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Article

UV-B irradiation-induced electron transfer between 3-hydroxykynurenine and tryptophan

Yosuke Nakazawa[a], Makoto Takehana[b*], Mikako Oka[b], Fukuko Shibuya[a], Jun'ichi Katakawa[a] and Yoh Sano[a]

[a]Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan
[b]Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan.

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Kynurenine metabolites are derived from the oxidation of tryptophan (Trp) and are involved in many diseases. Previous reports have suggested that some kynurenine metabolites produce superoxide radicals and singlet oxygen molecules under UV illumination. We used electron spin resonance (ESR) spectroscopy to analyze the formation of radicals following ultraviolet-B (UV-B) irradiation of Trp and kynurenine metabolites. As the results, strong phenoxyl radical formation was detected following UV-B irradiation of 3-hydroxy-kynuirenine (3HK), a kynurenine metabolite. Alpha-crystallin, the major lens protein, undergoes many post-translational modifications, including oxidation. Oxidation occurs selectively at methionine, histidine, Trp and lysine residues and associated with the development of age-related cataracts. We examined the effect of these amino acids, as well as tyrosine (which forms tyrosyl radicals *in vivo*) and aspartic acid (which undergoes racemization following exposure to UV irradiation), on radical formation by 3HK. The combination of Trp and 3HK generated a stronger ESR signal than did 3HK alone. The phenoxyl radical formed by 3HK was strongly induced by UV illumination in the presence of Trp. These results indicated that 3HK could easily produce the phenoxyl radicals via electron-transfer interactions with Trp, which act as a photosensitizer induced UV excitation. In addition, this radical promoted photo-oxidation and may enhance the ability of 3HK to cause disease.

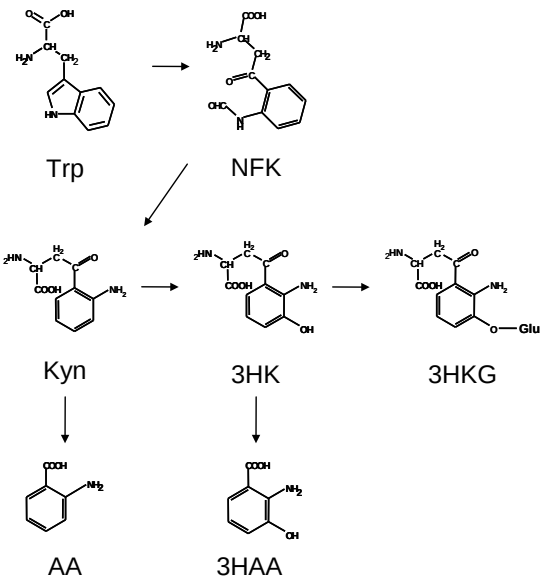
Keywords: 3-hydroxykynurenine; electron-transfer interaction; photo-oxidation

*** Corresponding author**: Makoto Takehana
Tel: +81-3-5400-2663. Fax: +81-3-5400-2693.
E-mail: takehana-mk@pha.keio.ac.jp

Abbreviations: UV, ultraviolet; ESR, electron spin resonance; 3HK, 3-hydroxy-kynurenine; NFK, N-formyl- kynurenine; Kyn, kynurenine; AA, anthranilic acid; 3HAA, 3-hydroxy-anthranilic acid; Trp, tryptophan; Met, methionine; His, histidine; Lys, lysine; Tyr, tyrosine; Asp, aspartic acid;

Introduction

While most of the pharmacological interest in tryptophan (Trp) has centered on the conversion of this amino acid to 5-hydroxytryptamine (5-HT), the majority of Trp is metabolized via a different route (i.e., through the kynuninine pathway). Ultraviolet-B (UV-B) and ultraviolet-C (UV-C) irradiation induce the oxidation of Trp. This process yields N-formyl-kynurenine (NFK), which is immediately hydrolyzed to kynurenine (Kyn). A series of enzymatic reactions convert Kyn to anthranilic acid (AA), 3-hydroxykynurenine (3HK) and 3-hydroxy-anthranilic acid (3HAA) [1]. Subsequently, 3HK is glucosylated to form 3-hydroxykynurenine *o*-glucoside (3HKG) (Scheme 1). Levels of 3HK are increased in the brains of people with Huntington's disease [2, 3], human immunodeficiency virus [4] and Parkinson's disease [5]. In addition, bladder cancer and cataracts are induced by 3HK [6, 7], and investigators have proposed that these diseases stem in part from the ability of kynurenine metabolites to interact with and modify other proteins, such as glutamate receptors or lens proteins.



