Melatonin Is a Potent Inhibitor for Myeloperoxidase†

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ABSTRACT: Myeloperoxidase (MPO) catalyzes the formation of potent oxidants that have been implicated in the pathogenesis of various diseases including atherosclerosis, asthma, arthritis, and cancer. Melatonin plays an important part in the regulation of various body functions including circadian sleep rhythms, blood pressure, oncogenesis, retinal function, seasonal reproduction, and immunity. Here, we demonstrate that melatonin serves as a potent inhibitor of MPO under physiological-like conditions. In the presence of chloride (Cl^-), melatonin inactivated MPO at two points in the classic peroxidase cycle through binding to MPO to form an inactive complex, melatonin−MPO−Cl, and accelerating MPO compound II formation, an inactive form of MPO. Inactivation of MPO was mirrored by the direct conversion of MPO−Fe(III) to MPO compound II without any sign of compound I accumulation. This behavior indicates that melatonin binding modulates the formation of MPO intermediates and their decay rates. The Cl^- presence enhanced the affinity of MPO toward melatonin, which switches the enzyme activity from peroxidation to catalase-like activity. In the absence of Cl^-, melatonin served as a $1e^-$ substrate for MPO compound I, but at higher concentration it limited the reaction by its dissociation from the corresponding complex. Importantly, melatonin-dependent inhibition of MPO occurred with a wide range of concentrations that span various physiological and supplemental ranges. Thus, the interplay between MPO 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 means through which melatonin can control MPO and its downstream inflammatory pathways.

Melatonin or 5-methoxy-*N*-acetyltryptamine is a neurohormone secreted by the pineal gland in the brain synthesized from the amino acid tryptophan. It plays an important role in the function of the neuroendocrine system and the circadian rhythm (*1*−*4*). Its synthesis and release are stimulated by darkness and suppressed by light (*5*−*7*). Synthetic melatonin is available commercially, and its supplements have been used in a variety of medical conditions such as jet lag, conditions resulting from shift work, and circadian rhythm sleep disorders. Melatonin has important antioxidant effects (*8*−*10*). It can inhibit H_2O_2[1]-induced lipid peroxidation and lipoprotein modification. However, the possible in vivo reaction pathways for these properties of melatonin have not been identified yet (*11*, *12*).

MPO-mediated lipid peroxidation plays a crucial role in the pathogenesis of many disorders including arthritis and pulmonary and heart-related diseases (*13*−*18*). MPO is a 150−165 kDa protein synthesized during myeloid differentiation and constitutes the major component of neutrophil azurophilic granules (*19*). MPO displays a unique ability to catalyze H_2O_2-dependent peroxidation of halides and pseudohalides to produce antimicrobial agents, hypohalous acids, through the formation of a ferryl π cation radical (E−Fe(IV)═$O^{\bullet+\pi}$) intermediate, compound I (*20*−*24*). Hypohalous acids are potent cytotoxic oxidants which directly oxidize reactive groups, including sulfhydryls, iron−sulfur centers, and hemes, or react with amines, forming chloramines (*25*, *26*). Mammalian peroxidases also oxidize multiple organic and inorganic molecules by two successive sequential $1e^-$ transitions, generating oxidant and diffusible radical species through the activated intermediates compound II (E−Fe(IV)═O) and MPO−Fe(III), respectively (*27*−*32*). MPO compound II is a long-lasting intermediate whose decay to the ground state (MPO−Fe(III)) is considered to be the rate-limiting step during steady-state catalysis (*33*, *34*). A variety of spectroscopic techniques, with a combination of structure analysis and advanced computer modeling, have shown that heme pockets of mammalian peroxidases are envisioned to form the catalytic site where the stepwise reduction of H_2O_2 takes place (*35*−*39*). Structural studies have demonstrated that the heme of MPO lies at the base of a deep and narrow cleft (*35*−*37*). Therefore, blocking the entrance of the heme pocket, ligand-binding to MPO heme iron, or perturbation in the heme pocket geometry may promote the inhibition of the enzyme (*37*−*41*).

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[1] Abbreviations: Cl^-, chloride; H_2O_2, hydrogen peroxide; MPO, myeloperoxidase.

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Despite the potential significance of MPO to both human health and disease, little is known about the factors that down-regulate its activity and function. In the present study, we utilized a combination of H_2O_2-selective electrode, optical absorbance, and rapid kinetics measurements to assess the effect of melatonin on the catalytic activity and function of MPO.

MATERIALS AND METHODS

Material. The melatonin used was of the highest purity grade obtained from Sigma Chemical Co. (St. Louis, MO).

Enzyme Purification. MPO was purified from detergent extracts of human leukocytes as described (*42*−*44*). Trace levels of contaminating eosinophil peroxidase were then removed by passage over a sulfopropyl Sephadex column (*45*). The purity of isolated MPO was established by demonstrating a Reinheitzal (RZ) value of >0.85 (A_{430}/A_{280}); SDS−PAGE analysis was achieved with Coomassie blue staining and in-gel tetramethylbenzidine peroxidase staining to confirm the absence of observable contaminating eosinophil peroxidase activity (*46*). The enzyme concentration was determined spectrophotometrically utilizing an extinction coefficient of 89 000 (M^{-1} cm^{-1})/heme of MPO (*46*).

H_2O_2 Selective Electrode Measurement. H_2O_2 measurements were carried out using a H_2O_2-selective electrode (Apollo 4000 free radical analyzer, World Precision Instruments, Sarasota, FL). Experiments were performed at 25 °C by immersing the electrode in 3 mL of 0.2 M sodium phosphate buffer, pH 7.0. A 20 μM concentration of H_2O_2 was added to a continuously stirred buffer solution containing various levels of melatonin and/or Cl^- (100 mM) during which the rise and fall of the H_2O_2 concentration was continuously monitored. Where indicated, 20 μL of MPO (30 nM final) was added to the reaction mixture. To determine the effect of melatonin on H_2O_2 consumption by MPO, similar experiments were repeated by adding 20 μL of the enzyme solution (300 nM final) to a continuously stirred H_2O_2 solution in the absence and presence of various melatonin concentrations.

