Benoxaprofen-photosensitization Decomposition of Tryptophan Peptides in Agueous Micellar Systems

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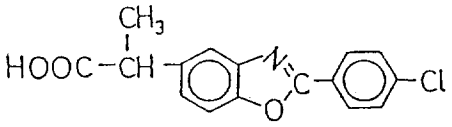
Benoxaprofen (2-(4-chlorophenyl)-α-methyl-5-benzoxazole acetic acid) is a nonsteroidal anti-inflammatory drug that causes acute cutaneous phototoxicity. The ability of benoxaprofen (BXP) and its photoproduct, decarboxybenoxaprofen (DBXP) to photosensitize the decomposition of tryptophan was evaluated in various media such as water, ethanol and aqueous micellar dispersions of surfactants. The weak photosensitization of BXP in water was found to be enhanced in cationic CTAB micelle system, but yielded little difference in anionic SDS micelles. In ethanol solution, BXP was determined to photosensitize the decomposition of tryptophan, but no photosensitization was observed with DBXP. All of these results implicate that the anion radical of BXP may play a major role in the photosensitization in hydrophobic micellar phase, forming superoxide through interaction with oxygen as demonstrated by observation that the photosensitization was inhibited by superoxide dismutase.

Introduction

Benoxaprofen (2-(4-chlorophenyl)- α-methyl-5-benzoxazole acetic acid) (BXP)[1], a non-steroidal and anti-inflammatory drug has more prominent effect than aspirin in therapeutic function.[1,2]



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However, exposure of the skin of BXP-treated human to UV-A irradiation results in development of urticaria.[3] The phototoxic urticaria provoked by BXP has been attributed to a direct photosensitization effect on degranulation of dermal mast cells.[3,4] As a result of investigation in the system of red blood cell (RBC) membranes, a possible mechanism was proposed in which BXP induces cell degranulation by photosensitizing nonspecific damage to cell membrane components.[5,6] It was also suggested that initial photodecarboxylation of BXP forms a lipophilic photoproduct, decarboxybenoxaprofen (DBXP) which subsequently photosensitize membrane hemolysis.

Nonetheless, no attempt has been made to investigate molecular mechanism of BXP (or DBXP) photosensitization of specific damage to the membrane components such as lipid and protein. Additionaly, photochemistry of BXP has not been extensively studied in the membrane mimetic system like micelles or liposomes, although BXP undergoes type I and type II reactions for photolysis in organic solvents.[7] The sufactant micelles or lipid liposomes have been useful as both functional and structural models for complex biomembranes.[8]

This paper describes a study of the photosensitizing action of BXP or DBXP for the decomposition of tryptophan peptides in the aqueous dispersion of surfactant micelles as well as in aqueous and ethanol solutions. Our results suggest that the anion radical of BXP is important for the function of photosensitization, which is more reactive in hydrophobic environment than in aqueous one.

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Experimental

Reagents. Benoxaprofen (BXP) and decarboxybenoxaprofen (DBXP) were generous gifts from Lilly Research Laboratories, Indianapolis. N-acetyl-L-tryptophan (NAT) and N-acetylphenylalanyl-L-tryptophan (NAPT) were purchased from Research Plus, Inc.. Sodium azide and superoxide dismutase (SOD) (bovine) were obtained from Sigma Chemical Co.. Phosphate buffered saline (PBS) was 0.05M in phosphate (Gibco). Water was purified by triple distillation and the absolute ethanol was used as obtained from Merck Co.. The surfactants used were cetyltrimethyl ammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) which were purchased from Merck Co..

Irradiation. As a light source, low power mercury lamp (Bausch & Lomb 100w medium pressure) was used to minimize time requirement for the precipitation of DBXP by decarboxylation in aqueous solution. Irradiation was performed in a modified merry-go-round apparatus.[9] 3 m*l* samples were irradiated in the matched Kimax test tubes which transmits less than 1% at 300 nm and lower wavelength so that auto-photolysis of tryptophan was minimized. Irradiation of NAT was also performed in parallel with all samples to correct day-to-day variation in the fluence rate, and NAT-autophotolysis was not observed at all. If necessary, the sample solutions were deoxygenated by gentle bubbling with 99% nitrogen in septum-capped test tubes for 120 min prior to irradiation.

