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Reaction of *para*-hydroxybenzoic acid esters with singlet oxygen in the presence of glutathione produces glutathione conjugates of hydroquinone, potent inducers of oxidative stress

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Abstract

The determination and toxicological characterization of products of the reaction between *p*-hydroxybenzoic acid esters (parabens) and singlet oxygen ($^1O_2$) are very important because of the frequent use of parabens in cosmetics and possible generation of $^1O_2$ in the skin. We observed $^1O_2$-dependent production of mono-, di-, and tri-substituted glutathione (GSH) conjugates of hydroquinone (HQ) during visible light-irradiation of a mixture of methyl or ethyl paraben and GSH in the presence of rose bengal (RB). 1,4-Benzoquinone (BQ) and HQ were produced during the irradiation in the absence of GSH. While a mixture of BQ and GSH produced only mono-substituted conjugate, irradiation of the mixture with RB produced mono-, di-, and tri-substituted conjugates. These observations indicate that $^1O_2$ is involved both in the production of BQ and HQ from parabens and in the formation of multi-substituted GSH conjugates from mono-substituted conjugate. Tri-substituted conjugate generated larger amounts of hydrogen peroxide in an aqueous solution than mono-substituted conjugates or HQ did. Detection of semiquinone radical suggests that the autoxidation of conjugates is related to the generation of hydrogen peroxide. The results obtained in this study indicate that parabens may induce oxidative stress in the skin after conversion to GSH conjugates of HQ by reacting with $^1O_2$ and GSH.

Keywords: *Parabens, singlet oxygen, glutathione conjugates, hydrogen peroxide, semiquinone radical, EPR*

Abbreviations: *BQ, 1,4-benzoquinone; 2,5-DGHQ, 2,5-(diglutathion-*S*-yl)hydroquinone; 2,6-DGHQ, 2,6-(diglutathion-*S*-yl)hydroquinone; GSH, reduced glutathione; HBA, p-hydroxybenzoic acid; H_2O_2, hydrogen peroxide; HQ, hydroquinone; HQ-GSH conjugates, glutathione conjugates of hydroquinone; MGHQ, 2-(glutathion-*S*-yl)hydroquinone; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, superoxide anion radical; $^{\cdot}OH$, hydroxyl radical; PB, 20 mM sodium phosphate buffer pH 7.4; RB, rose bengal; TGHQ, 2,3,5-(triglutathion-*S*-yl)hydroquinone*

Introduction

Parabens are esters of *p*-hydroxybenzoic acid (HBA), having a variety in their ester group. Parabens are widely used as antimicrobial preservatives in cosmetics, foods, and drugs. Concentrations of parabens in cosmetics are usually 0.3–1%, about two orders of magnitude higher than in the foods in which the compounds are permitted for use [1–3]. Methyl paraben is the most frequently used antimicrobial preservative in cosmetics [1]. The metabolism and potential toxicity of parabens have been extensively investigated *in vitro* and *in vivo*. When parabens are administered orally, they are readily hydrolyzed to HBA, metabolized to conjugates of glucuronic acid, sulfuric acid and glycine in the liver and kidney, and

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then excreted in the urine [1–3]. They do not accumulate in the body. For this reason, along with the results of acute, subchronic and chronic *in vivo* studies, parabens are considered practically non-toxic. On the other hand, they sometimes induce eczema, dermatitis or allergies [1–3], and several cases of excessive hyperpigmentation following exposure to methyl paraben and sunlight have also been reported [4].

Propionibacterium acnes, a bacterium on the surface of the skin, contains a photosensitizer called coproporphyrin. In addition, there are endogenous photosensitizers in the skin such as a few kinds of porphyrins and flavins. A great deal of protoporphyrin accumulates in the skin of patients with protoporphyria [5]. Singlet oxygen ($^1O_2$) is generated in human skin and the skin of healthy or porphyria mice exposed to ultraviolet or visible light [6–8]. Briviba et al. reported that 1,4-benzoquinone (BQ) and hydroquinone (HQ) were selectively produced by the reaction of phenol with $^1O_2$ generated by photosensitization of methylene blue [9]. However, the reaction of parabens, phenolic compounds having a substituent at para-position, with $^1O_2$ is at present unclear. Furthermore, the products of the reaction in the presence of biological substances and the toxicity of the products are not known at all.

