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MELATONIN MEDIATES CLASSIC LACTOPEROXIDASES CATALYTIC MECHANISM

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ABSTRACT

Lactoperoxidase (LPO) found in saliva, tears and airways protects system against bacterial and viral attack. It utilized hydrogen peroxide in the presence of halides or pseudohalide forming hypohalous acid a potent biocidal and virocidal agent. Melatonin is an important biomolecule that controls a number of functions including circadian sleep rhythms, blood pressure, oncogenesis, retinal function, seasonal reproduction, and immunity. Additionally, melatonin is frequently used as a supplement in a variety of medical conditions such as jet lag, shift work, and circadian rhythm sleep disorders, cancer, longevity and antioxidant therapy. Here, we demonstrate that melatonin modulates classic catalytic mechanism of LPO under physiological-like conditions. In the presence of chloride (SCN-), melatonin inactivated LPO classic peroxidase cycle by formation of compound II or two different intermediate compounds that are "dead end" products of LPO system. This behavior indicates that melatonin modulates the formation of LPO intermediates and their decay rates to the ground state this efficiently slowing down or inhibiting the cycling of the enzyme. Importantly, melatonin-dependent inhibition of LPO depends on hydrogen peroxide concentrations. Thus, the interplay between LPO and melatonin can have a broader implication especially in the cases of supplemental melatonin therapy.

Keywords: Lactoperoxidase, melatonin, hydrogen peroxide, inhibition

INTRODUCTION

Lactoperoxidase (LPO), a hemoprotein that uses H_2O_2 in the presence of halides or pseudohalides, generates the corresponding hypohalous acid, as a final end product in 2 electron transfer step of LPO catalytic cycle (1). Newly form hypohalous acid has a biocidal and biostatic activity thus efficiently providing the defense mechanism for the living system. LPO can be found in human fluids such as saliva and tears and is part of an airways defense system (2).

Catalytic mechanism of LPO is similar to one of myeloperoxidase, well known inflammatory enzyme, where, in classic catalytic cycle by consumption of H_2O_2, Fe(IV)=O group and a resonance-stabilized porphyrin π cation radical intermediate, Compound I is formed. In the presence of thiocyanate ions, compound I is reduced to the native state forming thiocyanous acid, a potent bactericidal agents. LPO can oxidize multiple organic and inorganic molecules such as phenols (3), catecholamines, and catechols (4), polychlorinated biphenyls (5), steroid hormones (6), that enhance the catalytic cycle of peroxidases by accelerating Compound II (LPO-Fe(IV)-O) formation and generating oxidant and diffusible radical species. In the presence of an excess of hydrogen peroxide, compound-I and compound-II may also react further with hydrogen peroxide, resulting in the superoxide adduct compound-III. Its formation and decay might have possible role in the removal of an excess of hydrogen peroxide. **Figure 1**

Melatonin 5-methoxy-*N*-acetyltryptamine is a neurohormone secreted by the pineal gland in the

brain synthesized from the amino acid tryptophan. It is a strong free radical scavenger and antioxidant involved in different biological and physiological regulation such as modulation of circadian rhythms and seasonal reproduction, retinal physiology and sleep regulation. Synthetic melatonin is available commercially, and its supplements have been used clinically in a variety of medical conditions such as jet lag, shift work, and circadian rhythm sleep disorders, cancer, longevity and antioxidant therapy (7). 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 doses ranging from 0.3 to 1000 mgs/day given for 1-4 weeks (8). A wide range of melatonin doses have been used, including "low-dose" (0.1 to 1.0 mgs) for jet lag and insomnia in the elderly, “moderate-doses” (5 and 10 milligrams), often taken by mouth 30 to 60 minutes prior to sleep time for disturbances in children with neuro-psychiatric disorders and bipolar disorder, or “high dose” (50 to 1200 milligrams) for treating cancer and migraine headaches (7, 9). Antioxidative ability of melatonin is based on its role as a scavenger of reactive oxygen species including hydroxyl radical, superoxide ion, peroxy radicals, singlet oxygen, nitric oxide, peroxynitrate and its metabolites. Recent studies demonstrated that melatonin serves as a potent inhibitor of MPO under physiological-like conditions. Apparently, interaction of enzyme binding site with melatonin in the presence of chloride ions results in inhibition of MPO activity in classic peroxidase cycle (10, 11). Additionally, melatonin can serve as a substrate for compound I and II thus efficiently balancing between enzyme inhibition and enzyme reactivation through classic catalytic cycle. LPO and MPO share similar structural features and kinetic behavior but there is difference in heme pocket reactivity under different conditions. Studies showed that upon reduction of MPO heme pocket collapses while LPO adopts two spectroscopically and kinetically distinguishable forms (one partially open and the other relatively closed).Thus, there is a possibility of different kinetic behavior of these two enzymes could be expected especially in the presence of potential inhibitors. However, if the activity of the enzymes is governed only by reactivity of the active metal centers or mediated by the substrate binding then similarities in inhibitory action of the same molecule will be visible. Since inhibition of MPO with melatonin has been detected and follows very specific mechanism, then melatonin as a biological molecule and pharmacological supplement, could affect LPO classic catalytic cycle in a mechanism that might differ from the one with MPO.

