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Protective Effect of Stilbenes on Oxidative Damage

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Abstract – Oxidative stress induced by reactive oxygen species (ROS) has been suggested to be the cause of various degenerative diseases as well as aging. To evaluate the antioxidant potential of stilbenes, we have investigated the cytoprotective effect of 10 stilbenes derived from plants on the oxidative stress induced by tertiary butyl hydroperoxide (*t*-BuOOH). Of the stilbenes tested, piceatannol (**3**) showed the most potent activity, which was further investigated using an animal model. When **3** (30 or 10 mg/kg) was topically administered prior to UVB irradiation, the amount of the thiobarbituric acid reactive substances (TBARS) was significantly reduced compared to that of the control (vehicle). Our findings suggest that piceatannol is capable of protecting cells and tissues from oxidative stress.

Keywords – oxidative stress, stilbenes, tertiary butyl hydroperoxide, UVB irradiation, piceatannol, TBARS

Introduction

Stilbenes are a group of plant polyphenols that are found in many families of higher plants. They have recently attracted a great deal of attention for their biological activities. Although several studies on the antioxidant activity of stilbenes have been reported (Fauconneau, *et al*., 1997; Wang, *et al*., 1999; Ryu, *et al*., 1999), previous studies used only a limited assay system. It is believed that various pathologies including cancer are directly related to intracellular oxidative stress such as lipid peroxidation and DNA damage (Black, *et al*., 1997; Spiteller, 2001). Therefore, an evaluation of the protective effect on oxidative stress induced cell damage is warranted. In this study, we investigated the protective effect of 10 stilbenes derived from plants on the oxidative stress induced by *t*-BuOOH and the structure-activity relationship. An *in vivo* study of piceatannol (**3**), which exhibited the most potent activity among the tested stilbenes was also performed using an oxidative skin damage model induced by UVB irradiation.

Experimental

Stilbenes – Resveratrol (**1**) and piceatannol (**3**) were isolated from *Cercis chinensis*, which were identified by comparison with reported data (Wang, *et al*., 1999; Kashiwada, *et al*., 1984). Oxyresveratrol (**2**) was obtained from a twig of *Morus alba* (Jin, *et al*., 2002). Rhaponticin (**6**) and deoxyrhaponticin (**7**) were purchased from Sigma (USA). Rhapontigenin (**4**) and deoxyrhapontigenin (**5**) were obtained from the acid hydrolysis of **6** and **7**, respectively (Kashiwada, *et al*., 1984). Piceid (**8**), piceid-2"-gallate (**9**) and piceid-2"-coumarate (**10**) were obtained from a root of *Pleuropterus ciliinervis* (Lee, *et al*., 2003). The purity of these stilbenes was > 98%.

Cell Culture – Human epidermal keratinocytes-Neonatal/Foreskin (HEK-N/F) were purchased from Modern Tissue Technologies, Inc. (MC1312, Korea). The HEK-N/F cells were cultured on a type IV collagen coated plate with KGM® Bulletkit medium (CC-3111, Clonetics) in humidified atmosphere of 5% CO_2/95% air at 37 °C, and cultured to 90% confluence.

***t*-BuOOH induced oxidative stress** – The HEK-N/F cells (1×10^4 cells/100 mL) were seeded on a 96 well microplate and were precultured for 24 h. They were then treated with 1 μL of the sample (30 μM) and 10 μl of *t*-BuOOH (1.5 mM) dissolved in Hank's balanced salt solution (HBSS, Gibco. BRL) for 3 h to induce cellular peroxidation. The cell viability was measured using the MTT method. The inhibitory activity of lipid peroxidation was also determined using a thiobarbituric acid (TBA) method as previously described (Park, *et al*., 2000).

***In vivo* study** – Female SKH-1 hairless mice (6 weeks old) were housed under standard conditions (temperature 24 ± 2 °C; relative humidity 50 ± 10%; 12 h/day light/dark

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cycle) and given a commercial diet and water *ad libitum*. The mice were divided into four groups containing five mice each. **3** was administered topically at dose of 30 or 10 mg/kg on their dorsal skin, and the dorsal skin of the mice was irradiated with UVB within 30 min of administration as described previously (Kobayashi, *et al*., 1996). After 24 h, the dorsal skin was collected from each mouse and frozen at −70 °C until used.

