ELSEVIER

European Journal of Pharmacology 438 (2002) 189–196

ejp

www.elsevier.com/locate/ejphar

CX-659S: a novel diaminouracil derivative that has antioxidative and acute anti-inflammatory activities

Yuso Goto [a], Nobuo Watanabe [a], Noriaki Kogawa [a], Masami Tsuchiya [a], Osamu Takahashi [a], Hiroshi Uchi [b], Masutaka Furue [b], Hideya Hayashi [a,*]

[a] *Pharmaceuticals and Biotechnology Laboratory, Japan Energy Corporation, 3-17-35, Niizo-Minami, Toda, Saitama 335-8502, Japan*
[b] *Department of Dermatology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan*

Received 29 November 2001; received in revised form 29 January 2002; accepted 1 February 2002

Abstract

We investigated the antioxidative activities and the effects on acute inflammation in mice of a novel diaminouracil derivative, CX-659S ((*S*)-6-amino-5-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)-3-methyl-1-phenyl-2,4(1*H*,3*H*)-pyrimidinedione). CX-659S showed potent scavenging activities against the hydroxyl radical and peroxynitrite and inhibited lipid peroxidation in rat brain homogenates in vitro. Topically applied CX-659S dose-dependently inhibited arachidonic acid- and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ear edema in mice. Consistent with its antioxidative properties in vitro, CX-659S dramatically attenuated the accumulation of lipid peroxides in the mouse ear elicited by repeated application of TPA. Previously, we reported the effectiveness of CX-659S against contact hypersensitivity reactions in both mouse and guinea pig models. These present results further suggest the therapeutic potential of CX-659S for acute skin inflammation that may involve oxidative tissue damage.

Keywords: Antioxidant; Hydroxyl radical; Peroxynitrite; Lipid peroxidation; Uracil derivative

1. Introduction

We recently reported that a novel diaminouracil derivative, CX-659S ((*S*)-6-amino-5-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)-3-methyl-1-phenyl-2,4(1*H*,3*H*)-pyrimidinedione, Fig. 1A) had inhibitory activities against contact hypersensitivity reactions in both mice and guinea pigs when topically applied, and that one of the anti-inflammatory mechanisms in vivo was the inhibition of the expression of messenger RNAs for proinflammatory cytokines interleukin-1β and tumor necrosis factor (TNF)-α (Goto et al., 2000).

It has been reported that reactive oxygen species such as superoxide anion, hydroxyl radical and peroxynitrite participate in the process of inflammation in various tissues including the skin (Trenam et al., 1992). In skin, reactive oxygen species can be produced not only by chemical ionization and/or UV radiation (Black, 1987) but also enzymatically by polymorphonuclear leukocytes that infiltrate the sites of infection (Thelen et al., 1993). In both cases, the excessively produced reactive oxygen species can injure cellular biomolecules such as nucleic acids, proteins, carbohydrates, and lipids, causing cellular and tissue damage, which in turn augments the state of inflammation (Cochrane, 1991). Among many reactive oxygen species, the hydroxyl radical and peroxynitrite have strong oxidizing properties towards biomolecules, especially lipids of cell membranes (Halliwell and Chirico, 1993; Beckman and Crow, 1993). Therefore, compounds that have scavenging activities toward these radicals and/or suppressive activities on lipid peroxidation may thus be expected to have therapeutic potentials for several skin inflammatory diseases (Rice-Evans and Diplock, 1993; Trenam et al., 1992). CX-659S has a vitamin E-related chroman moiety, and showed antioxidative activity against lipid peroxidation in our preliminary experiment (Tobe et al., 2000). However, the detailed antioxidative properties and acute anti-inflammatory activities of this compound were not elucidated at that time.

In this study, we first characterized the antioxidative activity of CX-659S toward two reactive oxygen species (hydroxyl radical and peroxynitrite) and toward lipid peroxidation in vitro and compared it with that of its related

* Corresponding author. Tel.: +81-48-433-2194; fax: +81-48-443-1605.
E-mail address: hhayashi@j-energy.co.jp (H. Hayashi).