Photodecomposition of Tryptophan in Peptides. BXP (or DBXP)-sensitized photodecomposition of tryptophan in peptides was monitored by analysis of decrease in absorption of tryptophan at 280 nm. Since the absorption spectra of BXP (DBXP) were partially overlapped with those of tryptophan peptides, the absorption spectra of the mixture of tryptophan peptides and BXP (DBXP) were measured by using BXP (DBXP) solution irradiated for the same period as that for the mixture, as a reference, to obtain spectral changes of tryptophan only.

The absorption spectra of the irradiated NAT-BXP

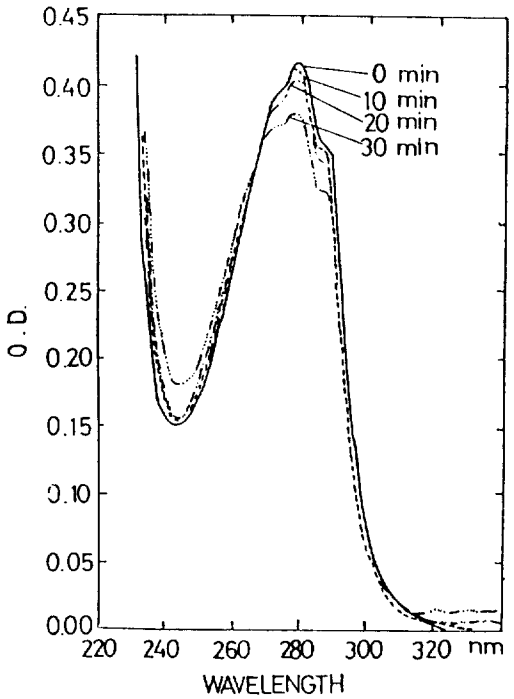

Figure 1. Absorption Spectra of NAT in the presence of BXP (5 ug/m*l*) as a function of irradiation time. NAT solutions (0.075 mM in PBS buffer, pH 7.00) were irradiated with UV-A for 10, 20 and 30min. BXP solutions irradiated in parallel with the mixture of NAT and BXP were used as reference solutions, since the absorption spectrum is partially overlapped with that of NAT.

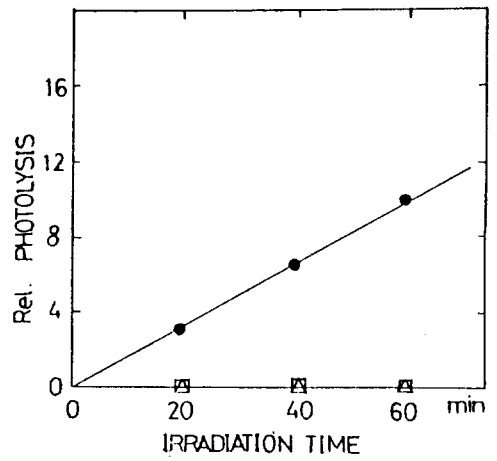

Figure 2. Relative Photolysis of NAPT sensitized by BXP and DBXP in ethanol as a function of irradiation time. BXP (DBXP) = 4 ug/m*l*. NAPT = 0.075 mM. △—△ NAPT in EtOH. □—□ NAPT + DBXP in EtOH. ●—● NAPT + BXP in EtOH.

(DBXP) mixtures were also measured with respect to the irradiated NAT solution as a reference. The changes in BXP absorption measured in this way were the same as those measured when BXP was irradiated alone, indicating that there is no interaction between the irradiated BXP and NAT.

Absorption and Fluorescence Spectra. Absorption spectra were recorded on a Beckman UV-5260 spectrophotometer. Fluorescence measurements were performed in 1cm quartz cuvette on a Jovin-Yvon spectrofluorimeter. When the fluorescence of BXP was measured, a narrow excitation slit (less than 5 nm) was used to minimize its photolysis. All spectral measurements were made at ambient temperature.

