Effects of UV-Sensitization of Hematoporphyrin on Lipid Hydroperoxides in Erythrocytes and on Their Hemolysis

Tadashi UCHINO,* Hiroshi TOKUNAGA, Keiji KIJIMA, and Masanori ANDO

*Division of Environmental Chemistry, National Institute of Health Sciences,
1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan*

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We have already reported the effect of ultraviolet-A (UVA)-sensitization of hematoporphyrin (HP) on the production of lipid hydroperoxides in erythrocytes and on their hemolysis. In this report, we investigated these effects under ultraviolet-B (UVB) irradiation and compared the results with those under UVA irradiation. It was found that an increase in lipid hydroperoxide preceded hemolysis, and that the UVB irradiation resulted in an increase in phosphatidylethanolamine hydroperoxide (PEOOH) and 2-thiobarbituric acid reactive substances (TBA-RS) at an earlier stage in comparison with UVA irradiation. Under either UVA or UVB irradiation, hemolysis was inhibited by anti-oxidants such as sodium azide and ascorbic acid (singlet oxygen scavengers), but not by mannitol, sorbitol (hydroxyl radical scavengers) or superoxide dismutase (SOD) (superoxide radical scavenger).

These results suggest that singlet oxygen ($^1\text{O}_2$) produced by UV irradiation peroxidized the lipids of erythrocyte membranes, and therefore, the hemolysis of erythrocytes occurred when the amount of hydroperoxides increased to a constant level, but auto-oxidation of lipids did not affect the hemolysis.

Key words — UVA ; UVB ; lipid hydroperoxide ; hematoporphyrin

Introduction

Among UV radiation emitted from the sun, ultraviolet-A (UVA, 320 — 400 nm) and ultraviolet-B (UVB, 280 — 320 nm) reach the surface of the earth and cause various biologically harmful effects¹⁻⁶⁾ such as sunburn, sun tan, wrinkles, spots and skin cancer. The effects are thought to be caused mainly by the production of activated oxygens such as $^1\text{O}_2$ and super oxide anion (O_2^-),^{7,8)} but the mechanisms of these phenomena has not yet been clarified. In our previous report,⁹⁾ we showed that UVA (350 — 380 nm, max. 370 nm) could cause the hemolysis of rabbit erythrocytes in the presence of a photosensitizer such as hematoporphyrin (HP), and the production of both phosphatidylcholine hydroperoxide (PCOOH) and PEOOH was demon-

strated before the hemolysis of erythrocytes. In addition, singlet oxygen scavengers such as NaN_3 inhibited the hemolysis. These results strongly suggested that $^1\text{O}_2$ produced with UVA in the presence of HP caused the hemolysis.

In this report, in order to clarify whether $^1\text{O}_2$ participates in the mechanisms of the bioeffects of UVB, the effect of UVB (300 — 330 nm, max. 317 nm), instead of UVA, against the erythrocytes was investigated in the presence of HP, and the results were compared with those obtained by UVA irradiation.

Materials and Methods

Reagents — Standards of PCOOH and PEOOH were kindly given by assistant professor T. Miyazawa, Tohoku University. Mannitol and

Fig. 1. Absorption Spectra of UVA Absorber (DDS) and UVB Sunscreen Product (SaA)

DDS was dissolved in a phosphate buffer and SaA in *n*-hexane, both at a concentration of 0.02%.

NaN₃ were purchased from Kanto Chemicals, Japan. Superoxide dismutase and 2-thiobarbituric acid were obtained from Sigma Chemical Company, U.S.A. 2,2'-Dihydroxy-4,4'-dimethoxybenzophenone-5-sulfonic acid (DDS) was a product of Matsumoto Koushyo, Japan. UVB sunscreen product sample A (SaA) (waterproof lotion, sun protection factor (SPF)⁸⁾ was obtained from H & R Company, U.S.A. Other reagents were purchased from Wako Chemicals (Japan). The absorption spectra of DDS and SaA are shown in Fig. 1.

UV Sources — UVA: UV radiator (Kinitsu-kun, Yamashita Densho, Japan), equipped with a mercury-xenon lamp and BPF365 filter, was utilized. Its maximum irradiation wavelength was around 370 nm, and the UVB, ultraviolet-C (UVC) and visible light were mostly eliminated. UVB: The radiator described above was used with a BPF313 filter at a maximum irradiation wavelength of about 317 nm.

