

RESEARCH PAPER

Analysis of the mechanism by which melatonin inhibits human eosinophil peroxidase

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Background and purpose: Eosinophil peroxidase (EPO) catalyses the formation of oxidants implicated in the pathogenesis of various respiratory diseases including allergy and asthma. Mechanisms for inhibiting EPO, once released, are poorly understood. The aim of this work is to determine the mechanisms by which melatonin, a hormone produced in the brain by the pineal gland, inhibits the catalytic activity of EPO.

Experimental approach: We utilized H₂O₂-selective electrode and direct rapid kinetic measurements to determine the pathways by which melatonin inhibits human EPO.

Key results: In the presence of plasma levels of bromide (Br⁻), melatonin inactivates EPO at two different points in the classic peroxidase cycle. First, it binds to EPO and forms an inactive complex, melatonin-EPO-Br, which restricts access of H₂O₂ to the catalytic site of the oxidation enzyme. Second, melatonin competes with Br⁻ and switches the reaction from a two electron (2e⁻) to a one electron (1e⁻) pathway allowing the enzyme to function with catalase-like activity. Melatonin is a bulky molecule and binds to the entrance of the EPO haem pocket (regulatory sites). Furthermore, Br⁻ seems to enhance the affinity of this binding. In the absence of Br⁻, melatonin accelerated formation of EPO Compound II and its decay by serving as a 1e⁻ substrate for EPO Compounds I and II.

Conclusions and implications: The interplay between EPO and melatonin may have a broader implication in the function of several biological systems. This dual regulation by melatonin is unique and represents a new mechanism for melatonin to control EPO and its downstream inflammatory pathways.

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Abbreviation: EPO, eosinophil peroxidase

Introduction

Eosinophils are white blood cells produced in the bone marrow and migrate to various tissues on a regular basis (Klebanoff *et al.*, 1983; Ackerman *et al.*, 1985). They play an essential role in host defences through their ability to release toxic substances to kill parasites (Klebanoff *et al.*, 1983; Ackerman *et al.*, 1985). They also play a role in tissue surveillance and allergic responses (Rothenberg 1998; Holt *et al.*, 1999). Eosinophils are found in higher numbers in asthmatic airways (Klebanoff *et al.*, 1983). Eosinophil peroxidase (EPO) is secreted from eosinophilic granules during phagocyte activation (Jong *et al.*, 1980; Klebanoff

et al., 1983, 1989; Weiss *et al.*, 1986; Agosti *et al.*, 1987; Mayeno *et al.*, 1989; Slungaard and Mahoney, 1991; Samoszuk *et al.*, 1994; Rothenberg 1998). EPO is a monomeric haemoprotein comprised of light and heavy chains with molecular masses of 15.5 and 50 kDa, respectively (Bolscher *et al.*, 1984a). EPO and related mammalian peroxidases (such as myeloperoxidase (MPO) and lactoperoxidase) use H₂O₂ as the electron acceptor in the catalysis of oxidative reactions, which have a role in generating inflammatory injury and cardiovascular diseases (Jong *et al.*, 1980; Klebanoff *et al.*, 1984; Mayeno *et al.*, 1989; Slungaard and Mahoney, 1991). The enzyme catalyses the formation of a cytotoxic and antimicrobial chemical, hypobromous acid (HOBr), by oxidation of bromide (Br⁻) as a favoured substrate (Bolscher *et al.*, 1984a; Arlandson *et al.*, 2001). Indeed, EPO is the only human enzyme known to selectively generate reactive brominating species in physiological concentration of halides (Wu *et al.*, 1999;

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MacPherson *et al.*, 2001). Brominated products can serve as markers to identify sites of EPO-mediated oxidative damage (Henderson *et al.*, 2001).

Eosinophil peroxidase-Fe(III) reacts rapidly with H_2O_2 and generates a ferryl π -cation radical (EPO-Fe(IV) = $\text{O}^{+\pi}$) intermediate Compound I (Weiss *et al.*, 1986; Mayeno *et al.*, 1989; Wu *et al.*, 1999; Mitra *et al.*, 2000). Compound I is capable of oxidizing either Br^- through a two-electron ($2e^-$) transition, generating EPO-Fe(III) and HOBr, or oxidizing multiple organic and inorganic molecules by two successive sequential one-electron ($1e^-$) transitions, generating their corresponding free radicals and the peroxidase intermediates Compound II (E-Fe(IV) = O) and E-Fe(III), respectively (Weiss *et al.*, 1986; Mayeno *et al.*, 1989; Mitra *et al.*, 2000). Compound II is a longer lasting intermediate whose decay to EPO-Fe(III) is considered to be the rate-limiting step during steady-state catalysis (Mitra *et al.*, 2000). Acceleration and decay of Compound II formation has been noted with a series of organic and inorganic substrates and physiological reductants like nitric oxide and superoxide (Mitra *et al.*, 2000; Abu-Soud *et al.*, 2001; Abu-Soud and Hazen, 2001; Galijasevic *et al.*, 2007).

Melatonin (5-methoxy-*N*-acetyltryptamine) is a ubiquitous neurohormone, synthesized from the amino acid tryptophan and secreted by the pineal gland in the brain. It is involved in numerous aspects of biological and physiological regulation, including circadian entrainment, blood pressure regulation, oncogenesis, retinal physiology, seasonal reproduction, ovarian physiology and immunity (Gastel *et al.*, 1998). Its synthesis and release are stimulated by darkness and suppressed by light (Wurtman *et al.*, 1963; Brown, 1994; Arendt *et al.*, 2000; Herxheimer and Waterhouse, 2003; Herxheimer and Petrie, 2004; Malhotra *et al.*, 2004; Hardeland, 2005; Schaffazick *et al.*, 2005). It can inhibit H_2O_2 -induced lipid peroxidation and lipoprotein modification. However, the possible *in vivo* reaction pathways for these properties have not yet been identified (Hardeland, 2005; Schaffazick *et al.*, 2005).

Melatonin is thought to act as a potent anti-inflammatory reagent through its action as an antioxidant and free-radical scavenger in both *in vivo* and *in vitro* (Hardeland, 2005). It plays an important role in protecting cell membranes from lipid peroxidation, neutralizing hydroxyl radicals and may bind to DNA, promoting further protection beyond antioxidant activity. Melatonin may stimulate some important antioxidative enzymes such as superoxide dismutase, glutathione peroxidase and glutathione reductase (Hardeland, 2005).

Synthetic melatonin supplements, administered orally or by intramuscular injection, have been used for a variety of medical conditions, most notably for disorders related to sleep (Goldman, 1995; Altun and Ugur-Altun, 2007). These uses have been tested in humans or animals. Safety and effectiveness have not always been proven. A wide range of melatonin doses has been used, including 'low dose' (0.1–1.0 mg) for jet lag and insomnia in the elderly, 'moderate doses' (5 and 10 mg), often taken by mouth 30–60 min prior to sleep time for sleep disturbances in children with neuropsychiatric disorders and bipolar disorder, or 'high dose' (50–1200 mg) for treating cancer

and migraine headaches (Goldman, 1995; Altun and Ugur-Altun, 2007).

Despite the potential significance of EPO to both human health and disease, little is known about the factors that downregulate its activity and function. In this report, we use a combination of H_2O_2 -selective electrode, optical absorbance and rapid kinetics measurements to show that melatonin is an effective inhibitor of EPO.

Methods

Enzyme purification

Human EPO was purchased from Lee Biosolutions Inc. (St Louis, MI, USA). The enzyme was isolated using a modification of the method of Jorg *et al.* (1982), using guaiacol oxidation as the assay. Purity of isolated EPO was established by demonstrating a Reinheitszahl (RZ) value of >0.85 (A_{412}/A_{280}). SDS-polyacrylamide gel electrophoresis with Coomassie blue staining and gel tetramethylbenzidine peroxidase staining was performed to confirm no contaminating MPO activity (van Dalen *et al.*, 1997). An extinction coefficient of $112\,000\text{ M}^{-1}\text{ cm}^{-1}$ per haem of EPO was used to determine the final EPO concentration used in each experiment (Bolscher *et al.*, 1984b; Carlson *et al.*, 1985).