Results and Dicussion

Benoxaprofen-sensitized photodamage of cell membrane may occur by specific damage to membrane protein. To evaluate the effect of BXP-photosensitization on the photolysis of protein, some small peptides or derivatives of tryptophan such as N-acetylphenylalanyl-L-tryptophan (NAPT), N-acetyl-L-tryptophan (NAT) and tryptamine were employed as a model substrate. Figure 1 shows the absorption spectra of NAT (0.075 mM) mixed with BXP (5 ug/m*l*) in aerated PBS buffer (PH 7.00), which were measured after 0 to 30 min irradiation with UV-A light. It has shown that the absorption at 230-260nm increases upon irradiation while the maximum peak at 280 nm decreases, demonstrating the decomposition of tryptophan by UV-A. However, even under the same condition of irradiation, these changes were not observed with NAT alone. These results indicate that BXP photosensitizes the decomposition of NAT. It is noteworthy that the small increase in the absorption at 320-340 nm is also shown in Figure 1. indicating that a N-formylkynurenine (NFK) derivative, the photoproduct of tryptophan peptide is formed.[10]. Since NFK-derivative is also known to be a photodynamic sensitizer[10], it could be expected to contribute to acceleration of the photosensitized decomposition of tryptophan. However, the NFK-photosensitized decomposition of tryptophan was not observed without BXP in our experimental condition.

Recently, two step mechanism for the BXP photosensitization has been proposed by the investigation of BXP-sensitized photolysis of red blood cell (RBC).[6] According to this proposal, the photoexcited BXP forms a lipophilic decarboxybenoxaprofen (DBXP), which is incorporated into the lipid bilayer of the RBC membrane and subsequently initiate cell lysis by hydrogen abstraction from lipid or $^1O_2$-formation. Thus, we tried to compare the photosensitization ability of BXP on tryptophan lysis with that of DBXP under the same experimental condition. Since DBXP is insoluble in water, ethanol was used as a solvent. Figure 2 shows the relative lysis of NAPT photosensitized by BXP and DBXP in ethanol solution which concentrations (ca. 4 ug/m*l*) were adjusted to absorb the same amount of light at wavelengths longer than 300nm. The relative photolysis of NAPT was increased in the presence of BXP as a function of irradiation time, whereas in the presence of DBXP the photolysis was not observed as in the case of NAPT alone. Similar results were observed in oxidation of methyl linoleate (unpublished results). These results indicate that lysis of tryptophan in peptides is photosensitized only by BXP.

The BXP-sensitized photolysis of NAT was inhibited in the presence of nitrogen significantly but not completely. The partial inhibition of BXP-photosensitization has been also observed in the BXP-photosensitized hemolysis of RBC by other investigators.[3,6] It would appear that oxygen independent mechanism as well as oxygen dependent one may be important in BXP-photosensitized lysis of tryptophan, too. Nonetheless, oxygen is quite necessary to increase the efficiency of BXP photosensitization.

Singlet oxygen and superoxide have been implicated as the active oxygen species in the oxygen dependent photosen-

Table 1. Inhibition of BXP-photosensitized Decomposition of NAT by sodium azide and SOD

Medium	Azide, mM			SOD, ug/ml	
	15	30	45	20	30
Buffer (pH 7.00)	−	−	+	+	+ +(90%)
CTAB micelle	−	−	+	+	+ +