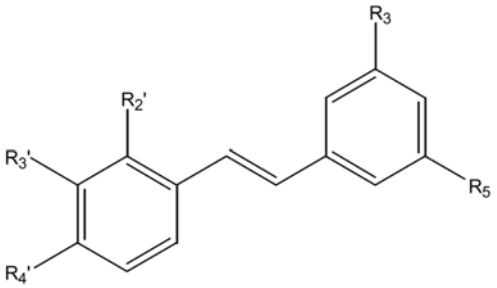
Assay of lipid peroxidation – The dorsal skin was homogenized in 10 volumes of 50 mM phosphate buffer (pH 7.8) under 4 °C. The extent of lipid peroxidation was measured using the TBA method (Ohkawa, *et al*., 1979). The proteins were determined using the dye binding method using a Bio-Rad protein assay kit (Bio-Rad Lab., USA).

Statistical analysis – The results are expressed as a mean ± SD, and the differences between the control and test groups were analyzed using a two-tailed Student's t-test. Differences $P < 0.05$ were considered to be statistically significant.

Results and Discussion

t-BuOOH is a hydroperoxidant that can be metabolized to free radical intermediates, which can subsequently initiate lipid peroxidation resulting in cell damage (Nishida, *et al*., 1997). The cytoprotective effect of the stilbenes (**1 - 10**) induced by *t*-BuOOH is shown in Table 1. The cell viability of the HEK-N/F cells was significantly reduced to 11.2 ± 1.6% when they were treated with 1.5 mM *t*-BuOOH for 3 h. The cytoprotective activity was increased according to the electron donating ability. Of the stilbenes tested, piceatannol (**3**), 3,3',4',5-tetrahydroxy stilbene, exhibited the most potent cytoprotective activity (74.8 ± 6.9%). Despite having four hydroxyl groups, oxyresveratrol (**2**) showed much low activity. This suggests that the *ortho*-dihydroxyl structure (catechol group) is essential for the protective activity against *t*-BuOOH-induced cell damage. It is known that *ortho* substitution with an electron donating group increases the stability of the phenoxy radical by facilitating electron

Table 1. Cytoprotective effect of stilbenes on *t*-BuOOH-induced oxidative damage



***t*-BuOOH**

	R_3	R_5	R_2'	R_3'	R_4'	Cell Viability (%)	TBARS (pmol/mg protein)
Blank						100	370.2 ± 51.2**
t-BuOOH						11.2 ± 1.6[e]	8721.5 ± 809.5
Resveratrol (**1**)	OH	OH	H	H	OH	18.1 ± 1.6*	2792.4 ± 259.1**
Oxyresveratrol (**2**)	OH	OH	OH	H	OH	22.3 ± 1.6*	1645.6 ± 200.2**
Piceatannol (**3**)	OH	OH	H	OH	OH	74.8 ± 6.9**	591.2 ± 60.7**
Rhapontigenin (**4**)	OH	OH	H	OH	OCH_3	20.0 ± 1.5*	2517.2 ± 285.3**
Desoxyrhapontigenin (**5**)	OH	OH	H	H	OCH_3	11.9 ± 1.0	7935.7 ± 642.5
Rhaponticin (**6**)	Oglc[a]	OH	H	OH	OCH_3	14.8 ± 1.6	5003.4 ± 429.2*
Deoxyrhaponticin (**7**)	OGlc	OH	H	H	OCH_3	12.0 ± 1.2	8182.3 ± 764.7
Piceid (**8**)	OGlc	OH	H	H	OH	12.1 ± 0.9	8255.1 ± 576.4
Piceid-2"-gallate (**9**)	OglcG[b]	OH	H	H	OH	14.9 ± 1.1*	4337.2 ± 230.9**
Piceid-2"-coumarate (**10**)	OglcC[c]	OH	H	H	OH	13.0 ± 1.1	5981.1 ± 412.6*
Quercetin						70.7 ± 1.6*	NT[d]

[a-d]Glc: glucose, G: galloyl, C: *p*-coumaroyl, NT: not tested.
[e]Values are expressed as the mean ± SD of three replicates.
*$P < 0.05$, **$P < 0.001$, were significantly different from the group treated *t*-BuOOH.

