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Melatonin nitrosation promoted by $NO_2^{\cdot}$ radical; comparison with the peroxynitrite reaction

FABIENNE PEYROT[1], CHANTAL HOUÉE-LEVIN[2], & CLAIRE DUCROCQ[1]

[1]*Institut de Chimie des Substances Naturelles, CNRS, F-91198 Gif-sur-Yvette, France, and* [2]*Laboratoire de Chimie Physique, Université Paris XI, F-91405 Orsay, France*

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Abstract

N-nitroso species have recently been detected in animal tissues. Protein *N*-nitrosotryptophan is the best candidate for this *N*-nitroso pool. *N*-nitrosation of *N*-blocked trytophan derivatives like melatonin (MelH) by N_2O_3 or peroxynitrite ($ONOOH/ONOO^-$) has been observed under conditions of pH and reagent concentrations similar to *in vivo* conditions. We studied the reaction of $NO_2^{\cdot}$ with MelH. When $NO_2^{\cdot}$ was synthesized by γ-irradiation of aqueous neutral solutions of nitrate under anaerobic conditions, detected oxidation and nitration of MelH were negligible. In the presence of additional nitrite, when $NO^{\cdot}$ was also generated, formation of 1-nitrosomelatonin increased with nitrite concentration. Nitrosation is not due to N_2O_3 but could proceed via successive additions of $NO_2^{\cdot}$ and $NO^{\cdot}$. For comparison, peroxynitrite was infused into a solution of MelH under air leading to the same products as those detected in irradiated solutions but in different proportions. In the presence of additional nitrite, the formation of nitroderivatives increased significantly while *N*-formylkynuramine and 1-nitrosomelatonin were maintained at similar levels. Mechanistic implications are discussed.

Keywords: *Nitrosation, radiolysis, nitrogen dioxide, dioxidonitrogen(·), nitric oxide, oxidoperoxidonitrate(1 −)*

Abbreviations: *MelH, melatonin; MelNO, 1-nitrosomelatonin; $MelNO_2$, nitromelatonin*

Introduction

Nitric oxide ($NO^{\cdot}$) and nitrogen dioxide (dioxidonitrogen(·), $NO_2^{\cdot}$) are two environmental compounds whose levels rise in polluted air. Both are also generated in living organisms by enzymatic routes. The stable and diffusible $NO^{\cdot}$ radical, synthesized by NO synthases, is a biological mediator via its interaction with metal center proteins and its reactions with molecular dioxygen and radicals [1]. The reaction of $NO^{\cdot}$ with O_2 is the source of nitrosating agents such as dinitrogen trioxide (N_2O_3) in physiological aqueous solutions, leading to the formation of nitrosothiols, nitrosoamines and nitrite [2]. In hydrophobic regions such as protein domains or cell membranes, local concentrations of $NO^{\cdot}$ and O_2 may increase, facilitating $NO_2^{\cdot}$ and/or N_2O_3 formations [3].

In pathological situations, the level of $NO^{\cdot}$ rises up and is generally accompanied by an increase in superoxide radical anion ($O_2^{\cdot-}$) [4]. Coupling of $O_2^{\cdot-}$ with $NO^{\cdot}$ forms the transient peroxynitrite ($ONOOH/ONOO^-$, $pKa = 6.8$) [4–6], which decomposes into nitrate, $NO_2^{\cdot}$ and $HO^{\cdot}$ radicals [7–10]. The major alternative pathway for $ONOO^-$ decomposition in the presence of physiological CO_2

Correspondence: C. Ducrocq, Institut de Chimie des Substances Naturelles, CNRS, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France. Fax: 33 1 69077247. E-mail: claire.ducrocq@icsn.cnrs-gif.fr

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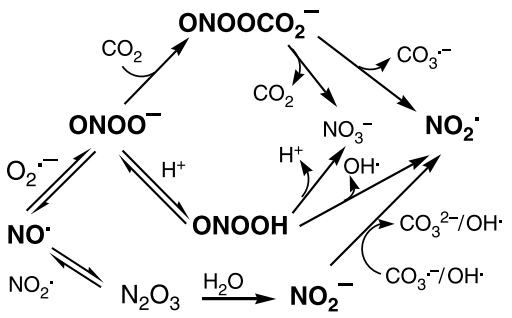


Scheme 1. Different biologically relevant pathways for $NO_2^{\cdot}$ radical formation.

concentrations, is the formation of unstable $ONOOCO_2^-$, yielding in part, $NO_2^{\cdot}$ and $CO_3^{\cdot-}$ [10–12] (Scheme 1).

Another important route for $NO_2^{\cdot}$ formation is the peroxidase-catalysed oxidation of nitrite [13–14]. This pathway and the decomposition of peroxynitrite are likely to give more $NO_2^{\cdot}$ than $NO^{\cdot}$ autoxidation, which is considered to be slow under physiological conditions. The pathochemistry of $NO_2^{\cdot}$ has been reviewed: it is generally accepted that $NO_2^{\cdot}$ radical performs hydrogen abstraction, intermolecular addition on double bonds, monoelectronic oxidations and radical recombinations [12,15]. Its ability to be scavenged by some antioxidants, like thiols, phenols, urate or ascorbate, has been demonstrated on several occasions [12,16–19].

These various nitrogen and oxygen species are more reactive towards bioorganic compounds than $NO^{\cdot}$ and $O_2^{\cdot-}$. Thiols are generally the major targets, but indolic amines also react readily, as described for melatonin (MelH), an endocrine hormone derived from tryptophan [20–22]. MelH and other indolic structures have been shown to scavenge reactive oxygen and nitrogen species such as hydroxyl, peroxyl radicals, N_2O_3, $NO_2^{\cdot}$ and peroxynitrite [23–28]. Notably, pulse radiolysis studies have been performed to characterize these reactions [29,30]. The rate constant for the reaction of MelH with $NO_2^{\cdot}$ was measured by Mahal et al. [30] ($k = 3.7 \times 10^6\,M^{-1}\,s^{-1}$ at pH 7), without identification of the products. In this study, our aim was to identify MelH reaction products in irradiated solutions containing nitrite or/and nitrate and to compare with the reaction of MelH with peroxynitrite in the presence of CO_2.