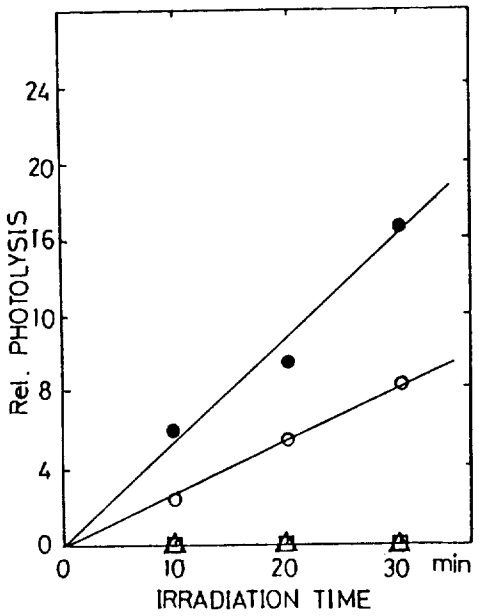

Figure 3. BXP-sensitized photolysis of NAT (0.075 mM) in the presence and absence of micellar solution (pH 8.00) as a function of irradiation time. BXP = 5 ug/ml. ●—● BXP + NAT in the presence of CTAB. ○—○ BXP + NAT in the absence of micelles. △—△ NAT in the presence of CTAB. □—□ BXP + NAT in the presence of SDS.

sitization.[11] In the present experiment, singlet oxygen could be detected by using a quencher, sodium azide only at high concentration (45mM). On the other hand, superoxide dismutase (SOD) (20-30ug/ml) inhibited BXP-sensitized photolysis of NAT significantly (90%, see Table 1). These results indicate that BXP photosensitization may be processed by Type I mechanism through formation of superoxide.

Reszka and Chignell[7] have shown from the spin trapping studies that the excited state of BXP in ethanol yields alkoxy or hydrogenated radicals via one-electron transfer reactions as shown in the following equations.

$$BXP \xrightarrow{h\nu} BXP^* \quad (1)$$

$$BXP^* + ROH \rightarrow BXP^{\cdot -} + RO^{\cdot} + H^+ \quad (2)$$

$$BXP^* + ROH \rightarrow (BXPH)^{\cdot} + RO^{\cdot} \quad (3)$$

Reaction of the BXP radicals with oxygen would then generate superoxide.

$$BXP^{\cdot -}(BXPH^{\cdot}) + O_2 \rightarrow BXP + HO_2^{\cdot} \rightarrow H^+ + O_2^{\cdot -} \quad (4)$$

BXP-photosensitized lysis of NAT was also performed in the presence of aqueous dispersions of CTAB and SDS above the critical micellar concentraion (CMC). Figure 3 shows the relative photolysis of NAT sensitized by BXP in

Table 2. Fluorescence Emission Maxima of BXP and Tryptophan Peptides

Medium	Emission maxima, nm		
	BXP	NAT	Tryptamine
Buffer	378	358	350
CTAB micelle	355	347	345
SDS micelle	360	358	342

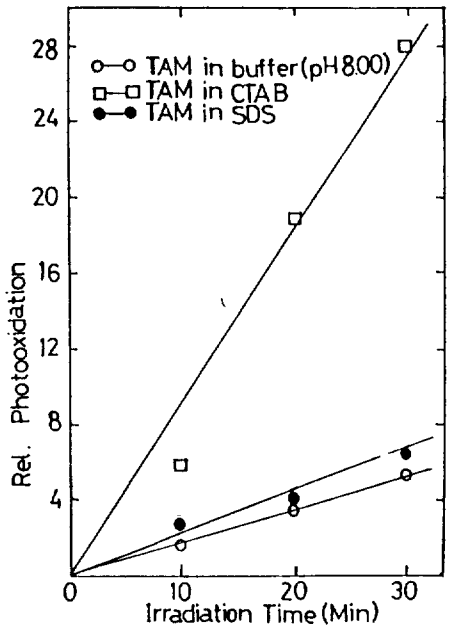

Figure 4. BXP-sensitized photolysis of tryptamine in the presence and absence of micelles (pH 8.00) as a function of erradiation time. BXP = 5 ug/ml. Tryptamine = 0.075 mM. □—□ in athe presence of CTAB. ●—● in the presence of SDS. ○—○ in the absence of micelles.