0014-2999/02/$ - see front matter
PII: S0014-2999(02)01340-7

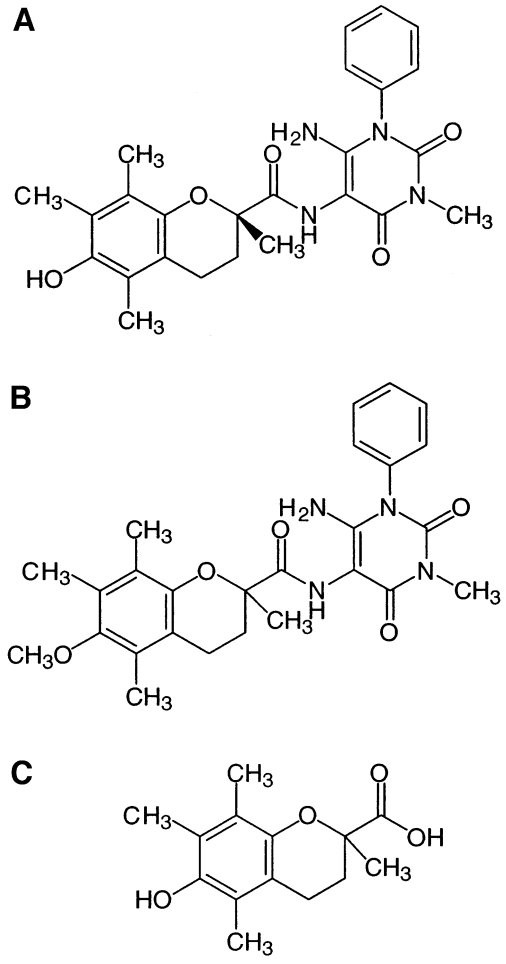

Fig. 1. Chemical structures of CX-659S and its related compounds used in this study. (A) CX-659S (MW: 464.5), (B) CX-659-052 (RS form, MW: 478.5), (C) Trolox® (MW: 250.3).

compounds and antioxidants. Next, we investigated the topical anti-inflammatory effect of CX-659S on two mouse models of acute inflammation, one using arachidonic acid and the other, 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Finally, to evaluate the role of the antioxidative activity in the anti-inflammatory effect of CX-659S, we investigated the correlation between the degree of edema formation and the amounts of lipid peroxides in the mouse ear following a repeated application of TPA.

2. Materials and methods

2.1. Animals

Male 6-week-old ICR mice and male 10-week-old Sprague–Dawley rats were purchased from Clea Japan (Tokyo, Japan). The animals were maintained on a 12-h light/12-h dark cycle, and the room temperature was set at 23 °C. They were fed food and tap water ad libitum. The study protocol was approved by the Japan Energy Animal Care and Use Committee.

2.2. Chemicals and reagents

CX-659S and CX-659-052 ((*RS*)-6-amino-5-(6-methoxy-2,5,7,8-tetramethylchroman-2-carboxamido)-3-methyl-1-phenyl-2,4(1*H*,3*H*)-pyrimidinedione, Fig. 1B) were synthesized by the methods previously described (Tobe et al., 2000). The other chemicals and reagent and their sources were as follows: diethylenetriaminepentaacetic acid, 2-thiobarbituric acid, malonaldehyde bis (dimethyl acetal), ascorbic acid, indomethacin, dimethyl sulfoxide (DMSO), and gelatin, from Nacalai Tesque (Kyoto, Japan); ferrous chloride, from Wako (Osaka, Japan); 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), from Aldrich Chemical (Milwaukee, WI, USA); arachidonic acid, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and betamethasone 17-valerate, from Sigma (St. Louis, MO, USA); rhodamine 123 and dihydrorhodamine 123, from Molecular Probes (Eugene, OR, USA); peroxynitrite, from Dojin Chemical Laboratory (Kumamoto, Japan); hydrogen peroxide (30%), from Santoku Chemical Industries (Tokyo, Japan); and butyl hydroxy toluene, from Tokyo Kasei Kogyo (Tokyo, Japan).

2.3. Hydroxyl radical-scavenging activity

Hydroxyl radicals were generated via the Fenton reaction with hydrogen peroxide and ferrous iron–diethylenetriaminepentaacetic acid complex (DETAPAC–Fe^{2+}; Yamazaki and Piette, 1990) and detected by the oxidation of dihydrorhodamine to its fluorescent form, rhodamine (Miles et al., 1996). The test compounds were dissolved in dimethylformamide at a 1000 times concentration, and all solutions used were purged by nitrogen. The reaction was started by mixing 100 μl of solution A [100 mM sodium phosphate buffer (pH 7.4), 0.3 mg/ml of gelatin, 50 μM dihydrorhodamine, 400 μM DETAPAC-Fe^{2+}, and various concentrations of test compounds] with 100 μl of solution B [100 mM sodium phosphate buffer (pH 7.4), 0.3 mg/ml of gelatin, and 200 μM hydrogen peroxide]. After a 5-min incubation at 37 °C, rhodamine fluorescence was measured with a fluorescence microplate reader (Corona, MTP-32) with excitation at 490 nm and emission at 530 nm. The resulting increase in fluorescence intensity was then converted into generated rhodamine by an external standard method. In this assay system, dihydrorhodamine oxidation was also inhibited by DMSO, a well-known hydroxyl radical scavenger (Babbs and Griffin, 1989).

2.4. Peroxynitrite-scavenging activity

As peroxynitrite has been reported to be stable in alkaline solution and to oxidize directly dihydrorhodamine to rhodamine (Kooy et al., 1994), we used the system with dihydrorhodamine as a probe for examining the scavenging

activities of the test compounds. Peroxynitrite was diluted with 0.3 N NaOH to give a 7 mM solution. A mixture containing 100 mM sodium phosphate buffer (pH 7.4), 0.3 mg/ml of gelatin, 25 μM dihydrorhodamine, and various concentrations of test compounds was preincubated at 37 °C for 5 min, and reaction was then started by adding 200 μl of the mixture to 2 μl of peroxynitrite solution. After incubation at 37 °C for 5 min, generated rhodamine was estimated by the above-described fluorescence measurement. Peroxynitrite-dependent increase in the fluorescence was then converted into rhodamine concentration from the external rhodamine standard. In this assay system, dihydrorhodamine oxidation was not inhibited by a hydroxyl radical scavenger, DMSO.

