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The Reaction of Melatonin with Peroxynitrite: Formation of Melatonin Radical Cation and Absence of Stable Nitrated Products

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Peroxynitrite is capable of hydroxylating and nitrating aromatic species. However, nitromelatonin is not found as a final product when melatonin was allowed to react with peroxynitrite either in the presence or absence of added bicarbonate. In the absence of bicarbonate, the two major products formed are 6-hydroxymelatonin and 5-methoxy-2-hydro-pyrroloindole, and the latter is the only major product with excess bicarbonate. A transient purple intermediate with a maximum absorbance at about 520 nm is observed upon mixing solutions containing peroxynitrite and melatonin. These observations indicate that the melatoninyl radical cation is formed in the peroxynitrite/melatonin reaction, providing a direct evidence for the one-electron oxidation ability of peroxynitrite. The melatoninyl radical cation also is observed with excess bicarbonate.

Key Words: **peroxynitrite; melatonin; hormone; radicals; electron transfer; radical cation.**

Peroxynitrite ($ONOOH/ONOO^-$), produced by endothelial cells, neutrophils, and macrophages via the rapid reaction of superoxide anion with nitric oxide (1, 2), is capable of oxidizing a variety of biological species, such as thiols, lipids, DNA, ascorbate, and sulfides (3–9). Hydroxylation and nitration are known reactions of peroxynitrite with aromatic species such as tyrosine and phenol (8, 10). In physiological fluids such as blood plasma, peroxynitrite first reacts with CO_2 due to the abundance of CO_2 and the large rate constant of this reaction (11–15); thus, the intermediates from the peroxynitrite/CO_2 system generally govern peroxynitrite chemistry.

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Melatonin (MLT), an indolyl hormone produced by the pineal gland, is a potent antioxidant and radical scavenger (16–19). A number of research findings suggest that nocturnally produced MLT affords some protection against oxidative damage induced by carcinogens (17). Recently MLT is shown to cause a dose-dependent inhibition of the oxidation of dihydrorhodamine 123 by peroxynitrite, indicating a scavenger role for MLT against peroxynitrite (20). In support to this proposition, the maximal levels of MLT in animals are about 100–150 pg/ml serum, and there are some cells in organisms where MLT concentrations could be much higher (17).

Thus, we investigated the reaction of peroxynitrite with MLT, both in the absence and presence of added bicarbonate (CO_3^-, HCO_3^-, H_2CO_3 and CO_2 are collectively referred as bicarbonate). Herein we report the involvement of the melatoninyl radical cation ($MLT^{\bullet +}$) in the reaction and the products that are formed.

MATERIALS AND METHODS

Melatonin (97%), sodium bicarbonate (99.7%), and the TLC (C_{18}) plates were obtained from Aldrich (Milwaukee, WI). Sodium azide (99%) and 6-hydroxy-MLT were from Sigma (St. Louis, MO). Water was purified with a MilliQ system from Millipore (Bedford, MA).

The buffer solutions containing MLT were made by first dissolving MLT in 5 M HCl and then diluting/adjusting the pH using 50 mM potassium phosphate buffer and 50% (w/v) NaOH. Peroxynitrite was synthesized by ozonation of an aqueous solution of sodium azide (21).

The color change of the reaction mixture was observed against a white background. The transient absorption spectra (Fig. 1) of the reactions were recorded using a stopped-flow instrument equipped with a rapid scan UV/Vis spectrophotometer and a 2 cm flow mixing cell (On Line Instrument Systems, Jefferson, GA). Product analysis was performed using a 300 MHZ NMR (Bruker, Billerica, MA) and a HP5890/5970 GC/MS (Hewlett Packard, Wilmington, DE) with an SGE BPX5 column (25 m). Before the ^1H NMR and GC/MS analysis, the water was evaporated from the reaction mixture on a rotary evaporator at 60 °C. The organic residue was dissolved in methanol, and was further separated using reversed-phase TLC.