the presence and absence of surfactant micelles at pH 8.00. When the cationic surfactant CTAB was used above its CMC (0.85mM), the BXP-photosensitized lysis of NAT was markedly greater than observed in buffer alone. On the other hand, when the anionic surfactant SDS was used above its CMC (8mM), it was rather lower than observed in buffer. These results indicate that charges of the photoexcited state of BXP is a major factor in photosensitization. In other words, a "cage effect" appears to occur where anion radicals of BXP are formed (Eq. 2), being stabilized to a greater extent in cationic surfactants as a result of charge interaction. Then, the anion radical reactivity of the photosensitizer with oxygen would be enhanced to generate superoxide in cationic micellar phase (Eq. 4). Actually both BXP and NAT appears to be trapped in the hydrophobic micellar phase of CTAB, since their incorporation into micellar structures brings about a clearly detectable blue shift of the emission maxima, compared to the emission maxima in aqueous solution (see Table 2). The superoxide formation by BXP in micelles could be also identified by observation that NAT photolysis sensitized by BXP is inhibited by SOD (see Table 1).

However, in the presence of SDS micelles, NAT shows the same fluorescence emission maximum as that in aqueous solution, indicating that NAT is located in aqueous phase of

SDS suspension. Thus, lowering BXP photosensitization of NAT lysis in SDS micellar suspension from that in homogeneous aqueous solution may be due to separation of NAT from BXP trapped in the micellar phase, where the superoxide is formed by the photoexcited BXP and diffuse out for the oxidation of NAT in aqueous phase.

In order to compare the effect of micellar composition on the BXP-sensitized photolysis of tryptophan under the same condition, tryptamine instead of NAT was used as a substrate. The tryptamine is located in both CTAB and SDS micellar phase as demonstrated by blue shift of emission maxima from that in aqueous solution (Table 2). This is in good agreement of previous results obtained by Rossi *et al.*[12] Under this condition, the relative photolysis of tryptamine sensitized by BXP was greater in SDS micellar phase than in aqueous solution, but much lower than in CTAB micelles (Figure 4). These results again implicate that the anion radical of BXP may play a major role in the photosensitization in micellar phase, and that the sensitizer is more reactive in hydrophobic environment. Similar results have been obtained for the photosensitizing action in surfactant solutions of chlorpromazine and furosemide.[13]

The mechanism of anion radical formation of BXP in micellar phase may be different from that in ethanol (Eqs. 1,2), since the surfactants would not function as electron donor.[14] It might be rather possible that the photoexcited molecule of BXP may accept electron from BXP remained in the group state and change into BXP as shown in the following equation.

$$BXP^* + BXP \rightleftharpoons [BXP \cdots BXP]^* \rightarrow BXP^{\cdot -} + BXP^{\cdot +} \quad (5)$$

The micelle may serve as a "super cage" for maintaining high local concentrations of reactants in a restricted space for a longer time than in aqueous phase and subsequently induce efficient formation of some excimer. In fact, the photo-induced [2 + 2] dimerization of 2-phenylbenzoxazole, an analog of BXP, was detected in degassed cyclohexane by Roussilhe *et al*[15]., although the quantum yield of this reaction is low. Thus, it is not impossible to postulate that the excimer formation of BXP would be more efficient in micellar phase, and induce efficient electron transfer from BXP* to BXP. Nevertheless, the direct detection of transients produced from BXP in micellar phase should be necessary to explore this possibility. Such investigation is in progress in our laboratory by using laser flash photolysis technique.

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Cationic Cyclizations to Tricyclene Structures†

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Various carbocation-mediated cyclizations to tricyclene structure (basically, tricyclo [2,2,1,$0^{2,6}$] heptane skeleton) were carried out, starting from protonated species of either 3-methyl-2,5-norbornadiene-2-carboxylic acid (**10**) or 3-methylene-5-norbornene-2-carboxylic acids (**18** and **19**). The resulting products were individually converted to π-iodotricyclene (**35**), a pivotal intermediate in almost all syntheses of tricyclene terpenes.

Introduction

The highly prized fragrance from isolate of *Santa ablum* Linn, East Indian sandalwood oil, is known to be derived from α-and β-santalols, **1** and **2**. Due to the highly condensed ring structures of these tricyclic sesquiterpenes and their importance, many synthetic achievements have been reported thus far[1].

† Dedicated to Professor Nung Min Yoon on the occasion of his 60th birthday.