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Para-aminobenzoic acid scavenges reactive oxygen species and protects DNA against UV and free radical damage

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Para-aminobenzoic acid (PABA), once considered a vitamin, is a precursor of folate for certain bacteria and is used as a sunscreen because of its UV-absorbing property. In addition to its mild anti-inflammatory activity, recent studies demonstrate that PABA protects against photocarcinogenesis in hairless mice and nephrotoxicity of cis-*diamminedichloroplatinum(II) in rats. However, little is known regarding the mechanism by which PABA exerts these effects. We have hypothesized that PABA may be an effective scavenger of reactive oxygen species. In this report, we showed that PABA reacted with hypochlorite in a ratio of reaction of 1:2, as measured by a spectrofluorometric method developed in this study. PABA reacted with hydroxyl radicals (·OH) by inhibiting deoxyribose oxidation induced by a Fenton-type reaction system (Fe^{3+} + EDTA/H_2O_2/ascorbic acid). The second-order rate constant for the reaction of PABA with ·OH was approximately $1.07 \times 10^{10}\ M^{-1}\ s^{-1}$. Electron spin resonance studies further demonstrated the reaction of PABA with ·OH. The Fenton-type reaction system also caused damage to calf thymus DNA, and concurrent treatment with UV (254 nm) enhanced the damage by 3 fold. PABA tested at 1.0 mM afforded 58% protection against such damage. Using rose bengal as a singlet oxygen ($^1O_2$) generator and ESR techniques, we showed that PABA scavenged $^1O_2$ more effectively than sodium azide, a known quencher of $^1O_2$. However, PABA did not scavenge superoxide anions or react with H_2O_2. Thus, in addition to its UV absorbing ability, PABA effectively scavenges certain reactive oxygen species, an effect that may be relevant to its protection against photocarcinogenesis, inflammation, and drug toxicity.* (J. Nutr. Biochem. 6: 504–508, 1995.)

Keywords: para-aminobenzoic acid; reactive oxygen species; hypochlorous acid; antioxidant; UV irradiation; DNA damage

Introduction

Reactive oxygen species (ROS) have been implicated to play an important role in human aging and various pathological states including many types of cancer.[1-3] Para-aminobenzoic acid (PABA), once known as vitamin B_x, is an essential cofactor for the production of folic acid in many species of bacteria.[4] This compound has mild anti-inflammatory and antifibrotic activities[5,6] and has been used empirically for more than 50 years in sun-blocking creams (commonly contains 2 to 10% PABA)[7,8] because of its high absorbance in the UVB region (280 to 320 nm).[4,9] Several studies have demonstrated that PABA is protective against skin cancer induced by UV irradiation[8-11] and by UV and 7,12-dimethylbenzanthranzene treatment[12] in hairless mice. In rats injected i.p. with *cis*-diamminedichloroplatinum(II), PABA suppresses cisplatin-DNA adducts and nephrotoxicity without compromising the antitumor activity.[13] However, the mechanism(s) of PABA in these actions are unclear. Sagone et al.[14] have shown that PABA is metabolized

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Received December 13, 1994; revised February 10, 1995; accepted March 23, 1995.

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655 Avenue of the Americas, New York, NY 10010

0955-2863/95/$10.00
SSDI 0955-2863(95)00082-B

by the myeloperoxidase system in stimulated polymorphonuclear neutrophils and have suggested that the metabolism is responsible for the mild anti-inflammatory activity of PABA. Since ROS have been implicated in inflammation,[3] UV-induced damage to biological systems including skin tissues,[15–17] and *cis*-platin toxicity,[18,19] we have hypothesized that the protection of PABA may involve scavenging of ROS.

In this report, we studied the reaction of PABA with ·OH generated by a Fenton-type reaction system using deoxyribose oxidation electron spin resonance (ESR), and DNA damage with and without UV irradiation. We also determined the reaction of PABA with singlet oxygen ($^1O_2$), sodium hypochlorite, superoxide anions ($O^-\cdot_2$), and H_2O_2.