H_2O_2 -selective electrode measurements

H_2O_2 measurements were carried out using an H_2O_2 -selective electrode (Apollo 4000 Free Radical Analyzer; World Precision Instruments, Sarasota, FL, USA). Experiments were performed at 25°C by immersing the electrode in 3 mL of 200 mM sodium phosphate buffer, pH 7, under air. H_2O_2 at a concentration of $20\ \mu\text{M}$ was added to a continuously stirred buffer solution containing various concentrations of melatonin and/or Br^- ($70\ \mu\text{M}$) during which the rise and fall of H_2O_2 concentration was continuously monitored. Where indicated, $20\ \mu\text{L}$ EPO (15 nM final) was added to the reaction mixture. To determine the effect of melatonin on H_2O_2 consumption by EPO, similar experiments were repeated by adding $20\ \mu\text{L}$ of the enzyme solution (15 or 60 nM final) to a continuously stirred H_2O_2 solution in the absence and presence of various concentrations of melatonin.

Absorbance measurements

Spectra were recorded with a Cary 100 Bio UV-visible spectrophotometer, at 25°C , in the phosphate buffer pH 7 (see above). Experiments were performed with a 1 mL cuvette containing EPO (0.7 – $1.0\ \mu\text{M}$) preincubated with increasing concentrations of melatonin (6 – $200\ \mu\text{M}$), in the absence and presence of $70\ \mu\text{M}$ Br^- . Concentrated volumes of H_2O_2 solution were added to the sample cuvette ($20\ \mu\text{M}$ final) and absorbance changes were recorded from 300 to 700 nm.

Rapid kinetic measurements

The kinetic measurements of EPO Compound I and/or Compound II formation and decay in the absence and presence of different melatonin and/or Br^- concentrations

were performed with a dual-syringe stopped-flow instrument obtained from Hi-Tech Ltd (model SF-61, Tgk Scientific Limited, Bradford on Avon, UK). Experiments were initially performed under conditions identical to those recently reported for EPO and other related haemoproteins to facilitate comparison (Galijasevic *et al.*, 2004, 2006; Proteasa *et al.*, 2007). Measurements were carried out under an aerobic atmosphere at 10 °C following rapid mixing of equal volumes of an H₂O₂-containing phosphate buffer solution and a peroxidase solution containing 70 μM Br⁻ and/or different melatonin concentrations. Reactions were monitored at both 412 and 432 nm. The time course of the absorbance change was fitted to a single-exponential, $y = 1 - e^{-kt}$, or a double-exponential $Y = Ae^{-k_1t} + Be^{-k_2t}$ function as indicated. Signal-to-noise ratios for all kinetic analyses were improved by averaging at least six to eight individual traces.

In some experiments, the stopped-flow instrument was attached to a rapid scanning diode array device (Hi-Tech) designed to collect multiple numbers of complete spectra (200–800 nm) at specific time ranges. The detector was automatically calibrated relative to a holmium oxide filter, as it has spectral peaks at 360.8, 418.5, 446.0, 453.4, 460.4, 536.4 and 637.5 nm, which were used by the software to correctly align pixel positions with wavelength. Rapid scanning experiments involved mixing solutions of peroxidase (1–2 μM) preincubated with 200 μM Br⁻ in the absence or in the presence of increasing (7–200 μM) melatonin concentrations with the phosphate buffer containing 40 μM H₂O₂, at 10 °C.

Solution preparation

A stock solution of melatonin was dissolved in dimethylformamide and then diluted to the required concentration with the phosphate buffer (pH = 7). The final concentration of dimethylformamide in all melatonin solutions used was less than 1% and had no effect on EPO reactions.

Materials

Melatonin, H₂O₂, sodium bromide and dimethylformamide used were of the highest purity grades and obtained from Sigma-Aldrich (St Louis, MO, USA).

Results

Ability of melatonin to inhibit EPO catalytic activity

We initially used an H₂O₂-selective electrode to determine how melatonin regulates the catalytic activity of EPO and its role in substrate switching, steering the catalytic activity of EPO from a 2e⁻ to a 1e⁻ oxidation pathway. As a control, we first tested whether melatonin alone and in combination with a plasma level of Br⁻ has an effect on H₂O₂ auto-reduction rate. Following addition of 20 μM H₂O₂ (final) to the continuously stirred reaction mixture, the H₂O₂ signal rose rapidly, achieved a maximum after ~30 s and fell gradually as H₂O₂ was depleted by auto-reduction (Figure 1, dashed line). Addition of different concentrations of

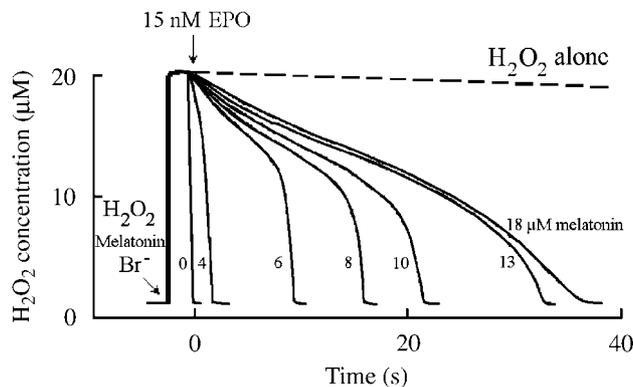


Figure 1 Melatonin inhibits H₂O₂ consumption by EPO in the presence of Br⁻. A typical recording by an H₂O₂-selective electrode demonstrating auto-reduction of H₂O₂ (20 μM) following addition to stirred phosphate buffer (200 mM; pH 7) supplemented with 70 μM Br⁻, at 25 °C (dashed line). Addition of 20 μL (15 nM final concentration) of EPO solution to the H₂O₂/Br⁻ stirred buffer caused rapid H₂O₂ consumption. Addition of 20 μL (15 nM final concentration) EPO solution to the H₂O₂/Br⁻ solution supplemented with increasing melatonin concentrations 4, 6, 8, 10, 13 and 18 μM (solid lines from left to right, respectively) causing slower H₂O₂ removal. Tracings shown are from a typical experiment performed at least three times. EPO, eosinophil peroxidase.

melatonin (7, 14, 28, 50 and 100 μM) and/or plasma concentration of Br⁻ (70 μM) to a stirred H₂O₂ buffer solution had no or little effect on H₂O₂ auto-reduction rates, indicating that melatonin alone did not significantly consume H₂O₂ (data not shown).

Addition of EPO (20 μL, 15 nM final concentration) solution to a continuously stirred H₂O₂ (20 μM) buffer solution supplemented with 70 μM Br⁻ caused rapid consumption of H₂O₂ (Figure 1). Under these circumstances, EPO metabolized Br⁻ through a 2e⁻ oxidation pathway generating HOBr. The rapid disappearance of H₂O₂ did not occur in the presence of saturating amounts of melatonin (>20 μM melatonin). Instead a much slower rate of H₂O₂ consumption (Figure 1) indicated that melatonin might have a role in inhibiting the catalytic activity of EPO. In the presence of sub-saturating amounts of melatonin (<20 μM melatonin), EPO catalysed a slow reduction of H₂O₂, which did accelerate upon melatonin oxidation. When the same experiments were repeated by adding higher EPO concentrations (for example, 60 nM), the H₂O₂ consumption rates were significantly increased, the duration of the reactions decreased and the trend by which melatonin inhibited EPO stayed the same (data not shown).