In the present study, we examined products formed by the reaction of methyl and ethyl parabens or their hydrolysate, HBA, with $^1O_2$ in the presence of reduced glutathione (GSH). The result is the formation of GSH conjugates of HQ (HQ–GSH conjugates), which generate hydrogen peroxide (H_2O_2) in their aqueous solutions.

Materials and methods

Materials

Rose bengal (RB), reduced GSH, methyl *p*-hydroxybenzoate, ethyl *p*-hydroxybenzoate, xylenol orange, D-sorbitol, and deutrium oxide (D_2O) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), HBA and BQ from Sigma Inc. (St Louis, MO, USA), and HQ from Nakarai Chemicals, Ltd. (Kyoto, Japan). HQ–GSH conjugates were synthesized as previously described [10]. In brief, a mixture of GSH and equimolar BQ in water was stirred at room temperature for 2 h. After removal of residual BQ by extraction with ethyl acetate, 2-(glutathion-*S*-yl)hydroquinone (MGHQ), 2,5-(diglutathion-*S*-yl)hydroquinone (2,5-DGHQ), 2,6-(diglutathion-*S*-yl) hydroquinone (2,6-DGHQ), and 2,3,5-(triglutathion-*S*-yl)hydroquinone (TGHQ) were purified by preparative HPLC (HPLC system, CCP & 8020 system, Tosoh, Tokyo, Japan; column, Whatman magnum 9 ODS-3 reverse phase semipreparative column, 9.4 × 250 mm; eluate, methanol/water/acetic acid (5:94:1); flow rate, 3 ml/min) and lyophilized. Structural characterization of the products were performed by ^{1}H-NMR spectroscopy as described in Ref. [10]. All other reagents were of the highest purity commercially available. Pure water was freshly prepared with a Millipore Milli-Q Labo (Bedford, MA, USA).

Reaction of parabens or HBA with $^1O_2$

A sample solution containing 1 mM parabens or HBA and RB with or without GSH in 20 mM sodium phosphate buffer, pH 7.4 (PB) was transferred into a quartz flat cell (Labotec, Tokyo, Japan) and irradiated (0.7 W/m^2) with visible light (tungsten bulb, Philips AP-12, 750 W) at room temperature. In some experiments, reactions were performed in PB prepared with 90% D_2O. Addition of sodium azide and bubbling with argon gas was carried out before irradiation.

HPLC analysis of products

HQ, BQ and HQ–GSH conjugates were analyzed by HPLC with an electrochemical detector (Coulochem II, ESA, USA) equipped with a Model 5011 analytical cell. HPLC was carried out using a CCP & 8020 system (Tosoh) with a TSK-GEL Octyl 80-Ts reverse phase column (4.6 × 250 mm, Tosoh). The potential of the first electrode was set at −350 mV, and that of the second at 400 mV in the oxidative mode. The HPLC mobile phase consisted of methanol/water/acetic acid (5:94:1). Elution was performed at a flow rate of 1.0 ml/min and at a column temperature of 25°C. Retention times of products were checked every time with authentic standards.

H_2O_2 assay

A solution of HQ–GSH conjugates, parabens, HQ or BQ (2 mM) was prepared with saline purged with argon to prevent autoxidation before use. The solution was diluted 10-fold with air-saturated Dulbecco's phosphate buffered saline (DPBS) and incubated at 37°C for 30 min. The concentration of H_2O_2 in the solutions was measured by a modified method of ferrous oxidation in xylenol orange (FOX1 assay) [11]. Fifty microlitre of sample solution was added to 950 µl of FOX1 reagent (100 µM xylenol orange, 250 µM $FeSO_4$, 100 mM sorbitol and 25 mM H_2SO_4), vortexed and incubated for 30 min at room temperature. The absorbance of the blue–purple complex that formed was read at 560 nm. The H_2O_2-specificity of the complex formation was checked with catalase. The concentration of standard H_2O_2 solution was determined spectrophotometrically ($\epsilon = 43.5\ M^{-1}\ cm^{-1}$ at 240 nm).