Despite the potential significance of LPO to both human health and disease, and its significant use in biotechnology little is known about the factors that down-regulate its activity and function. In the present study, we studied LPO catalytic mechanism in the presence of physiological concentrations of SCN^- using a melatonin as a substrate and/or inhibitor that could possibly limit and control classics LPO cycle or initiate new mechanistic pathways. Different melatonin concentrations, that are much higher than normal biological ones, were used considering the fact that most of pharmacological uses of melatonin are based on increased concentrations but clear regulation for it use is not yet established.

EXPERIMENTAL

Materials: Highly purified LPO with purity index (A439/280) of at least 0.85 was obtained from Sigma Aldrich Chemical Co. Enzyme concentration was determined spectrophotometrically using extinction coefficient λ_{412}=112000 M^{-1} cm^{-1} (12). Hydrogen peroxide solutions were prepared before every experiment by diluting 30 % H_2O_2 solution (SigmaAldrich) and diluted solution concentration was determined spectrofotometrically (λ_{240} = 43.6 $M^{-1}cm^{-1}$) (12). Used melatonin was of the highest purity grade and obtained from Sigma Co (St. Louis, MO). Solutions were prepared by dissolving melatonin in minimum amount of dimetilyeneformaide (DMF) and diluting it with phosphate buffer (pH= 7.2). The final DMF concentration was 2% or less in all assays and did not have any effect on catalytic reactions of enzyme. All other reagents and materials were of the highest purity grades available and obtained from Sigma Aldrich Chemical Co. or the indicated source.

UV-Visible Spectroscopy. - LPO was incubated with thiocyante and/or different concentrations of melatonin and reactions was triggered by addition of H_2O_2 in 0.01 M phosphate buffer, pH= 7.2. Spectral changes were monitored by recording UV-visible absorption in the Soret region (350-450 nm) for 0.2- or 0.5-s cycles in 20 minutes total time.

RESULTS AND DICUSSION

Lactoperoxidase reaction with H_2O_2 in the presence of melatonin: Lactoperoxidase was incubated with 10μM melatonin solution, at room temperature and pH of 7.2 for 5 minutes. LPO catalytic reaction was triggered by addition of 40 μM H_2O_2, and spectrum recorded every 30 seconds for 1200 seconds. As a shown in **Figure 2A,** upon initiation of the reaction detected spectrum showed Soret absorbance at 430 nm, which is typical spectrum of compound II. The

formation of compounds I (with a maximum absorbance at 410 nm) was not detected, meaning that formation of compound I and its conversion into Compound II occurs in less than 30 seconds. Another possible mechanistic pathway was direct formation of compound II without 2e transfer and the formation of compound I. The spectrum of compound II in the next 20 minutes slowly converted into a form with the maximum peak of 423 nm.

According to available literature, this maximum is characteristic of compound III, LPO-(II)-O_2. Compound III can be formed in the presence of slight excess of H_2O_2 relative to the LPO native enzyme. No decay of compound III to the ground state or any other form was detected after its formation. These findings differ significantly from the classical peroxidase mechanism (in the absence of SCN^- ions), where formation and accumulation of compound II was detected without decay to other LPO forms in measured time. **Figure 2B** shows the absorption spectrum lactoperoxidase and 5μM melatonin in reaction with, 40μM H_2O_2, where scanning was done as function of time (40 scans per 30 seconds) at the wavelength of 400-750 nm. After starting the reaction, the first form created under these conditions is compound II with peak absorption at 430 nm. The widening of the peak indicates small amounts of other LPO forms that could not be detected under these conditions. Even after 20 minutes of reaction, compound II was still present with slightly lower absorption, and no trace of compound II decay was observed. Apparently, melatonin did not act as a substrate for LPO compound II and cycling of the enzyme back to the ground state was inhibited through "dead end" mechanism as an accumulation of compound II.