Methods and materials

Potassium PABA, deoxyribose, calf thymus DNA, mannitol, cytochrome c, xanthine oxidase, thiobarbiturate acid (TBA), and sodium hypochlorite (NaOCl) were from Sigma Chemical Co. (St. Louis, MO USA) and 2,2,6,6-tetramethypiperidine (TEMP) was from Aldrich. Hydrogen peroxide was obtained from Merk (Darmstadt, Germany); 5,5-dimethyl-1-pyrroline N-oxide (DMPO) from Labotec (Tokyo, Japan). All other chemicals used were of reagent grade.

Reaction of PABA with HOCl, H_2O_2, and superoxide anions

Consumption of PABA by NaOCl and H_2O_2 was measured using a fluorescent method that was developed in this study. We found that PABA in aqueous solution (water and phosphate buffer, pH 7.4) showed strong excitation and emission peaks at 280 and 340 nm, respectively (*Figure 1*). A Hitachi spectrofluorometer (Tokyo, Japan, Model 650-40) having a ratio photometer was used. NaOCl or H_2O_2 at various concentrations was quickly mixed with 0.1 mM PABA in potassium phosphate buffer, pH 7.4 (final concentration 37.5 mM) in a volume of 3.0 mL. The mixture of PABA with H_2O_2 was incubated at room temperature at various intervals. The concentration of NaOCl was standardized using $\epsilon_{290} = 350\ M^{-1}\ cm^{-1}$ at pH 12.[20] At pH 7.4, OCl^{-1} is approximately 50% protonated to HOCl. For convenience, HOCl was used to represent the protonated and the dissociated forms. H_2O_2 was standardized using $\epsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$.[21] Reaction of PABA with $O^-\cdot_2$ was determined using a xanthine oxidase-hypoxanthine system coupled with ferricytochrome c reduction.[22]

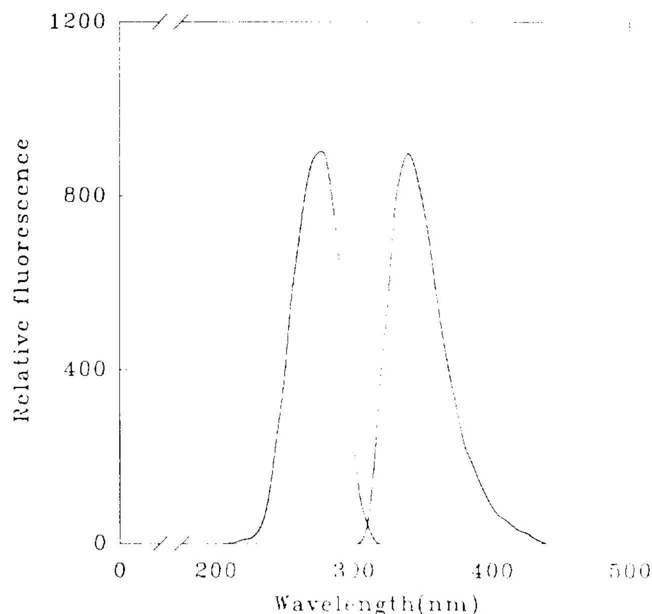


Figure 1 Fluorescence spectra of PABA (30 μM) in phosphate buffer (37.5 mM), pH 7.4. Excitation and emission peaks were at 280 and 340 nm, respectively.

Deoxyribose oxidation assay

Hydroxyl radicals, which are known to damage deoxyribose and form TBA-reactive chromogens,[23] were generated by a Fenton-type reaction system (Fe^{3+} + EDTA/H_2O_2/ascorbic acid)[24] with slight modification. The reaction system (1.0 mL) contained 2.8 mM deoxyribose, 2.8 mM H_2O_2, 20 μM ferric chloride premixed with 100 μM EDTA, and appropriate amounts of PABA in 25 mM phosphate buffer, pH 7.4. The reaction was initiated by the addition of ascorbic acid (0.10 mM final concentration), and the mixture was incubated for 1 hr at 37°C followed by the addition of 1.0 mL of TBA (0.7% in 0.05 N KOH) and 1.0 mL of 2.5% TCA. The mixture was heated at 100°C for 8 min, cooled, and the color formed was measured at 532 nm. The second-order rate constant (k_2) for the reaction of PABA with ·OH was obtained based on competition of PABA with deoxyribose for ·OH.[25]

ESR studies of ·OH and $^1O_2$

Reaction of PABA with ·OH was also determined using ESR techniques. Hydroxyl radicals were produced by the Fenton-type system as described previously except that deoxyribose was omitted. Ten microliters of DMPO (3 mM final concentration) was added as a radical trap. The mixture was incubated at 37°C for 15 min before ESR measurement.