UV intensity was measured with a UV Checker (Yamashita Densho, Japan) which was equipped with two different sensors, an SY-365 sensor for UVA and SY-313 sensor for UVB.

Erythrocytes — Rabbit erythrocytes were prepared as described previously.⁹⁾

Measurement of Hemolysis: The isolated erythrocytes were suspended in 10 mM phosphate buffer containing 0.85% NaCl (pH 7.4) to give a 0.2% (v/v) erythrocyte suspension. Two ml of this 0.2% erythrocyte suspension and 4 ml of HP solution were added to a sample vial, and the mixture was irradiated with UVA (350–380 nm) or UVB (300–330 nm) at room temperature (r.t.) for the indicated periods. The intensities of irradiation were 7.0 mW/cm² in both cases. The percentage of hemolysis of the erythrocytes was calculated according to the following equation:

$$\text{hemolysis}(\%) = (A_{740} - A'_{740}) / A_{740} \times 100 \quad (1)$$

A'_{740} = absorbance at 740 nm after irradiation

A_{740} = absorbance at 740 nm before irradiation

Determination of PCOOH, PEOOH, Phosphatidylcoline (PC) and Phosphatidylethanolamine (PE) — The method was basically the same as was described in the previous report.⁹⁾

PCOOH and PEOOH were analyzed by HPLC with a Chemiluminescence (CL) detector, and both PC and PE were analyzed by HPLC with a UV detector.

Analytical conditions were as follows:

CL-HPLC conditions: column, Lichrosorb NH₂ (250 × 4.6 mm i.d. Hibar) or Lichrosorb NH₂-5 (250 × 4.6 mm i.d. GL Sciences Inc.); column temp., 40°C; mobile phase, a mixture of *n*-hexane–2-propanol–H₂O–methanol (9:27:5:4, v/v/v/v, flow rate 1.0 ml/min); post column CL reagent, 10 μg/ml cytochrome c and 2 μg/ml luminol in 50 mM borate buffer (pH 10), flow rate 1.1 ml/min; UV detector: UV spectrophotometric detector (Shimadzu SPD-M6A); detection wavelength: 210 nm; CL detector: Chemiluminescence Analyzer (Soma S-3400).

Measurement of TBA-RS — TBA-RS in erythrocytes were measured according to the method of Ohisi¹⁰⁾ with a slight modification, and the concentration of TBA-RS was calibrated using the absorbance at 532 nm, then the content of TBA-RS per protein was calculated. Protein content was measured according to an improved

Lowry method.¹¹⁾

Results and Discussion

Goder *et al.*, after irradiation of UVB (36 J/cm²) alone onto the sheep erythrocytes, described that hemolysis of the erythrocytes did not occur.¹²⁾ Vargas *et al.* reported that no hemolysis of erythrocytes was observed by irradiation with UVB alone onto the mixture of human erythrocytes, but that hemolysis was almost completely induced in the presence of photosensitizers such as Fenofibrate.¹³⁾ We have already reported that the hemolysis caused by irradiation of UVA (6.3 J/cm²) onto the rabbit erythrocytes in the absence of HP as a photosensitizer was less than 5%, but that complete hemolysis was observed in its presence.⁹⁾

As shown in Fig. 2, UVB irradiation (50.4 J/cm²) for 120 min produced hemolysis of about 26%, which was slightly higher than that in the case of UVA irradiation. It was presumed that under strong UVB irradiation, hemolysis was induced to a certain level by the energy of UVB itself, but under weak

Fig. 2. Effect of Irradiation Time on Hemolysis

Erythrocyte suspension was irradiated with UVB (300–330 nm). Hemolysis was immediately determined after the irradiation. Each point represents the mean of three data.

Fig. 3. Effect of HP Concentration on Hemolysis

Erythrocyte suspension was irradiated for 15 min with UVB (300–330 nm, ○) or UVA (350–380 nm, ●). Hemolysis was determined immediately after the end of irradiation. Each point represents the mean of three data.

Fig. 4. Absorption Spectra of HP

HP was dissolved in a phosphate buffer (pH 7.4).

UVB irradiation, UVB alone did not induce hemolysis, as was the case for UVA irradiation. The hemolysis was measured after irradiating UVB onto the erythrocytes for 15 min at several concentrations of HP. The hemolysis increased, depending on the HP concentration, and 100% hemolysis was obtained with 50 μM (which was a higher concentration compared to UVA irradiation) HP (Fig. 3). These results demonstrated that the sensitizing efficacy of HP with UVB became lower than that with UVA. We considered that the reason is that the absorption maximum of HP was at about 380 nm and its ϵ_{365} value was about 8-fold larger than its ϵ_{313} value (Fig. 4).