Figure 2C shows the spectrum of already formed compound II in the presence of low concentrations of melatonin and hydrogen peroxide (1 μ M) with a peak maximum at 430 nm. After 20 minutes, Compound II did not convert to any of the possible transient forms of LPO or to the "ground state". This mechanistic pathway implies that both melatonin and hydrogen peroxide were consumed in the first step of formation of compound II. However, the absence of compound II decay in this time frame could be explained by the formation of LPO-Melatonin inactive complexes that essentially traps compound II and regulates its rate of decay.

Lactoperoxidase reaction with H_2O_2 in the presence of melatonin and thiocyante ions: **Figure 3A** shows the absorption spectrum lactoperoxidases ground state (412 nm), compound II with the absorption maximum at 430 nm, and intermediate compound with maximum at 418 nm. After incubation of LPO with thiocyante, and addition of 1μM melatonin, the reaction was stared with addition of 1μM H_2O_2, and spectra collected over period of 1200 seconds. LPO incubated with melatonin and SCN ions showed, just after addition of H_2O_2, formation of compound II as judged by the peak maximum at 430 nm.

However, under these reaction conditions no accumulation of Compound II was observed and after 60 secnds, Sorets peak with maximum at 418 nm was detected. These changes in Soret absorbance were a result of a decay of compound II to a ground state that occurs through generation of intermediate LPO compound. Low H_2O_2 concentrations efficiently diverted the mechanism from formation of compound III. Molecular UV-Vis absorption spectrum LPO/SCN^- with of 1μM melatonin and 20μM of hydrogen peroxide, is shown in **Figure 3B.** Here, too, was apparent the formation of compound II with no signs of formation of compound I. However, the formation of this compound was somewhat slower and transition of compound II to transitional intermediate with maximum peak at 418 nm was followed over first 90 seconds. However, the reaction of formation of intermediates was faster than the intermediate formation in the previous experiment, suggesting that the formation of this intermediate is direct related controlled by hydrogen peroxide and any inhibitory step by melatonin in a classic lactoperoxidase mechanism can be overcome through increased concentration of hydrogen peroxide. The same concentration of LPO incubated with and SCN ions and 10μM melatonin just after addition of H_2O_2 (**Figure 3C)** did not show a prominent compound II peak. After 30 seconds of the initiation reaction, we could detect only the so-called compound II "shoulder" that converted to intermediate at 418 nm without any further conversion to 20-minute observation of the reaction.

It is obvious that the increased concentrations of melatonin caused rapid transition to compound II in transitional form but also inhibit the conversion of transitional form to LPO ground state thus forming essentially "dead end" of catalytic cycle of LPO in the presence of melatonin. It is clear that in a given time, in the presence of melatonin, recovery to the ground state of LPO and thus initiation of a new cycle cannot be achieved. Based on collected data we proposed the catalytic mechanism of LPO modulated by a presence of different melatonin concentration that is shown **Figure 4** C. The most important part is the existence of LPO intermediate at

418 nm whose formation completely blocks the catalytic activity of an enzyme.

CONCLUSION

Collectively, our results show that a different melatonin concentration can modulate LPO catalytic mechanism and even block it by formation of transition form that is considered as a "dead end" of reaction. The main role of LPO system in the presence of physiological concentrations of thiocyanate ions is defense against pathogens. However, it is apparent that melatonin can either slow down or even block cycling of the LPO forming compound III or different intermediates thus rendering LPO system inactive for longer periods of time. Melatonin effect can be overcomed only by increased hydrogen peroxide concentrations. Normal physiological concentrations of melatonin are in picomolar range and any supplementations by high doses of melatonin could disturb normal host defense system that involves LPO. Thus, additional melatonin treatment should be monitored carefully considering the role that LPO system has in human airway defense mechanism.

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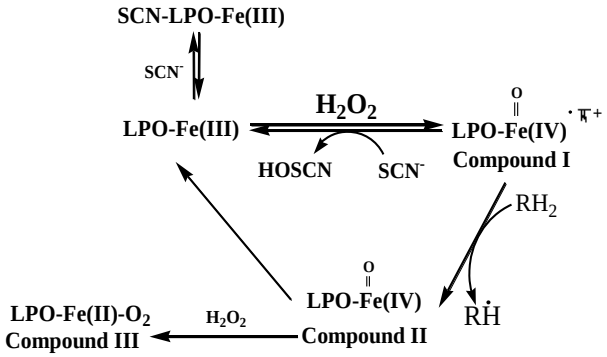