Materials and methods

Materials

MelH ($C_{13}H_{16}N_2O_3$), diethylene triamine pentaacetic acid (DTPA, $C_{14}H_{23}N_3O_{10}$), sodium nitrate-^{15}N ($Na^{15}NO_3$) and sodium nitrite-^{15}N ($Na^{15}NO_2$) were purchased from Sigma-Aldrich. Sodium nitrite-^{14}N ($NaNO_2$) and sodium nitrate-^{14}N ($NaNO_3$) were purchased from Fluka (purity > 99%). Disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$) were purchased from Prolabo (France). Dipotassium hydrogen phosphate trihydrate ($K_2HPO_4{\cdot}3H_2O$) was purchased from Merck. 2-Methyl-2-propanol (*t*-butanol, $C_4H_{10}O$) and acetonitrile were purchased from Carlo Erba-SDS (France). Ultrapure water was obtained using a Millipore reverse osmosis system (Waters). Argon and N_2O were purchased from Air Liquide (France).

Sample preparation for radiolysis

MelH was first dissolved in 5 M HCl before further dilution in a 10 mM potassium phosphate-buffered solution to a final concentration of 580 μM (unless otherwise stated). The pH was adjusted to 7.4. MelH integrity and concentration were checked by absorption spectroscopy and HPLC. Sodium nitrite or a mixture of the two salts, and when necessary *t*-butanol, were added. Ten millilitre Erlenmeyers containing 1 ml of solution were flushed for 1 h with argon or N_2O with constant stirring before γ-irradiation. For each condition, a non-irradiated control sample was prepared.

Steady-state γ-radiolysis

γ-Irradiations were performed with a Cobalt-60 source at the Laboratoire de Chimie Physique, Université Paris XI, Orsay, France. Irradiation times varied from 30 to 75 min, with dose rates between 15 and 38 Gy min^{-1}. All experiments were carried out at 25°C.

Dosimetry was based on the Fricke dosimeter [31].

The radiolytic yield, or G-value expressed in μmol. J^{-1}, for a given X species was calculated from the following formula:

$$G(X) = \frac{[X]}{\text{Dose} \times \rho}$$

where ρ is the density of the solution, assumed to be equal to that of the Fricke dosimeter ($\rho_{\text{Fricke}} = 1.024$ kg l^{-1}), and with the dose expressed in J kg^{-1} or Gy.

The primary species produced in the radiolysis of water are e_{aq}^-[$G(e_{aq}^-) = 0.27\,\mu mol\,J^{-1}$], $HO^{\cdot}$ [$G(HO^{\cdot}) = 0.28\,\mu mol\,J^{-1}$] and $H^{\cdot}$ [$G(H^{\cdot}) = 0.06\,\mu mol\,J^{-1}$] at pH 7. Their concentrations are at a steady state and the total amount formed is proportional to the dose: with a dose rate of 20 Gy min^{-1}, the rate of formation of hydrated electrons is equal to 5.4 μM min^{-1} and for a dose of 1000 Gy, the total concentration of hydrated electrons is 270 μM. Radicals were generated by irradiation of aqueous solutions of nitrate and/or nitrite, in 10 mM potassium phosphate buffer at pH 7.4.

Table I. Reactions involved in the radiolysis experiments and their rate constants at 25°C when known. Reactions 1–11 were taken into account for the calculations of $G(NO_2^{\cdot})$, $G(NO^{\cdot})$ and $G(MelH^{\cdot+})$ (cf. Table III and supporting information).

No.	Reaction		Reference
1	$NO_3^- + e_{aq}^- + 2H^+ \rightarrow NO_2^{\cdot} + H_2O$	$9.7 \times 10^9\,M^{-1}\,s^{-1}$	[31,32]
2	$NO_3^- + H^{\cdot} \rightarrow NO_2^{\cdot} + HO^-$	$(1.4–4.4) \times 10^6\,M^{-1}\,s^{-1}$	[33,34]
3	$NO_2^- + HO^{\cdot} \rightarrow NO_2^{\cdot} + HO^-$	$1.0 \times 10^{10}\,M^{-1}\,s^{-1}$	[32]
4	$N_2O + e_{aq}^- \rightarrow N_2O^{\cdot-} \rightarrow N_2 + HO^- + HO^{\cdot}$	$9.1 \times 10^9\,M^{-1}\,s^{-1}$	[32]
5	$NO_2^- + e_{aq}^- \rightarrow NO_2^{\cdot 2-} \xrightarrow{H_2O} NO^{\cdot} + 2HO^-$	$3.5 \times 10^9\,M^{-1}\,s^{-1}$	[35]
6	$NO_2^- + H^{\cdot} \rightarrow HNO_2^{\cdot-} \rightarrow NO^{\cdot} + HO^-$	$7.1 \times 10^8–1.6 \times 10^9\,M^{-1}\,s^{-1}$	[33,38]
7	$N_2O + H^{\cdot} \rightarrow N_2 + HO^{\cdot}$	$2.1 \times 10^6\,M^{-1}\,s^{-1}$	[37]
8	$MelH + e_{aq}^- \rightarrow$ products	$6.4 \times 10^9\,M^{-1}\,s^{-1}$	[30]
9	$MelH + HO^{\cdot} \rightarrow MelH^{\cdot+} + HO^-$	$1.3 \times 10^{10}\,M^{-1}\,s^{-1}$	[30]
10	$tBuOH + H^{\cdot} \rightarrow tBuOH^{\cdot} + H_2$	$1.7 \times 10^5\,M^{-1}\,s^{-1}$	[33]
11	$tBuOH + HO^{\cdot} \rightarrow tBuOH^{\cdot} + H_2O$	$6 \times 10^8\,M^{-1}\,s^{-1}$	[30]
12	$NO_2^{\cdot} + NO_2^{\cdot} \rightleftharpoons N_2O_4$	$k_{12} = 1 \times 10^8\,M^{-1}\,s^{-1}$; $k_{-12} = 6.9 \times 10^3\,s^{-1}$	[12,15,41]
	$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$	$18\,s^{-1}$	[15]
13	$NO^{\cdot} + NO_2^{\cdot} \rightleftharpoons N_2O_3$	$k_{13} = 1.1 \times 10^9\,M^{-1}\,s^{-1}$; $k_{-13} = 8.4 \times 10^4\,s^{-1}$	[43]
	$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$	$2 \times 10^3 + 10^8[OH^-] + (6.4–9.4) \times 10^5$[phosphate] s^{-1}	[43]