EPR measurement

HQ–GSH conjugate or HQ was dissolved in air-saturated DPBS at a concentration of 800 μM and transferred into a 100 μl disposable micropipette (Drumond Scientific Co., Broomall, PA). X-band EPR spectra were recorded using a JEOL JES TE-100 spectrometer at 25°C. Spectrometer settings were microwave power, 4 mW; amplitude of 100 kHz field modulation, 0.079 mT. Spectral simulation was performed with an isotropic EPR spectrum simulation system for JES FE series, ver. 1.01 (JEOL).

Results

Formation of HQ–GSH conjugates by the reaction of paraben with $^1O_2$ in the presence of GSH

An aqueous solution of methyl paraben containing GSH and RB, a photosensitizer, was irradiated with visible light for 2 min, and the products were analyzed by HPLC. As shown in Figure 1A, HQ–GSH conjugates such as TGHQ, MGHQ, 2,5-DGHQ and 2,6-DGHQ were detected. TGHQ and MGHQ were major products. The formation of HQ–GSH conjugates was not observed in the dark or under illumination in the absence of RB (data not shown). Either argon-bubbling or addition of sodium azide, a quencher of $^1O_2$, before irradiation almost completely suppressed the formation of any HQ–GSH conjugates (Figure 1B, C). The replacement of 90% of the H_2O with D_2O (which increases the lifetime of $^1O_2$ [12]) resulted in a remarkable increase in the formation of HQ–GSH conjugates (Figure 1D). Similarly, the GSH conjugates were obtained by using ethyl paraben or HBA instead of methyl paraben (Table I). The production of HQ–GSH conjugates from these compounds responded to the argon-bubbling, the presence of sodium azide, or the replacement of H_2O with D_2O, as observed in the case of methyl paraben (data not shown). These results indicate that $^1O_2$ is involved in the formation of HQ–GSH conjugates from parabens or HBA in the presence of GSH.

Products obtained by the reaction of parabens with $^1O_2$ in the absence of GSH

To analyze the mechanism for the formation of HQ–GSH conjugates, an aqueous solution of parabens or HBA was irradiated with RB in the absence of GSH. BQ and HQ were produced in both cases (Figure 2A). The amount of BQ produced during 2 min irradiation was much larger than that of HQ. Neither BQ nor HQ was observed in the dark or under illumination in the absence of RB (data not shown). The replacement of 90% of the H_2O with D_2O resulted in a remarkable increase in the generation of BQ and HQ (Figure 2B).

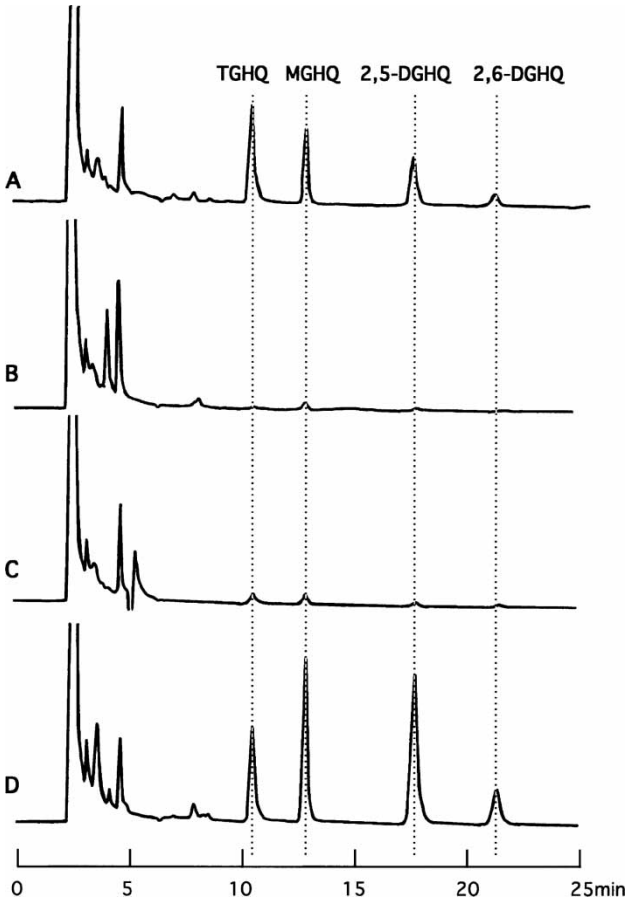


Figure 1. HPLC chromatogram of products formed during irradiation of methyl paraben and GSH in the presence of RB. A, A sample solution containing 1 mM methyl paraben, 1 mM GSH and 14 μM RB in air-saturated PB was irradiated with visible light for 2 min. B, The same as A except for argon-bubbling for 3 min before irradiation. C, The same as A except for the addition of sodium azide (5 mM) before irradiation. D, The same as A except for the replacement of 90% of H_2O with D_2O.