Figure 1: Classic LPO catalytic mechanism with formation of HOSCN

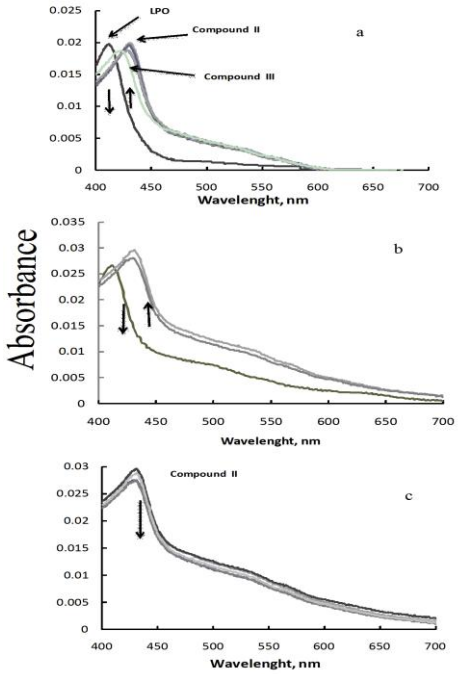

Figure 2. UV/Vis spectra showing LPO catalytic reactions in the presence of a.) 10 µM melatonin and 40 µM H_2O_2 b.) 5 µM melatonin and 40 µM H_2O_2 c.) 1 µM melatonin and 10 µM H_2O_2

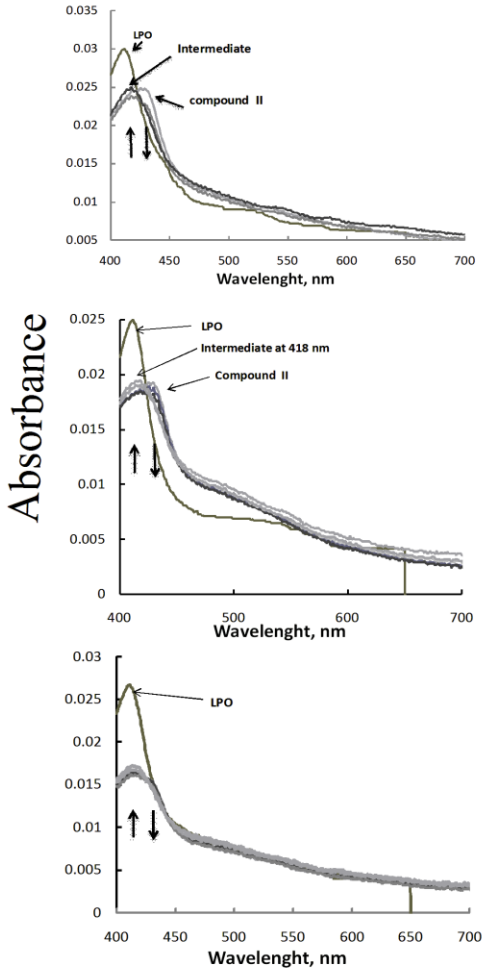

Figure 3. UV/Vis spectra of showing formed intermediates when LPO was incubated with SCN^- in the presence of a.) 1 µM melatonin and 1 µM H_2O_2 b.) 1 µM melatonin and 20 µM H_2O_2 c.) 10 µM melatonin and 20 µM H_2O_2

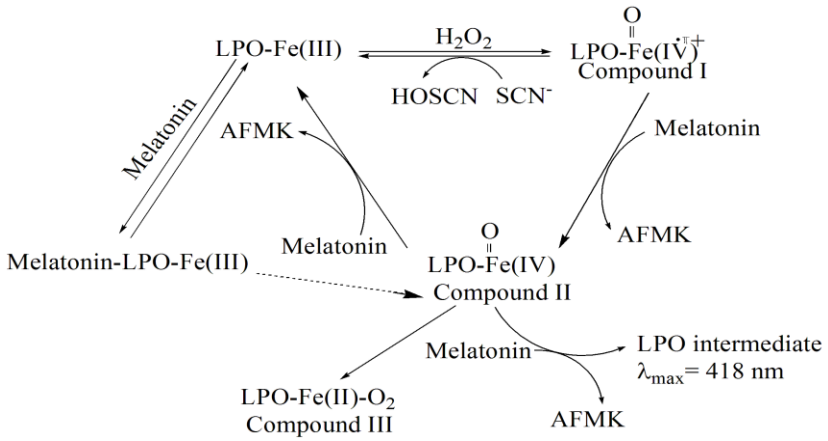

Figure 4. Modified LPO catalytic mechanism in the presence of melatonin

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