2.5. Lipid peroxidation in rat brain homogenate

Rat brain was homogenized in 9 volumes of 0.1 M phosphate buffer (pH 7.4) at 4 °C with a Polytron homogenizer. After elimination of tissue debris by centrifugation at 120×*g* for 5 min, the supernatant was used for the assay. The desired test compound in DMSO was added to the homogenate on ice, and the mixture was incubated for 1 h at 37 °C. The detection of lipid peroxidation products in the homogenate was performed by monitoring thiobarbituric acid-reactive substances according to the method of Ohkawa et al. (1979). Sodium thiobarbiturate solution (1.2%, v/v) was added to the mixture (final 2.0 ml), and the solution was heated for 1 h at 95–97 °C. After cooling, 0.5 ml of distilled water and 2.5 ml of *n*-butanol/pyridine (15:1, v/v) were added to the solution, which was then mixed vigorously. The absorbance of the thiobarbituric acid-reactive substances extracted in the organic layer was determined at 532 nm, and the level of lipid peroxides was expressed as malondialdehyde concentration by using an external malondialdehyde standard.

Antioxidant activities of test compounds were calculated using the following formula:

$$\text{Inhibition } \% = \{1 - (C_3 - C_1)/(C_2 - C_1)\} \times 100$$

where C_1=internal malondialdehyde concentration, C_2= malondialdehyde concentration in control sample, C_3=malondialdehyde concentration in test sample.

2.6. Arachidonic acid-induced ear edema in mice

Arachidonic acid-induced ear edema was determined by the method of Young et al. (1984). Arachidonic acid (0.5 mg) dissolved in 20 μl acetone was applied to the inner and outer surfaces of the left ear of mice. Test compounds in acetone or vehicle in a volume of 40 μl was painted on the left ear immediately before the application. The ear thickness was measured with a calibrated digital thickness gauge (Mitutoyo, Tokyo) before and 1 h after the challenge, and the difference in thickness was calculated.

2.7. TPA-induced ear edema in mice

TPA-induced ear edema was performed by the method previously reported by Rao et al. (1993). TPA (1.0 μg) dissolved in 20 μl acetone was applied to the inner and outer surfaces of the left ear of mice. Test compounds in acetone or vehicle in a volume of 20 μl was painted on the left ear 5 min before the TPA administration. The ear thickness was measured with a calibrated digital thickness gauge before and 6 h after the challenge, and the difference in thickness was calculated.

2.8. Edema formation and lipid peroxidation in mice repeatedly treated with TPA

Five mice in a group were used. TPA (1.0 μg) or TPA (1.0 μg) plus various concentrations of test compounds dissolved in 20 μl acetone was applied to the inner and outer surfaces of both ears of mice every 24 h for 4 days. The thickness of the left ear was measured with a digital thickness gauge before each application and 20 h after the final application. Then the mice were sacrificed by cervical dislocation. Both ears were excised, minced with scissors, and weighed. The skin pieces were pooled in 20 mM Tris–HCl buffer, pH 7.4, containing 5 mM butyl hydroxy toluene, and homogenized with a glass homogenizer. The homogenate was centrifuged at 3000×*g* for 10 min at 4 °C, and the supernatant was centrifuged again. The resultant supernatant was used for the measurement of lipid peroxidation. The measurement was carried out with BIOXYTECH® LPO-586™ (OXIS International, Portland, OR, USA) according to the manufacturer's protocol. In this system, the presence of malonaldehyde and 4-hydroxyalkenals was used as an indicator of lipid peroxidation (Esterbauer et al., 1991). The protein concentration in the homogenate was assayed by a colorimetric assay (Smith et al., 1985) using a bicinchoninic acid (BCA) Protein Assay Reagent (Pierce Chemical, Rockford, IL, USA).

2.9. Statistical analysis

The results of in vivo experiments were expressed as the mean±S.E.M. Comparisons among the groups were performed by Dunnett's test. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Antioxidative activities in vitro (Table 1)

The free radical-scavenging activities of CX-659S against hydroxyl radical or peroxynitrite and its effect on lipid peroxidation in vitro were investigated along with its related compounds (CX-659-052 and Trolox) and the antioxidant ascorbic acid for comparison. CX-659-052 is an analogue of CX-659S and has a methoxy group instead of a

hydroxyl group in the chroman structure (Fig. 1B, RS form). Table 1 summarizes the results, given as the IC_{50} values for these compounds.

The hydroxyl radical-scavenging activity was investigated by using the DETAPAC–Fe^{2+}/hydrogen peroxide system (Yamazaki and Piette, 1990) to generate hydroxyl radical and dihydrorhodamine 123 as a detector molecule. CX-659S, Trolox, and ascorbic acid all inhibited the production of rhodamine in a concentration-dependent manner, whereas CX-659-052 remained inactive at 200 μM (Table 1). In this experiment, none of the compounds interfered with either the coordination state of DETAPAC–Fe^{2+} or the fluorescence of rhodamine. The compounds did not change the UV spectrum of DETAPAC–Fe^{2+}, suggesting that they did not change the coordination state of ferrous iron or its oxidation state. Also, the compounds did not affect the fluorescence of rhodamine when added after the reaction, suggesting that neither reduction of once-formed rhodamine to dihydrorhodamine nor interference effect on the fluorescence of rhodamine occurred.