Absorbance Measurements. Spectra were recorded with a Cary 100 Bio UV−vis spectrophotometer, at 25 °C, in phosphate buffer, pH 7.0. Experiments were performed with a 1 mL cuvette containing MPO (0.7−1.0 μM) preincubated with increasing concentrations of melatonin (6−200 μM), in the absence and presence of 100 mM Cl^-. Concentrated volumes of H_2O_2 solution were added to the sample cuvette (20 μM final), and absorbance changes were recorded from 300 to 700 nm.

Rapid Kinetic Measurements. The kinetic measurements of MPO compound I and/or compound II formation and decay in the absence and presence of different melatonin and/or Cl^- concentrations were performed using a dual-syringe stopped-flow instrument obtained from Hi-Tech, Ltd. (model SF-61). Experiments were initially performed under conditions identical to those recently reported for MPO and other related hemoproteins to facilitate comparison (*47*−*49*). Measurements were carried out under an aerobic atmosphere at 10 °C following rapid mixing of equal volumes of a H_2O_2-containing buffer solution and a peroxidase solution that contained 100 mM Cl^- and/or different melatonin concentrations. Reactions were monitored at both 432 and 450 nm.

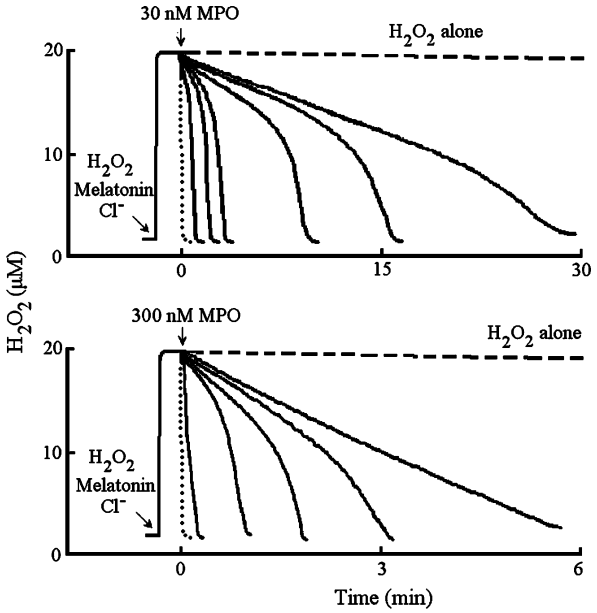

FIGURE 1: Melatonin/Cl^- inhibits H_2O_2 consumption by MPO. (A) A typical recording by a H_2O_2-selective electrode demonstrating the autoreduction of H_2O_2 (20 μM) following addition to a stirred 0.2 M phosphate buffer (pH 7.0) supplemented with 100 mM Cl^- at 25 °C (dashed line). Addition of 20 μL (30 nM in 3 mL of solution, final) of MPO solution to the H_2O_2/Cl^- stirred buffer caused rapid MPO consumption (dotted line). Addition of 20 μL (30 nM final) of MPO solution to the H_2O_2/Cl^- solution supplemented with increasing melatonin concentrations, 5, 8, 10, 16, 21, and 32 μM (solid lines from left to right, respectively), caused slower H_2O_2 removal. (B) The same reactions were repeated by adding 20 μL (300 nM final) of MPO to a buffer solution supplemented with 100 mM Cl^- (dotted line) and increasing concentrations of melatonin, 5, 10, 16, 21, and 50 μM (solid lines from left to right, respectively). Tracings shown are from a typical experiment performed at least three times.

The time course of the absorbance change was fit to a single-exponential ($Y = 1 - e^{-kt}$) or a double-exponential ($Y = Ae^{-k_1 t} + Be^{-k_2 t}$) 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 involve mixing solutions of peroxidase (1−2 μM) preincubated with 200 mM Cl^- in the absence or in the presence of increasing (7−200 μM) melatonin concentrations with buffer solutions containing 40 μM H_2O_2 at 10 °C.

RESULTS

Ability of Melatonin To Inhibit MPO Catalytic Activity. Our initial experiments utilized a H_2O_2-selective electrode to determine whether melatonin serves as a potent inhibitor for MPO. Reactions were performed under conditions where MPO was present in catalytic amounts. The results are illustrated in Figures 1 and 2. As a control, we first examined whether melatonin alone can consume H_2O_2. In the absence of MPO, following addition of 20 μM H_2O_2 (final) to the

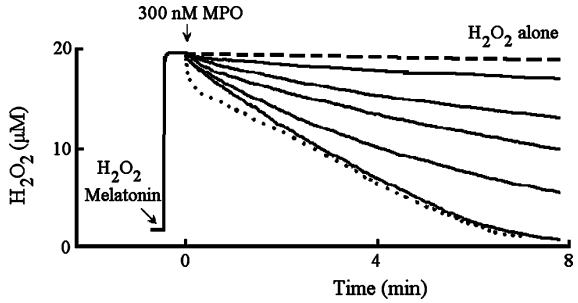

FIGURE 2: Effect of melatonin on H_2O_2 consumption by MPO in the absence of Cl^-. A typical recording by a H_2O_2-selective electrode demonstrating the autoreduction of H_2O_2 (20 μM) following addition to a stirred 0.2 M phosphate buffer (pH 7.0) at 25 °C (dashed line). The dotted line represents a H_2O_2-selective electrode trace after the addition of MPO (300 nM) to the H_2O_2 solution in the absence of melatonin. Solid lines represent the H_2O_2-selective electrode traces after the addition of MPO to the H_2O_2 solution in the presence of 1.5, 3, 12, 40, and 80 μM melatonin (from top to bottom, respectively). Note that the increasing melatonin concentration is associated with a decreased rate of H_2O_2 consumption. Tracings shown are from a typical experiment performed at least three times.