Scheme 1. Schematic representation of the biochemical pathway from Try to AA, 3HKG and 3HAA. All steps are enzymatically catalyzed.

The optical lens is exposed to sunlight every day, making this tissue highly conducive to photochemical events such as direct photo-oxidation (i.e., a type 1 event) and indirect oxidation (i.e., a type 2 event) [8]. The lens has an efficient UV protection system that acts to ameliorate the effects of photo-oxidation. The major UV filters in the lens are kynurenine metabolites (e.g., Kyn and 3HK). These molecules prevent light below 400 nm from reaching the retina. Proteins containing 3HK are oxidized by phenoxyl, hydroperoxyl and superoxide anion radicals [9]. It has been suggested that the photodynamic actions of kynurenine metabolites contribute to the development of cataracts. One of the best-characterized interactions of kynurenine metabolites with proteins is the lens protein α-crystallin, which comprises up to 50% of the total protein mass of the mammalian lens and this protein is comprised from two subunits, αA- and αB-crystallins. The protein has been many reports of post-translational modifications associated with age, including phosphorylation [10], racemization [11 - 13], deamination [14] and oxidation. Oxidation

Table 1. Oxidative modifications of αA- and αB-crystallins in human and bovine.

Amino acid	Modification	Reference
αA-crystallin		
Met1	*in vivo* oxidation	[15 - 17]
His7, 97, 154	photooxidation	[15, 17]
Trp9	photooxidation	[15, 17]
Lys166	photooxidation	[17]
αB-crystallin		
Trp9	photooxidation	[15, 17]
Met68	*in vivo* oxidation	[15]

occurs selectively at methionine (Met) [15 - 17], histidine (His) [15, 17], Trp [15, 17] and lysine (Lys) [17] residues (Table 1). Tyrosine (Tyr) possesses an aromatic side chain that can directly absorb UV light and serves as a target for a type 1 and a type 2 photo-oxidation [18, 19]. In addition, aspartic acid (Asp) undergoes racemization and isomerization in response to UV-B and UV-C irradiation, forming D-isomers and β-aspartic acid [12, 13]. Thus, Tyr and Asp residues may interact via mechanisms other than photo-oxidation or *in vivo* oxidation in response to UV irradiation.

Here, we used ESR spectroscopy to investigate the formation of radicals following exposure of Trp and kynurenine metabolites to UV-B irradiation. We also compared the ESR spectra of 3HK alone or in mixtures with various amino acids to determine which amino acids could modulate the ESR spectrum of 3HK in response to UV-B irradiation. Finally, we sought to characterize the electron-transfer interactions between various amino acids and 3HK.

Materials and methods

Materials

L-tryptophan, L-methionine, L-histidine, L-lysine, L-tyrosine and L-aspartic acid were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). DL-kynurenine, anthranilic acid, DL-3-hydroxykynurenine and 3-hydroxy-anthranilic acid were purchased from Sigma Chemical Co. (MO, USA). Sodium phosphate buffer (pH 7.2) was prepared using analytical reagent grade NaH_2PO_4 and Na_2HPO_4, which were both obtained from Wako Pure Chemical Ind. Ltd.

Ultraviolet-B (UV-B) irradiation

Trp, kynurenine metabolites, and mixtures of 3HK and amino acids were dissolved in 50 mM sodium phosphate buffer (pH 7.2) and irradiated with UV-B light (312 nm) using Spectroline (EB280C/J; Spectronics co., NY, USA) at 4°C.

Electron spin resonance (ESR) spectroscopy

The ESR spectra were measured 10 minutes after UV irradiation using an X-band ESR spectrometer (JES-TE300, JEOL Datum co. Ltd., Tokyo, Japan) and recorded using an ESR monitor (ES-PRIT425, Ver. 1.9, JEOL Datum co. Ltd.), as described previously [20], with minor modifications. All measurements were carried out at 18°C with a radio frequency of 100 kHz, a microwave power of 5 mW, a modulation amplitude of 0.1 mT, a time constant of 1 second, a scan speed of 10 mT/ 4 minutes, and an amplitude of 4 x 1000.