Thus, parabens and HBA reacted with $^1O_2$ to produce both BQ and HQ in the absence of GSH.

HQ can be converted to BQ by oxidation, although determination of products is necessary to characterize the mechanism of the reaction between parabens and $^1O_2$. Therefore, the production of BQ and HQ was traced over time under three different conditions of $^1O_2$ to determine initial products of this reaction. The

Table I. MGHQ and TGHQ formed by irradiation of methyl paraben, ethyl paraben, or HBA with RB in the presence of GSH. A sample solution containing 1 mM parabens or HBA, 1 mM GSH and 14 μM RB in air-saturated PB was irradiated with visible light for 2 min. The values are indicated as mean ± standard deviation ($n = 3$).

	MGHQ (μM)	TGHQ (μM)
Methyl paraben	4.7 ± 0.6	5.2 ± 0.2
Ethyl paraben	4.7 ± 0.6	4.9 ± 0.8
HBA	8.1 ± 0.2	1.8 ± 0.0

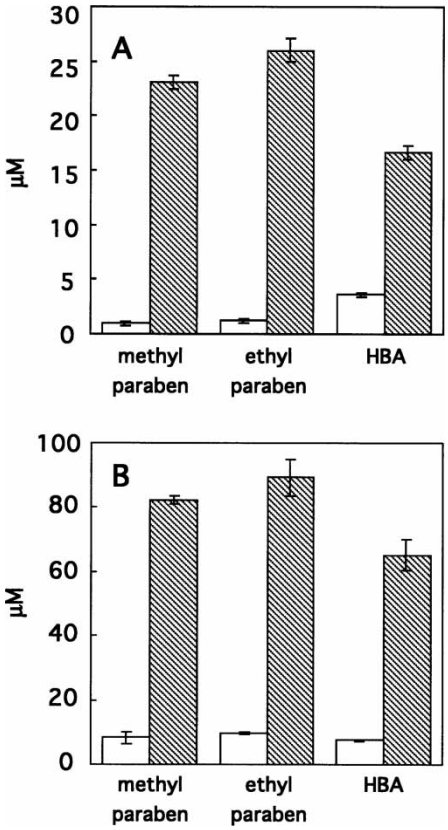


Figure 2. Production of HQ and BQ during irradiation of methyl paraben in the presence of RB. Sample solutions containing 1 mM methyl paraben, ethyl paraben or HBA and 14 μM RB in PB prepared with H_2O (A) or 90% D_2O (B) were irradiated with visible light for 2 min, and HQ (open column) and BQ (hatched column) were determined. The values are the average of three experiments, and the bars indicate standard deviation.