continuously stirred reaction mixture, the H_2O_2 signal rose rapidly, achieved a maximum after ~30 s, and fell gradually as H_2O_2 was depleted by autoreduction (Figure 1A, dashed line). Addition of different melatonin levels (5, 8, 10, 16, 21, and 32 μM) and/or a plasma level of Cl^- (100 mM) to a stirred H_2O_2 buffer solution had no or little effect on H_2O_2 autoreduction rates, indicating that melatonin alone did not significantly consume H_2O_2 (data not shown). Addition of MPO (20 μL, 30 nM final) solution to a continuously stirred H_2O_2 (20 μM) solution supplemented with 100 mM Cl^- caused a rapid consumption of H_2O_2 (Figure 1A, dotted line). Under these experimental conditions, MPO metabolized Cl^- through a $2e^-$ oxidation pathway, generating HOCl. The rapid disappearance of H_2O_2 did not occur in the presence of saturated amounts of melatonin (e.g., 32 μM). Instead, a much slower rate in H_2O_2 consumption (Figure 1A) indicated that melatonin inhibited the MPO catalytic activity. Because MPO is thought to utilize melatonin as a $1e^-$ substrate, we next examined whether oxidation of melatonin could restore MPO catalytic activity. Experiments were performed under conditions where melatonin concentrations were lower than the H_2O_2 concentrations used. The results are shown in Figure 1A. MPO catalyzed a slow reduction of H_2O_2, which accelerated upon melatonin oxidation. The transformation that is apparent from our kinetic analysis may represent a switch in subcycles in which the first step in H_2O_2 consumption is limited by the rate of melatonin dissociation from the Cl−MPO−melatonin complex when the reaction is initiated by adding an MPO solution to a melatonin/Cl^-/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−MPO−Cl complex by either oxidation or dissociation to generate inhibitor-free MPO. Under the latter circumstances, the enzyme generates HOCl. Thus, the variation in the binding affinity of MPO−Fe(III) toward melatonin and the release of melatonin from the MPO−melatonin complex due to its oxidation accounts for the inflection point in the H_2O_2 consumption rate. The IC_{50} value, the concentration of melatonin that inhibits H_2O_2 consumption by 50%, calculated from the initial rate of H_2O_2 consumption, as a function of the melatonin concentration was 3 μM. The maximum stoichiometry value obtained by plotting the melatonin concentration used versus the H_2O_2 concentrations corresponding to the cross points of the extrapolation of the limiting slopes of the two H_2O_2 consumption phases for the H_2O_2 traces shown in Figure 1A provided a maximum stoichiometry of 2.0 ± 0.3.

When the above experiments were repeated by adding 300 nM MPO, the H_2O_2 consumption rates were significantly increased, the duration of the reactions decreased, and the trend by which melatonin inhibits MPO stayed the same (Figure 1B). Neither the maximum stoichiometry nor the IC_{50} values were affected by the MPO concentration. Together, these results show how different melatonin and MPO concentrations can control H_2O_2 consumption rates in the presence of Cl^-.

In the absence of Cl^-, addition of 20 μL of MPO (300 nM final) to a continuously stirred H_2O_2 (20 μM) solution caused an immediate rapid decay in the level of free H_2O_2, followed by a slower decay, indicating that H_2O_2 was consumed as a substrate by MPO during catalysis (Figure 2, dotted line). The first step occurred immediately after the enzyme addition and was attributed to the formation of MPO compound I. The second step was much slower and was attributed to the reaction of MPO with H_2O_2 after the conversion of MPO compound II to MPO−Fe(III) (*44*). The first phase was significantly attenuated when an MPO solution was added to a stirred H_2O_2 solution supplemented with 2 μM melatonin (Figure 2, top solid line). This indicates that melatonin may have served as a $1e^-$ substrate for MPO compound I and reduced the multiple cycles of H_2O_2 consumption that occur prior to the conversion of MPO compound I to compound II. Increasing the melatonin concentration increased the rate of H_2O_2 consumption, indicating that melatonin serves as a $1e^-$ substrate for MPO compound II (Figure 2).

Rapid Kinetic Measurements. Under substrate-free conditions, MPO−Fe(III) displayed a Soret absorbance peak centered at 432 nm and visible peaks centered at 573, 630, and 694 nm. Upon addition of H_2O_2 to MPO−Fe(III), compound II accumulation was observed at 452 nm, and its formation occurred through rapid initial formation of compound I. Compound I was characterized by a significant decrease in the extinction coefficient of the Soret absorbance peak at 432 nm, as previously reported (*33*). Figure 3A shows MPO compound I formation and its conversion to compound II when an MPO solution was rapidly mixed with the same volume of a H_2O_2 solution at 10 °C, using the stopped-flow diode array spectrophotometer. Similar results were obtained when the MPO solution was preincubated with 100 μM melatonin prior to initiation of the reaction, except that the conversion rate constants of compound I to compound II were progressively accelerated (Figure 3B). At higher melatonin concentrations, the rate of the absorbance decrease at 432 was essentially identical to the rate of absorbance increase at 452 nm (data not shown), suggesting that MPO binds to melatonin and its dissociation from the melatonin−MPO complex is the rate-limiting step of the overall reaction.