*Assay **1***: A buffered solution of 50 mM sodium nitrate and 50 mM *t*-butanol under argon was irradiated. NO_3^- reacted with e_{aq}^- to yield $NO_2^{\cdot}$ radical [32]. No reaction of $HO^{\cdot}$ with nitrate has been observed. According to the reactions presented in Table I, *t*-butanol trapped most of the $OH^{\cdot}$ radical and 4–11% of $H^{\cdot}$ radical [33,34]. MelH trapped 20% of $HO^{\cdot}$ radical leading to $MelH^{\cdot+}$ radical cation formation [30] and oxidation products. Radiolytic yield of $NO_2^{\cdot}$ radical formation is shown in Table III (detailed calculation is given in the supporting information).

*Assay **2***: A 50 mM sodium nitrite solution under N_2O was irradiated leading mainly to $NO_2^{\cdot}$ radical [32]. Though N_2O reacts rapidly with the hydrated electrons, its saturating concentration (25 mM) was not sufficient to prevent the reaction of the hydrated electrons with nitrite, leading to NO_2^{2-} ion [35]. This ion undergoes a rapid protonation, which is either simultaneous with or is very rapidly followed by decomposition into $NO^{\cdot}$ [36].

The hydrogen atom reacts partially with N_2O [37] and with nitrite, leading to $HNO_2^{\cdot-}$, which is assumed to decompose to $NO^{\cdot}$ [33,38].

Taking into account that MelH competes with nitrite for $HO^{\cdot}$ scavenging [30], the radiolytic yields of $NO_2^{\cdot}$ and $NO^{\cdot}$ radical formation reached $0.43\,\mu mol\,J^{-1}$ and $0.18\,\mu mol\,J^{-1}$, respectively (Table III).

*Assays **3** and **4***: Mixtures of 50 mM sodium nitrate and 10 mM nitrite (assay 3) or 50 mM nitrite (assay 4) containing MelH were irradiated under argon. Under such conditions, $HO^{\cdot}$ radical was essentially scavenged by nitrite (>93%). The radiolytic yields of $NO_2^{\cdot}$ and $NO^{\cdot}$ radical formation are given in Table III.

Alternatively, conditions similar to those used in assay 4 were applied using labelled nitrate or labelled nitrite.

Spectrophotometric analysis

All absorption data were recorded with a double-beam device (Uvikon 942, Kontron Instruments). The concentrations were estimated from absorbance values at 280 nm for MelH ($\varepsilon = 6300\,M^{-1}\,cm^{-1}$), and at 346 nm for 1-nitrosomelatonin (MelNO, $\varepsilon = 10900\,M^{-1}\,cm^{-1}$).

Reverse-phase HPLC (RP-HPLC) devices, mass spectrometry and NMR measurements

The equipment for analytical HPLC and mass spectrometry has been described [21]. Yields were evaluated by 215 nm integration using external standards of MelNO, which we synthesized following the method of Bravo [39], and MelH derivatives obtained by reaction with peroxynitrite [21].

For MelNO, the molecular mass peak could not be observed by LC-MS. Thus, in order to identify unambiguously this product obtained in assay 2, the same preparation was performed on a greater scale (10 or 100 ml, 5×10^{-4} M MelH, 1300 Gy), and the irradiated solution was injected directly into an HPLC column. The yellow compound was collected, lyophilized (3.6 mg, 28% yield) and analyzed by NMR and mass spectrometry by direct infusion in the mass spectrometer Navigator under a 10 V cone voltage.

^{1}H NMR spectra were recorded with a Bruker spectrometer. The characteristic mixture of the two conformers of MelNO displayed chemical shifts expressed as ppm relative to $SiMe_4$: δ_H (600 MHz, CD_3OD, 300 K) 1.90/1.93 (3H, s, $-NHC(O)CH_3$), 2.88/2.94 (2H, td, $\mathcal{J}_{HH} = 1.0$, 7.1 Hz, H_2), 3.49/3.56 (2H, t, $\mathcal{J}_{HH} = 7.1$ Hz, H_1), 3.88/3.90 (3H, s, $-OCH_3$), 6.95/7.08 (1H, dd, $\mathcal{J}_{HH} = 2.4$, 8.8 Hz, $H_{6'}$),

Table II. Yields of melatonin (MelH) consumption and MelH derivatives formed by γ-irradiation (1000-Gy dose). Phosphate-buffered solutions at pH 7.4 contained 580 μM MelH with: 50 mM nitrate and *t*-butanol in assay 1, 50 mM nitrite in assay 2, 10 mM nitrite and 50 mM nitrate in assay 3, and 50 mM nitrate and nitrite in assay 4. Solutions in assay 1, 3 and 4 were saturated with argon, while in assay 2 was under N_2O. Analysis was performed by HPLC and yields were evaluated using external standards. ND means not detected.

			Nitration products			Oxidation products	
Assay	Consumed MelH μM (%)	MelNO μM (%)	On N-1 μM (%)	On C-6 μM (%)	On C-4 μM (%)	2,3-Epoxide μM (%)	*N*-formylkynuramine μM (%)
1	110 ± 16 (19%)	ND	ND	ND	ND	6 (1%)	8 ± 5 (1%)
2	226 ± 1 (39%)	110 ± 1 (19%)	6 ± 1 (1%)	6 ± 1 (1%)	11 ± 1 (2%)	1 (0.2%)	5 ± 1 (1%)
3	221 ± 10 (38%)	38 ± 1 (7%)	11 ± 4 (2%)	13 ± 1 (2%)	23 ± 1 (4%)	3 (0.5%)	11 ± 1 (2%)
4	219 ± 1 (38%)	74 ± 5 (13%)	11 ± 1 (2%)	9 ± 2 (2%)	18 ± 3 (3%)	2 (0.5%)	12 ± 4 (2%)

7.13/7.21 (1H, d, $\mathcal{J}_{HH} = 2.4$ Hz, $H_{4'}$), 7.66/8.12 (1H, s, $H_{2'}$), 8.02/8.23 (1H, d, $\mathcal{J}_{HH} = 8.8$ Hz, $H_{7'}$).