For comparison, the above experiments had been repeated in the absence of the competing co-substrate, Br⁻. Addition of 60 nM EPO to the reaction mixture caused an immediate rapid decay in the level of free H₂O₂, followed by a slower decay, indicating that H₂O₂ was consumed as a substrate by EPO during steady-state catalysis, as previously reported (Figure 2a; Tahboub *et al.*, 2005). The first step occurred immediately after enzyme addition and is attributed to the formation of EPO Compound I. The second step is much slower and is attributed to the reaction of EPO with H₂O₂ after the conversion of EPO Compound II to EPO-Fe(III). Decreasing the concentration to 15 nM EPO caused slower

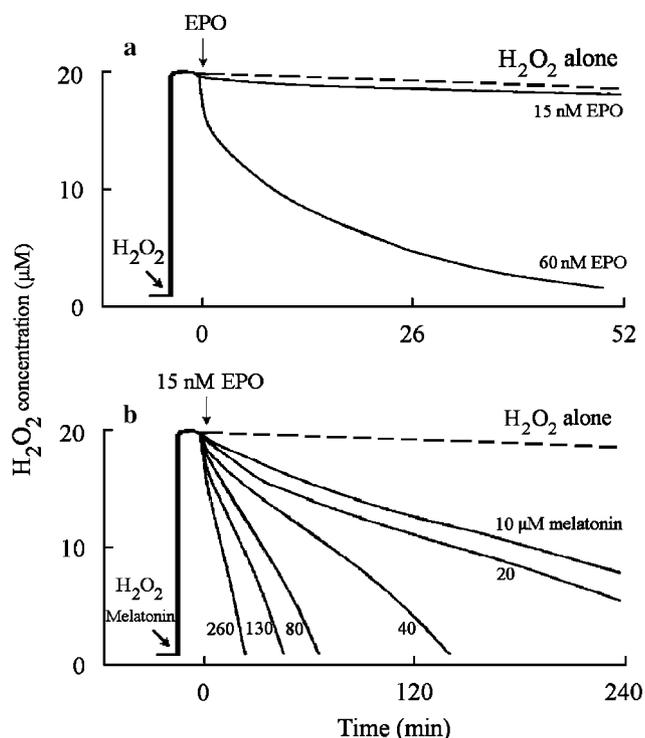


Figure 2 Effect of melatonin on H₂O₂ consumption by EPO in the absence of Br⁻. (a) A typical recording by an H₂O₂-selective electrode demonstrating the auto-reduction of H₂O₂ (20 μM) following addition to stirred phosphate buffer (200 mM; pH 7), at 25 °C (dashed line). Solid lines represent the H₂O₂ consumption as a function of time after the addition of 15 and 60 nM EPO. (b) The effect of increasing melatonin concentration on H₂O₂ consumption rate after the addition of 15 nM EPO to a stirred buffer solution supplemented with 20 μM H₂O₂ and the indicated concentration of melatonin on the solid lines. Tracings shown are from a typical experiment performed at least three times. EPO, eosinophil peroxidase.

disappearance of H₂O₂ (Figure 2a). Addition of 20 μL EPO (15 nM final concentration) to continuously stirred H₂O₂ (20 μM) buffer solutions supplemented with increasing melatonin concentrations increased the rate of H₂O₂ consumption (Figure 2b), indicating that melatonin served as a 1e⁻ substrate for both EPO Compounds I and II.

Rapid kinetic measurements

We used stopped-flow spectroscopy to examine the effect of melatonin on the distribution of EPO intermediates during steady-state catalysis. The reactions were initiated at 10 °C by mixing an aerobic solution of EPO-Fe(III) in the absence and presence of increasing concentrations of melatonin with a solution of H₂O₂. Upon initiating the reaction, we observed an initial rapid decrease in absorbance in the first 10 ms that can be attributed to EPO Compound I formation. This was followed by an increase in absorbance at 432 nm over the next 3 s that can be attributed to the formation of EPO Compound II (Figure 3a). Similar results were obtained in the presence of 10 μM melatonin (final), except that the conversion rate constants of Compound I to Compound II were progressively accelerated with increasing melatonin concentration (Figure 3b). At higher melatonin concentrations, the

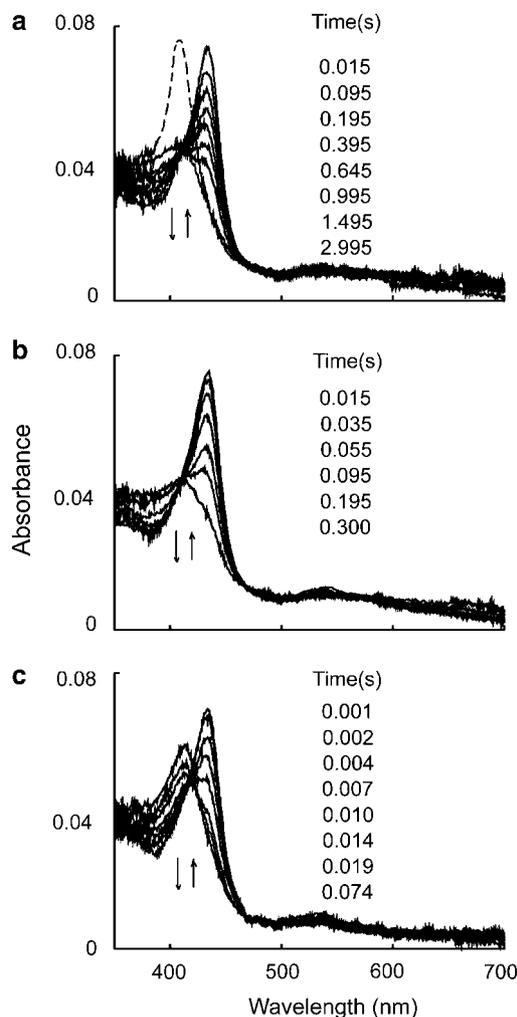


Figure 3 Melatonin accelerates the conversion rates of EPO Compound I to Compound II. Absorbance spectra of EPO recorded by diode array stopped-flow in the absence (a) and in the presence of 10 μM (b) and 160 μM melatonin (c). Experiments were performed under aerobic conditions when a phosphate buffer solution (200 mM, pH 7) containing 1.6 μM EPO in the absence and presence of 20 and 320 μM melatonin was rapidly mixed with a buffer solution supplemented with 40 μM H₂O₂ at 10 °C as described under Methods. The time of each collected spectrum after initiating the reaction is indicated in each figure in seconds. Arrows indicate the direction of spectral change over time. The dashed line spectrum represents the enzyme as isolated. EPO, eosinophil peroxidase.

rate of the absorbance decrease at 412 nm was essentially identical to the rate of absorbance increased at 432 nm (data not shown), suggesting that EPO binds to melatonin and its dissociation from the melatonin–EPO complex is the rate-limiting step of the overall reaction (Figure 3c).

Single-wavelength stopped-flow spectroscopy was also used to highlight melatonin's influences on EPO Compound II formation, complex duration and decay. Investigations were carried out by rapid mixing of EPO-Fe(III) solution preincubated with increasing concentrations of melatonin (for example, 5, 10, 20, 40 and 80 μM) against 40 μM H₂O₂. Figure 4 shows actual stopped-flow traces for the build-up, duration and decay of EPO Compound II collected at 432 nm (absorbance of EPO Compound II) in the presence of

increasing melatonin concentrations. In the presence of 5 μM melatonin, there was a fast build-up of EPO Compound II, which remained at the same level for around 800 s, and decayed through a biphasic reaction to the ferric form with apparent rate constants of 0.08 s^{-1} for the faster phase and 0.005 s^{-1} for the slower phase (Figure 4). Between 5–7% of the total absorbance changes at 452 nm were associated with the fast phase. The amplitude of the first phase increased, while that of the second phase decreased upon increasing melatonin concentration. Only the faster phase was observed at higher melatonin concentrations (Figure 4). In all cases, conversion of EPO Compound I to Compound II was best fitted to a single-exponential function. Figure 5 shows the relationship between melatonin concentration