Results

Analysis of tryptophan metabolites by ESR spectroscopy

We dissolved Trp and kynurenine metabolites (e.g., Kyn, 3HK, AA and 3HAA) in 50 mM sodium phosphate buffer (pH 7.2) and subjected them to UV-B irradiation (i.e., 54 kJ/m^2) at 4°C. Radical formation was measured 10 minutes after UV irradiation, using ESR spectroscopy. Radicals were not detected following UV irradiation of Trp, Kyn and AA. However, UV irradiation of 3HK produced a strong ESR signal, while irradiation of 3HAA produced a relatively weaker ESR signal (Fig. 1). The g-values and hyperfine coupling constants detected in this study are identical to those previously determined for the phenoxyl radicals of 3HK, indicating that these molecules are the same species (i.e., as determined with g = 2.004 and hyperfine coupling constant = 0.53 mT) [20 - 22].

To determine if the height of the ESR peaks of the 3HK spectrum correlated with UV dose or 3HK concentration, we irradiated 1 mM 3HK with varying doses of UV-B light (i.e., 18, 54 and 90 kJ/m^2) and measured radical formation by ESR. Radical formation increased gradually with increasing doses of UV (Fig. 2). When we exposed different concentrations of 3HK (i.e., 0.25, 0.5 and 1.0 mM) to a single dose of UV-B irradiation (i.e., 54 kJ/m^2), the heights of the ESR peaks increased linearly with increasing 3HK concentration (Fig. 3) and were directly proportionate with 3HK concentrations (Fig. 4).

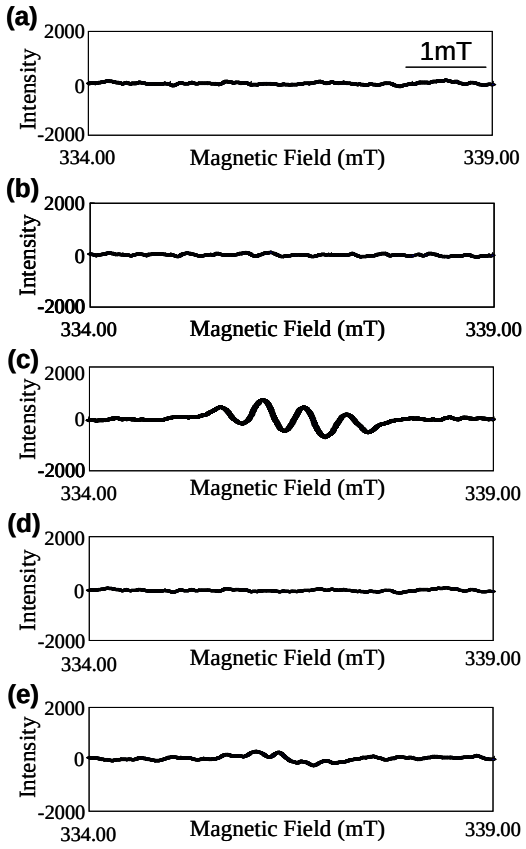


Figure 1. ESR spectra of the Trp and kynurenine metabolites. ESR spectra were detected following UV-B irradiation (312 nm, 54 kJ/m^2) at 4°C of (a) Trp (1 mM), (b) Kyn (1 mM), (c) 3HK (1 mM), (d) AA (1 mM) or (e) 3HAA (1 mM) in 50 mM sodium phosphate buffer (pH 7.2). Spectra were averages of two scans. Recording conditions: temperature, 18 °C; radio frequency, 100 kHz; microwave power, 5 mW; modulation amplitude, 0.1 mT.