Reactions of peroxynitrite with melatonin

Peroxynitrite was synthesized following the method of Uppu and Pryor [40] and stored at −20°C. Its concentration was measured spectrophotometrically ($\varepsilon_{302\,nm} = 1670\,M^{-1}\,cm^{-1}$) in fresh NaOH solutions (0.025 M). The fresh solution containing 3 M peroxynitrite was contaminated by 5% nitrite (evaluated by Griess reaction of acid-diluted solution of peroxynitrite).

A total of 700 μM peroxynitrite was added through a syringe at a flow rate of $0.4\,\mu M\,s^{-1}$ into the stirred 1 mM MelH solution in 400 mM phosphate-buffered solution at pH 7.4. When stated, sodium nitrite was added before peroxynitrite injection. Experiments were carried out at 25°C.

The final mixture was then analyzed by HPLC and spectrophotometry. The same transformations were obtained with 2 mM MelH.

Results

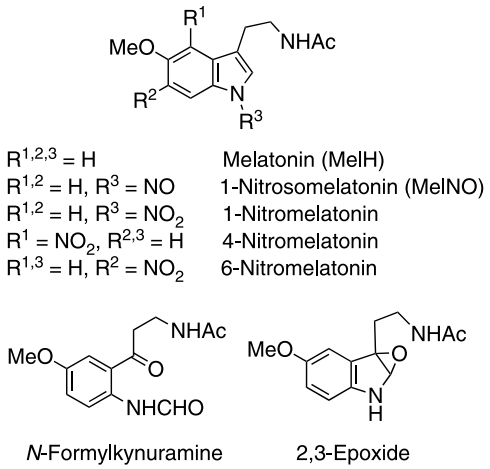
Nitrite-dependent nitrosation of melatonin induced by γ-radiolysis

MelH aqueous solutions were irradiated in the presence of various nitrite and/or nitrate concentrations. MelH concentration was chosen to avoid direct scavenging of e_{aq}^- prior to its reaction with NO_2^-, NO_3^- or N_2O and in as much as possible to allow detection of most of the MelH derivatives produced. The reactions that took place during irradiation are given in Table I with their rate constants when known.

Generation of $NO_2^{\cdot}$ radical from nitrate (Assay 1, Table II) induced a consumption of MelH (19% with a 1000 Gy dose), but only low levels of oxidation products were detected (2,3-dihydro-2,3-epoxymelatonin and *N*-{3-[2-(formylamino)-5-methoxyphenyl]-3-oxopropyl}acetamide, hereafter referred to as epoxide and *N*-formylkynuramine, respectively) (Scheme 2). In assay 1, oxidation of MelH is due, at least in part, to the reaction of $OH^{\cdot}$ that has not been trapped by *t*-butanol (Table I, Equation 11) leading to $MelH^{+\cdot}$ (Table I, Equation 9) and subsequent oxidation products.

Generation of $NO_2^{\cdot}$ and $NO^{\cdot}$ radicals from nitrite (Assay 2, Table II) led to a completely different transformation of MelH (39% consumption with a 1000 Gy dose). 1-Nitrosomelatonin (MelNO) was detected as the main product (19% with a 1000 Gy dose), together with only traces of oxidation and nitration products (1-, 4-, and 6-nitromelatonins).

The formation of 1-nitrosomelatonin in the presence of nitrite was revealed by its characteristic 346 nm absorption and confirmed by NMR and mass



Scheme 2. Structures of MelH derivatives.

spectrometry after synthesis on a preparative scale (data reported in experimental section). The mass spectrum of MelNO is difficult to obtain due to the labile NO function. Only by direct infusion of a methanolic solution in the mass spectrometer ion source did the spectrum exhibit the molecular mass besides fragmentation peaks: *m/z* 254 $[M - NO + Na]^+$, 284 $[M + Na]^+$, 295 $[M - NO + CH_3CN + Na]^+$, 485 $[2(M\text{-}NO) + Na]^+$, 515 $[M + (M - NO) + Na]^+$, 545 $[2M + Na]^+$.

In order to unravel the source of the nitroso group in MelNO, we successively generated, mixtures of $^{14}NO_2^{\cdot}$ and $^{15}NO_2^{\cdot}$ radicals in the presence of $^{14}NO_2^-$ or $^{15}NO_2^-$. A solution of 580 μM MelH containing 50 mM $Na^{15}NO_3$ and 50 mM $NaNO_2$ was irradiated, and MelNO isolated from the irradiated MelH solution. The quasimolecular ion $[M + Na]^+$ *m/z* 284 was similar to that obtained with authentic MelNO. In the mass spectrum, the following signals at *m/z* 285 (15%), 286 (3%) corresponded to the natural abundance of ^{13}C in the molecule. In a similar experiment, where MelH was irradiated in the presence of 50 mM $Na^{15}NO_2$ and 50 mM $NaNO_3$, the signal of the quasimolecular ion of MelNO shifted to *m/z* 285 with a very low residual peak at *m/z* 284 (<3%) showing clearly that the nitroso group of MelNO was fully labelled (Figure S1 in the supporting information).

Variation of $NaNO_2$ concentration (10–50 mM) in argon-saturated 50 mM $NaNO_3$, phosphate-buffered solution (Assays 3 and 4, Table III) further evidenced the role of nitrite in the nitrosation of MelH. Under these conditions, the radiolytic yield of MelH consumption (0.21 μmol J^{-1}) remained constant while radiolytic yields of MelNO formation increased from 0.04 to 0.07 μmol J^{-1} for 10 and 50 mM of NO_2^-, respectively. Non-irradiated references did not show any transformation of MelH.