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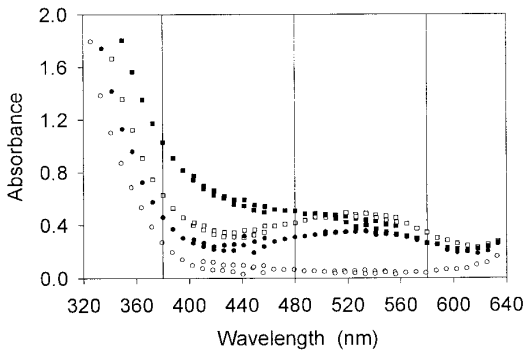
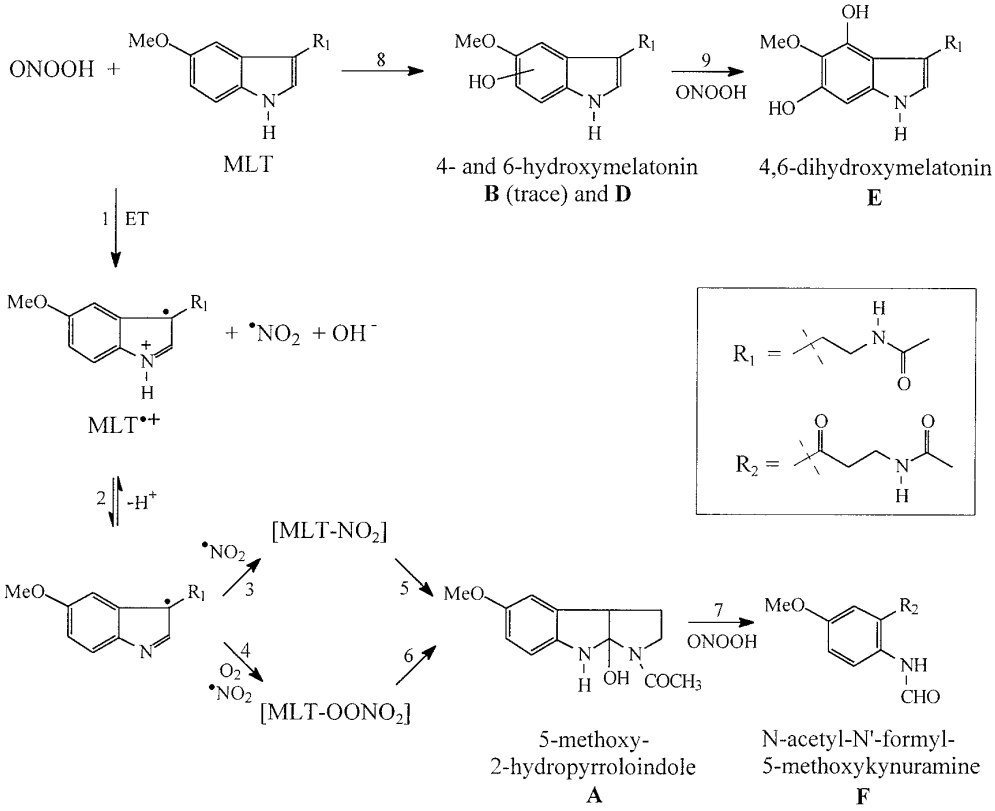


FIG. 1. Transient absorption spectra of the reaction of peroxynitrite with melatonin at 7°C. Each set is made up of three sub-spectra centered at 380, 480 and 580 nm, respectively. Immediately after mixing the reaction mixture contained 2 mM of peroxynitrite, 2 mM of melatonin, 25 mM of potassium phosphate buffer. The ionic strength was adjusted to 0. 15 M using sodium chloride, and the pH was 6. 18. Reaction time in ms: (○) 0, (●) 436, (□) 936, and (■) 4000.

RESULTS AND DISCUSSION

Melatoninyl radical cation. Upon vortex-mixing a solution of peroxynitrite (4 mM) with an equal volume of a phosphate buffer containing 4 mM of MLT, the color of the mixture rapidly changed to purple for about 2–3 seconds. When the same reaction was carried out using the stopped flow instrument, the transient spectra recorded at a speed of 1000 scans/s revealed a maximum absorbance at about 520 nm during the first few seconds of the reaction (Fig. 1). These observations suggest the involvement of an intermediate (step 1; Scheme 1), whose structure is assigned to $MLT^{\bullet+}$, since the transient spectra in Fig. 1 are similar to other radical cations of the indole family (22–24). In addition, similar transient spectra to what we observed were reported for the reaction of MLT with $^{\bullet}OH$ and were assigned to the melatonin radical cation (25). We suggest that $MLT^{\bullet+}$ arises from one-electron transfer to peroxynitrite, although it is difficult to rule out electron transfer to $CO_3^{\bullet-}$ (which could be formed from the reaction of peroxynitrite with adventitious CO_2 even when bicarbonate is not added). However, the yield of $MLT^{\bullet+}$ does not change upon purging the working solutions for 30 min with argon. In addition, we have determined that the overall peroxynitrite/MLT reaction is first order in MLT and first order in peroxynitrite. (Detailed kinetic analysis of the reaction will be given in a separate paper.) Since hydroxylation of MLT (see below) is likely zero order in MLT as we had observed for hydroxylation of phenol (26), the formation of $MLT^{\bullet+}$ has to be first order in MLT. These findings suggest that $MLT^{\bullet+}$ is formed by electron