Interaction between 3HK and amino acids

Previous reports have shown that the Met, His, Trp and Lys residues of α-crystallin undergo oxidative modification (Table 1) and that the oxidation of α-crystallin by 3HK may contribute to cataractogenesis [6]. Tyr

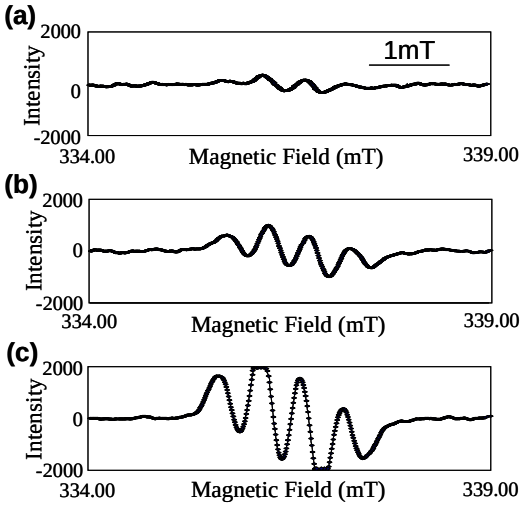


Figure 2. ESR spectra of 3HK (1 mM) in 50 mM sodium phosphate buffer (pH 7.2) exposed to different doses of UV-B irradiation (312 nm). The doses of UV-B used were (a) 18, (b) 54 and (c) 90 kJ/m^2. Radicals were detected 10 minutes after UV irradiation, and spectra were averages of two scan. Recording conditions: temperature, 18 °C; radio frequency, 100 kHz; microwave power, 5 mW; modulation amplitude, 0.1 mT.

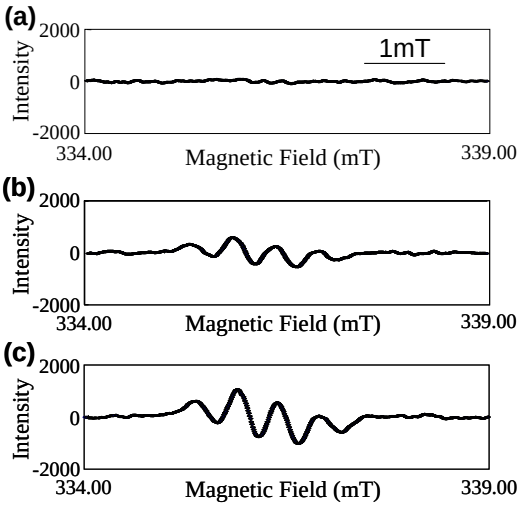


Figure 3. ESR spectra of different concentrations of 3HK in 50 mM sodium phosphate buffer (pH 7.2) exposed to UV-B irradiation (312 nm, 54 kJ/m^2). The concentrations of 3HK were (a) 0.25 mM, (b) 0.5 mM and (c) 1.0 mM. Radicals were detected 10 minutes after UV irradiation, and recording conditions: temperature, 18 °C; radio frequency, 100 kHz; microwave power, 5 mW; modulation amplitude, 0.1 mT. Spectra were averages of two scans.

has been shown to form the tyrosyl radicals and act as an electron generator [23], and Asp undergo *in vivo* racemization in response to UV irradiation [12, 13]. To determine which amino acids influenced radical formation by 3HK, we compared the ESR spectra of 3HK alone to the spectra of 3HK mixtures containing Met, His, Trp, Lys, Tyr or Asp in 50 mM phosphate buffer (pH 7.2), in response to UV irradiation (i.e., 54 kJ/m^2). We did not detect radical formation by any individual amino acids in response to UV irradiation (data not shown). The ESR spectrum of 3HK alone was similar to that of 3HK mixtures containing Met, Lys, Tyr or Asp. The mixture of His and 3HK displayed a weak ESR spectrum, compared with that of 3HK alone. His is oxidated during the initial formation of one or more endoperoxides and via the consumption of radicals during His ring opening [19, 23]. In contrast, the ESR spectra of 3HK mixtures containing Trp were stronger than that of 3HK alone (Fig. 5), indicating that a strong electron-transfer interaction occurs between 3HK and Trp in response to UV illumination.