Effects of nitrite in the peroxynitrite reaction with melatonin

$ONOO^-$ (700 μM) was flow-injected over 30 min into a phosphate-buffered 1 mM MelH solution at pH 7.4 under stirring. The peroxynitrite/MelH ratio only allowed a partial conversion of MelH. The reaction of peroxynitrite led to a complex mixture of products which can be classified under three categories: oxidation, nitration and nitrosation products (Scheme 2). The main product of the reaction was *N*-formylkynuramine (75 μM) with a significant amount of MelNO (15 μM) and minor yields of epoxide (8 μM) and 1-, 4-, and 6-nitromelatonins (≤3 μM) (Figure 1, first columns). Addition of nitrite did not change epoxide and *N*-formylkynuramine levels but increased the nitration by 3–4-fold. The 1.3-fold increase in MelNO was not significant (Figure 1, second and third columns).

A larger excess of MelH (2 mM) in the reaction did not modify the transformation yields.

Discussion

Nitrite-dependent nitrosation of melatonin induced by γ-irradiation

Interpretation of the results requires evaluation of the amount of each free radical that might react with MelH in each assay. Thus we calculated the yields assuming all the free radicals are at a steady state (Table III).

Our experiments demonstrated that the reaction of MelH with $NO_2^{\cdot}$ radical did not produce significant amounts of oxidized and nitroderivatives of MelH.

Table III. Radiolytic yields of formation for $NO_2^{\cdot}$, $NO^{\cdot}$, $MelH^{\cdot+}$ and MelNO according to assay conditions (1000 Gy dose). Calculations of $G(NO_2^{\cdot})$, and $G(NO^{\cdot})$, and $G(MelH^{\cdot+})$ are given in supporting information. G(MelNO) was derived from HPLC analyses using an external standard.

Assay	[MelH] (mM)	$[NO_2^-]$ (mM)	$[NO_3^-]$ (mM)	$[N_2O]$ (mM)	[*t*-butanol] (mM)	$G(NO_2^{\cdot})$ (μmol J^{-1})	$G(NO^{\cdot})$ (μmol J^{-1})	$G(MelH^{\cdot+})$ (μmol J^{-1})	G(MelNO) (μmol J^{-1})
1	0.58	0	50	–	50	0.32	0	0.06	0
2	0.58	50	–	25	–	0.42	0.18	0.01	0.11
3	0.58	10	50	–	–	0.51	0.08	0.02	0.04
4	0.58	50	50	–	–	0.47	0.13	0.00	0.07

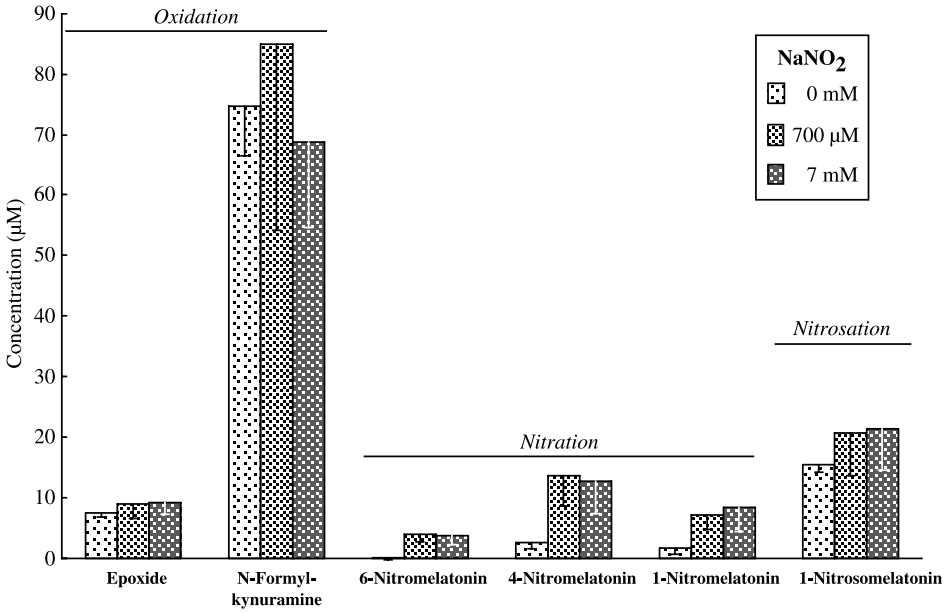


Figure 1. Effect of nitrite on the transformation of MelH by peroxynitrite. Peroxynitrite (700 μM) was added slowly over 30 min with stirring to the phosphate-buffered solutions of MelH (1 mM) and nitrite, pH 7.4. The products were analyzed and quantified by HPLC using external standards.

It is noteworthy that the pulse radiolysis study carried out by Mahal et al. under similar conditions to ours shows no sign of $MelH^{\cdot+}$ radical cation. $NO_2^{\cdot}$ radical reaction with MelH is slow ($k = 3.7 \times 10^6\ M^{-1}\ s^{-1}$ at pH 7) and incomplete [30]. A transient spectrum with two narrow absorption bands at $\lambda_{max} = 335$ and 370 nm was observed, which differed from the spectrum of $MelH^{\cdot+}$ radical cation obtained after oxidation reactions by $OH^{\cdot}$, $Br_2^{\cdot-}$ or $N_3^{\cdot}$ ($\lambda_{max} = 335$ and 500–515 nm at pH 7). The authors did not speculate on the nature of the new transient species they observed, but the absorption at 370 nm is reminiscent of the characteristic absorption of a nitro-adduct. Thus, the reaction of $NO_2^{\cdot}$ radical is likely to be an addition on the C-2 of MelH.