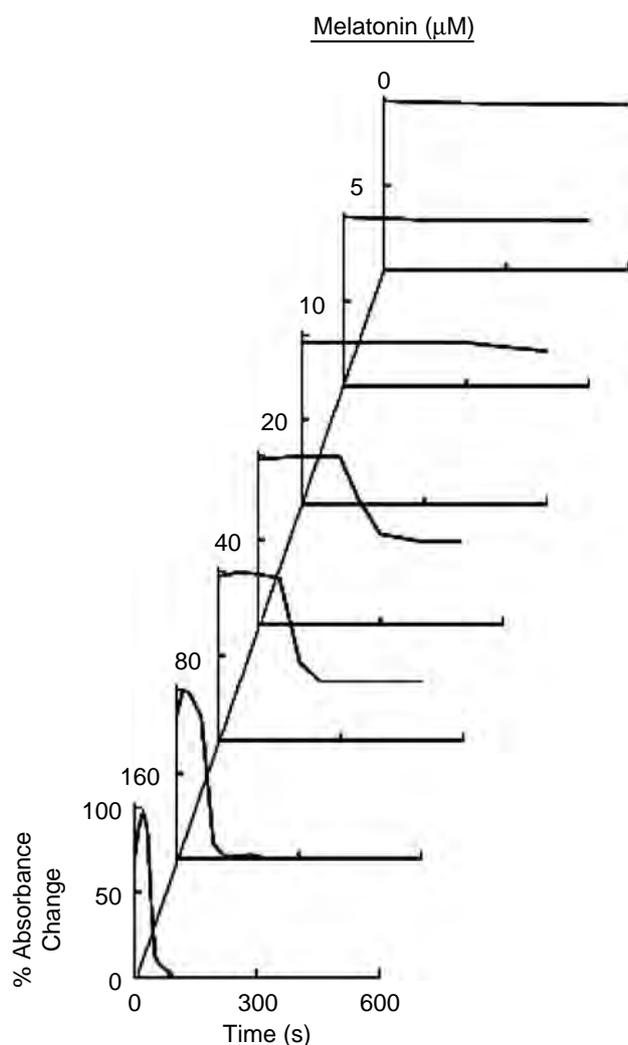


Figure 4 Effect of melatonin concentration on EPO-Fe(III) Compound II formation, duration and decay during steady-state catalysis. Formation and decay of Compound II of EPO-Fe(III) were monitored as a function of time by observing spectral changes at 432 nm. A solution containing sodium phosphate buffer (200 mM, pH 7) supplemented with H_2O_2 (40 μM) was rapidly mixed with an equal volume of buffer containing 2.0 μM of EPO-Fe(III), 200 M Br^- , and differing concentrations of melatonin at 10 °C. The final concentration of melatonin in the mixtures is indicated (0–160 μM). EPO, eosinophil peroxidase.

and the rate of EPO Compound II formation (Figure 5a), duration of its formation (Figure 5b) and the rate of complex decay (Figure 5c). It was evident as melatonin's concentration increased the formation of EPO Compound II and its decay rate constant progressively increased in a linear manner. The second-order rate constant of EPO Compound II formation (k_{on}) calculated from the slope was $1.60 \times 10^6\text{ M}^{-1}\text{ s}^{-1}$, while first-order rate, calculated from the intercept, was similar to that measured in the absence of melatonin (3 s^{-1}). The second-order rate constant of EPO Compound II decay calculated from the slope of Figure 5c was $1.00 \times 10^3\text{ M}^{-1}\text{ s}^{-1}$, while the first-order rate calculated from the intercept was similar to that found in the absence of melatonin (0.001 s^{-1}). EPO Compound II duration decreased in a linear and saturable manner. Collectively, these results may suggest that melatonin serves as a $1e^-$ substrate for both EPO Compounds I and II.

We next investigated how a plasma level of Br^- in the reaction changes the distribution of EPO intermediates during steady-state catalysis. Experiments were performed

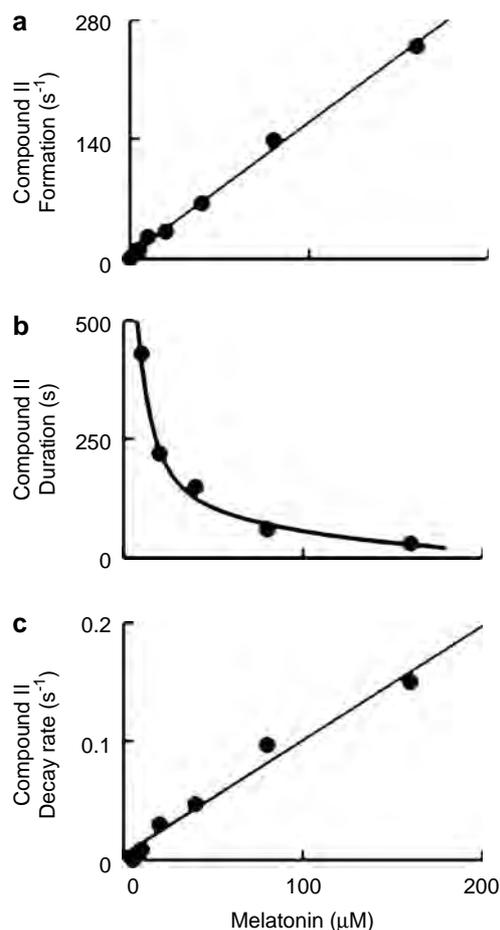


Figure 5 Rate of EPO-Fe(III) Compound II formation, duration and decay as a function of melatonin concentration. The observed rates of EPO-Fe(III) Compound II formation (a), decay (b) and duration (c) monitored at 432 nm observed in Figure 4 were plotted as a function of melatonin concentration. EPO, eosinophil peroxidase.

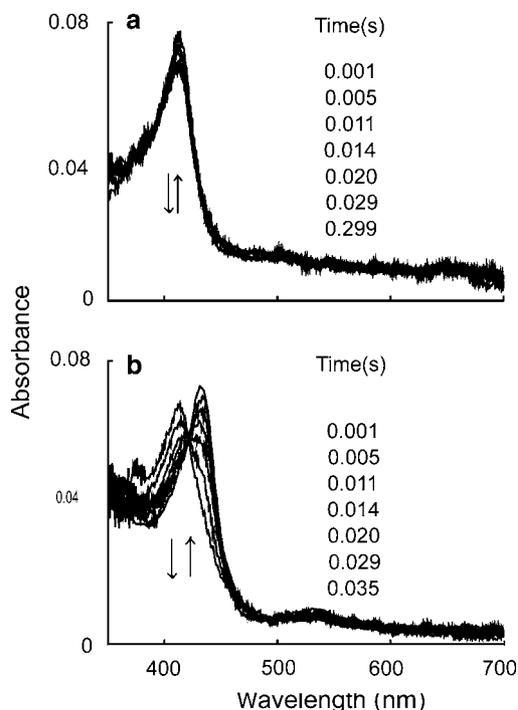


Figure 6 Melatonin facilitates direct conversion of EPO-Fe(III) to Compound II. Absorbance spectra of EPO recorded by diode-array, stopped-flow in the absence (a) and the presence of 100 μM melatonin (b). Experiments were performed under aerobic conditions when phosphate buffer (200 mM, pH 7) containing 1.9 μM EPO and 140 μM Br^- in the absence (a) and the presence of 200 μM melatonin (b), was rapidly mixed with a buffer solution supplemented with 40 μM H_2O_2 at 10 $^\circ\text{C}$. The time of each collected spectrum after initiating the reaction is indicated in each figure in seconds. Arrows indicate the direction of spectral change over time. EPO, eosinophil peroxidase.

in the absence and presence of melatonin at concentrations that were either higher or lower than H_2O_2 concentrations. Addition of H_2O_2 to EPO solution in the presence of 100 μM Br^- caused rapid small decreases in absorbance at 412 nm, followed by instant recovery (Figure 6a), indicating that the decay of EPO Compound I is faster than its formation. When the same reactions were repeated in the presence of saturating amounts of melatonin (200 μM), EPO-Fe(III) converted directly to Compound II without any sign of Compound I accumulation (Figure 6b). When the melatonin concentration used was less than H_2O_2 concentration (for example, 10 and 20 M), the majority of EPO-Fe(II) was converted to Compound II, which then decayed to EPO-Fe(III) upon melatonin oxidation.