To investigate the mechanism by which melatonin exerts its effect, we utilized single-wavelength stopped-flow spectroscopy to examine the buildup, duration, and decay of MPO compound II during steady-state catalysis at five different

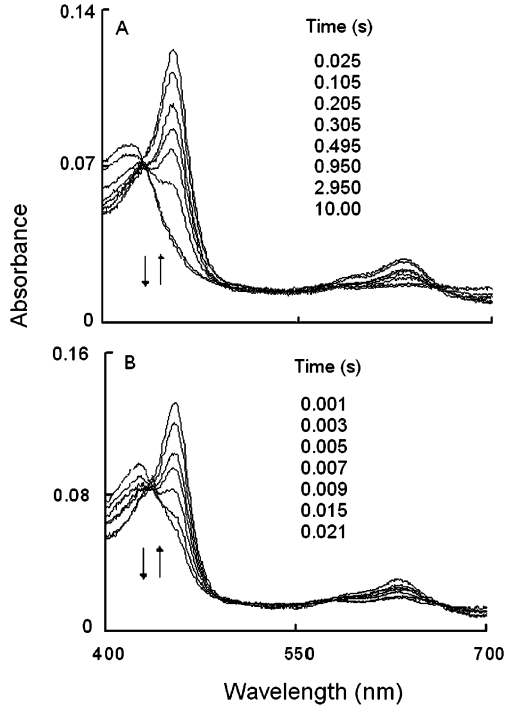

FIGURE 3: Melatonin accelerates the conversion rates of MPO compound I to compound II. Absorbance spectra of MPO recorded by a diode array stopped-flow spectrophotometer in the absence (A) and the presence (B) of 100 μM melatonin. Experiments were performed under aerobic conditions when a phosphate buffer solution (200 mM, pH 7.0) containing 2.4 μM MPO in the absence and presence of 200 μM Cl^- was rapidly mixed with a buffer solution supplemented with 40 μM H_2O_2 at 10 °C as described in the Materials and Methods. Panel A contains spectra collected 0.025, 0.105, 0.205, 0.305, 0.495, 2.950, and 5 s after mixing, while panel B contains spectra collected 0.001, 0.003, 0.005, 0.007, 0.009, 0.015, and 0.021 s after initiation of the reaction. Arrows indicate the direction of spectral change over time.

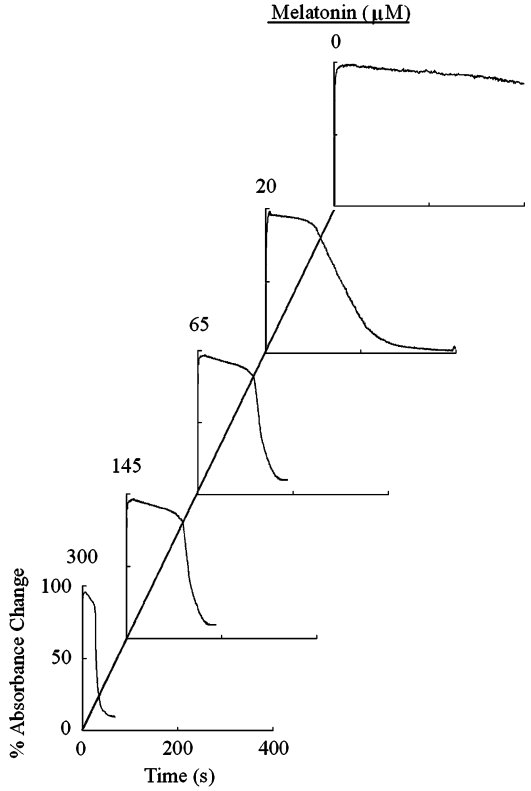

FIGURE 4: MPO compound II formation, duration, and decay during MPO catalysis at five different melatonin concentrations. MPO compound II formation and decay were monitored at 452 nm and 10 °C. Initial melatonin concentrations were 0, 20, 65, 145, and 300 μM. Experiments were carried out by rapidly mixing an enzyme solution (2.2 μM) preincubated with increasing concentrations of melatonin against a buffer solution supplemented with 40 μM H_2O_2.

melatonin concentrations (0, 20, 65, 145, and 300 μM). The reactions each received an equivalent amount of H_2O_2 (20 μM, final concentration). As shown in Figure 4, we observed a rapid buildup of MPO compound II at all five melatonin concentrations, followed by a steady state in which the amount of complex remained relatively constant, and finally a decay of the complex after MPO had reduced all of the available H_2O_2. The rate of compound II accumulation was enhanced nearly 75-fold in the presence of melatonin and increased in a concentration-dependent and saturable manner (Figure 5A). The presence of melatonin had a variable effect on the steady-state concentration of compound II that develops following H_2O_2 addition (Figure 5B). Finally, melatonin significantly accelerated the rate of MPO compound II decay in a concentration-depended fashion (Figure 5C). A plot of melatonin concentration versus rate of compound II decay demonstrated linear kinetics (Figure 5C) and yielded a second-order rate of 1.9×10^2 M^{-1} s^{-1}. The first-order rate constant derived from the *y* intercept was similar to the one in the absence of melatonin. This behavior may suggest that MPO compounds I and II readily used melatonin as a $1e^-$ substrate and, at higher concentrations, melatonin binds to MPO and limits the reaction by either its dissociation from the melatonin−MPO complex or its oxidation.

To examine how melatonin affects the formation of MPO compound I, we studied H_2O_2 binding in the presence of increasing melatonin concentration at 432 nm. In the absence of melatonin, complex buildup was relatively fast and was best described by a single-exponential equation with a pseudo-first-order rate of 390 s^{-1}. The MPO compound I formation rate was decreased by approximately a factor of 0.5 at higher melatonin concentrations and matched the rate of MPO compound II formation observed at 452 nm (Figure 5D). Melatonin may decrease the rate of H_2O_2 binding by constraining H_2O_2 within the distal pocket of MPO or limiting the reaction by either the dissociation of melatonin from the complex or melatonin oxidation. Observing two kinetically distinct heme irons at unsaturated melatonin concentration suggests that at least two populations of MPO exist in the samples: free and melatonin-bound enzyme.

