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SINGLET OXYGEN REACTS WITH INHIBITORS OF ULTRAVIOLET MEDIATED DAMAGE TO SKIN: p-AMINOBENZOIC ACID AND ITS DERIVATIVES

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SUMMARY

P-Dimethylaminobenzoate, p-aminobenzoate, and ethyl-p-dimethylaminobenzoate react with singlet molecular oxygen, photochemically generated with hematoporphyrin as a sensitizer, to yield spectrally distinguishable products. The reaction is potentiated by D_2O and is competitively inhibited by methionine. p-Aminobenzoic acid and its derivatives are often used topically to prevent actinic damage to skin. They are thought to act solely as physical screens to absorb UV light. Their reactivity toward singlet oxygen, however, may also play an important role in the protective effect.

The prevention of ultraviolet (UV) light-mediated skin damage is a topic of considerable clinical importance. With an incidence of 300,000 new cases per year, cancer of the skin occurs with a greater frequency than cancer of any other organ (1). The great preponderance of these cases are mediated by UV light in the 290-320 nm region of the solar spectrum.

PABA and its derivatives, when topically applied, have been shown to afford considerable protection against UV-induced damage (2-7). Heretofore these compounds have been thought to act as "sunscreens" (8); i.e. the compounds are supposed to exert their photoprotective effect solely by absorbing the appropriate photons of UV light and thus prevent the action of the light on cutaneous cells. Since the mechanism of UV-mediated damage to

Abbreviations: NBT = nitroblue tetrazolium; HP = hematoporphyrin; PABA = p-aminobenzoate; dimethylPABA = p-dimethylaminobenzoate; DMSO = dimethylsulfoxide.

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skin is unknown and since singlet oxygen ($^1O_2$) has been shown to be the mediator of many photosensitized oxidations (9) we felt it worthwhile to determine if PABA and its derivatives react with $^1O_2$ and to exclude some of the other reactive oxygen species by studying the effects of various inhibitors and activators.

MATERIALS AND METHODS

Chemicals were purchased from the following suppliers as indicated: deuterium oxide (Bio-Rad Laboratories); DL-methionine sulfoxide, DL-methionine sulfone, NBT, and HP (Sigma Chemical Co.); potassium superoxide (Research Organic Inorganic Chemical Co.); PABA and ethyl p-dimethylaminobenzoate (Aldrich Chemical Co.); dimethylPABA (Eastman Kodak Co.); DL-methionine (Mann Research Laboratories); dicyclohexyl-18-crown-6 ether (Tridom-Fluka Chemical Co.); DMSO (J.T. Baker Chemical Co.).

Photooxidation was performed in an apparatus containing two 15-watt Sylvania 15T8-BL black light fluorescent bulbs which emit maximally at 375 nm (10). The apparatus was designed to protect personnel from exposure to ultraviolet radiation in order to avoid possible ocular cataract formation. Light intensity was determined with a Blak-Ray long wave UV meter (Ultraviolet Products Inc.). A typical reaction mixture contained 8 µM HP, 10 mM potassium phosphate, pD 7.5, 40 µM PABA or PABA derivative. Unless otherwise indicated, all solutions were prepared with 99.8% D_2O. All photooxidations were carried out in quartz cuvettes. The KO_2 was prepared as a saturated solution in DMSO containing 0.5% dicyclohexyl-18-crown-6 ether (11). The DMSO was previously dried with molecular sieves 3A. The concentration of O_2^- was assayed by sequential 5 µl additions of KO_2 solution to a 0.1 µM solution of NBT in 10 µM potassium phosphate, pD 7.5, prepared with 99.8% D_2O. The extinction coefficient at 560 nm for the reduction of NBT to diformazan was taken to be 3×10^3 M^{-1} cm^{-1} (12,13).

RESULTS AND DISCUSSION

We have chosen to generate singlet oxygen with UV light longer than 340 nm, using HP as a photosensitizer (14-16):

$$HP \xrightarrow{h\nu} HP^1$$

$$HP^1 \longrightarrow HP^3$$

$$HP^3 + {}^3O_2 \longrightarrow HP + {}^1O_2$$

Ground state HP is excited by the absorption of a photon and is transformed into a relatively shortlived HP singlet (HP^1). HP^1 undergoes spin inversion to a relatively longlived HP triplet (HP^3). HP^3 is then quenched by ground state triplet molecular oxygen. This energy transfer reaction results in the formation of singlet molecular oxygen ($^1O_2$) and ground state HP. Control experiments have shown that UV light alone (in the absence of HP) does not cause significant changes in the absorption spectra of PABA and its derivatives.

HP-catalyzed photooxidation of dimethylPABA in D_2O.