Next, we investigated the effects of compounds on peroxynitrite-scavenging activity. As expressed in Table 1, CX-659S, Trolox, and ascorbic acid all showed strong scavenging activities against peroxynitrite. These compounds showed no reducing activity against once-formed rhodamine in this system (data not shown). In contrast, CX-659-052 was inactive under this experimental condition.

The antioxidative activities of the compounds were also examined by conducting an experiment on lipid peroxidation. As previously reported (Tobe et al., 2000), CX-659S showed strong inhibitory activities against lipid peroxidation in rat brain homogenate (Table 1). CX-659-052 was inactive at doses of up to 200 μM. Trolox inhibited lipid peroxidation, whereas ascorbic acid did not, in agreement with a previous report by Hara et al. (1992).

3.2. Topical anti-inflammatory activities (Figs. 2 and 3)

The topical anti-inflammatory activities of CX-659S were determined by assessing the effects of the drug on mouse ear edema induced by arachidonic acid or TPA.

As shown in Fig. 2A, in the arachidonic acid-induced ear edema model, when CX-659S (0.1–1.0 mg/ear) was topically applied immediately before the treatment with arachidonic acid, ear edema formation was inhibited in a dose-dependent manner. The inhibitory effect of this compound at a dose of 1.0 mg/ear was statistically significant. Indomethacin, which is a well-known cyclooxygenase inhibitor, also suppressed ear edema at a dose of 1.0 mg/ear in this model. Next, the effect of CX-659S was compared with that of its related compounds, CX-659-052 and Trolox. CX-659-052, which had showed no antioxidative activities (Table 1), at a dose of 1.0 mg/ml had a subtle inhibitory effect on the ear edema, but the effect was not statistically significant (Fig. 2B). Trolox at a dose of 3.0 mg/ear inhibited ear edema in this model (Fig. 2B).

We also examined the effect of CX-659S on TPA-induced ear edema, which is known to be a different type of acute inflammatory model. The application of TPA onto mouse ears rapidly induced an edema that was maximal at 6 h. Therefore, we measured the ear thickness 6 h after the TPA

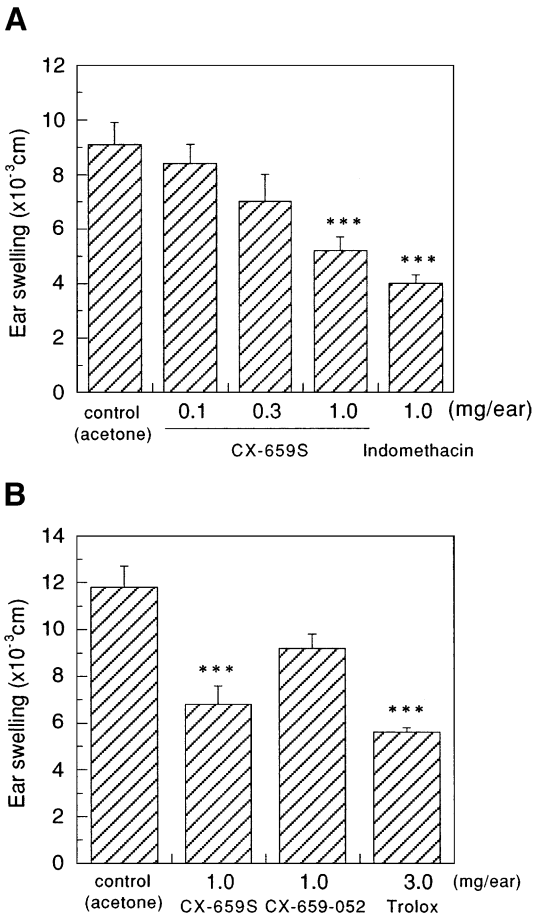

Fig. 2. Effects of compounds on arachidonic acid-induced ear edema in mice. (A) Dose-dependent effect of CX-659S. (B) Effects of CX-659S, CX-659-052, and Trolox. Arachidonic acid (0.5 mg) dissolved in 20 μl acetone was applied to the left ear of mice. Test compounds in acetone or vehicle in a volume of 40 μl, was painted on the left ear immediately before the application. The ear thickness was measured with a digital thickness gauge before and 1 h after the challenge, and the difference in thickness was calculated. The results are expressed as the mean±S.E.M. of 14 (A) or 5 (B) mice. ***$P < 0.001$ vs. control.

Table 1
Antioxidative activities of CX-659S, its related compounds, and a known antioxidant

Compound	IC_{50} (μM)		
	Hydroxyl radical	Peroxynitrite	Lipid peroxidation
CX-659S	44	8	4.6
CX-659-052	>200	>100	>200
Trolox	24	9	12
Ascorbic acid	54	11	>300

The antioxidative activities of test compounds were determined as described under Materials and methods. Concentrations causing 50% inhibition (IC_{50} values) are presented.