We next utilized diode array spectrophotometry to examine the effect of melatonin on the formation, duration, and decay of MPO intermediates as they occur during steady-state catalysis in the presence of plasma levels of Cl^- (100 mM). Investigations were carried out for three different conditions: in the presence of subsaturated and saturated levels of melatonin as well as in the absence of melatonin. Figure 6 shows the change in absorbance over time after addition of H_2O_2 to a solution of MPO containing 100 mM Cl^- in the absence and presence of melatonin. Addition of H_2O_2 to a solution of MPO in the presence of 100 mM Cl^- caused small decreases in absorbance (Figure 6A), indicating that the decay of MPO compound I is faster than its formation. Therefore, compound I did not accumulate. When the same reactions were repeated in the presence of saturated amounts

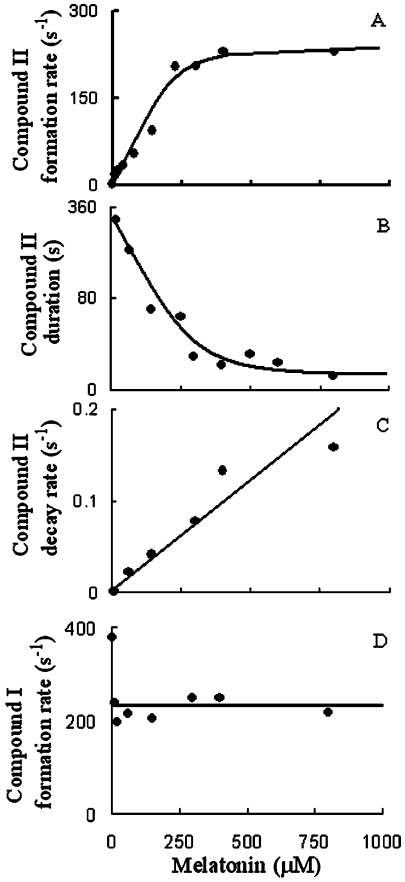

FIGURE 5: Rate of MPO compound II formation, duration, and decay as a function of the melatonin concentration in the absence of Cl^-. The observed rates of MPO compound II formation (A), duration (B), and decay (C) (monitored at 452 nm) observed in Figure 4 were plotted as a function of the melatonin concentration. Data represent the mean of triplicate determinations from an experiment performed three times. The formation rate monitored at 432 nm is also shown as a function of the melatonin concentration (D).

of melatonin (200 μM), MPO−Fe(III) converted directly to compound II without any sign of compound I accumulation (Figure 6B). Figure 6B shows spectra recorded 0.006, 0.03, 0.062, 0.106, 0.142, 0.202, 0.242, and 0.398 s after initiation of the reaction.

In a parallel series of experiments, single-wavelength stopped-flow spectroscopy was also utilized to investigate how melatonin interacts with MPO and switches the reaction from a $2e^-$ to a $1e^-$ oxidation pathway during the metabolism of Cl^-. Reactions were again performed at 10 °C, and the absorbance change at 455 nm was monitored following rapid mixing of enzyme incubated with various concentrations of melatonin (5, 10, 20, 30 and 60 μM final) in the presence of Cl^- (100 mM final) against a fixed amount of H_2O_2 (20 μM final). In all cases, MPO compound II formation occurred without any indication of the buildup of compound I. Figure 7 shows actual stopped-flow traces for the buildup, duration, and decay of MPO compound II collected at 452 nm in the presence of increasing melatonin concentrations. Analysis of stopped-flow traces collected indicated the formation and decay of MPO compound II were both monophasic and accurately fitted by a single-exponential function. Figure 8 shows the relationship between the melatonin concentration and the rate of MPO compound II formation (A), the duration

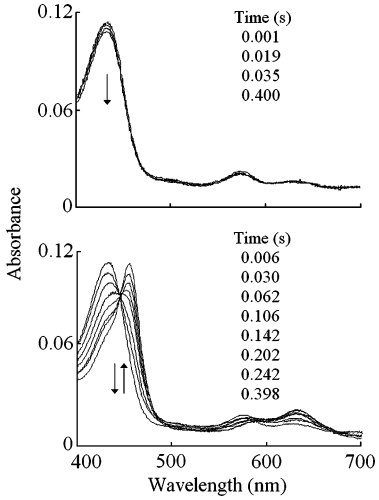

FIGURE 6: In the presence of plasma levels of Cl^-, melatonin facilitates the direct conversion of MPO−Fe(III) to compound II. Absorbance spectra of MPO recorded by a diode array stopped-flow spectrophotometer in the absence (A) and the presence (B) of 200 μM melatonin. Experiments were performed under aerobic conditions when a phosphate buffer (200 mM, pH 7.0) containing 1.9 μM MPO and 200 mM Cl^- in the absence (A) and the presence (B) of 200 μM melatonin was rapidly mixed with a buffer solution supplemented with 40 μM H_2O_2 at 10 °C. Panel A contains spectra collected 0.001, 0.019, 0.035, and 0.400 s after mixing, while panel B contains spectra collected 0.006, 0.030, 0.062, 0.106, 0.142, 0.202, 0.242, and 0.398 s after initiation of the reaction. Arrows indicate the direction of spectral change over time.