DimethylPABA was studied in greatest detail as the changes in its absorption spectrum were the most dramatic as compared with PABA and ethyl-p-dimethylaminobenzoate. Fig. 1 shows that the absorption spectrum of dimethylPABA in a singlet oxygen-generating system (HP-D_2O) changes steadily with increasing length of irradiation, indicating a HP-catalyzed photoreaction of dimethylPABA. Under these conditions no significant changes in HP were detected from 230 nm to 340 nm. The loss of dimethylPABA as indicated by the decrease in absorbance at 290 nm is accompanied by the appearance of the product(s) with an absorption maximum near 245 nm. Upon prolonged irradiation after 28 minutes, when most of the starting material has been transformed, a further decrease at 290 nm is no longer compensated by an increase at 245 nm, suggesting that the product(s) may also be destroyed by singlet oxygen.

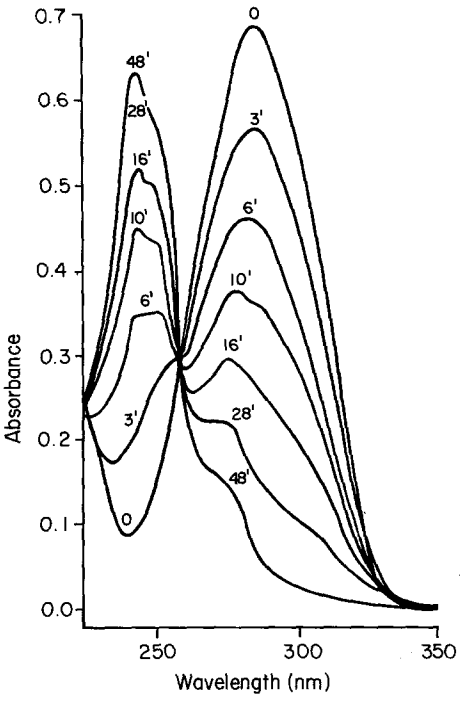

Fig. 1. Time course of photooxidation of p-dimethylaminobenzoate. The reaction mixture and photooxidation procedure are as described under Materials and Methods. The absorbance of the solution indicated for varying time intervals was measured in a Cary 14 spectrophotometer against a reference cell containing all components except p-dimethylaminobenzoate.

Singlet oxygen as the mediator in the photooxidation of PABA and its derivatives.

The results in Table I support the hypothesis that singlet oxygen is the mediator in the photochemical conversion of PABA and its derivatives. Singlet oxygen has a mean lifetime of 20 μs in D_2O as compared to 2 μs in H_2O (17-19). As can be seen in Table I, the reaction carried out in D_2O shows a 4 to 9-fold potentiation over that in H_2O. The effect of additions of methionine, a well known scavenger of singlet oxygen was also

Table I. Effects of singlet oxygen reactive compounds on the hematoporphyrin-catalyzed photooxidation of dimethylPABA, PABA and ethyl-p-dimethylaminobenzoate.

Addition	DimethylPABA	PABA	Ethyl-p-dimethylamino-benzoate
	(% ΔA_{290})	(% ΔA_{280})	(% ΔA_{315})
None	(100)	(100)	(100)
H_2O in place of D_2O	25	14	11
Methionine, 2.5 mM	53	14	9
Methionine, 0.5 mM	92	63	45

The experimental conditions are described under Materials and Methods. Change in absorbance is expressed as percentage of control. Duration of irradiation for dimethylPABA is 5 min; PABA, 30 min; ethyl-p-dimethylaminobenzoate, 70 min. The initial concentrations of PABA and its derivatives are 40 µM. The decreases in absorbance in the control experiments without additions are 0.27, 0.14 and 0.19 for dimethylPABA, PABA, and ethyl-dimethylaminobenzoate respectively. These values are designated as 100%.

tested. Methionine reacts with single oxygen with a rate constant of 3×10^7 M^{-1} s^{-1} (20). Table I shows that methionine can effectively inhibit the HP-catalyzed photooxidation of PABA and its derivatives. These data indicate not only that singlet oxygen is a mediator in the reaction, but also that PABA and its derivatives are very reactive with singlet oxygen (e.g. when methionine is used as an inhibitor of PABA oxidation, over 12 times the concentration ratio is needed to bring about 37% inhibition). When the methionine containing reaction mixture was analyzed on the amino acid analyzer, methionine sulfoxide was identified as a major photooxidation product similar to that observed by Sysak et al. (21).

Table II. Effects of various compounds on the photooxidation of p-dimethylaminobenzoate.

Experiment	Addition	% ΔA_{290}
1.	None	(100)
2.	H_2O_2, 2.5 mM	90
3.	Mannitol, 2.5 mM	92
4.	Formate, 2.5 mM	90
5.	Benzoate, 2.5 mM	90
6.	Isopropanol, 100 mM	97
7.	EDTA, 0.1 mM	108

Photooxidation was performed as described under Materials and Methods. Change in absorbance is expressed as percentage of control. The duration of irradiation was 5 min. The ΔA_{290} for the control cuvettes ranged from 0.19 to 0.27. Appropriate control experiments were used to negate the possible contribution of the added compounds (or their oxidation products) to the optical density. Experimental and control cuvettes were run concomitantly.

Exclusion of $OH^{\bullet}$, $O_2^{\bullet-}$, H_2O_2, and metal ion complexes as mediators in the photooxidation.