Table 2. Effect of piceatannol (**3**) on UVB-induced lipid peroxidation in SKH-1 mice

	Amount of TBARS (nmol/mg protein)
Control	0.32 ± 0.05*
UVB irradiated	0.68 ± 0.10[a]
Piceatannol (**3**) 30 mg/kg + UVB irradiated	0.07 ± 0.03**
Piceatannol (**3**) 10 mg/kg + UVB irradiated	0.16 ± 0.08*
MAP[b] 50 mg/kg + UVB irradiated	0.16 ± 0.05*
MAP[b] 30 mg/kg + UVB irradiated	0.28 ± 0.06*

[a] Values are expressed as mean ± SD.
[b] Positive control: magnesium-L-ascorbyl-2-phosphate
* $P < 0.05$, ** $P < 0.001$, were significantly different from the UVB irradiated group.

delocalization, and a catechol group enhances the antioxidant activity (Heim, *et al*., 2002; Cos, *et al*., 2001). Rhapontigenin (**4**), which has a methoxyl group at the C-4' of **3**, also showed a low activity. Although the methoxyl group is a good electron donating group, the cytotoxic effect should be considered. Indeed, **4** inhibited the proliferation of the keratinocytes at high concentrations (> 50 μM). Glycosylation of a hydroxyl group reduces the cytoprotective activity. The aglycone, **1**, exhibited higher activity than its glycoside piceid (**8**). This result is in accordance with those reported in other assay systems (Heim, *et al*., 2002; Cos, *et al*., 2001). Although, piceid-2''-gallate (**9**) and piceid-2''-coumarate (**10**) exhibited strong antioxidant activity on radical scavenging and lipid peroxidation due to the residual galloyl and *p*-coumaryl groups, respectively (Lee, *et al*., 2003), they did not show a high activity in this assay system. This indicates that the cytoprotective effect of the stilbenes on the *t*-BuOOH-induced oxidative stress is slightly different from the radical scavenging and lipid peroxidation activity. The cytoprotective effect of the stilbenes against the *t*-BuOOH-induced oxidative stress was also confirmed by measuring the extent of lipid peroxidation (Table 1). The TBARS of the cells treated with *t*-BuOOH (1.5 mM) for 3 h was increased 23.6-fold compared to the blank. As shown in Table 1, the amount of TBARS was in good accordance with the cell viability. The TBARS of **3** was also significantly lower than the control, which indicates that **3** can protect cells from oxidative stress.

In order to evaluate the protective effect on oxidative stress, **3** was further investigated to determine the protective effect against the UVB-induced skin lipid peroxidation. UVB radiation (in the range of 290 - 320 nm) is believed to be a major cause of skin cancer (Black, *et al*., 1997). UVB is also known to induce excessive ROS generation, and lipid peroxidation is a marker of oxidative stress (Black, *et al*., 1997; Valenzuela, 1991). As shown in Table 2, the UVB exposed mice skin had a significantly higher amount of TBARS (0.68 ± 0.10 nmol/mg protein) in 24 h after UVB irradiation. In contrast, the TBARS concentration was significantly reduced to 0.07 ± 0.03 nmol/mg protein ($P < 0.001$) when **3** was topically administered at a dose of 30 mg/kg. The amount of TBARS was significantly decreased by the topical administration of **3** even at dose as low as 10 mg/kg. That is, **3** exhibited potent inhibitory activity on the UVB-induced skin lipid peroxidation in SKH-1 mice. As a result, it is demonstrated that **3** affords protection against the oxidative damage induced by UVB irradiation due to its potent antioxidant properties that efficiently reduce oxidative stress.

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