Reaction of PABA with $^1O_2$ was determined using ESR spectroscopy according to Das and Misra.[26] Singlet oxygen was produced by rose bengle (40 mM) irradiated for 5 min at room temperature using a slide projector (Cabin, AF-250) and detected using TEMP (22 mM final concentration) as the spin trap.

ESR spectra were recorded at room temperature on a JES-EE3X spectrometer (JEOL, Tokyo, Japan; X-band) with 100 kHz field modulation and the following settings: microwave power, 10 mV; magnetic field, 336 mT; scan range, ±5 mT; modulation width, 0.079 mT; scan speed, 4 min; response, 0.3 sec.

DNA damage by Fenton reagents and UV irradiation

Replacement of deoxyribose with calf thymus DNA (0.50 mg/mL of reaction mixture) in the Fe^{3+} + EDTA/H_2O_2/ascorbic acid system resulted in the appearance of TBA-reactive chromogens with a maximal absorption at 532 nm. The reaction mixture (1.0 mL) placed in a small beaker was exposed to a UV lamp (main output at 254 nm) in a laminar flow with the surface of the mixture at a distance of 3 cm from the lamp. The beakers were placed and rotated every 15 min in the middle part of the lamp where the light intensity was highest (1.9 mW/cm^2 at 3 cm distance). During the irradiation (24°C, 1 hr) the control (unirradiated) samples were wrapped in aluminum foil and the laminar flow was covered with dark cloth.

Results

PABA reacts with HOCl but not H_2O_2 and superoxide anions

PABA reacted with HOCl in a concentration-dependent manner with the reaction being completed within 2 min, as

a measure using the spectrofluorometric method. The stoichiometry of the reaction of PABA with HOCl was approximately 1:2 (*Table 1*). There was no detectable loss in PABA (0.1 mM) when incubated with 5.0 mM H_2O_2 for up to 30 min. PABA at a concentration up to 5 mM did not react appreciably with superoxide anions (data not shown).

PABA Scavenges ·OH

Figure 2A compares the effect of PABA with mannitol on deoxyribose oxidation. Both PABA and mannitol inhibited the oxidation in a concentration-dependent manner. The concentrations required for 50% decrease (IC_{50}) were approximately 0.25 and 3.3 mM for PABA and mannitol, respectively. Control experiments (minus deoxyribose) showed that neither PABA nor mannitol interfered with the assay.

The slope of a plot of the reciprocal of absorbance ($A_{532\ nm}$) against concentrations of PABA or mannitol (*Figure 2B*) was used to calculate k_2 of reaction with ·OH.[25] The approximate k_2 thus obtained was $1.07 \times 10^{10}\ M^{-1}\ s^{-1}$ (SD = 0.34, n = 3) and $2.35 \times 10^9\ M^{-1}\ s^{-1}$ (SD = 0.28, n = 3) for PABA and mannitol, respectively.

ESR studies further demonstrated scavenging of ·OH by PABA. *Figure 3* shows that PABA decreased the DMPO-OH adduct signal in a concentration-dependent manner. As in the deoxyribose oxidation assay, PABA was more effective in scavenging of ·OH than mannitol (50 mM, line F; *Figure 3*).