Finally, we examined the influence of melatonin on the kinetics of EPO Compound II build-up, duration and decay during steady-state catalysis in the presence of plasma levels of the competing substrate Br^- . Reactions were performed by following rapid mixing of EPO preincubated with various concentrations of melatonin (0, 5, 10, 20, 40, 80 and 160 μM final) and physiologically relevant levels of the competing substrate Br^- (100 μM final) against 40 μM H_2O_2 . Initiation of the reaction resulted in a significant increase in the amount of Compound II formed (amplitude at 432 nm) during steady-state catalysis (Figure 7). Increasing melatonin's concentration not only increased the amplitude of

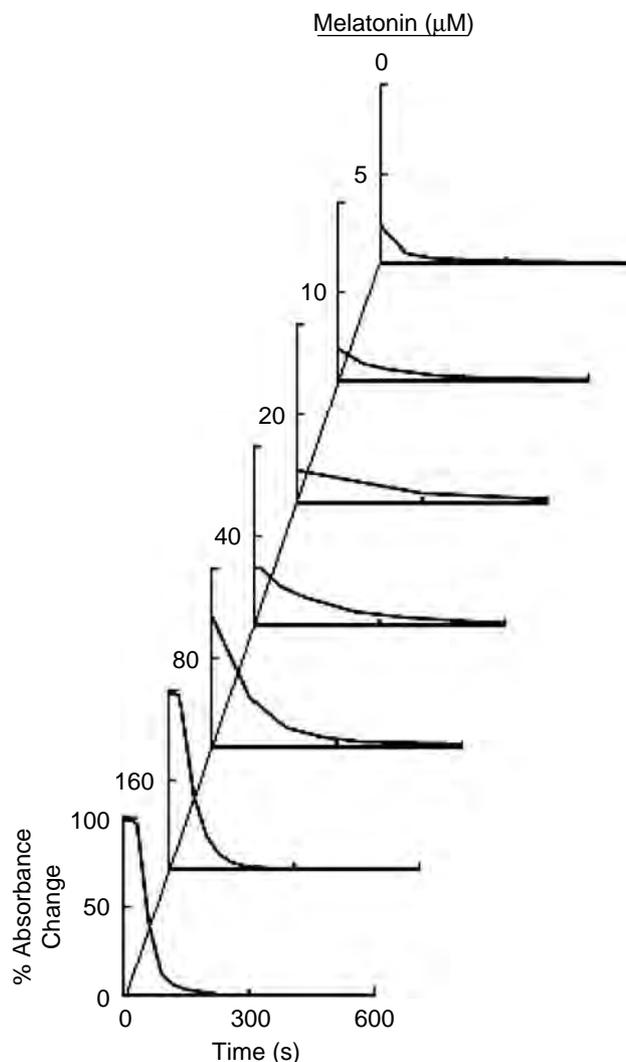


Figure 7 Effect of melatonin concentration on EPO Compound II formation during steady-state catalysis in the presence of plasma levels of Br^- . A solution containing sodium phosphate buffer (200 mM, pH 7) supplemented with 2 μM EPO, 200 μM NaBr, and increasing concentrations of melatonin, was rapidly mixed with an equal volume of buffer containing 40 μM of H_2O_2 EPO-Fe(III) at 10 $^\circ\text{C}$. The time course of EPO Compound II formation during steady-state catalysis was monitored by observing spectral changes at 432 nm. The final concentration of melatonin in the mixture is indicated. Note that even in the presence of physiological levels of the competing substrate Br^- , melatonin significantly enhances the overall level of EPO Compound II formed during steady-state catalysis. Data represent the mean of triplicate determinations from an experiment performed three times. EPO, eosinophil peroxidase.

Compound II formation, but also increased the rate of complex formation, duration of its formation and complex decay rate (Figure 8). Further stopped-flow analysis of melatonin concentration dependence on both the amount of Compound II formed and the rate of Compound II formation and decay showed that melatonin serves as a substrate for EPO Compounds I and II during steady-state catalysis. Comparison of the second-order rate constant in the presence and absence of Br^- showed a fivefold decrease in the second-order formation rate of Compound II, while a

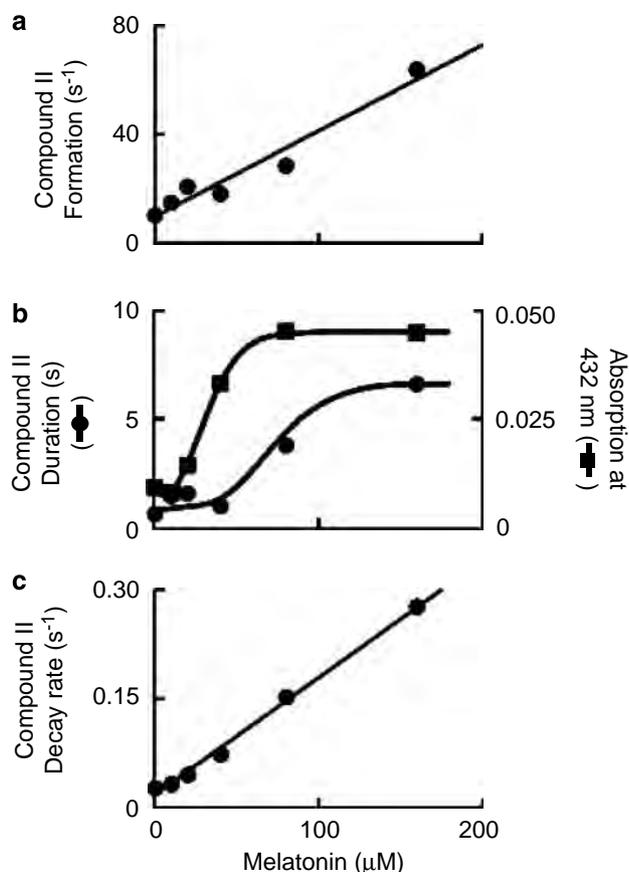


Figure 8 Rate of EPO Compound II formation, duration, and decay, and overall content of EPO Compound II formed, as a function of melatonin concentration. The effect of varying melatonin concentration on the observed rate of EPO Compound II formation (a) and decay (c), and steady-state levels and duration (b) of EPO compound II formed were monitored at 432 nm as described under experimental conditions identical to those described in Figure 7. Data represent the mean of three independent experiments. EPO, eosinophil peroxidase.

~0.64-fold decrease in the decay rate of Compound II was observed.

In addition, we observed a significant increase in EPO Compound II duration at higher melatonin concentrations. These results clearly demonstrate that melatonin competes with a plasma level of Br⁻ and switches the reaction from a 2e⁻ to a 1e⁻ oxidation. When the same reactions were monitored at 412 nm, the direction of absorbance change reversed, but otherwise proceeded with identical kinetics (not shown).

Discussion

Evidence suggests EPO-mediated reactive oxidants promote oxidative tissue damage in a variety of inflammatory conditions, including lung diseases (Venge *et al.*, 1987; Wu *et al.*, 2000; MacPherson *et al.*, 2001; Aldridge *et al.*, 2002). Thus, inhibition of EPO and its downstream inflammatory byproducts can be attractive targets in the development of biomarkers and therapeutic interventions of various inflam-

matory conditions such as allergy and asthma. Melatonin may have a beneficial role in modulating the pathogenesis of several disorders by inhibiting EPO catalytic activity and function. Our current results not only highlight the potential role of melatonin in inhibiting EPO, but also emphasize the role of Br⁻ in this inhibition. In the presence of Br⁻, the efficiency of melatonin in inhibiting EPO is enhanced probably due to stabilization of the Br-EPO-melatonin complex. Formation of such a complex restricts the access of H₂O₂ to the catalytic site of the enzyme and limits the overall EPO catalytic activity by the removal of melatonin from the complex by either dissociation or oxidation. This behaviour was evident by direct conversion of EPO-Fe(III) to Compound II, decrease in the second-order rate constant of Compound II accumulation and restoration of catalytic activity upon melatonin exhaustion. When melatonin dissociated from the complex, it accelerated the formation of EPO Compound II and its decay to the ground state by serving as a 1e⁻ substrate. The ability of melatonin to compete with plasma levels of Br⁻ and serve as a 1e⁻ substrate of EPO Compounds I and II drives the enzyme to function with catalase-like activity.