$$MelH + NO_2^{\cdot} \rightarrow [MelH{-}NO_2]^{\cdot} \quad (1)$$

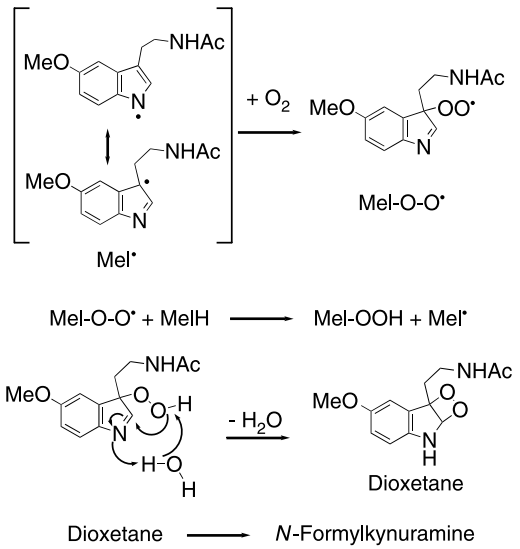
This first step accounted for MelH consumption increasing with the radiolytic yield of $NO_2^{\cdot}$ radical formation (Assay 2 vs. Assay 1, Tables II and III). Under assay 1 conditions (nitrate solution), products formed by dimerization, disproportionation and radical rearrangement of $[MelH{-}NO_2]^{\cdot}$ were not detected by the techniques used here, probably due to their dispersion on the chromatogram.

However, when nitrite was present, we noted a nitrite-dependent nitrosation of MelH which accounted for half of the transformed MelH. As suggested several times in literature [34–36], the product of both reactions of nitrite with the hydrated electrons and $H^{\cdot}$ radical would be $NO^{\cdot}$ (Equations 5 and 6, Table I). The correlation between the radiolytic yields of $NO^{\cdot}$ and that of MelNO formation (Table III) suggests $NO^{\cdot}$ takes part in the nitrosation. Neither nitrite, nor $NO^{\cdot}$ alone is reactive towards MelH at pH 7.4 and 25°C [20,42], but Kirsch et al. described that the *N*-nitrosation of tryptophan by N_2O_3 was highly effective at pH 7.4, provided that the primary amine was blocked [27]. The formation of N_2O_3 by the coupling of $NO^{\cdot}$ with $NO_2^{\cdot}$ radical (Equation 13, Table I) is unlikely under the assay conditions, given the low radical concentrations involved in steady-state radiolysis. Confirmation was brought by experiments with labelled nitrite and nitrate. If N_2O_3 was formed as a mixture of $O^{15}NONO$ and $O^{15}NO^{15}NO$ from labelled nitrite and a mixture of ONONO and $O^{15}NONO$ from labelled nitrate, a mixture of labelled and unlabelled 1-nitrosomelatonin could be expected under both conditions. This was not observed. We saw a clear-cut shift of the mass spectrometry peak when using labelled nitrate vs. labelled nitrite.

The reaction of nitric oxide with the melatoninyl radical cation produced by the reaction of residual hydroxyl radicals with MelH could explain the nitrosation, but the calculated radiolytic yield of $MelH^{\cdot+}$ is significantly lower than that measured for MelNO (Table III, columns $G(MelH^{\cdot+})$ and G(MelNO)), which rules out this possibility.

One possible mechanism for the nitrosation is shown in Equation 2. Though radical–radical reactions are relatively unfavoured under steady-state radiolysis conditions, the absence of competing reactions with non-radical species would authorize this process.

$$[MelH{-}NO_2]^{\cdot} + NO^{\cdot} \rightarrow MelNO + NO_2^{-} + H^{+} \quad (2)$$



Scheme 3. Proposed mechanism for the formation of *N*-formylkynuramine.

Experiments performed with labelled nitrate and nitrite provided evidence for this reaction, as labelling of MelNO was observed only when $^{15}NO^{\cdot}$ was produced from labelled nitrite (Equations 5 and 6, Table I).

Comparison with the reaction of peroxynitrite with melatonin

Synthetic $ONOO^-$ was flow-injected under air over 30 min into a phosphate-buffered MelH solution at pH 7.4 ($ONOOH/ONOO^-$, p$K = 6.8$) to establish a radical flux [43–45] as in the radiolysis experiment.

The direct reaction of peroxynitrite with MelH has not been observed [20], even though tryptophan has been described to react directly with peroxynitrite at a slow rate ($k = 37\,M^{-1}\,s^{-1}$ at pH 7.4 and 37°C) [46]. The products detected in the reaction of peroxynitrite with MelH at neutral pH are the same as those identified in the radiolysis experiments. The variation in their proportions between the two experiments can be rationalised by taking into account the nature and amount of the radicals formed. Under air, the presence of dioxygen and CO_2 differed from radiolysis conditions. In the $ONOO^-$ infusion experiments, the adventitious CO_2 converted around 47% of peroxynitrite into $ONOOCO_2^-$ (calculated from the rate constants at pH 7.4 and 25°C of peroxynitrite decay ($0.26\,s^{-1}$) and of its reaction with CO_2 [10], assuming 10 μM constant CO_2 [47]). Homolyses of ONOOH and $ONOOCO_2^-$ have been reported to produce $NO_2^{\cdot}$ and $HO^{\cdot}$ with a free radical yield of ca. 30% [43] and $CO_3^{\cdot-}$ [11,12,48] and $NO_2^{\cdot}$ radicals with 33% yield, respectively (Scheme 1) [43–45]. Both $OH^{\cdot}$ and $CO_3^{\cdot-}$ radicals are stronger oxidants than $NO_2^{\cdot}$, which allows the formation of MelH radical cation ($MelH^{\cdot+}$) [20,49]. $MelH^{\cdot+}$ radical cation deprotonates readily to $Mel^{\cdot}$ at pH 7.4 according to its pKa value of 4.7 [30]. Only the rate constant of oxidation of MelH by $OH^{\cdot}$ is known ($k_9 = 1.3 \times 10^{10}\,M^{-1}\,s^{-1}$ at pH 7) and is identical to that of tryptophan [30,12]. For reference, the rate constants of tryptophan oxidation by $CO_3^{\cdot-}$ and $NO_2^{\cdot}$ are reported to be $7 \times 10^8\,M^{-1}\,s^{-1}$ at pH 7 and $1 \times 10^6\,M^{-1}\,s^{-1}$ at pH 6.5, respectively [12].