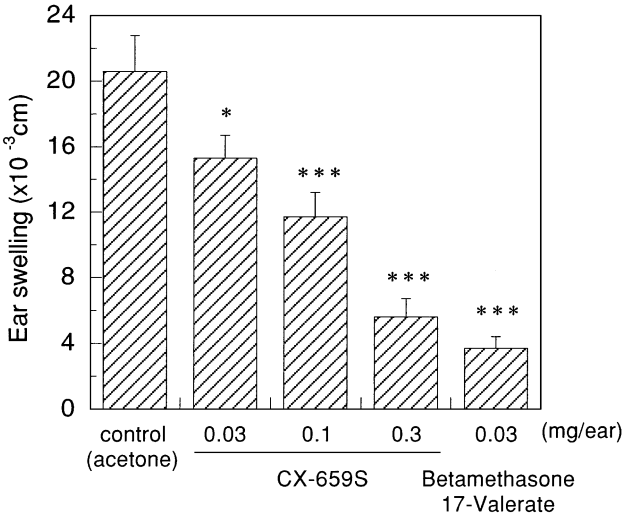

Fig. 3. Effect of CX-659S on TPA-induced ear edema in mice. TPA (1.0 μg) dissolved in 20 μl acetone was applied to the left ear of mice. Test compounds in acetone or vehicle in a volume of 20 μl was painted on the left ear 5 min before the TPA administration. The ear thickness was measured with a digital thickness gauge before and 6 h after the TPA treatment, and the difference in thickness was calculated. The results are expressed as the mean±S.E.M. of 10 mice. *$P<0.05$; ***$P<0.001$ vs. control.

application. As shown in Fig. 3, topical application of CX-659S inhibited the ear edema in a dose-dependent manner ranging from 0.03 to 0.3 mg/ear. A representative steroid, betamethasone 17-valerate, at a dose of 0.03 mg/ear, strongly inhibited ear edema in this model.

3.3. Anti-inflammatory and antioxidative activities in vivo (Fig. 4)

To test the correlation between the anti-inflammatory activities and the antioxidative properties of CX-659S, we chose the TPA-induced ear edema model, and determined the change in ear thickness and amounts of lipid peroxides in the same tissue. In our preliminary experiment, a single application of TPA in mouse ear did not produce detectable amounts of oxidized lipids in the lesions (data not shown). Therefore, we sought for the suitable experimental conditions in which TPA would induce a detectable amount of lipid peroxides in the mouse ear. As a result, we found that four consecutive applications of TPA onto the mouse ear at 24-h intervals resulted in not only increased ear edema but also a detectable amount of oxidized lipids in the sites. The amount of oxidized lipids after these four applications was

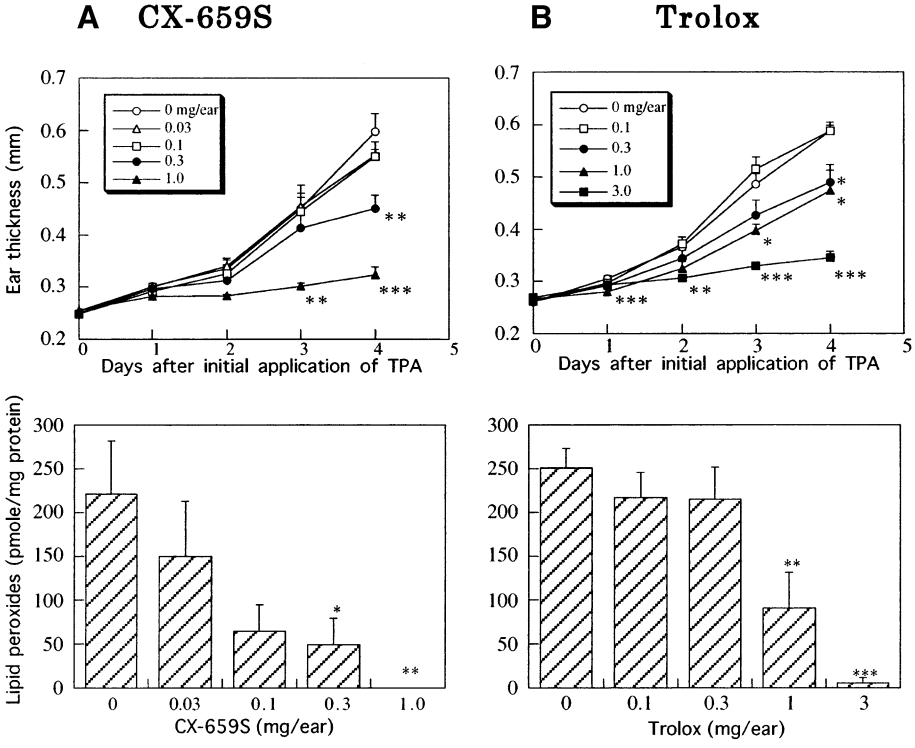

Fig. 4. Effects of CX-659S (A) and Trolox (B) on ear edema and lipid peroxidation in mice with repeated application of TPA. (Upper) Effect on ear edema. (Lower) Effect on lipid peroxidation. TPA (1.0 μg) or TPA (1.0 μg) plus various concentrations of test compounds dissolved in 20 μl acetone was applied to both ears of mice four times at 24-h intervals. The thickness of the left ear was measured with a digital thickness gauge before each application and 20 h after the final application. Then, both ears were excised, minced and weighed. The skin pieces were pooled and homogenized with a glass homogenizer. The homogenate was centrifuged twice, and the resultant supernatant was used for a measurement of lipid peroxidation. The measurement was carried out with BIOXYTECH® LPO-586™. The results are expressed as the mean±S.E.M. of five mice. *$P<0.05$; **$P<0.01$; ***$P<0.001$ vs. nontreatment (0 mg/ear) group.