Our results did not exclude the possibility that the Br-EPO-melatonin complex might have reacted directly with H₂O₂ generating Compound II and the oxidized form of melatonin. In the absence of Br⁻, melatonin served as a 1e⁻ substrate of EPO Compound I and accelerated its conversion to Compound II. Collectively, our results suggest a bidirectional relationship that links EPO and melatonin, which may have a much broader application in biological systems. The present studies support the notion that the formation of the Br-EPO-melatonin complex during steady-state catalysis is a fundamental feature of the kinetic reactions of EPO under these experimental conditions. Its formation and decay accelerate the removal of the H₂O₂ from the EPO milieu. This is particularly important in the human airway, where catalase, a major H₂O₂ scavenger, is present at very low levels (Erzurum *et al.*, 1993), thus leaving mammalian peroxidases to serve as the major H₂O₂ scavenger at this site.

The X-ray structure of EPO is presently unavailable. However, using rapid kinetic measurements, NO binding to EPO haem iron and a theoretical EPO three-dimensional model built on the scaffold of the lactoperoxidase X-ray structure, we have recently shown that Br⁻ binds at two different binding sites on EPO (Galijasevic *et al.*, 2007). Both sites have distinct effects on the EPO haem iron microenvironment. Melatonin is a bulky aromatic molecule that may influence EPO steady-state catalysis by binding to the entrance of the hydrophobic pocket of the distal haem cavity, preventing access of H₂O₂ to the catalytic site of the enzyme. Our current results are consistent with this hypothesis, since inhibition of EPO requires occupation of both catalytic sites of EPO. Previous electron paramagnetic resonance (EPR) and X-ray structure simulations have demonstrated that aromatic molecules bind near the haem centre of MPO (Hori *et al.*, 1994). The hydroxamic side chains of salicylhydroxamic acid and benzohydroxamic acid seem to interact with the haem iron (Hori *et al.*, 1994). The docking calculations carried out by Hallingbäck *et al.* (2006) also showed that the indole substituent and/or the indole

ring moieties of the substrate positioned at the MPO haem edge. These findings are consistent with MPO being able to oxidize melatonin through $1e^-$ pathway reactions (Allegra *et al.*, 2001; Ximenes *et al.*, 2005). Our current findings provide further evidence for an allosteric coupling between melatonin, EPO haem pocket entrance binding (regulatory) site and the catalytic centre that induces detectable inhibition of EPO. They also provide a new role for Br^- in facilitating melatonin binding that mediates EPO inhibition.

The apparent ability of Br^- to stabilize the EPO–melatonin complex is unprecedented, and it may be governed by a mechanism involving communication between the two binding sites of the enzyme. Melatonin significantly inhibited H_2O_2 consumption in levels that govern both (patho)-physiological, and supplemental ranges (Van den Heuvel *et al.*, 1998; Altun and Ugur-Altun, 2007). A steady-state competition model that describes the modulating effects of melatonin on EPO inhibition in the presence of Br^- is shown in Figure 9. EPO Compound I was the first intermediate that was formed in the catalytic cycle. Its formation is relatively fast, with a second-order rate constant of $4.3 \times 10^7 M^{-1} s^{-1}$ at $10^\circ C$ (Ximenes *et al.*, 2005). EPO Compound I oxidizes Br^- , the physiological substrate for EPO (Furtmuller *et al.*, 2000), in a single $2e^-$ reaction, leading to formation of EPO-Fe(III) and HOBr. The high rate of Br^- catalysis was associated with undetectable Compound I build-up and minimal decrease in the Soret absorbance peak during the steady-state catalysis. In the absence of Br^- and during turnover, Compound I is converted to Compound II both spontaneously by $1e^-$ leak (from endogenous electron donors) and more rapidly in the presence of melatonin, which serves as an exogenous $1e^-$ reductant. In the absence of Br^- , EPO consumes H_2O_2 by immediate rapid decay in the level of free H_2O_2 , followed by a much slower decay (Tahboub *et al.*, 2005). The first step occurs immediately after EPO addition and is attributed to multiple cycles of H_2O_2 consumption prior to conversion of EPO Compound I to Compound II. Our results showed that melatonin was able to constrain this reaction by accelerating conversion of Compound I to Compound II. Formation of Compound II, the inactive form of EPO, limits H_2O_2 consumption rate by the slow conversion rate of EPO

Compound II to EPO-Fe(III). In the presence of Br^- , only the first phase is observed, indicating that EPO catalyses the formation of HOBr. HOBr is a highly active compound, possessing potent bactericidal and viricidal activities (Weiss *et al.*, 1986; Mayeno *et al.*, 1989; Mitra *et al.*, 2000).

In the presence of sub-saturating amounts of melatonin, the transformation that is apparent from our H_2O_2 -selective electrodes may represent a switch in subcycles (Figure 9). The first step in H_2O_2 consumption is limited by the rate of melatonin dissociation from the Br -EPO-melatonin complex when the reaction is initiated by adding EPO solution to melatonin/ Br^- / H_2O_2 mixture. In this case, the second step of H_2O_2 consumption is the peroxidation reaction after the release of melatonin from the melatonin-EPO- Br complex by either oxidation or dissociation to generate inhibitor-free EPO. Under the latter circumstances, the enzyme generates HOBr. Thus, the variation in the binding affinity of EPO-Fe(III) towards melatonin and the release of melatonin from the EPO-melatonin complex due to its oxidation accounts for the inflection point in the H_2O_2 consumption rate.

The finding that oxidation of melatonin can restore the catalytic activity of EPO is very intriguing. More importantly, oxidized melatonin had no biological harmful sequelae and no effect on the EPO activity. The maximum stoichiometry value obtained for melatonin in H_2O_2 consumption was approximately a 1:1 ratio, suggesting that EPO Compound I partitions between two subcycles as during catalysis. It could oxidize Br^- and melatonin by a $2e^-$ and $1e^-$ pathway, respectively. The rate of melatonin oxidation and degree of Compound II formation may both depend on melatonin bioavailability. Indeed, increasing melatonin concentration not only increases the amplitude of EPO Compound II formation, it also increases the duration of its formation. Our data indicated that formation of Compound II occurs much faster than its decay. Thus, the bioavailability of melatonin versus Br^- , the affinity of EPO Compound I towards melatonin versus Br^- and the decay rate of EPO Compound II are all likely to determine the amount of EPO Compound II that builds up during steady state. The different activities of EPO are best appreciated in the context of Figure 9. Compound II complex formation is intrinsic to catalysis and

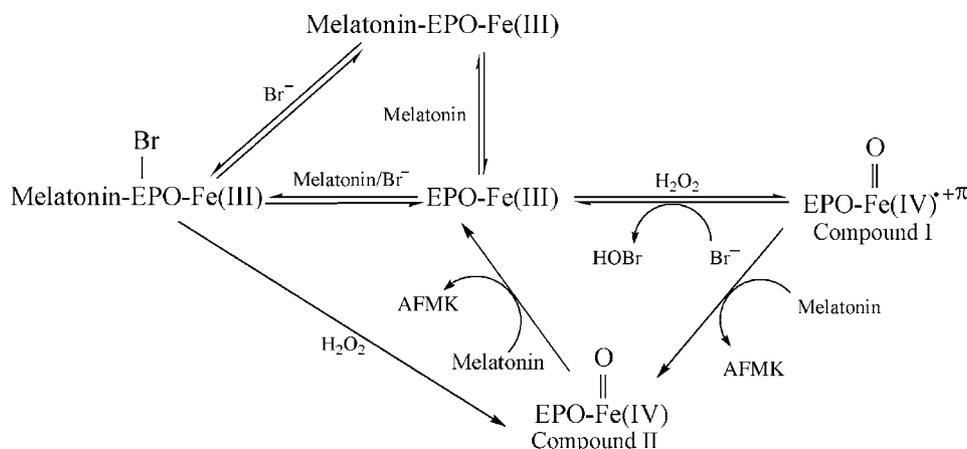


Figure 9 Working kinetic model for melatonin interactions with EPO. AFMK, *N*(1)-acetyl-*N*(2)-formyl-5-methoxykynuramine, the major metabolite of melatonin; EPO, eosinophil peroxidase.

causes a majority of the enzyme to partition into catalase-like activity. Thus, inactivation of EPO and its duration can be controlled effectively by melatonin supplementation.