Aerobic conditions favoured the formation of *N*-formylkynuramine by the reaction of $Mel^{\cdot}$ with O_2 via a proposed chain mechanism (Scheme 3).

Tryptophanyl radical was described to react with O_2 in a similar way ($k < 5 \times 10^6\,M^{-1}\,s^{-1}$) to give a hydroperoxyl radical which does not release superoxide [50]. In both cases, the hydroperoxides decompose to form additional radicals which add to the chain process. This proliferation of radicals causes chain reactions to develop.

When excess of nitrite was added to the phosphate-buffered solution of MelH, nitrite anion competed with MelH for $HO^{\cdot}$ (Equations 3 and 9, Table I) and $CO_3^{\cdot-}$ radicals [51,52]:

$$NO_2^- + CO_3^{\cdot-} \rightarrow NO_2^{\cdot} + CO_3^{2-} \qquad k = 4 - 6.6 \times 10^5 M^{-1}s^{-1} \tag{3}$$

$$MelH + CO_3^{\cdot-} \rightarrow MelH^{\cdot+} + CO_3^{2-} \qquad k \approx 7 \times 10^8 M^{-1}s^{-1} \tag{4}$$

The formation of *N*-formylkynuramine was not significantly affected (Figure 1), which confirms that the initiation of the chain mechanism (Table I, Equations 9 and 4) is not rate-limiting in the mechanism proposed in Scheme 3. However, nitration was significantly increased (Figure 1, second and third columns) following $NO_2^{\cdot}$ concentration. Nitration occurred by the most probable termination reaction for $Mel^{\cdot}$ radical produced by the chain reaction.

$$Mel^{\cdot} + NO_2^{\cdot} \rightarrow MelNO_2 \tag{5}$$

This confirms that formation of nitroderivatives of MelH or tryptophan derivatives requires the presence of a stronger oxidant besides $NO_2^{\cdot}$ radical. The same was observed for the reaction of $NO_2^{\cdot}$ with tyrosine at pH 7.4 [12].

Concerning the nitrosation, we observed that the formation of MelNO remained relatively stable upon nitrite addition. Production of $NO^{\cdot}$ is the rate limiting step of nitrosation. $ONOO^-$ homolytic rupture ($k = 0.020 \pm 0.003\,s^{-1}$) and $ONOO^-$ reaction with $HO^{\cdot}$ and $CO_3^{\cdot-}$ radicals ($k = 4.8 \times 10^9$ and $10^7\,M^{-1}\,s^{-1}$, respectively) are the only possible sources of production of $NO^{\cdot}$ [43]. The amount of

$NO^{\cdot}$ formed is not modified by nitrite-driven reactions.

Three mechanisms are possible: (i) the formation of MelNO by a direct combination of $Mel^{\cdot}$ and $NO^{\cdot}$; (ii) involvement of N_2O_3 formed by the reaction proposed in literature (Table I, Equation 13) [43] occurring as previously described in the nitrosation of *N*-blocked-tryptophan derivatives [27]; (iii) successive addition of $NO_2^{\cdot}$ and $NO^{\cdot}$ on the indole ring (Equations 1 and 2) as proposed in the radiolysis experiments.

Conclusion: Biological implications

Oxidative stress is characterized *in vivo* by an increased level of transformations by inorganic oxidants ($ONOO^-$, $CO_3^{\cdot-}$, $HO^{\cdot}$ and $NO_2^{\cdot}$). A large variety of oxidation and nitration products have repeatedly been detected in tissues under stress conditions [53,54]. More recently, *in vivo* formation of *S*- and *N*-nitrosocompounds has also been described in some similar models [55,56]. A thorough identification of these *N*-nitrosamines was not performed but we suspect protein tryptophan residues to be a major target of nitrosation.

The most frequently considered nitrosation pathways involve N_2O_3 and NO_2^- in acidic compartments [53,57]. Under hypoxia, the nitrosation of indoles might also proceed in two steps: initial formation of an indolic radical (through monoelectronic oxidation and/or $NO_2^{\cdot}$ addition) followed by addition of $NO^{\cdot}$. Considering these pathways, MelH is shown to be a free radical scavenger able to divert some reactive nitrogen species into others. Its biological activities could be related to such chemical properties [58].

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Supporting information

Supporting information available: calculations of $G(NO_2^{\cdot})$, $G(NO^{\cdot})$, and $G(MelH^{\cdot+})$ under the radiolysis experiment conditions (the reaction numbers refer to the Equations given in Table I). Mass spectrometry results of the experiment with labelled nitrate and nitrite (Figure S1).

From the radiolytic dose rate of ca. 20 Gy min^{-1} (or 0.33 Gy s^{-1}), the formulas given below and the G-values given in Table III, the flux of $NO_2^{\cdot-}$ radical in the radiolysis experiments was estimated to be 0.19–0.29 μM s^{-1} per mM MelH, while the flux of $OH^{\cdot}$ radical available for reaction with MelH (equivalent to the flux in $MelH^{\cdot+}$) was 0.002–0.03 μM s^{-1} per mM MelH.