In the presence of 10 μM HP, which did not cause hemolysis during UV irradiation, we determined the effect of temperature at r.t. and 0°C on hemolysis. Hemolysis of erythrocytes was not observed during UV irradiation but gradually increased up to 60 min, and hemolysis was observed after termination of the irradiation (Fig. 5). The maximal level of hemolysis was approximately 90% at r.t., and

Fig. 5. Effect of Temperature on Hemolysis of Erythrocytes after UVB (300–330 nm) or UVA (350–380 nm) Irradiation for 15 min

Erythrocyte suspension was irradiated for 15 min with UVB (300–330 nm) in the presence of 10 μM HP or with UVA (350–380 nm) in the presence of 0.5 μM HP. Each point represents the mean of three data.

similar results were obtained also at 0°C. Comparing the time to reach the plateau phase of hemolysis by UVB with that by UVA, the former was shorter than the latter, as shown in Fig. 5.

In our previous report, we suggested that the harmful effect of UVA on erythrocytes in the presence of HP may be caused by the autooxidation of membrane lipids. In order to examine the effects of UVA and UVB, several scavengers such as mannitol, sorbitol, NaN_3 , histidine and ascorbic acid were used. In the case of UVA (Table 1), mannitol, NaN_3 , histidine and ascorbic acid suppressed

Table 1. Effects of Anti-oxidants and Radical Scavengers on Hemolysis in the Presence of HP

Chemicals	(mM)	Hemolysis (%)	Inhibition rate (%)
—	—	95	—
NaN_3	10	43	55
	50	18	81
Histidine	10	94	1
	50	79	17
Mannitol	10	77	19
	50	18	81
Ascorbic acid	1	76	16
	10	3	97

Erythrocyte suspension was irradiated for 15 min by UVA (350–380 nm) with 0.5 μM HP.

Table 2. Effects of Anti-oxidants and Radical Scavengers on Hemolysis in the Presence of HP

Chemicals	(mM)	Hemolysis (%)	Inhibition rate (%)
—	—	91	—
NaN_3	10	92	0
	50	20	78
Histidine	10	93	0
	50	69	24
Mannitol	50	88	3
Sorbitol	50	79	13
Ascorbic acid	1	76	16
	10	11	88
SOD	3825 U/ml	92	0

Erythrocyte suspension was irradiated for 15 min by UVB (300–330 nm) with 10 μM HP.

Table 3. Effect of UVA Irradiation on TBA-RS Content Using 0.2% Rabbit Erythrocytes

		TBA-RS (nmol/mg protein)		
		r.t.	0°C	DDS 0.4 mm
Control			5.80 ± 0.32 ^{a)}	
UVA 15 min irradiation	After 0 min		6.00 ± 0.26 ^{a)}	5.74 ± 0.32 ^{b)}
	After 10 min	6.74 ± 0.60 ^{**b)}	5.80 ± 0.44 ^{#b)}	5.68 ± 0.43 ^{#b)}
	After 20 min	6.86 ± 0.46 ^{***b)}	6.16 ± 0.46 ^{#b)}	5.77 ± 0.50 ^{##b)}
	After 30 min	7.00 ± 0.46 ^{***b)}	6.16 ± 0.36 ^{#b)}	5.81 ± 0.32 ^{##b)}

a) Data are means ± S.D. of ten measurements. b) Data are means ± S.D. of five measurements. Significantly different from control, * $p < 0.01$, *** $p < 0.001$. Significantly different from r.t. group, # $p < 0.05$, ## $p < 0.01$.

the hemolysis of erythrocytes. As for UVB (Table 2), NaN_3 and ascorbic acid completely blocked the hemolysis, but the suppressive effect of the other scavengers was less potent. SOD showed no effect on hemolysis. Since mannitol and sorbitol cause the oxygen radical to disappear, NaN_3 eliminates the singlet oxygen, and ascorbic acid has both actions, we concluded that a certain amount of $^1\text{O}_2$ produced by UVB in the presence of HP directly attached to the erythrocyte membranes. In addition, it is conceivable that

histidine suppressed hemolysis less than NaN_3 because of less potent $^1\text{O}_2$ scavenging compared with NaN_3 , but that was not clarified.