SCHEME 1

TABLE 1

Products from the Reaction of Melatonin with Peroxynitrite[a]

PN[b]:MLT:CO_2 Molar ratio (mM)[c]	Percentage yields (loss) with respect to PN						
	Loss of MLT	**A**	**B**	**D**	MLT-NO_2[e]	Other	pH
2:1:0	11	2	Trace	6	[d]	3	6.3
2:1:$NaHCO_3$[d]	15	12	Trace	[d]	[d]	3	7.2

Note. (*a*) Measurements based on integration of peaks of GC/MS chromatograms. (*b*) PN stands for peroxynitrite. (*c*) All concentrations are after mixing values. (*d*) Saturated solutions of sodium bicarbonate are used. (*e*) Not detected.

transfer to peroxynitrite, with little or no contribution from $CO_3^{\bullet -}$ derived from adventitious CO_2.

Peroxynitrite has been suggested to be both a one-electron and a two-electron oxidant (9). Examples of substrates oxidized by peroxynitrite through one-electron transfer (ET) include methionine, phenol, thiols and ascorbate (27, 28). However, our observation of the $MLT^{\bullet +}$ provides a direct evidence for the one-electron transfer to peroxynitrite. The reaction of peroxynitrite with CO_2 generates a number of intermediate species including $CO_3^{\bullet -}$ and $^{\bullet}NO_2$ (Eq. 1) (29–32). The melatoninyl radical cation is observable in a system containing peroxynitrite, carbon dioxide and MLT (Table 1) under conditions in which peroxynitrite is entirely trapped by carbon dioxide. Thus, $MLT^{\bullet +}$ also must be formed by a one-electron oxidation of MLT by $CO_3^{\bullet -}$ (Eq. 2); the carbonate radical is known to react with indolyl species to give indolyl radical cations (24). In the peroxynitrite/CO_2/MLT system, $MLT^{\bullet +}$ is observed only briefly (< 1 s), and its rate of formation reflects the short period of time in which peroxynitrite is consumed, providing further evidence for the formation of $MLT^{\bullet +}$ by oxidation of MLT by $CO_3^{\bullet -}$.

$$ONOO^- + CO_2 \rightleftarrows CO_3^{\bullet -} + {}^{\bullet}NO_2 \qquad [1]$$

$$CO_3^{\bullet -} + MLT \rightarrow CO_3^{=} + MLT^{\bullet +} \rightleftarrows \mathbf{A} \qquad [2]$$

Reaction products. In the peroxynitrite/MLT reaction, two major products, **A** and **D,** and some minor products were detected using GC/MS (Fig. 2). An estimation of the overall product yields is summarized in Table 1. We assign structures to these products as follows. The structure of **D** is assigned to 6-hydroxymelatonin by matching its molecular ion (248), mass fragmentation pattern, and the retention time on the GC/MS chromatogram to those of the commercially available 6-hydroxy-MLT (Fig 2). **A** (Fig. 2) also has a molecular ion of 248, but its ^{1}H NMR spectrum[2] reveals that it is 5-methoxy-2-hydropyrroloindole. Traces of another isomer of hydroxymelatonin (molecular ion 248) occasionally were detected (**B** in Fig. 2). The ^{1}H NMR spectrum suggests this product is 4-hydroxymelatonin (data not shown). The structures of two secondary products, **E** and **F** (Fig. 2) are assigned to 4,6-dihydroxymelatonin (molecular ion 264), and to *N*-acetyl-*N*-formyl-5-methoxykynuramine (molecular ion 260), respectively, based on their molecular ions and fragmentation patterns. In the reaction of peroxynitrite with tryptophan, *N*-formylkynurenine is produced (33, 34), probably from a similar set of reactions in which **F** is formed. Traces of other unidentified products have been observed (i. e., **G, H** in Fig. 2), but these are not nitromelatonin derivatives, based on their mass spectra.

The formation of **D** is expected, since hydroxylation of aromatics is a known reaction of peroxynitrite (8, 26). This metabolite of MLT has been reported to be a free radical scavenger (35) and is a precursor of 4,6-dihydroxymelatonin (**E**) (step 9; Scheme 1).