PABA protects DNA against damage by UV and Fenton reagents

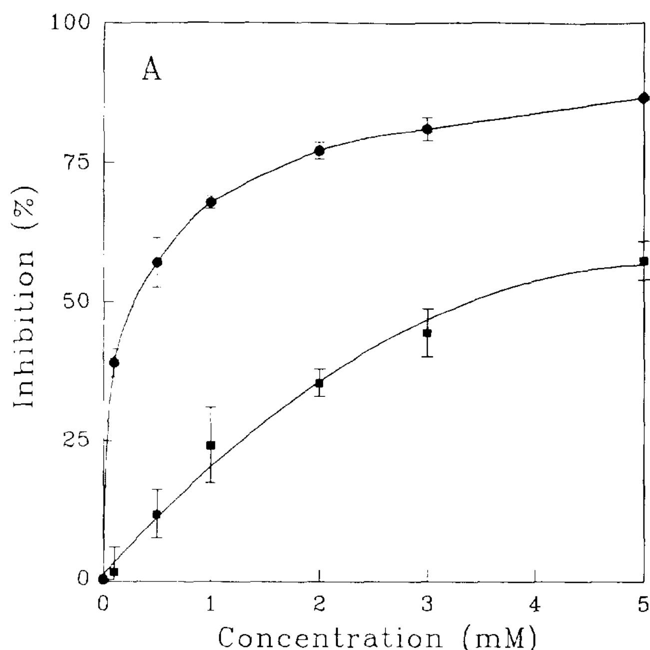
Incubation with the Fenton reagents alone (i.e., without UV irradiation) for 1 hr resulted in damage to calf thymus DNA ($A_{532\ nm}$ = 0.11) (*Table 2*). Inclusion of 1.0 mM PABA and mannitol decreased the damage by 55 and 40%, respectively.

Concurrent treatment with UV enhanced the ·OH-induced damage to DNA by 3 fold. Inclusion of 1.0 mM PABA and mannitol decreased the damage by 58 and 46%, respectively. UV irradiation alone (i.e., without Fenton components) resulted in no appearance of TBA-reactive products of DNA either in the absence of presence of PABA

Table 1 Stoichiometry of the reaction of PABA with NaOCl*

NaOCl added (μM)	PABA (μM)		Stoichiometry
	Remaining	Loss	(NaOCl/loss of PABA)
0	100†	0	–
25	90.4	9.6	2.6
50	77.3	22.7	2.2
100	50.3	49.7	2.0
150	21.6	78.4	1.9
180	10.9	89.1	2.0
200	5.2	94.8	2.1

*PABA was measured at 280 nm excitation and 340 nm emission as described in the text.
†Means of two separate experiments each having duplicate samples.



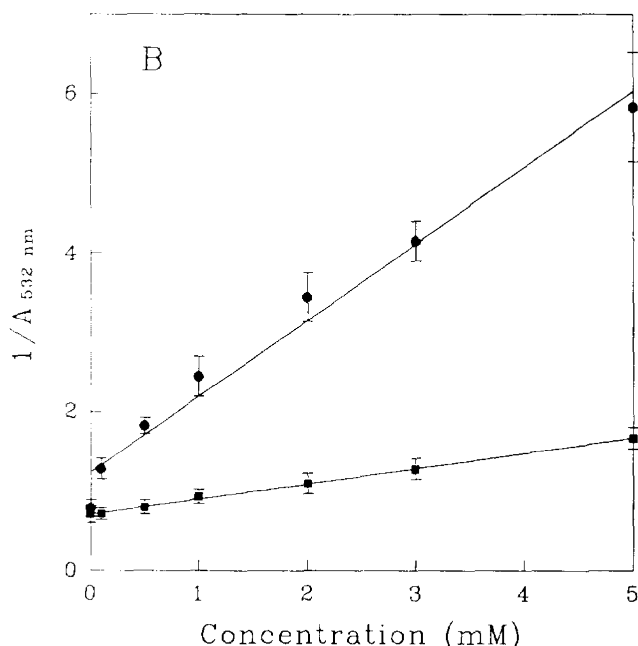


Figure 2 (A) Inhibition (%) of deoxyribose oxidation by PABA (circle) and mannitol (square). (B) A plot of reciprocal of absorbance of TBA-reactive chromogens at 532 nm against the concentration of PABA (circle) and mannitol (square) for the calculation of rate constants. Values are means of 2 or 3 experiments; bars denote standard deviation.

(which turned yellowish but did not interfere with the TBA assay).