Discussion

Here, it was examined the formation of

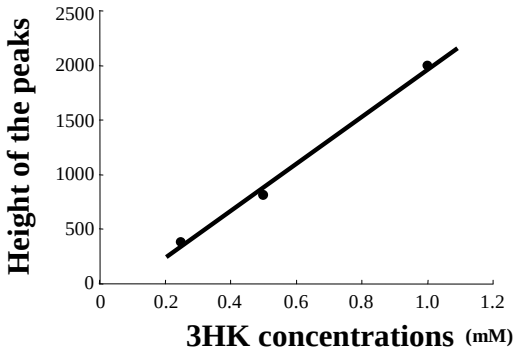


Figure 4. Quantification of ESR spectra. The heights of the ESR peaks at each 3HK concentration were calculated by determining the difference between the maximum intensity and the minimum intensity of the spectra. The height of ESR peaks and the 3HK concentrations were correlated.

radicals by Trp and kynurenine metabolites following UV-B irradiation. In addition, we investigated the interactions of a specific metabolite, 3HK, with various amino acids to determine which amino acids modulated UV-B induced radical formation. To detect the formation of radicals in response to the UV irradiation with oxygen of Trp and kynurenine metabolites using ESR spectroscopy, because the lens is continually exposed to UV rays with oxygen *in vivo*. Strong radical formation was detected following the UV-B irradiation of 3HK at 54 kJ/m^2 (Fig. 1c). The extent of radical formation was dependent on UV dose (Fig. 2) and 3HK concentration (Figs. 3 and 4) and the ESR spectra were similar to those of 3HK, H_2O_2 and horseradish peroxidase (HRP) [20]. Ichijima and Iwata speculated that radical-related reaction are readily induced and mediated by the phenoxyl groups of 3HK and 3HAA [24]. Our data reveal that 3HAA generates a weak radical (Fig. 1e). In general, the presence of carboxyl group in a phenyl ring will weaken the phenoxyl radical and render it unstable, because radicals are often abstracted by reactions in which protons are present in the carboxyl group.

Age-related cataracts are associated with the radical-related oxidation of lens proteins [25]. Several studies have reported the photo-oxidation and *in vivo* oxidation of α-crystallin (Table 1). The effects of various amino acids, including Tyr and Asp, on radical formation by 3HK in response to UV-B irradiation was examined. We found that Met, Lys, Tyr and Asp had no effect on the ESR spectrum of 3HK (Fig. 5a, 5d, 5e and 5f).

Takemoto et al. previously reported that the N-terminus Met residue of αA-crystallin is modified *in vivo* by oxidation [16]. This modification occurs soon after synthesis of the protein, and has also been reported in fetal bovine lens tissues. These results suggest that the *in vivo* oxidation of Met is not related to aging or UV irradiation. Our results are consistent with those of previous studies demonstrating that Met does not participate in the radical-mediated reactions of 3HK in response to UV irradiation. Previous studies, performed in the dark, reported that 3HK binds to Lys residues via Michael addition [6, 25]. In our study, Lys did not affect the ESR spectrum of 3HK (Fig. 5d), suggesting that Lys residues and 3HK do not interact via a