almost the same as that induced by eight consecutive applications of TPA (data not shown). Therefore, we conducted the study on the effects of the compounds after the four applications. As shown in Fig. 4, CX-659S not only dose-dependently inhibited ear swelling (Fig. 4A, upper) but also suppressed lipid peroxides production in the ear at 4 days after the initial TPA application (Fig. 4A, lower). The effects of CX-659S at doses of 0.3–1.0 mg/ear at day 4 were statistically significant for both parameters. At the dose of 1.0 mg/ear, CX-659S completely inhibited the production of lipid peroxides and suppressed ear edema formation by 80%. Trolox, which was effective in the in vitro lipid peroxidation assay (Table 1), also inhibited both ear swelling (Fig. 4B, upper) and lipid peroxidation (Fig. 4B, lower), but the potency of this compound was only about one-third of that of CX-659S.

4. Discussion

The present study demonstrates that CX-659S had potent scavenging activities against both hydroxyl radicals and peroxynitrite, and that its potency was the same as that of the well-known antioxidants such as Trolox and ascorbic acid (Table 1). Although both the hydroxyl radical and peroxynitrite play an important role in host defense against infection by virtue of their potent oxidizing ability, they are also responsible for the cellular and tissue damage in inflammation and other various pathological processes (Rice-Evans and Diplock, 1993). The hydroxyl radical can directly or indirectly increase vascular permeability and thus augment edema formation; that is, in addition to injuring vascular endothelial cells directly, the hydroxyl radical participates in the nonenzymatic generation of possible permeability-stimulating factors such as isoprostanoids (Morrow et al., 1996) and leukotriene-like molecules (Harrison and Murphy, 1995), and activates chemotactic factors such as complement C5a (Oldham et al., 1988). Peroxynitrite also has the capacity to induce lipid peroxidation (Radi et al., 1991). We consider the potent scavenging activity of CX-659S against these oxidants, as shown in this study, to be possibly responsible for the anti-inflammatory mechanism of this compound. The antioxidative property of CX-659S was further confirmed by our experiments on peroxidation of lipids from biological sources. We found that CX-659S had potent inhibitory activity on lipid peroxidation in rat brain homogenates (Table 1), as previously reported (Tobe et al., 2000), and the effect of this compound was more potent than that of Trolox under our experimental conditions. The importance of the hydroxyl group in the chroman moiety for these antioxidative (reactive oxygen species scavenging and lipid peroxidation inhibitory) activities of CX-659S was clearly reflected in our results showing that the effects of CX-659S disappeared when the hydroxyl group was protected as a methyl ether (CX-659-052). However, we cannot yet rationalize the slight difference in potency between CX-659S and Trolox, which have the same 6-hydroxy-2-chroman moiety.

We also showed in this study that topically applied CX-659S dose-dependently inhibited ear edema induced by either arachidonic acid or TPA in mice. Although the synthesis of leukotrienes and prostaglandins has been reported to be involved in arachidonic acid- and TPA-induced ear edema (Rao et al., 1993; Goulet et al., 1994; Chen et al., 1994), it has been assumed that reactive oxygen species also play an important role in the edema formation in these models. As evidence in support of this hypothesis, several antioxidative compounds have been reported to show anti-inflammatory action either on arachidonic acid-induced ear edema (Crummey et al., 1987) or on TPA-induced ear edema (Hara et al., 1992; Sugiyama et al., 1994). The result that CX-659-052, which showed no antioxidative activity in vitro, failed to inhibit arachidonic acid-induced ear edema (Fig. 2B) is an additional data supporting the correlation between reactive oxygen species and edema formation.

To gain further insight into the role of antioxidative activity in the prevention of inflammation, we also evaluated the antioxidative effect of CX-659S in vivo. Various stimuli including ozone (Thiele et al., 1997), UV radiation (Yuen and Halliday, 1997), and TPA (Perchellet and Perchellet, 1989; Beckman et al., 1994) can induce lipid peroxidation in the skin. In this study, we chose TPA as a stimulant and ear skin as a reaction site. As previously reported (Beckman et al., 1994), repetitive applications of TPA were needed to induce detectable amounts of lipid peroxide end products in the ear. In this model, CX-659S dramatically inhibited both lipid peroxidation and ear edema (Fig. 4). The activity of this compound toward both parameters was superior to that of the well-known antioxidant Trolox, consistent with the result from the arachidonic acid-induced edema model (Fig. 2B). These results demonstrate that CX-659S had potent antioxidative activities in vivo, which may account for the anti-inflammatory activities of this compound in acute inflammatory models induced by arachidonic acid (Fig. 2) or TPA (Fig. 3).