PABA quenches $^1O_2$

Figure 4 shows that PABA inhibited TEMP-$^1O_2$ adduct formation in a dose-dependent manner. PABA scavenged

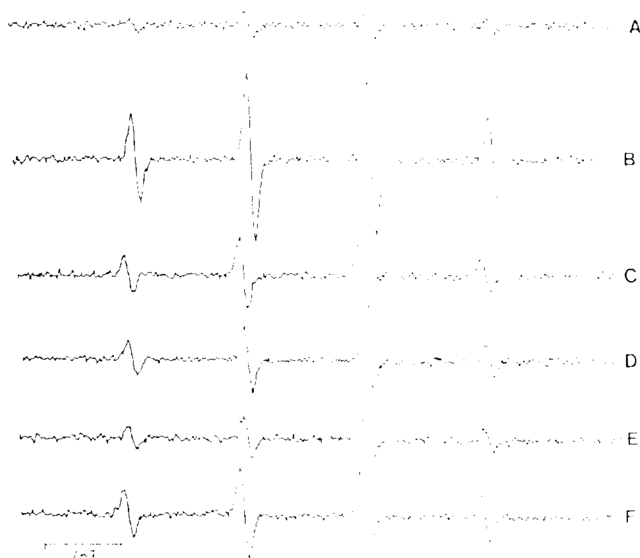


Figure 3 ESR spectra of the DMPO-OH adduct and the effect of PABA and mannitol. The reaction mixture containing Fenton-type reagents, DMPO and PABA was incubated at 37°C for 15 min before ESR measurement. Line A was without Fe^{3+} + EDTA, lines B, C, D, and E were 0, 1, 2, and 3 mM PABA, respectively, and line F was 50 mM mannitol.

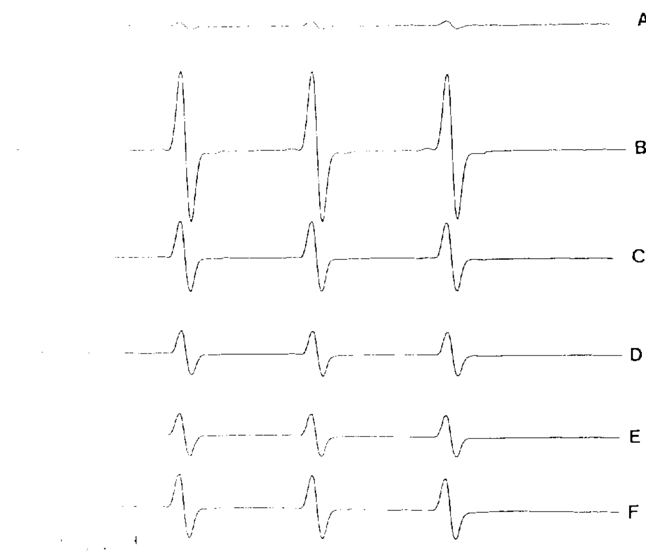


Figure 4 ESR spectra of TEMP-$^1O_2$ adduct and the effect of PABA and sodium azide. $^1O_2$ was generated from rose bengal by light irradiation. Line A was without rose bengal; lines B, C, D, and E were 0, 1, 5, and 10 mM PABA, respectively, and line F was 10 mM sodium azide.

$^1O_2$ more effectively than sodium azide (10 mM, line F; *Figure 4*), a known $^1O_2$ quencher.

Discussion

Free radicals are known to be involved in UV-induced skin damage,[15–17] and chronic exposure of skin to UV results in free radical generation, possibly through iron deposition, which can be detected by ESR.[27] The ability of PABA to absorb strongly at UVB range (280 to 320 nm) may largely account for its protection against photocarcinogenesis. However, the UV-absorbing ability does not account for other effects of PABA such as anti-inflammation,[5,6] and protection against *cis*-diamminedichloroplatinum(II) toxicity in rats.[13]