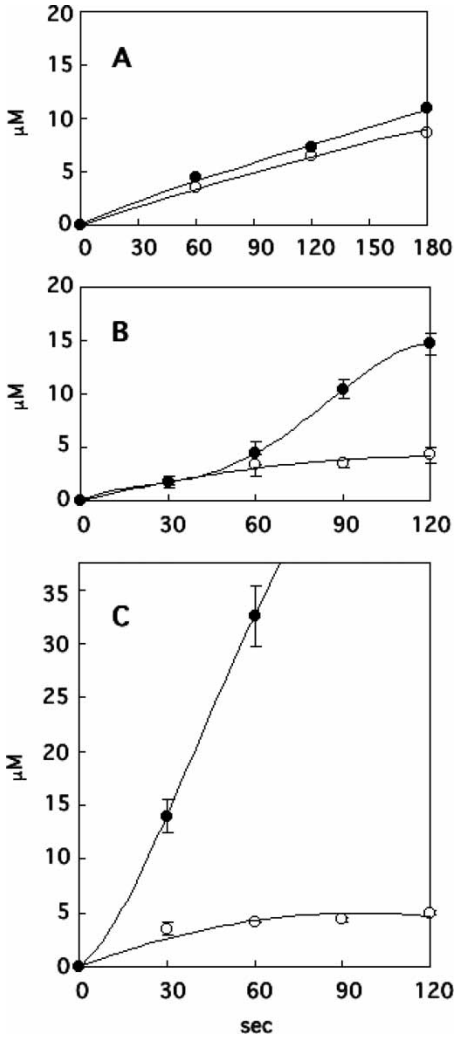


Figure 3. Effect of $^1O_2$ production on time course of HQ and BQ production during irradiation of HBA in the presence of RB. A sample solution containing 1 mM HBA and 10 μM RB in PB (A) or 1 mM HBA and 14 μM RB in PB (B and C) was irradiated with visible light. PB for C contained 90% D_2O. Open circle, HQ; filled circle, BQ.

amount of BQ was almost the same as that of HQ for at least 180 s after starting irradiation in the presence of 10 μM RB (lower RB concentration than that used above) (Figure 3A). However, when the reaction occurred in the presence of 14 μM RB, the amount of BQ increased and was higher than that of HQ at 60 s after starting irradiation (Figure 3B). The increase in BQ was remarkable from the beginning of the irradiation, when 90% of H_2O was replaced with D_2O (Figure 3C). Methyl paraben, ethyl paraben and HBA all showed similar characteristics. A mechanism for this reaction will be discussed later.

Relationship between BQ production and formation of HQ–GSH conjugates

An aqueous solution of methyl paraben containing RB was irradiated for 2 min in the presence of various concentrations of GSH, and then reaction products were determined. The amount of BQ decreased with an increase in GSH concentration up to 50 μM, while the amount of HQ was almost constant (Table II). In accordance with the decrease in BQ, the amount

Table II. GSH dose-dependence of products formed during irradiation of methyl paraben and GSH in the presence of RB. Samples containing 1 mM methyl paraben, 14 μM RB, and GSH in PB were irradiated with visible light for 2 min. The products were quantified with HPLC. The experiments were repeated 5 times independently, and the similar results were obtained.

GSH (μM)	HQ (μM)	BQ (μM)	MGHQ (μM)	TGHQ (μM)
0	4.1	22.0	0	0
10.0	3.4	20.0	0.3	0
25.0	3.3	6.7	0.7	0
37.5	4.0	1.2	3.0	0.6
50.0	2.5	0	5.1	2.7
62.5	1.0	0	5.5	4.8
75.0	0.6	0	5.4	6.1
100.0	0.4	0	4.8	7.4
250.0	0	0	4.2	8.3

of MGHQ increased. 2,5-DGHQ, 2,6-DGHQ and TGHQ were clearly detected besides MGHQ after the reaction in the presence of GSH at a concentration of more than 37.5 μM, suggesting that multi-substituted conjugates are produced after formation of MGHQ.

The GSH dose-dependency of production of GSH conjugates leads to the hypothesis that the HQ–GSH conjugates may result from a reaction of GSH with BQ produced by a reaction of parabens with $^1O_2$. To examine this hypothesis, a mixture of BQ and GSH was left for 2 min, a period corresponding to that of the irradiation carried out above. As shown in Figure 4A, only MGHQ was formed in the dark. No reaction occurred in a mixture of HQ and GSH (data not shown). It is noteworthy that both RB and irradiation were necessary to form multi-substituted HQ–GSH conjugates such as 2,5-DGHQ, 2,6-DGHQ and TGHQ in a mixture of BQ and GSH (Figure 4B–D). These observations indicate that the formation of multi-substituted HQ–GSH conjugates from MGHQ is also $^1O_2$-dependent.