It has been well documented that various reactive oxygen species are interconvertible (16). Moreover, in addition to singlet oxygen, methionine can be oxidized by other oxidants such as $OH^{\bullet}$ and peroxide. We have attempted to exclude the involvement of some of these species in this system.

The hydroxyl radical reacts very rapidly with mannitol, benzoate or formate, which are often used as inhibitors to detect involvement of $OH^{\bullet}$ (22). Table II shows that additions of any of these scavengers at a concentration over 40 times that of dimethylPABA caused only a slight inhibition in the photo-oxidation of the PABA derivative. Another $OH^{\bullet}$ scavenger,

isopropanol, also did not show any significant effect. Experiment 2 shows that 2.5 mM H_2O_2 did not potentiate the photooxidation. Addition of a metal ion chelator, EDTA, in Experiment 7 was also without inhibitory effect.

Exclusion of O_2^- as an initiator of the reaction was carried out by direct addition of a KO_2 solution to a similar reaction mixture without irradiation. 200 µl of the KO_2 (in crown ether-DMSO) solution was slowly added to 1.0 ml of a 48 µM solution of dimethylPABA in D_2O buffer (with magnetic stirring). The final concentration of O_2^- added was estimated to be 190 µM. A similar crown ether-DMSO solution without KO_2 was added to the control cuvette. No significant difference was detected between the absorption spectra of the experimental and control solutions. The results taken collectively are consistent with a singlet oxygen-mediated oxidation of PABA and its derivatives in this system.

Epstein (23) studied the effect of β-carotene on UV-induced skin tumors in mice. He noted that tumors appeared earlier and grew more rapidly in the placebo-treated animals as compared with the β-carotene-treated group. Although β-carotene is an excellent quencher of $^1O_2$ (24), Epstein noted that this compound can react with free radicals and also its absorption spectrum slightly overlapped the emission spectrum of the light source used to generate the tumors. Thus, although his data suggest a possibility of involvement of $^1O_2$ in tumor formation, he could draw no definitive conclusions regarding the mechanism of the protective effect. Our results indicate the possibility that if singlet oxygen is generated by UV in cutaneous cells, PABA and its derivatives may prevent UV-mediated skin damage via their ability to react with singlet oxygen in addition to their screening effect.

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REFERENCES

1. Silverberg, E.S. (1977) CA - A Cancer Journal for Clinicians 27, 26-41.
2. Snyder, D.S., and May, M. (1975) J. Invest. Dermatol. 65, 543-546.
3. Pathak, M.A., Fitzpatrick, T.B., and Frenk, E. (1969) New Eng. J. Med. 280, 1459-1463.
4. Katz, S.I. (1970) Arch. Derm. 101, 466-468.
5. Willis, I., and Kligman, A.M. (1970) Arch. Derm. 102, 405-417.
6. Langner, A., and Kligman, A.M. (1972) Arch. Derm. 105, 851-855.
7. MacLeod, T.M., and Frain-Bell, W. (1975) British J. Derm. 92, 417-425.
8. Magnus, I.A. (1976) Dermatological Photobiology, pp. 266-274, Blackwell, London.
9. Foote, C.S. (1976) in Free Radicals in Biology, Vol. II. Pryor, W.A., Ed., pp. 85-113, Academic Press, New York.
10. GTE Sylvania Engineering Bulletin, Publication 0-36.
11. Ohnishi, T., Hirata, F., and Hayaishi, O. (1977) J. Biol. Chem. 252, 4643-4647.
12. Miller, R.W., and Kerr, C.T. (1966) J. Biol. Chem. 241, 5597-5604.
13. Lee-Ruff, E. (1977) Chem. Soc. Rev. 6, 195-214.
14. Suwa, K., Kimura, T., and Schaap, A.P. (1977) Biochem. Biophys. Res. Commun. 75, 785-792.
15. Cannistraro, S., and Van de Vorst, A. (1977) Biochem. Biophys. Res. Commun. 74, 1177-1185.
16. Bodaness, R.S., and Chan, P.C. (1977) J. Biol. Chem. 252, 8554-8560.
17. Merkel, P.B., Nilsson, R., and Kearns, D.R. (1972) J. Am. Chem. Soc. 94, 1030-1031.
18. Merkel, P.B., and Kearns, D.R. (1972) J. Am. Chem. Soc. 94, 7244-7253.
19. Merkel, P.B., and Kearns, D.R. (1972) J. Am. Chem. Soc. 94, 1029-1030.
20. Nilsson, R., Merkel, P.B., and Kearns, D.R. (1972) Photochem. Photobiol. 16, 117-124.
21. Sysak, P.K., Foote, C.S., and Ching, T-Y. (1977) Photochem. Photobiol. 26, 19-27.
22. Anbar, M., and Neta, P. (1967) Int. J. Applied Radiat. Isot. 18, 493-523.
23. Epstein, J.H. (1977) Photochem. and Photobiol. 16, 117-119.
24. Foote, C.S., Denny, R.W., Weaver, L., Chang, Y., and Peters, J. (1970) Annals N.Y. Acad. Sci. 171, 139-148.