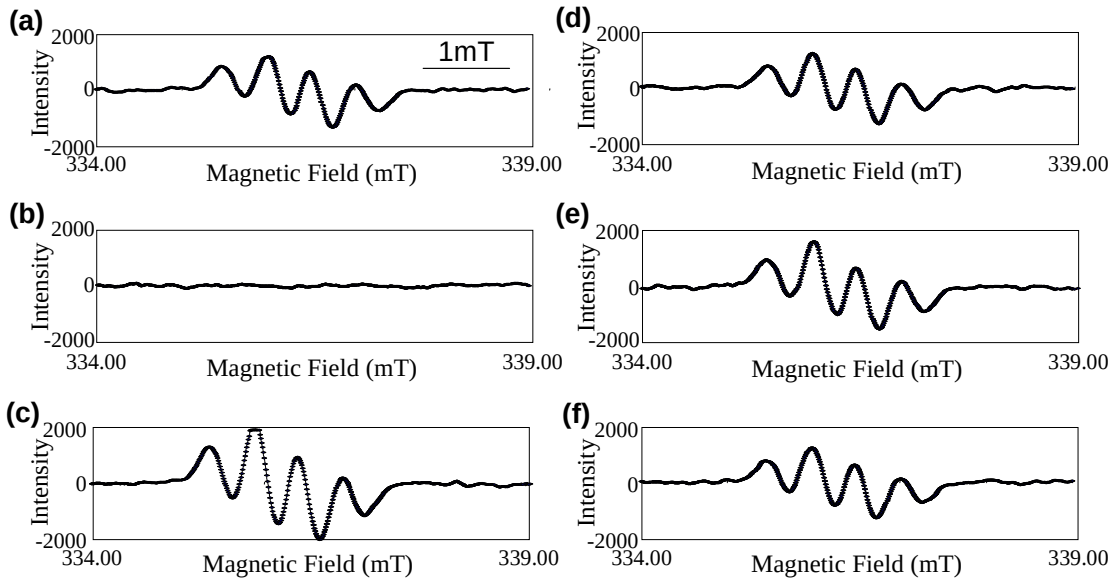


Figure 5. The ESR spectra of 3HK mixtures containing various amino acids in response to UV-B irradiation (i.e., 312 nm, 54 kJ/m^2). Mixtures of 1 mM 3HK and (a) 10 mM Met, (b) 10 mM His, (c) 10 mM Trp, (d) 10 mM Lys, (e) 1 mM Tyr or (f) 10 mM Asp were subjected to UV-B irradiation. Radicals were detected 10 minutes after UV irradiation at 18 °C with a radio frequency of 100 kHz, a microwave power of 5 mW and a modulation amplitude of 0.1 mT.

radical-mediated mechanism. Tyr is known to form the tyrosyl radical *in vivo*, however, we could not detected the radicals following UV irradiation by 3HK (Fig. 5e). This process may reflect the 10 μs lifespan of the transitional state of Tyr [26]. UV-B and UV-C irradiation have been reported to trigger the increased racemization and isomerization of Asp residues in α-crystallin, and suggested that the racemization of Asp is dependent on the rate of succinimide formation [12, 13]. It could not detect radical formation by Asp following UV-B irradiation (data not shown), suggesting that the conversion to succinimide is not related to radical reactions. These observations further suggest that the racemization and isomerization of Asp, although catalyzed by UV light, progress via pathways other than radical reactions. Various His residues of α-crystallin are known to undergo photo-oxidation (Table 1). Uroporphyrin, as a singlet oxygen generator, has contributes to the photo-oxidation of His residues [18]. His residues are readily oxidized in the presence of peroxide species and are known to consume peroxide species during the ring opening process [19, 23]. In the current study, the peak ESR spectra for a 3HK mixture containing His were lower than those of 3HK alone under UV illumination with oxygen (Fig. 5b). These data suggest that the unpaired electrons of the phenoxyl radicals generated by 3HK may be transferred to His residues and consumed during the His ring opening. In contrast, the peak ESR spectra of 3HK mixtures

containing Trp were higher than those of 3HK alone (Fig. 5c). In this case, singlet oxygen molecules were re-distributed to Trp to form tryptophanyl radicals. Radiolysis experiments have shown that two types of species are formed when phenoxyl radicals react with Trp: phenoxyl radical adducts [27] and tryptophanyl radicals in equilibrium with cation radicals [28]. We did not detect the formation of radicals in the presence of Trp alone under UV illumination; however, this may reflect the lifetime of tryptophanyl radicals (i.e., 8 to 20 μs) [29]. It was hypothesized that it may be easier for 3HK to generate phenoxyl radicals when the ESR spectra of 3HK mixtures containing Trp become stronger, because the excited state of Trp residues (i.e. or tryptophanyl radicals) act as photosensitizers to phenoxyl radical that allow the electron-transfer reaction to proceed between the excited Trp molecules and 3HK.

In summary, among the Trp and kynurenine metabolites tested in this study, 3HK was the only molecule capable of forming phenoxyl radicals in response to UV-B irradiation. The peak heights of the 3HK ESR spectra were higher when a mixture of Trp and 3HK was subjected to UV irradiation. However, Met, Lys, Tyr, and Asp had no effect on phenoxyl radical formation by 3HK. We suggest that the formation of phenoxyl radicals by 3HK occurs in response to daily sunlight exposure and that radical transformation occurs in the presence of Trp via electron-transfer interactions. This phenomenon can be induced by photo-oxidation and can subsequently contribute to disease.

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