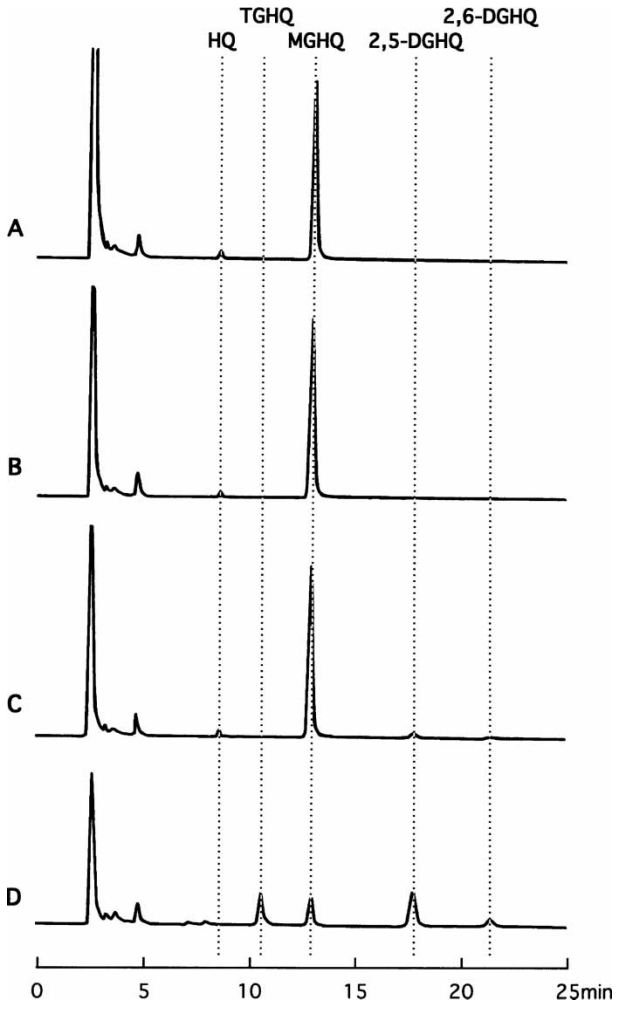


Figure 4. HPLC chromatogram of products formed by the reaction of BQ and GSH. A sample solution containing 25 μM BQ and 1 mM GSH in PB was left for 2 min without (A) or with (B) exposure to visible light. The same sample solution as above was left in the presence of 14 μM RB for 2 min without (C) or with (D) exposure to visible light.

Generation of H_2O_2 from HQ–GSH conjugates

Autoxidation of HQ generates $O_2^{\cdot-}$, which is converted to H_2O_2 by disproportionation [5,13]. H_2O_2 is capable of causing oxidative stress in biological systems through the generation of highly reactive $^{\cdot}OH$ [5] and the modification of signal transductions [5,14,15]. To elucidate whether or not HQ–GSH conjugates generate H_2O_2, aqueous solutions containing the conjugates were incubated for 30 min at 37°C. As shown in Figure 5, TGHQ and MGHQ generated H_2O_2 as observed with HQ. The amount of H_2O_2 from TGHQ was remarkably larger than that from MGHQ and HQ. On the other hand, parabens, HBA, and BQ hardly generated H_2O_2.

Aqueous solutions of TGHQ and MGHQ gave EPR signals reasonably assigned to their semiquinone-type radicals (Figure 6), suggesting that the H_2O_2 results from $O_2^{\cdot-}$ generated by autoxidation of HQ–GSH conjugates.

Discussion

In this study, we observed the generation of HQ–GSH conjugates by the reaction of parabens with $^1O_2$ generated by a photosensitizing reaction of RB in the presence of GSH. $^1O_2$ is probably involved in the reaction because the yield of the conjugates decreases with either the presence of sodium azide or removing oxygen by argon-bubbling, and increases with the replacement of H_2O by D_2O. BQ and HQ were products of the reaction in the absence of GSH. This indicates that the HQ–GSH conjugates are formed by the addition of GSH to BQ produced by the reaction of parabens with $^1O_2$, because it has been already reported that Michael addition of GSH to BQ forms