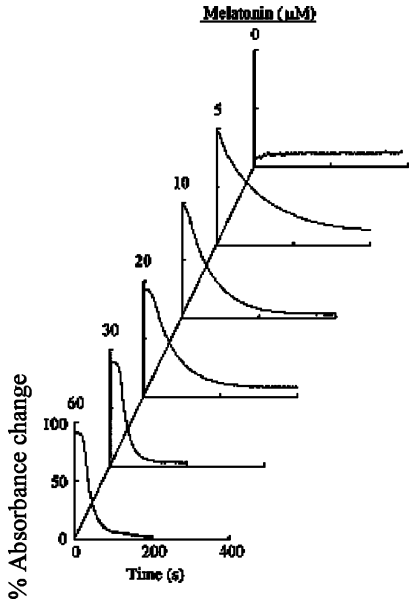

FIGURE 7: MPO compound II formation, duration, and decay during MPO catalysis at five different melatonin concentrations in the presence of a plasma level of Cl^-. MPO compound II formation and decay were monitored at 452 nm and 10 °C. Initial melatonin concentrations were 5, 10, 20, 30, and 60 μM. Experiments were carried out by rapidly mixing an enzyme solution (2.2 μM) preincubated with a plasma level of Cl^- (100 mM, final concentration) and increasing concentrations of melatonin against a buffer solution supplemented with 40 μM H_2O_2.

of its formation (B), and the rate of complex decay (C). It was evident as the melatonin concentration increased the MPO compound II formation rate constant and complex decay increased in linear and saturable manners (Figure 8A,C). The saturated value of MPO Compound II formation

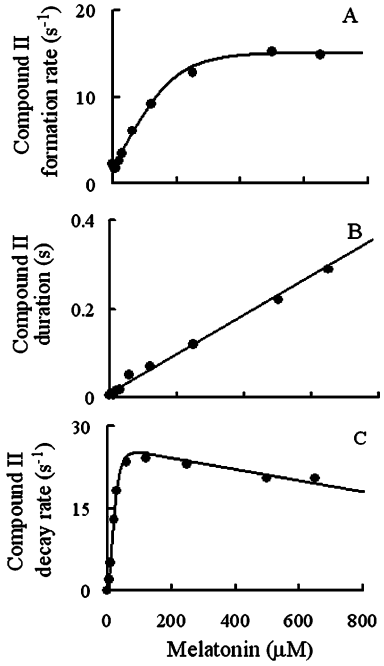

FIGURE 8: Rate of MPO compound II formation, duration, and decay as a function of the melatonin concentration in the presence of a plasma level of Cl^-. The observed rates of MPO compound II formation (A), duration (B), and decay (C) (monitored at 452 nm) observed in Figure 7 were plotted as a function of the melatonin concentration. Data represent the mean of triplicate determinations from an experiment performed three times.

rate constant at higher melatonin concentrations indicates that melatonin binds to MPO-Fe(III) in the presence of Cl^- and limits the reaction by its dissociation from the corresponding complex. The dissociation rate constant estimated from the saturation levels of compound II formation (15 s^{-1}) was ~15-fold slower than that observed in the absence of Cl^- (225 s^{-1}), while the first-order rate calculated from the intercept was similar to that measured in the absence of melatonin and Cl^-. The decrease of the MPO Compound II decay rate constant at higher melatonin concentrations confirms that melatonin binds to MPO and limits the MPO catalytic activity by the rate of its dissociation for the complex. The plot of the duration of MPO complex decay as a function of the melatonin concentration was linear. When the same reactions were monitored at 432 nm, the direction of absorbance change reversed and the signal amplitudes increased, but otherwise the reactions proceeded with identical kinetics (data not shown).

To assess the influence of H_2O_2 on the catalytic activity of MPO, the rates of MPO−Fe(III) conversion to compound II were determined as a function of the H_2O_2 concentration. Investigations were carried out by mixing a buffer solution sublimated with increasing H_2O_2 concentrations with a buffer solution sublimated with fixed amounts of melatonin (200 μM), Cl^- (100 mM), and MPO (1.2 μM). Again, the observed rate constants measured at both 432 and 450 were identical, and the plots of the apparent rate constants for H_2O_2 binding as a function of the H_2O_2 concentration were linear, consistent with a simple one-step mechanism. The k_{on} calculated from the slope was 3.9×10^5 M^{-1} s^{-1} with an intercept of 7 s^{-1} (Figure 9).

Solutions preparation. A stock solution of melatonin was dissolved in dymethylformamide (DMF) and then diluted to the required concentration with phosphate buffer (pH = 7.00). The final concentration of DMF in all melatonin solutions used was less than 1% and has no effect on MPO reactions.

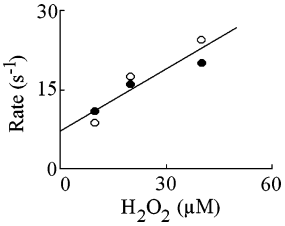

FIGURE 9: Rate of MPO−Fe(III) conversions to compound II as a function of the H_2O_2 concentration. The observed rates of MPO compound II formation monitored at both 430 nm (closed circles) and 455 nm (open circles) were plotted as a function of the melatonin. Data represent the mean of triplicate determinations from an experiment performed three times.