Melatonin is currently associated with improved outcome when used to treat jet-lag, disturbances of sleep, lowering of blood pressure, cancer, longevity and even improving sex life (Goldman, 1995; Altun and Ugur-Altun, 2007). Melatonin has been found to act as one of the most effective antioxidants through its ability to neutralize and scavenge hydroxyl radicals known to induce DNA damage (Reiter, 1995; Cuzzocrea and Reiter, 2001; Küçükakin *et al.*, 2008). Due to the relatively low physiological levels of melatonin in human tissue, melatonin has been recommended as a supplement for a wide variety of conditions in daily doses ranging from 0.3 to 1000 mg given for 1–4 weeks (Goldman, 1995; Zhdanova *et al.*, 1995). EPO, like MPO, is released into the extracellular space as eosinophils degranulate in the lung tissues during inflammation (Andreadis *et al.*, 2003). Inhibition of EPO by sufficient amounts of melatonin may have a beneficial effect *in vivo*. Our current study uses concentrations of melatonin that have been shown to induce a biological effect in human cells and tissues, without any sign of toxicity or side effects (Goldman, 1995). Thus, the results of this study suggest a potential role for melatonin in the treatment of various inflammatory disorders.

Normal plasma levels of melatonin in human subjects are approximately 2.5–50 pg mL⁻¹, but they increase to 1700 pg mL⁻¹ (7 nM) within 60 min of consumption of 5 mg orally (Mero *et al.*, 2006). As melatonin can be metabolized through several pathways to various components, it is important to measure the levels of melatonin and its metabolites in the blood (Hardeland and Pandi-Perumal, 2005). N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK) is the major metabolite of melatonin, accompanied by 6-hydroxymelatonin, sulphatoxymelatonin and glucuronic acid conjugates. The high variable first-pass metabolism of melatonin after oral administration can be one of the factors that limit measuring its serum levels. Various methods, such as immunoassays, liquid and gas chromatography and capillary electrophoresis, were used to measure serum melatonin concentrations. Only the sulphated fraction has been measured by immunoassay to determine the quantitative level of the metabolite. The hepatic metabolism of oral melatonin cannot be measured using this assay, because approximately 20% goes undetected as the glucuronidated fraction. Härtter *et al.* (2001) developed a method using liquid chromatography–mass spectrometry, which showed relatively lower melatonin concentration (19.2 ng mL⁻¹; 0.1 μM) versus a higher concentration of its 6-hydroxy metabolite (694 ng mL⁻¹; ~3 μM) in human plasma 1 h after ingestion of 20 mg of melatonin. Thus, melatonin and its metabolites should all be taken in consideration when evaluating the overall melatonin concentration in biological systems. The lower melatonin concentrations used in our current study are comparable to plasma 6-hydroxy metabolite concentrations detected by others (Härtter *et al.*, 2001). Higher doses of melatonin (>20 mg) can probably result in greater serum concentrations of melatonin and its metabolites, close to those we used in our experiment. We believe that the concentrations used in these studies may have

pharmacological relevance in inflammatory diseases. Further investigation is needed to elucidate the bidirectional relationship between various doses of melatonin and EPO *in vivo*.

Collectively, melatonin and peroxidase activities are apparently coupled through complex and interdependent pathways. Our results clearly show that Br⁻ facilitates melatonin binding to the enzyme, which in turn inhibits the catalytic activity of the enzyme. EPO may be functioning as a scavenger for melatonin limiting its bioavailability and function. This may subsequently modulate the negative effects of sleep loss on various body functions. On the other hand, melatonin may have an important role in the inhibition of EPO activity in various tissues during inflammation. The biological consequences of melatonin–peroxidase interactions may have broad implications in the regulation of sleep, inflammation, infectious and cardiovascular disorders *in vivo*.

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Conflict of interest

The authors state no conflict of interest.

References

- Abu-Soud HM, Hazen SL (2001). Interrogation of heme pocket environment of mammalian peroxidases with diatomic ligands. *Biochemistry* **40**: 10747–10755.
- Abu-Soud HM, Khassawneh MY, Sohn JT, Murray P, Haxhiu MA, Hazen SL (2001). Peroxidases inhibit nitric oxide (NO) dependent bronchodilation: development of a model describing NO–peroxidase interactions. *Biochemistry* **40**: 11866–11875.
- Ackerman SJ, Gleich GJ, Loegering DA, Richardson BA, Butterworth AE (1985). Comparative toxicity of purified human eosinophil granule cationic proteins for schistosomula of *Schistosoma mansoni*. *Am J Trop Med Hyg* **34**: 735–745.
- Agosti JM, Altman LC, Ayars GH, Loegering DA, Gleich GJ, Klebanoff SJ (1987). The injurious effect of eosinophil peroxidase, hydrogen peroxide, and halides on pneumocytes *in vitro*. *J Allergy Clin Immunol* **79**: 496–504.
- Aldridge RE, Chan T, van Dalen CJ, Senthilmohan R, Winn M, Venge P *et al.* (2002). Eosinophil peroxidase produces hypobromous acid in the airways of stable asthmatics. *Free Radic Biol Med* **33**: 847–856.
- Allegra M, Furtmüller PG, Regelsberger G, Turco-Liveri ML, Tesoriere L, Perretti M *et al.* (2001). Mechanism of reaction of melatonin with human myeloperoxidase. *Biochem Biophys Res Commun* **282**: 380–386.
- Altun A, Ugur-Altun B (2007). Melatonin: therapeutic and clinical utilization. *Int J Clin Pract* **61**: 835–845.
- Andreadis AA, Hazen SL, Comhair SA, Erzurum SC (2003). Oxidative and nitrosative events in asthma. *Free Radic Biol Med* **35**: 213–225.
- Arendt J, Stone B, Skene D (2000). Jet-lag and sleep disruption. In: Kryger MH, Roth TR, Dement WC (eds). *Principles and Practice of Sleep Medicine*, 3rd edn. W B Saunders: Philadelphia, pp 591–599.
- Arlandson M, Decker T, Roongta VA, Bonilla L, Mayo KH, MacPherson JC *et al.* (2001). Eosinophil peroxidase oxidation of thiocyanate. Characterization of major reaction products and a