We showed in this study that PABA was effective in reaction with HOCl. HOCl, a microbicidal agent produced by myeloperoxidase system in activated neutrophils,[28,29] can damage essential components of the host tissues,[28–32] and thus cellular and extracellular defense against HOCl-induced damage is crucial. PABA can be metabolized by the myeloperoxidase system in stimulated neutrophils, and such metabolism may account for its mild anti-inflammatory activity.[14] Although it is not known how much PABA can accumulate in the inflammatory sites, the 1:2 ratio of reaction of PABA with HOCl suggests that PABA is somewhat more favorable than –SH groups (from small and protein thiols) in reaction with HOCl (–SH:HOCl = approximately 1:1.5).[33]

Both deoxyribose degradation assay and ESR techniques showed that PABA was a powerful scavenger of ·OH. The k_2 (1.07×10^{10} M^{-1} s^{-1}) of the reaction of PABA with ·OH, which equalled diffusion-controlled limit, was higher than that (1.2×10^9 M^{-1} s^{-1}) reported by Anderson et al.[34] using pulse radiolysis. However, a k_2 of 2.35×10^9 M^{-1} s^{-1} (SD = 0.28, n = 3) for the reaction of mannitol with ·OH obtained in our study was in agreement with reported values (approximately 2×10^9 M^{-1} s^{-1}) based on the same deoxyribose assay or pulse radiolysis.[25] As PABA was much more effective than mannitol in scavenging ·OH, the k_2 obtained in our study appeared to be a reasonable figure.

The Fenton-type reaction system also caused damage to calf thymus DNA, and concurrent treatment with UVC (254 nm) enhanced the damage by 3 fold. PABA at 1.0 mM gave 58% protection, but the effect was only slightly better than that of 1.0 mM mannitol. This appeared to contradict the results from deoxyribose oxidation assay (and ESR studies) in which PABA was much more effective than mannitol. One possible explanation is that some iron ions may have been bound by DNA molecules leading to site-specific damage which cannot be prevented by PABA or mannitol. Since PABA also absorbs in the 254 nm region (data not shown),

Table 2 Inhibition by PABA of DNA damage induced by a Fenton-type reaction system and UV irradiation*

Addition to DNA	DNA damage ($A_{532\ nm} \times 10^2$) – UV	DNA damage ($A_{532\ nm} \times 10^2$) + UV
None	2.5 ± 1.0†	1.7 ± 0.5
+PABA (1.0 mM)	2.3 ± 0.7	2.3 ± 0.1
+Fenton system	11.2 ± 0.7	34.5 ± 1.4
+Fenton system + PABA (1.0 mM)	5.0 ± 0.5	14.4 ± 0.3
+Fenton system + mannitol (1.0 mM)	6.7 ± 0.3	18.6 ± 0.5

*Reaction mixture containing DNA (0.5 mg/mL) was treated with or without a Fenton-type system (Fe^{3+}-EDTA/H_2O_2/ascorbic acid) and/or UV (254 nm) at 24° for 1 hr.
†Means ± SD, n = 3.

the concurrent treatment could not differentiate the sunscreen effect from the antioxidant effect of PABA. Studies using UVA (320 to 380 nm where PABA does not absorb) are currently being planned.

$^1O_2$ is often produced in photodynamic actions of various photosensitizers (known as Type II mechanism).[3] The ability of PABA to scavenge $^1O_2$ more effectively than sodium azide suggests that PABA may protect human eyes and skin against oxidative damage by natural and synthetic photosensitizers. It is worth mentioning that PABA was not excited by UVC to damage DNA under our experimental conditions (*Table 2*, without addition of Fenton reagents), although our assay method for DNA damage does not measure other types of damage to DNA such as formation of pyrimidine dimers.

In summary, this study supports our hypothesis that, besides being a sunscreen agent and a precursor for folate synthesis in bacteria, PABA is an effective scavenger of certain ROS. Since little is known regarding the metabolism and levels of PABA that can be reached in vivo, it is difficult to relate the present findings to what PABA may do in vivo. Nevertheless, the reaction with HOCl may, in part, explain the anti-inflammatory effect of PABA, as has been suggested previously.[14] Scavenging of ·OH and $^1O_2$ may be related to the protection by PABA against photocarcinogenesis and drug toxicity.

Acknowledgments

This research was supported by the National Science Council, Republic of China (NSC 84-2321-B005-051). We thank Ming-Kuei Shih and Der-Young Chang for technical assistance.

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