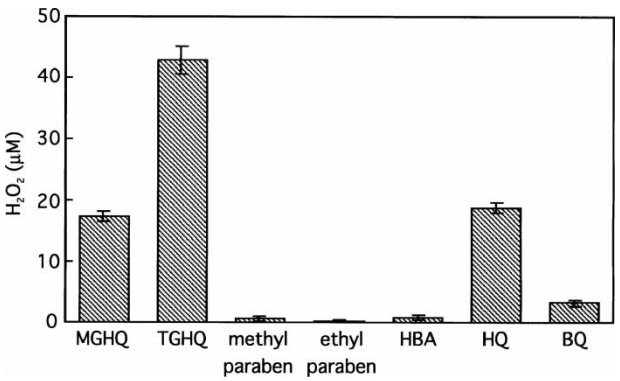


Figure 5. Generation of H_2O_2 in an aqueous solution of HQ–GSH conjugates, parabens, HBA, HQ or BQ. MGHQ, TGHQ, methyl paraben, ethyl paraben, HBA, HQ, or BQ (200 μM) in DPBS was incubated at 37°C for 30 min. The values are the average of three experiments, and the bars indicate standard deviation.

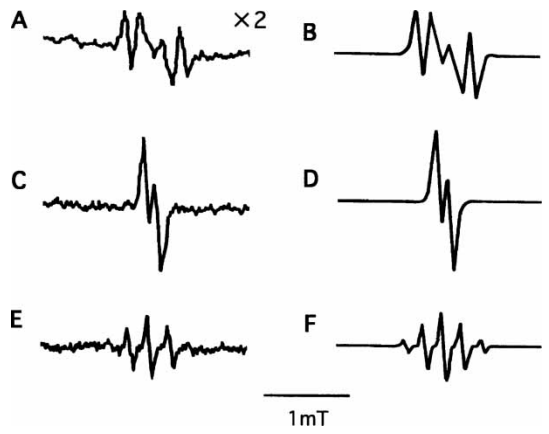


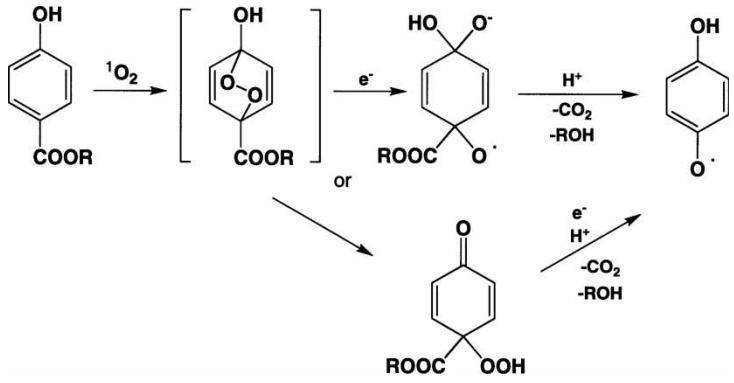
Figure 6. ESR spectra of semiquinone-type radicals generated from HQ–GSH conjugates and HQ. MGHQ (A), TGHQ (C), or HQ (E) was dissolved in DPBS at 800 μM and measured with an X-band EPR spectrometer. B, D, and F are simulated spectra for A, C, and E, respectively. Parameters for simulation are $a^{H} = 0.274$ mT (1H), $a^{H} = 0.212$ mT (1H), $a^{H} = 0.139$ mT (1H), $g = 2.0054$, and line width = 0.101 mT (100% gaussian) for B, $a^{H} = 0.126$ mT (1H), $g = 2.0056$, and line width = 0.101 mT (100% gaussian) for D, and $a^{H} = 0.222$ mT (4H), $g = 2.0054$, and line width 0.090 mT (100% gaussian) for F.

HQ–GSH conjugates [10,16]. HQ–GSH conjugates generated H_2O_2 in an aqueous solution at physiological pH. The ability of the H_2O_2 production for TGHQ was remarkably higher than that for HQ, a product of the reaction under the GSH-free condition. The detection of semiquinone radicals derived from HQ–GSH conjugates indicates that H_2O_2 is probably produced by disproportionation of $O_2^{\cdot-}$ which is generated by coupling with autoxidation of HQ–GSH conjugates and/or their semiquinone radicals as described elsewhere [5,13]. HQ–GSH conjugates are formed in the liver as metabolites of benzene, phenol and HQ and are nephrotoxicants [10,17]. The conjugates reportedly increase in cytotoxicity after cleavage of glutathionyl group by γ-glutamyl transpeptidase and other enzymes in renal proximal tubule epithelial cells [10,18]. In addition to the nephrotoxicity of the conjugates, the present study suggests that HQ–GSH conjugates may form in the skin and induce oxidative stress there.