$$G(NO_2^{\cdot}) = \frac{k_1[NO_3^-]G(e_{aq}^-)}{k_1[NO_3^-] + k_4[N_2O] + k_5[NO_2^-] + k_8[MelH]} + \frac{k_2[NO_3^-]G(H^{\cdot})}{k_2[NO_3^-] + k_6[NO_2^-] + k_7[N_2O] + k_{10}[tBuOH]} + \frac{k_3[NO_2^-]}{k_3[NO_2^-] + k_9[MelH] + k_{11}[tBuOH]} \times \left(G(OH^{\cdot}) + G(e_{aq}^-)\frac{k_4[N_2O]}{k_1[NO_3^-] + k_4[N_2O] + k_5[NO_2^-] + k_8[MelH]} + G(H^{\cdot})\frac{k_7[N_2O]}{k_2[NO_3^-] + k_6[NO_2^-] + k_7[N_2O] + k_{10}[tBuOH]} \right)$$

$$G(NO^{\cdot}) = \frac{k_5[NO_2^-]G(e_{aq}^-)}{k_1[NO_3^-] + k_4[N_2O] + k_5[NO_2^-] + k_8[MelH]} + \frac{k_6[NO_2^-]G(H^{\cdot})}{k_2[NO_3^-] + k_6[NO_2^-] + k_7[N_2O] + k_{10}[tBuOH]}$$

$$G(MelH^{+\cdot}) = \frac{k_9[MelH]}{k_3[NO_2^-] + k_9[MelH] + k_{11}[tBuOH]} \times \left(G(OH^{\cdot}) + G(e_{aq}^-)\frac{k_4[N_2O]}{k_1[NO_3^-] + k_4[N_2O] + k_5[NO_2^-] + k_8[MelH]} + G(H^{\cdot})\frac{k_7[N_2O]}{k_2[NO_3^-] + k_6[NO_2^-] + k_7[N_2O] + k_{10}[tBuOH]} \right)$$

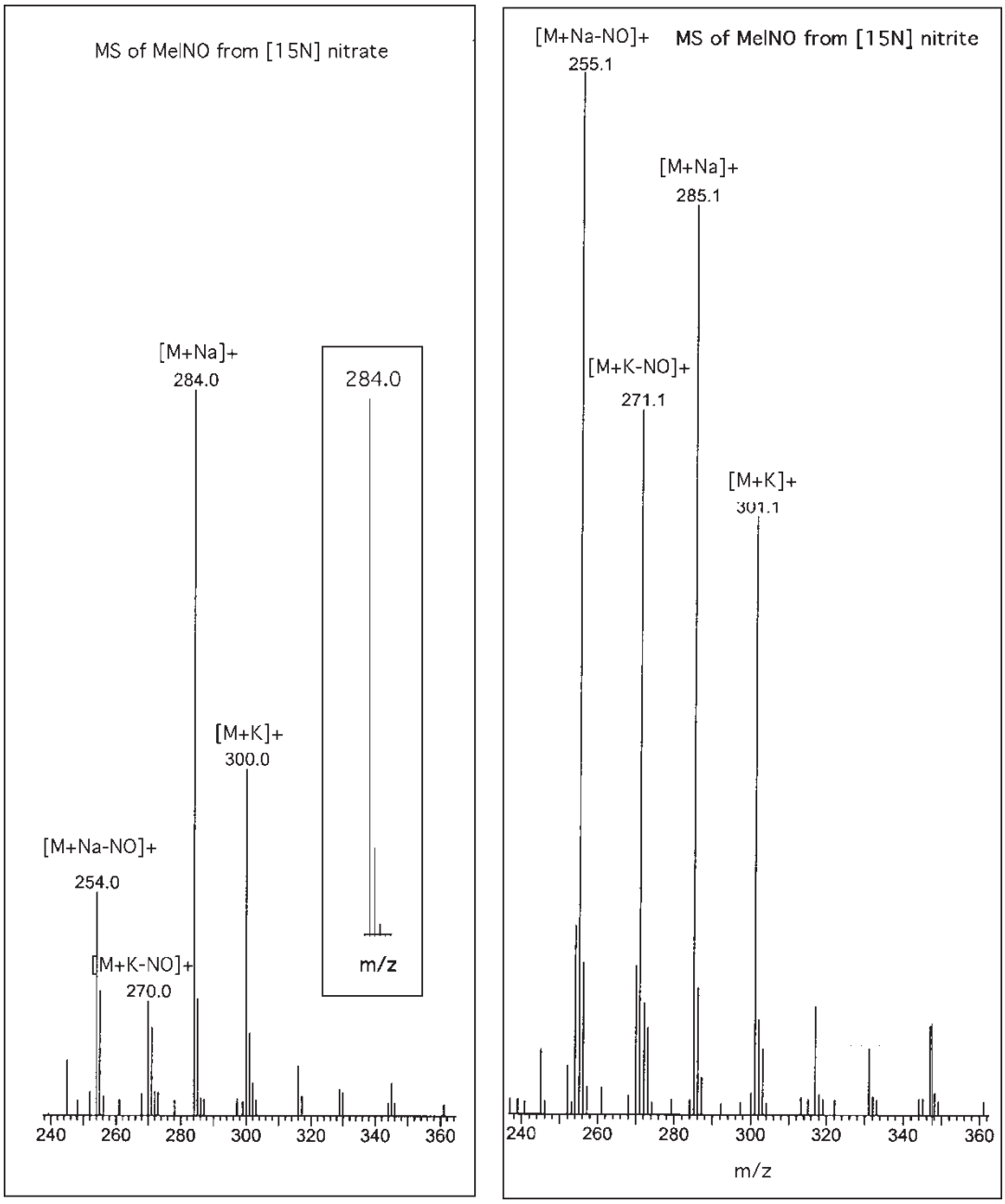

Figure S1. Mass spectra of MelNO obtained after γ-irradiation. Phosphate-buffered solutions at pH 7.4 under argon contained 500 mM MelH and: (a) 50 mM ^{15}N-nitrate and ^{14}N-nitrite or (b) 50 mM ^{14}N-nitrate and ^{15}N-nitrite. Analysis was performed by direct infusion of a methanolic solution in the mass spectrometer navigator under a 10 V cone voltage. Quasimolecular ions $[M + Na]^+$ and $[M + K]^+$ are indicated. Fragmentation peaks at *m/z* 254 ($[M - NO + Na]^+$) and 270 ($[M - NO + K]^+$) are present on both spectra. The signals at *m/z* 255 and 271 are very tall in spectrum (b). This suggests either that fragmentation occurs preferentially in the protonated molecule for ^{15}N–MelNO (peak assignments $[M + H - NO + Na]^+$ and $[M + H - NO + K]^+$) or that the half-life of ^{15}N–MelNO is shorter than that of ^{14}N–MelNO, allowing partial decomposition to MelH in solution before MS analysis (peak assignments $[MelH + Na]^+$ and $[MelH + K]^+$).