron-induced adverse effects.

Eight weeks old male *Mpo*KO mice and their WT littermates were injected intraperitoneally Fe²⁺ (25 mg/kg of body weight; ferrous sulfate) or sterile saline as vehicle. After 24h, mice were monitored for body weights (A). Serum was collected to measure levels of MPO (B), AST (C), ALT (D), ELA2 (E), Lcn2 (F), SAA (G), and KC (H). Data presented are means $\pm$ SEM, n=5–6. Bar without a common letter differ. $p < 0.05$

Figure 2. ABAH, an inhibitor of MPO enzyme activity, alleviates iron-induced toxicity. Eight weeks old female WT mice were injected Fe²⁺ (25 mg/kg of body weight; ferrous sulfate), intraperitoneally or sterile saline as vehicle. These mice were pretreated with 40 mg/kg of ABAH in PBS or vehicle control 1h before and 12h after iron administration. At 24h post iron injection, mice were monitored for body weight (A). Serum MPO (B), AST (C), ALT (D), ELA2 (E), Lcn2 (F), SAA (G), and KC (H) levels were measured. Values are means ± SEM, n=4–5. Bar without a common letter differ. $p < 0.05$

Figure 3. Excess dietary-iron induced hyperferremia in WT and *MpoKO* mice.

Seven weeks old male WT and *MpoKO* mice were maintained on 2% carbonyl iron diet or control diet for 8 weeks. Body weight was monitored (A). Serum total iron (B), liver non-heme iron (C), and liver mRNA expression of *Hamp* (D) were quantified. Iron staining (E) was performed to visualize liver iron accumulation (10x magnification). Values are means $\pm$ SEM (A-D), $n=4-5$. Bar without a common letter differ. $p < 0.05$

Figure 4. MPO deficiency reduces systemic inflammation and liver pro-inflammatory molecules in diet-induced chronic iron overload.

Seven weeks old male WT and *Mpo*KO mice were maintained on 2% carbonyl iron diet or control diet for 8 weeks. Serum MPO (A), ELA2 (B), Lcn2 (C) and KC (D) and liver MPO (E), ELA2 (F), Lcn2 (G), IL-6 (H), and IL-1 β (I) were analyzed by ELISA. Liver lipid peroxidation was measured as MDA (J). Values are means $\pm$ SEM, $n=4-5$. Bar without a common letter differ. $p < 0.05$

Figure 5. MPO deficient BMDN display reduced iron stimulated ROS generation. BMDN cells were isolated from 6–8 weeks old female WT and *MpoKO* mice. ROS generation was stimulated by supplementing the media with Fe^{2+} or Fe^{3+} (25 μ M) and analyzed by flow cytometry (A) and quantified as mean fluorescence intensity (MFI) (B). Values are means $\pm$ SEM, n=3. Bar without a common letter differ. p< 0.05.