Parabens and HBA were transformed to BQ and HQ by the reaction with $^1O_2$. The amount of BQ was almost the same as that of HQ under a relatively low concentration of $^1O_2$, while the amount of BQ was dominant under a relatively high concentration of $^1O_2$ (Figure 3). The reaction of HQ with $^1O_2$ resulted in production of BQ (data not shown). These observations imply involvement of two mechanisms in the production of BQ. BQ and HQ may be produced simultaneously at an initial step, and the reaction of HQ with $^1O_2$ at a subsequent step may produce more BQ. Briviba et al. [9] proposed that the reaction of HQ with $^1O_2$ produces an unstable endoperoxide, which is subsequently reduced (two-electron transfer) by another HQ to produce two BQ molecules with one water molecule.

We have to consider the involvement of $^1O_2$ in the formation of multi-substituted HQ–GSH conjugates such as TGHQ, because the mixture of BQ and GSH produces only MGHQ at relatively high concentrations after standing for 2 min. The most probable mechanism is as follows: the exposure of MGHQ to excess $^1O_2$ may produce benzoquinone-typed MGHQ by a mechanism similar to the one described above, and further GSH-addition may occur to form 2,5- or 2,6-DGHQ. Repetition of this reaction may result in the production of TGHQ.

The mechanism for the initial reaction, that is the reaction of parabens with $^1O_2$ to form HQ and BQ, is at present unclear, although a few mechanisms have been proposed for the production of BQ and HQ by reactions of phenolic compounds with $^1O_2$. Formation of an endoperoxide intermediate was postulated for the reaction of phenol or 4-substututed phenols [19–22]. Briviba et al. [9] proposed that an endoperoxide of phenol is reduced (transfer of two electrons) by phenol, a parent compound, resulting in the production of HQ, which subsequently reacts with $^1O_2$ to produce BQ. A mechanism of BQ production from the endoperoxide via a hydroperoxycyclohexadienone



Scheme 1. Proposed mechanism for a reaction of parabens with $^1O_2$ to produce semiquinone radical.

was also proposed [21,23]. However, these mechanisms do not account for our observation that HQ and BQ were simultaneously produced at the initial step. It is generally accepted that disproportionation of two semiquinone radicals produces HQ and BQ. Therefore, the generation of a semiquinone radical may be involved in the mechanism for the simultaneous production of HQ and BQ in the present reaction. Considering the formation of a semiquinone radical and elimination of substituents at para-position, a possible mechanism is shown in Scheme 1. A parent parabens/HBA may provide an electron to an endoperoxide or a hydroperoxycyclohexadienone, resulting in the generation of semiquinone radicals.

Parabens, especially methyl paraben, are extensively used in cosmetics at high concentrations (0.3–1%) and easily penetrate the skin [1–3]. The skin contains endogenous and exogenous porphyrins and other photosensitizers, sometimes at high levels [5]. The partial pressure of oxygen in the epidermis is estimated to be higher than in the other tissues because of direct exposure to air [24]. GSH content in the human epidermis is at the level of about $1\,mmol\,kg^{-1}$ [25]. Under such conditions, multi-substituted HQ–GSH conjugates such as TGHQ should probably be produced in the skin by exposure to visible or ultraviolet light. As demonstrated in the present study, TGHQ has the ability to generate large amounts of H_2O_2, compared with MGHQ and HQ, through autoxidation, which would be enhanced by the high concentration of oxygen in the skin. It is widely accepted that H_2O_2 readily penetrates biomembranes and decomposes to $^{\cdot}OH$ and other reactive oxidants through the action of ultraviolet light or trace metals. The involvement of oxidative stress caused by these reactive oxygen species in the inflammation and aging of the skin has been already reported [26,27]. On the other hand, the continuous consumption of GSH by the formation of HQ–GSH conjugates should enhance the oxidative stress in the skin, because GSH is an important antioxidant in biological systems. Thus, parabens are potential pro-oxidants in the skin. Attention should be paid to the use of paraben-containing cosmetics, especially by patients of protoporphyria.

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