In addition to their role in acute inflammation, reactive oxygen species may also contribute to several chronic cutaneous inflammatory diseases such as psoriasis (Das et al., 1992; Muller, 1997), atopic dermatitis (Polla et al., 1992), and contact dermatitis (Sharkey et al., 1991). Therefore, the antioxidative activity of CX-659S shown in this study may, at least in part, account for the inhibitory activities of this compound against contact hypersensitivity reactions in various animal models (Goto et al., 2000). Although the correlation between the antioxidatative properties and the inhibitory activities toward these reactions is not clear at present, some kind of interference of signal transduction systems may be involved. For example, reactive oxygen species are reported to be involved in the regulation of signaling pathways for gene expression in various tissues and cell types including keratinocytes (Tebbe et al., 1997; Valacchi et al., 2001), and we previously showed that CX-

659S inhibited the expression of messenger RNAs for proinflammatory cytokines, interleukin-1β, and TNF-α in a contact hypersensitivity model in mice (Goto et al., 2000). Therefore, some reactive oxygen species-sensitive components such as mitogen-activated protein (MAP) kinase (Chiu et al., 2001) and nuclear factor (NF)-κB (Tebbe et al., 2001) in signal transduction pathways may be one of the possible targets for CX-659S. The experiments for determining the detailed inhibitory activities of CX-659S on cytokine gene expression of dermal and epidermal cells and for evaluating the correlation between the activities and its antioxidative properties are now underway in our laboratories.

Taken together, our findings suggest that CX-659S may become a useful therapeutic agent not only for chronic inflammatory diseases but also for acute inflammation in the skin due to its antioxidative properties.

Acknowledgements

We wish to thank Dr. Y. Matsuda and Mr. T. Yumizaki for their helpful discussions and Mr. H. Yamashita for his excellent technical assistance.

References

Babbs, C.F., Griffin, D.W., 1989. Scatchard analysis of methane sulfinic acid production from dimethyl sulfoxide: a method to quantify hydroxyl radical formation in physiologic systems. Free Radical Biol. Med. 6, 493–503.

Beckman, J.S., Crow, J.P., 1993. Pathological implications of nitric oxide, superoxide and peroxynitrite formation. Biochem. Soc. Trans. 21, 330–334.

Beckman, J.K., Bagheri, F., Ji, C., Blair, I.A., Marnett, L.J., 1994. Phospholipid peroxidation in tumor promoter-exposed mouse skin. Carcinogenesis 15, 2937–2944.

Black, H.S., 1987. Potential involvement of free radical reactions in ultra violet light mediated cutaneous damage. Photochem. Photobiol. 46, 213–221.

Chen, X.S., Sheller, J.M., Johnson, E.N., Funk, C.D., 1994. Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372, 179–182.

Chiu, C., Maddok, D.A., Zhang, Q., Souza, K.P., Townsend, A.R., Wan, Y., 2001. TGF-beta-induced p38 activation is mediated by Rac1-regulated generation of reactive oxygen species in cultured human keratinocytes. Int. J. Mol. Med. 8, 251–255.

Cochrane, C.G., 1991. Cellular injury by oxidants. Am. J. Med. 91, 23S–30S.

Crummey, A., Harper, G.P., Boyle, E.A., Mangan, F.R., 1987. Inhibition of arachidonic acid-induced ear oedema as a model for assessing topical anti-inflammatory compounds. Agents Actions 20, 69–76.

Das, U.N., Vijaykumar, K., Madhavi, N., Suryaprabha, P., Sravankumar, G., Ramesh, G., Koratkar, R., Sagar, P.S., Padma, M., 1992. Psoriasis: current concepts and new approaches to therapy. Med. Hypotheses 38, 56–62.

Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radical Biol. Med. 11, 81–128.

Goto, Y., Inoue, Y., Tsuchiya, M., Isobe, M., Ueno, T., Uchi, H., Furue, M., Hayashi, H., 2000. Suppressive effect of topically applied CX-659S, a novel diaminouracil derivative, on the contact hypersensitivity reaction in various animal models. Int. Arch. Allergy Immunol. 123, 341–348.

Goulet, J.L., Snouwaert, J.N., Latour, A.M., Coffman, T.M., Koller, B.H., 1994. Altered inflammatory responses in leukotriene-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 91, 12852–12856.

Halliwell, B., Chirico, S., 1993. Lipid peroxidation: its mechanism, measurement, and significance. Am. J. Clin. Nutr. 57, 715S–725S.

Hara, H., Sukamoto, T., Ohtaka, H., Abe, K., Tatumi, Y., Saito, Y., Suzuki, A., Tsukamoto, G., 1992. Effects of baicalein and α-tocopherol on lipid peroxidation, free radical scavenging activity and 12-*O*-tetradecanoylphorbol acetate-induced ear edema. Eur. J. Pharmacol. 221, 193–198.

Harrison, K.A., Murphy, R.C., 1995. Isoleukotrienes are biologically active free radical products of lipid peroxidation. J. Biol. Chem. 270, 17273–17278.