DISCUSSION

A wealth of clinical and biochemical data support the notion that exogenic HOCl/OCl^- and hypochlorite produced by MPO catalysis are implicated in the initiation of lipid peroxidation, which plays important roles in the pathogenesis of atherosclerosis and other inflammatory processes (*50*−*52*). However, no effective inhibitors have been identified yet for the enzyme. Melatonin displays the potential capacity to resist oxidative processes both in vivo and in vitro and to combat oxidative damage not only to lipids, but also to DNA (*53*−*55*). Our current studies provide the first direct evidence that melatonin can inhibit the catalytic activity of MPO by multiple pathways. Melatonin significantly inhibited H_2O_2 consumption in levels that span both (patho)physiological and supplemental ranges (*56*, *57*). Preincubation of MPO with a Cl^-/melatonin mixture generates a more complex biological setting and suggests the possibility of the existence of two separate binding sites on MPO. The present results are the first to show a direct communication between the two binding sites. Indeed, Cl^- binding significantly enhances MPO affinity toward melatonin. In the absence of Cl^-, melatonin mainly serves as a $1e^-$ substrate for MPO compounds I and II. Thus, binding of MPO with its cosubstrate, Cl^-, may cause conformational changes that significantly enhance the affinity of the second binding site toward melatonin, which in turn inhibits the catalytic activity of MPO. There is already evidence to support the existence of two halide-binding sites that have a distinct impact on the heme iron microenvironment in MPO and regulate its function (*58*). Therefore, any structural changes in the MPO heme environment that arise due to binding of the cosubstrate to the active and inactive sites or to MPO heme iron are envisioned to potentially affect the heme iron microenvironment, its substrate binding, its reduction potential, and its catalytic activity. This may provide new insights into the biological role of Cl^- in facilitating the cross-talk between the two binding sites of MPO. Collectively, understanding the interplay between the two binding sites of MPO, in addition to the ability of melatonin to switch peroxidation catalytic activity to catalase-like activity, may help us further understand the catalytic activity of the enzyme and advance methods of inhibiting MPO and its downstream inflammatory byproducts. This alone may be important in biologic settings

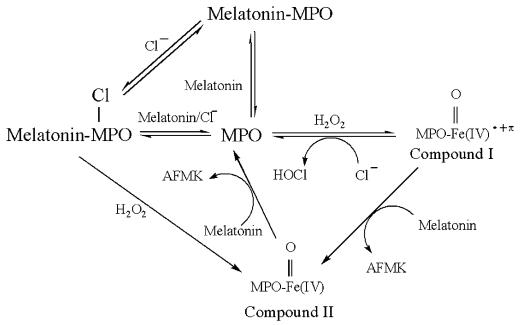

FIGURE 10: Working kinetic model for melatonin interactions with MPO.

where MPO is expressed. This process subsequently may lead to the development of biomarkers and therapeutic interventions of various conditions such as atherosclerosis and other diseases.

The MPO inhibition by melatonin is due to combination effects, which include allosteric binding to the entrance of the MPO heme pocket and accelerating the formation and the decay of MPO compound II, which switches the MPO catalytic activity from peroxidation to catalase-like activity. This allosteric binding to the MPO heme pocket entrance is enhanced by Cl^- binding to the halide-binding site, where HOCl is generated, and prevents the access of H_2O_2 to the catalytic site of the enzyme. Thus, melatonin competes with H_2O_2 and switches the reaction from free to melatonin-bound enzyme (active to inactive form), and melatonin also competes with the cosubstrate, Cl^-, and switches the reaction from a $2e^-$ oxidation to a $1e^-$ oxidation pathway. Rapid mixing of MPO preincubated with melatonin and Cl^- against the same volume of H_2O_2 solution caused immediate buildup of a transient intermediate, compound II, which then decays to MPO−Fe(III) through oxidation of another melatonin molecule, thereby closing the catalase-like cycle. The ability of melatonin to compete with H_2O_2 and Cl^- on the active sites of MPO−Fe(III) and MPO compound I, respectively, is a key feature that drives the enzyme to alter its function to catalase-like activity. Our kinetic parameters have indicated that the decay of compound I occurs faster than its formation and the decay of compound II occurs slower than its formation; thereby, as a replacement for compound I, compound II accumulated during steady-state catalysis. This mechanism of competitive inhibition has been shown to occur with tryptophan and several phenolic and aromatic amines.

A model that highlights melatonin interactions and inhibitions of MPO is illustrated in Figure 10. Previous rapid kinetic measurements have demonstrated that the reaction of MPO−Fe(III) with H_2O_2 to generate compound I is relatively fast with a second-order rate constant of 1.07×10^{-6} M^{-1} s^{-1} at 10 °C (*39*). Chloride at a plasma concentration of 100 mM is the physiological substrate for MPO (*45*, *59*). MPO compound I oxidizes halides in a single $2e^-$ reaction, leading to reduction of compound I to MPO−Fe(III). The high rate of Cl^- catalysis was associated with undetectable compound I buildup and a minimal decrease in the Soret absorbance peak during the steady-state catalysis. In the absence of Cl^- 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 (*33*, *60*−*62*).

In the absence of Cl^-, MPO consumes H_2O_2 by immediate rapid decay in the level of free H_2O_2 followed by a slow decay (*44*). The first step occurs immediately after MPO addition and is attributed to multiple cycles of H_2O_2 consumption prior to the conversion of MPO compound I to compound II (*44*). Our results showed that melatonin was able to constrain this reaction by accelerating the conversion of compound I to compound II. Formation of compound II, the inactive form of MPO, limits the H_2O_2 consumption rate by the slow conversion rate of MPO compound II to MPO−Fe(III) (*44*), which can be accelerated by melatonin. In the presence of Cl^-, only the first phase is observed, indicating that MPO catalyzes the formation of HOCl. HOCl is the active component of bleach, which possesses potent bactericidal and viricidal activities (*25*, *26*). The ability of melatonin to influence compound I rates of formation strongly supports the notion that melatonin competes with H_2O_2 and binds tightly to MPO−Fe(III), forming a dead end melatonin−MPO−Cl complex. Thus, Cl^- facilitates the binding of melatonin to MPO.