We have previously reported⁹⁾ a significant increase in PCOOH and PEOOH after UVA irradiation of a 20% erythrocyte suspension in the presence of 50 μM HP. In this report, when TBA-RS was examined with the mixture of 0.5 μM HP and a 0.2% erythrocyte suspension, a significant increase in TBA-RS was observed at the standing time of more than 10 min at r.t., but TBA-RS did not

Fig. 6. Effects of UVA and UVB Irradiation on PCOOH and PEOOH Content in 20% Rabbit Erythrocyte Suspension

Data are means ± S.D. of five experiments. The data were significantly different from the control group without UV irradiation, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. —○—, PEOOH (UVB); —△—, PCOOH (UVB); —●—, PEOOH (UVA); —▲—, PCOOH (UVA).

increase at 0°C because auto-oxidation was suppressed (Table 3).

When 0.4 mM DDS (UVA absorber), which suppressed hemolysis by approximately 80%, was added, the increase in PCOOH and PEOOH was significantly inhibited because UVA, and consequently, the production of lipid hydroperoxides with $^1\text{O}_2$, was blocked (Table 3).

In the next experiment, a 20% erythrocyte suspension was irradiated by UVB in the presence of 1 mM HP for 15 min, and the contents of both PCOOH and PEOOH were measured until 30 min after the irradiation of UVB. The content of PEOOH was higher than that of PCOOH. Both PCOOH and PEOOH started to increase immediately after the irradiation and returned to the control level at 20 min. The amount of PEOOH produced by UVB irradiation showed an earlier decrease compared to that produced by UVA irradiation (Fig. 6).

It is conceivable that the faster increase of PEOOH under UVB irradiation than under UVA irradiation may have caused the appearance of $^1\text{O}_2$ under UVB irradiation in the presence of 10 μM HP to be faster than that under UVA in the presence of 0.5 μM HP. However, the appearance of $^1\text{O}_2$ under UVB irradiation and that under UVA irradiation is not clear. Therefore, further study is required. When a 0.2% erythrocyte suspension was irradiated by UVB for 15 min in the presence of 10 μM HP and the contents of TBA-RS

were measured until 30 min, TBA-RS was observed to be increased at the end of the irradiation, and returned to the control level at 10 min (Table 4). By the addition of SaA at a concentration of 0.08%, which blocked about 80% hemolysis, the TBA-RS content was significantly decreased, similarly to the case of UVA irradiation. These results suggested that SaA blocked UVB and, consequently, the production of lipid hydroperoxides with $^1\text{O}_2$ (Table 4).

The present results suggest that hemolysis under UV irradiation could be caused by sequential events, *i.e.*, the production of $^1\text{O}_2$, the oxidation of membrane lipids, and an increase of PCOOH and PEOOH to a certain level. Since mannitol and sorbitol protected erythrocytes very little from hemolysis by UVB irradiation, and no hemolysis was observed at 0°C, it is conceivable that the difference in hemolysis by UVB irradiation and that by UVA irradiation could be explained if the production rate of $^1\text{O}_2$ under UVB irradiation in the presence of 10 μM HP was faster than that under UVA in the presence of 0.5 μM HP. However, the comparative production rate of $^1\text{O}_2$ under UVB irradiation and that under UVA irradiation is not clarified so we will do further investigation. It is conceivable that PEOOH may contribute to the hemolysis more than PCOOH. We will study the quantitative correlation between hemolysis and the contents of these hydroperoxides, as well as the production rate of $^1\text{O}_2$.

Table 4. Effect of UVB Irradiation on TBA-RS Content Using 0.2% Rabbit Erythrocytes

		TBA-RS (nmol/mg protein)		
		r.t.	0°C	SaA 0.08%
Control			6.26±0.89 ^{a)}	
UVB 15 min irradiation	After 0 min		7.24±0.92* ^{a)}	5.84±0.14** ^{b)}
	After 10 min	6.49±0.92 ^{b)}	5.85±0.90 ^{b)}	5.83±0.15 ^{b)}
	After 20 min	6.35±0.80 ^{b)}	6.14±0.87 ^{b)}	5.98±0.16 ^{b)}
	After 30 min	6.71±0.89 ^{b)}	6.17±0.86 ^{b)}	5.81±0.59 ^{b)}

a) Data are means±S.D. of ten measurements. b) Data are means±S.D. of five measurements. Significantly different from control, * $p < 0.05$. Significantly different from r.t. group, ## $p < 0.01$.

under UVB irradiation and that under UVA irradiation.

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