Kooy, N.W., Royall, J.A., Ischiropoulos, H., Beckman, J.S., 1994. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radical Biol. Med. 16, 149–156.

Miles, A.M., Bohle, D.S., Glassbrenner, P.A., Hansert, B., Wink, D.A., Grisham, M.B., 1996. Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. J. Biol. Chem. 271, 40–47.

Morrow, J.D., Awad, J.A., Wu, A., Zackert, W.E., Daniel, V.C. , Roberts II, L.J., 1996. Nonenzymatic free radical-catalyzed generation of thromboxane-like compounds (isothromboxanes) in vivo. J. Biol. Chem. 271, 23185–23190.

Muller, K., 1997. Antipsoriatic and proinflammatory action of anthralin: implications for the role of oxygen radicals. Biochem. Pharmacol. 53, 1215–1221.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Oldham, K.T., Guice, K.S., Till, G.O., Ward, P.A., 1988. Activation of complement by hydroxyl radical in thermal injury. Surgery 104, 272–279.

Perchellet, E.M., Perchellet, J.P., 1989. Characterization of the hydroperoxide response observed in mouse skin treated with tumor promoters in vivo. Cancer Res. 49, 6193–6201.

Polla, B.S., Ezekowitz, R.A., Leung, D.Y.M., 1992. Monocytes from patients with atopic dermatitis are primed for superoxide production. J. Allergy Clin. Immunol. 89, 545–551.

Radi, R., Beckman, J.S., Bush, K.M., Freeman, B.A., 1991. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch. Biochem. Biophys. 288, 481–487.

Rao, T.S., Currie, J.L., Shaffer, A.F., Isakson, P.C., 1993. Comparative evaluation of arachidonic acid (AA)- and tetradecanoylphorbol acetate (TPA)-induced dermal inflammation. Inflammation 17, 723–741.

Rice-Evans, C.A., Diplock, A.T., 1993. Current status of antioxidant therapy. Free Radical Biol. Med. 15, 77–96.

Sharkey, P., Eedy, D.J., Burrows, D., McCaigue, M.D., Bell, A.L., 1991. A possible role for superoxide production in the pathogenesis of contact dermatitis. Acta Derm.-Venereol. 71, 156–159.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Sugiyama, N., Akahoshi, F., Kuwahara, S., Kajii, M., Sakaue, Y., Yakamaru, H., Sugiura, M., Fukaya, C., 1994. Synthesis and topical anti-inflammatory and antiallergic activities of antioxidant *o*-aminophenol derivatives. J. Med. Chem. 37, 1977–1982.

Tebbe, B., Wu, S., Geilen, C.C., Eberle, J., Kodelja, V., Orfanos, C.E., 1997. L-ascorbic acid inhibits UVA-induced lipid peroxidation and secretion of IL-1alpha and IL-6 in cultured human keratinocytes in vitro. J. Invest. Dermatol. 108, 302–306.

Tebbe, B., Schwarz, C., Ruderisch, H.S., Treudler, R., Orfanos, C.E., 2001. L-ascorbic acid increases NFkappaB binding activity in UVA-irradiated HaCaT keratinocytes. J. Invest. Dermatol. 117, 154–156.

Thelen, M., Dewald, B., Baggiolini, M., 1993. Neutrophil signal transduction and activation of the respiratory burst. Physiol. Rev. 73, 797–821.

Thiele, J.J., Traber, M.G., Tsang, K., Cross, C.E., Packer, L., 1997. In vivo exposure to ozone depletes vitamins C and E and induces lipid perox-

idation in epidermal layers of murine skin. Free Radical Biol. Med. 23, 385–391.

Tobe, M., Isobe, Y., Goto, Y., Obara, F., Tsuchiya, M., Matsui, J., Hirota, K., Hayashi, H., 2000. Synthesis and biological evaluation of CX-659S and its related compounds for their inhibitory effects on the delayed-type hypersensitivity reaction. Bioorg. Med. Chem. 8, 2037–2047.

Trenam, C.W., Blake, D.R., Morris, C.J., 1992. Skin inflammation: reactive oxygen species and the role of iron. J. Invest. Dermatol. 99, 675–682.

Valacchi, G., Rimbach, G., Saliou, C., Weber, S.U., Packer, L., 2001. Effect of benzoyl peroxide on antioxidant status, NF-kappaB activity and interleukin-1alpha gene expression in human keratinocytes. Toxicology 165, 225–234.

Yamazaki, I., Piette, L.H., 1990. ESR spin-trapping studies on the reaction of Fe^{2+} ions with H_2O_2-reactive species in oxygen toxicity in biology. J. Biol. Chem. 265, 13589–13594.

Young, J.M., Spires, D.A., Bedord, C.J., Wagner, B., Ballaron, S.J., De Young, L.M., 1984. The mouse ear inflammatory response to topical arachidonic acid. J. Invest. Dermatol. 82, 367–371.

Yuen, K.S., Halliday, G.M., 1997. α-Tocopherol, an inhibitor of epidermal lipid peroxidation, prevents ultraviolet radiation from suppressing the skin immune system. Photochem. Photobiol. 65, 587–592.