- potential sulfhydryl-targeted cytotoxicity system. *J Biol Chem* **276**: 215–224.
- Bolscher BG, Plat H, Wever R (1984a). Some properties of human eosinophil peroxidase, a comparison with other peroxidases. *Biochim Biophys Acta* **784**: 177–186.
- Bolscher BG, Zoutberg GR, Cupeus RA, Wever R (1984b). Vitamin C stimulates the chlorinating activity of human myeloperoxidase. *Biochim Biophys Acta* **784**: 189–191.
- Brown GM (1994). Light, melatonin and the sleep-wake cycle. *J Psychiatry Neurosci* **19**: 345–353.
- Carlson MG, Peterson CG, Venge P (1985). Human eosinophil peroxidase: purification and characterization. *J Immunol* **134**: 1875–1879.
- Cuzzocrea S, Reiter RJ (2001). Pharmacological action of melatonin in shock, inflammation and ischemia/reperfusion injury. *Eur J Pharmacol* **426**: 1–10.
- Erzurum SC, Danel C, Gillissen A, Chu CS, Trapnell BC, Crystal RG (1993). *In vivo* antioxidant gene expression in human airway epithelium of normal individuals exposed to 100% O₂. *J Appl Physiol* **75**: 1256–1262.
- Furtmuller PG, Burner U, Regelsberger G, Obinger C (2000). Spectral and kinetic studies on the formation of eosinophil peroxidase compound I and its reaction with halides and thiocyanate. *Biochemistry* **39**: 15578–15584.
- Galijasevic S, Proteasa G, Abdulhamid I, Abu-Soud HM (2007). The potential role of nitric oxide in substrate switching in eosinophil peroxidase. *Biochemistry* **46**: 406–415.
- Galijasevic S, Saed GM, Diamond MP, Abu-Soud HM (2004). High dissociation rate constant of ferrous-dioxy complex linked to the catalase-like activity in lactoperoxidase. *J Biol Chem* **279**: 39465–39470.
- Galijasevic S, Saed GM, Hazen SL, Abu-Soud HM (2006). Myeloperoxidase metabolizes thiocyanate in a reaction driven by nitric oxide. *Biochemistry* **45**: 1255–1262.
- Gastel JA, Roseboom PH, Rinaldi PA, Weller JL, Klein DC (1998). Melatonin production: proteasomal proteolysis in serotonin N-acetyltransferase regulation. *Science* **279**: 1358–1360.
- Goldman A (1995). Melatonin: a review. *Br J Clin Pharmacol* **19**: 258–260.
- Hallingbäck HR, Gabdoulline RR, Wade RC (2006). Comparison of the binding and reactivity of plant and mammalian peroxidases to indole derivatives by computational docking. *Biochemistry* **45**: 2940–2950.
- Hardeland R (2005). Antioxidative protection by melatonin: multiplicity of mechanisms from radical detoxification to radical avoidance. *Endocrine* **27**: 119–130.
- Hardeland R, Pandi-Perumal SR (2005). Melatonin, a potent agent in antioxidative defense: actions as a natural food constituent, gastrointestinal factor, drug and prodrug. *Nutr Metab* **2**: 22–36.
- Härter S, Morita S, Bodin K, Ursing C, Tybring G, Bertilsson L (2001). Determination of exogenous melatonin and its 6-hydroxy metabolite in human plasma by liquid chromatography–mass spectrometry. *Ther Drug Monit* **23**: 282–286.
- Henderson JP, Byun J, Williams MV, McCormick ML, Parks WC, Ridnour LA *et al.* (2001). Bromination of deoxycytidine by eosinophil peroxidase: a mechanism for mutagenesis by oxidative damage of nucleotide precursors. *Proc Natl Acad Sci USA* **98**: 1631–1636.
- Herxheimer A, Petrie KJ (2004). Melatonin for the prevention and treatment of jet lag. In: *The Cochrane Library* Issue 4 John Wiley & Sons, Ltd: Chichester, UK.
- Herxheimer A, Waterhouse J (2003). The prevention and treatment of jet lag. *Br Med J* **326**: 296–297.
- Holt PG, Macaubas C, Stumbles PA, Sly PD (1999). The role of allergy in the development of asthma. *Nature* **402**: B12–B17.
- Hori H, Fenna RE, Kimura S, Ikeda-Saito M (1994). Aromatic substrate molecules bind at the distal heme pocket of myeloperoxidase. *J Biol Chem* **269**: 8388–8392.
- Jong EC, Henderson WR, Klebanoff SJ (1980). Bactericidal activity of eosinophil peroxidase. *J Immunol* **124**: 1378–1382.
- Jorg A, Pasquier JM, Klebanoff SJ (1982). Purification of horse eosinophil peroxidase. *Biochim Biophys Acta* **701**: 185–191.
- Klebanoff SJ, Agosti JM, Jorg A, Waltersdorff AM (1989). Comparative toxicity of the horse eosinophil peroxidase–H₂O₂–halide system and granule basic proteins. *J Immunol* **143**: 239–244.
- Klebanoff SJ, Locksley RM, Jong E, Rosen H (1983). Oxidative response of phagocytes to parasite invasion. *Ciba Found Symp* **99**: 92–112.
- Klebanoff SJ, Waltersdorff AM, Rosen H (1984). Antimicrobial activity of myeloperoxidase. *Methods Enzymol* **105**: 399–403.
- Küçükakin B, Gögenur I, Reiter RJ, Rosenberg J (2008). Oxidative stress in relation to surgery: is there a role for the antioxidant melatonin? *J Surg Res* January 10, e-pub ahead of print.
- MacPherson JC, Comhair SA, Erzurum SC, Klein DF, Lipscomb MF, Kavuru MS *et al.* (2001). Eosinophils are a major source of nitric oxide-derived oxidants in severe asthma: characterization of pathways available to eosinophils for generating reactive nitrogen species. *J Immunol* **166**: 5763–5772.
- Malhotra S, Sawhney G, Pandhi P (2004). The therapeutic potential of melatonin: a review of the science. *Med Gen Med* **6**: 46 (review).
- Mayeno AN, Curran AJ, Roberts RL, Foote CS (1989). Eosinophils preferentially use bromide to generate halogenating agents. *J Biol Chem* **264**: 5660–5668.
- Mero AA, Vähälummukka M, Hulmi JJ, Kallio P, von Wright A (2006). Effects of resistance exercise session after oral ingestion of melatonin on physiological and performance responses of adult men. *Eur J Appl Physiol* **96**: 729–739.
- Mitra SN, Slungaard A, Hazen SL (2000). Role of eosinophil peroxidase in the origins of protein oxidation in asthma. *Redox Rep* **5**: 215–224.
- Proteasa G, Tahboub YR, Galijasevic S, Raushel FM, Abu-Soud HM (2007). Kinetic evidence supports the existence of two halide binding sites that have a distinct impact on the heme iron microenvironment in myeloperoxidase. *Biochemistry* **46**: 398–405.
- Reiter RJ (1995). Oxygen radical detoxification processes during aging: the functional importance of melatonin. *Aging (Milano)* **7**: 340–351.
- Rothenberg ME (1998). Eosinophilia. *N Engl J Med* **338**: 1592–1600.
- Samoszuk MK, Nguyen V, Thomas C, Jacobson D (1994). Effects of sonicated eosinophils on the *in vivo* sensitivity of human lymphoma cells to glucose oxidase. *Cancer Res* **54**: 2650–2653.
- Schaffazick SR, Pohlmann AR, de Cordova CA, Creczynski-Pasa TB, Guterres SS (2005). Protective properties of melatonin-loaded nanoparticles against lipid peroxidation. *Int J Pharm* **289**: 209–213.
- Slungaard A, Mahoney Jr JR (1991). Bromide-dependent toxicity of eosinophil peroxidase for endothelium and isolated working rat hearts: a model for eosinophilic endocarditis. *J Exp Med* **173**: 117–126.
- Tahboub YR, Galijasevic S, Diamond MP, Abu-Soud HM (2005). Thiocyanate modulates the catalytic activity of mammalian peroxidases. *J Biol Chem* **280**: 26129–26136.
- Van Dalen CJ, Whitehouse MW, Winterbourn CC, Kettle AJ (1997). Thiocyanate and chloride as competing substrates for myeloperoxidase. *Biochem J* **327**: 487–492.
- Van den Heuvel CJ, Kennaway DJ, Dawson D (1998). Effects of daytime melatonin infusion in young adults. *Am J Physiol* **275**: E19–E26.
- Venge P, Hakansson L, Peterson CG (1987). Eosinophil activation in allergic disease. *Int Arch Allergy Appl Immunol* **82**: 333–337.
- Weiss SJ, Test ST, Eckmann CM, Ross D, Regiana S (1986). Brominating oxidants generated by human eosinophils. *Science* **234**: 200–203.
- Wu W, Chen Y, d' Avignon A, Hazen SL (1999). 3-Bromotyrosine and 3,5-dibromotyrosine are major products of protein oxidation by eosinophil peroxidase: potential markers for eosinophil-dependent tissue injury *in vivo*. *Biochemistry* **38**: 3538–3548.
- Wu W, Samoszuk MK, Comhair SA, Thomassen MJ, Farver CF, Dweik RA, Kavuru MS, Erzurum SC, Hazen SL (2000). Eosinophils generate brominating oxidants in allergen-induced asthma. *J Clin Invest* **105**: 1455–1463.
- Wurtman RJ, Axelrod J, Phillips LS (1963). Melatonin synthesis in the pineal gland: control by light. *Science* **142**: 1071–1073.
- Ximenes VF, Silva SO, Rodrigues MR, Catalani LH, Maghazal GJ, Kettle AJ *et al.* (2005). Superoxide-dependent oxidation of melatonin by myeloperoxidase. *J Biol Chem* **280**: 38160–38169.
- Zhdanova IV, Wurtman RJ, Lynch HJ, Ives JR, Dollins AB, Morabito C *et al.* (1995). Sleep-inducing effects of low doses of melatonin ingested in the evening. *Clin Pharmacol Ther* **57**: 552–558.