Nitromelatonin is not found in the peroxynitrite/MLT reaction, even though its formation should be

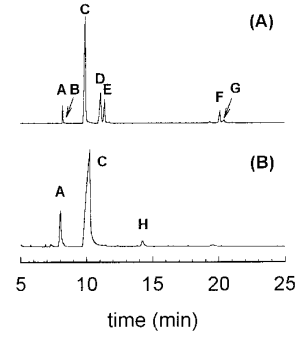


FIG. 2. GC/MS chromatogram of the reactions of MLT with peroxynitrite in the absence and presence of added bicarbonate. The reaction conditions: (A) A solution containing peroxynitrite (4 mM, pH 11) was vortex-mixed with equal volume of a buffer solution (pH 6. 85) containing melatonin (1 mM) at room temperature. (B) The buffer solution was saturated with sodium bicarbonate and the initial pH was 7. 20. The reaction conditions were otherwise the same as in (A). Peak identification: **A,** 5-methoxy-2-hydropyrroloindole; **B,** 4-OH-MLT; **C,** MLT; **D,** 6-OH-MLT; **E,** 4,6-dihydroxy-MLT; **F,** *N*-acetyl-*N*-formyl-5-methoxykynuramine. Other peaks are unidentified, but none of them can be assigned to MLT-NO_2.

[2] The NMR data (in $CDCl_3$) for **A** (δ in ppm and j in Hz): δ6. 900, 1H, d, j 2. 535; δ6. 768, 1H, d-d, j_1 2. 599, j_2 8. 591; δ6. 5679, 1H, d, j 8. 502; δ3. 763, 3H, s; δ3. 736-3. 652, 2H, m; δ3. 331, 1H, d-d, j_1 7. 054, j_2 9. 862; δ2. 560-2. 339, 2H, m; δ2. 021, 3H, s.

facilitated by the coupling of $MLT^{\bullet+}$ with $^{\bullet}NO_2$ formed *in situ* (step 1; Scheme 1). Nitration of tryptophan by peroxynitrite has been reported (33), but, Yato *et al.* report the absence of nitrotryptophan in the reaction of peroxynitrite with tryptophan (34).

We do not find nitrated MLT even when the reaction is conducted in the presence of excess amounts of added bicarbonate (Table 1), although CO_2 is a known catalyst for nitration of aromatic compounds by peroxynitrite (11, 13, 26). We suggest that nitration does occur, both in the absence and the presence of bicarbonate, to give rise to an unstable nitrated intermediate ([MLT-NO_2] in Scheme 1), which then undergoes further reactions and loss of nitrite to yield **A** (Scheme 1). Thus, $MLT^{\bullet+}$ (with a pK_a between 5 and 6 by comparison with the radical cations of other 5-methoxylated indoles (22)), is the key precursor of [MLT-NO_2], [MLT-$OONO_2$] and **A.** In the absence of bicarbonate, the two processes (steps 1 and 8; Scheme 1) compete for peroxynitrite, resulting in two major products, **A** and **D.** In the presence of bicarbonate, the carbonate radicals generated by the peroxynitrite/CO_2 reaction give rise only to $MLT^{\bullet+}$, resulting in the formation of **A** as the major product (Eq. 2). A portion of $MLT^{\bullet+}$ is expected to react with adventitious O_2 to form the peroxymelatoninyl radical cation, which apparently leads to the same final product as does $MLT^{\bullet+}$ (steps 3–6; Scheme 1).

The 5-methoxy-2-hydropyrroloindole (**A**) is identified here for the first time as a product of the peroxynitrite/MLT reaction. Some of **A** probably reacts further with peroxynitrite to give **F** (step 7; Scheme 1). In the presence of an excess of added bicarbonate, **F** is not formed because **A** is a less effective scavenger of free radicals than MLT (Eqs. 1 and 2).

CONCLUSION

The reaction of peroxynitrite with melatonin gives the melatoninyl radical cation ($MLT^{\bullet+}$), providing direct evidence that peroxynitrite is capable of one-electron oxidation. When the reaction is performed in the presence of added bicarbonate, $MLT^{\bullet+}$ is still produced, although the product distribution is different from that in the absence of added bicarbonate. In either case, no nitrated product of melatonin is detected. It is possible that nitromelatonin is formed but is subsequently converted to 5-methoxy-2-hydropyrroloindole. The potential biological activity of the products of melatonin/peroxynitrite reaction remains to be studied.

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