The ability of melatonin to serve as an inhibitor for MPO is illustrated by the dramatic slow dissociation rate of melatonin from the melatonin−MPO−Cl complex. As a result, the ability of melatonin to compete with Cl^- and serve as a $1e^-$ substrate can lead to a lack of accumulation of compound I and direct formation of compound II. Alternatively, the melatonin−MPO−Cl complex may interact directly with H_2O_2, leading to oxidation of melatonin (*63*) and generation of MPO compound II. Under these circumstances the catalytic activity of MPO appears to be limited by the slow conversion of MPO compound II to MPO−Fe(III). Whether the reaction of MPO occurs by the slow dissociation of melatonin from Cl−MPO−melatonin or through the direct reaction of the complex with H_2O_2 to form compound II, which in turn is converted to MPO−Fe(III), has not been established. The net results, however, are that melatonin inhibits MPO catalytic activity.

Taken together, our studies suggest a bidirectional relationship that links MPO and melatonin. This interplay between MPO and melatonin may have a much broader application in biological systems. Evidence suggests that MPO-mediated reactive oxidants can promote protein nitration, lipid peroxidation, amine chlorination, and thiol nitrosylation (*13*−*18*). These species can trigger various inflammatory events that affect immune defenses, atherosclerosis, asthma, and arthritis (*13*−*18*). Thus, inhibition of MPO and its downstream inflammatory byproducts could be attractive targets in the development of biomarkers and therapeutic interventions of various conditions such as atherosclerotic and cardiovascular diseases. Melatonin may have a beneficial role in modulating the pathogenesis of several disorders by inhibiting MPO catalytic activity and function. Since melatonin is a major player in the sleep−wake cycle, the interaction between melatonin and MPO may prove to be an important mechanism in the regulation of circadian rhythm and other sleep functions.

To date, the crystal structure of MPO with melatonin or other related aromatic molecules remains unknown. Recent studies by Hallingback et al. utilizing computational docking have shown that melatonin could be docked to MPO−Fe(III), compound I, and compound II forms (*64*). To achieve stacking, they have shown that the indole ring is docked

parallel to the heme plane and close enough to the D pyrrole ring. The distance of the closest indole atom to the center of the D ring is averaged between 3.3 and 3.5 Å. Comparison among MPO−Fe(III), compound I, and compound II modeling indicated that melatonin was pushed approximately 1 Å away from the ferryl oxygen in both compounds I and II. In all three cases, the side chain of the indole compound was directed toward the outside of the distal cavity (*64*). Thus, the heme iron cannot be reduced by melatonin binding under our experimental conditions. Melatonin, like salicylhydroxamic acid and benzohydroxamic acid (*40*), is a bulky aromatic molecule that may influence MPO steady-state catalysis by binding to the entrance of the hydrophobic pocket of the distal heme cavity and preventing the access of H_2O_2 to the catalytic site of the enzyme. Previous EPR and X-ray structure simulations have demonstrated that aromatic molecules bind near the heme center of MPO (*40*). The hydroxamic side chains of salicylhydroxamic acid and benzohydroxamic acid seem to interact with the heme iron (*40*). Recent studies utilizing rapid kinetic measurements and NO binding to MPO−Fe(III) heme iron have also supported the existence of two halide-binding sites that have a distinct impact on the heme iron microenvironment in MPO (*58*). Our current results are consistent with two binding sites since the inhibition of MPO requires the occupation of both MPO binding sites, one site with chloride and the other site with melatonin.

Understanding the circumstances that lead to MPO inhibition can provide important information and a general framework of understanding the role of MPO in certain diseases. The heme moiety of MPO plays a critical role in ligand binding and catalytic function of the enzyme (*33*, *65*, *66*). Its primary function is to support electron transfer within the catalytic domain of the enzyme during catalysis (*33*, *65*, *66*). Our current findings provide further evidence for an allosteric coupling among melatonin, the MPO entrance binding (regulatory) site, and the catalytic center that induces detectable inhibition of MPO. Consequently, oxidation of melatonin is required before it diffuses from the binding site of the enzyme and restores the catalytic activity of MPO. The finding that oxidation of melatonin can restore the catalytic activity of MPO is very intriguing. Because melatonin oxidation is H_2O_2 dependent, we calculated the ratio between melatonin consumed and H_2O_2 consumed and found it requires approximately a 2:1 ratio for MPO reactivation. This ratio is consistent with the fact that melatonin switches the peroxidase function from peroxidation to catalase-like activity (Figure 10). More importantly, oxidized melatonin had no biologically harmful sequela and no effect on MPO activity. Thus, inactivation of MPO and its catalytic duration can be controlled effectively by melatonin supplementation. Indeed, when the melatonin concentration is less than twice the H_2O_2 concentration, H_2O_2 consumption proceeds in a slower and linear manner and MPO decays rapidly to MPO−Fe(III) after melatonin oxidation. This behavior clearly demonstrates that MPO is capable of restoring its catalytic activity and rejoining the peroxidase cycle after melatonin exhaustion. On the other hand, when the melatonin concentration is greater than twice the H_2O_2 concentration, the initial slow phase of H_2O_2 consumption remains at the same rate through the progression of the reaction and ceases when H_2O_2 is completely consumed. The oxidation of melatonin is supported by the control studies that show no H_2O_2 consumption in the absence of MPO.

Melatonin and peroxidase activities are apparently coupled through complex and interdependent pathways. It appears that the affinity of MPO for melatonin is very high under physiological conditions. MPO may be acting as a chelator for melatonin and limiting its bioavailability and function. This may partly explain the harmful effects of sleep loss on various body functions. In addition, melatonin may have an important role in the inhibition of MPO activity in various tissues during inflammation. Hence, it is possible to speculate that increased inflammation and decreased immunity associated with lack of sleep are the result of a lack of inhibitory protective effects of melatonin on MPO during prolonged waking hours and chronic sleep loss. On the other hand, the pathogenesis of various disease processes can be very complicated and involves multiple inflammatory mediators and oxidizing agents. The biological consequences of melatonin−peroxidase interactions may have broad implications in the regulation of sleep, inflammation, infectious, and cardiovascular events in vivo.

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