Journal of Cellular Biochemistry 92:425–435 (2004)

FAST TRACK

Preventive Effects of a Water-Soluble Derivative of Chroman Moiety of Vitamin E on Lipid Hydroperoxide-Induced Cell Injuries and DNA Cleavages Through Repressions of Oxidative Stress in the Cytoplasm of Human Keratinocytes

Zhe Huang,[1] Yuichiro Senoh,[1] Shinya Katoh,[2] and Nobuhiko Miwa[1]*

[1]Laboratory of Cell Death Control Technology, Department of BioTechnology, Hiroshima Prefectural University School of BioSciences, Nanatsuka, Shobara, Hiroshima, Japan
[2]Development Department, Research and Development Division, Kuraray Co., Inc., Japan

Abstract ChrCrx (6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid) is a water-soluble analog in which 4′, 8′, 12′-trimethyltridecyl chain is deleted from an alpha-tocopherol molecule known as a hydrophobic antioxidant. Cell viability of human skin epidermal keratinocytes HaCaT was lowered by treatment with *tert*-butylhydroperoxide (*t*-BuOOH) of 50 μM for 48 h, designated as a subacute cytotoxicity, which was prevented by previous administration with ChrCrx in a dose-dependent manner as estimated by mitochondrial function-based WST-1 assay and cell morphological microscopy. In contrast an acute cytotoxicity due to treatment with *t*-BuOOH as dense as 200 μM for a period as short as 2 h could be also prevented with ChrCrx that was administered before and after, but was eliminated during, treatment with *t*-BuOOH. In contrast α-tocopherol was not cytoprotective against *t*-BuOOH. DNA strand cleavages were induced with *t*-BuOOH in the keratinocytes, and could be prevented by ChrCrx more effectively than α-tocopherol as assayed by TUNEL stain. The intracellular reactive oxygen species (ROS) was accumulated in a manner dependent on periods of *t*-BuOOH treatment in the cytoplasm more abundantly rather than the nucleus of keratinocytes, and was markedly diminished by ChrCrx as shown by fluorography using the redox indicator dye. Thus *t*-BuOOH-induced cell injuries and DNA cleavages of the keratinocytes can be prevented at least in part through efficient diminishment of ROS generated in the cytoplasm, to which the preferred distribution of ChrCrx may be advantageous over to the nucleus or membrane owing to its molecular hydrophilicity relative to α-tocopherol. J. Cell. Biochem. 92: 425–435, 2004.

Key words: vitamin E derivative; reactive oxygen species (ROS); keratinocytes; lipid hydroperoxide

Oxidative stress is one of the most important factors in the pathogenesis of skin diseases including skin senescence and skin cancer [Singh and Agarwal, 2002; Trouba et al., 2002]. Investigation of the mechanism of oxidative stress in the development of skin disease has drawn more attention. Furthermore, new non-cytotoxic antioxidative approaches to the prevention and therapy of skin disease are urgently needed. In vivo and in vitro biological studies have indicated that specific anti-apoptotic genes such as *Bcl-2* [Virgili et al., 1998; Saitoh et al., 2003], cytokines such as the platelet-activating factor acetylhydrolase II [Marques et al., 2002], and antioxidant compounds such as tocopherol analogs and ascorbic acid prevent oxidative stress-induced skin damages.

Alpha-tocopherol has a hydrophobic property, and is distributed most predominantly as the lipid chain-breaking antioxidant that is localized closely to the polyunsaturated fatty acid of phospholipids in the outer part of the cell membrane and nuclear membrane. It is believed to be the most important anti-oxidative lipophilic agent which is naturally occurred nonenzymatically. It transacts as a direct

*Correspondence to: Nobuhiko Miwa, PhD, Laboratory of Cell Death Control Technology, Department of BioTechnology, Hiroshima Prefectural University School of BioSciences, Nanatsuka 562, Shobara, Hiroshima 727-0023, Japan. E-mail: miwa-nob@bio.hiroshima-pu.ac.jp

Received 9 February 2004; Accepted 17 February 2004
DOI 10.1002/jcb.20081

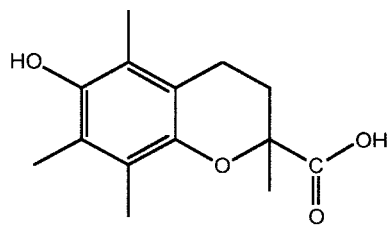
antioxidant toward $^1O_2$ and $O_2^{-\bullet}$, while it indirectly interrupts the reaction chain of lipid peroxidation [Serbinova et al., 1991; Nachbar and Korting, 1995]. ChrCrx (6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid), the water-soluble analog in which the phytyl moiety is deleted from alpha-tocopherol (Fig. 1), has been shown to have a potential for preventing oxidation in diverse cell systems. Some recent in vitro studies show that ChrCrx exerts anti-apoptotic activity through an anti-oxidative mechanism in varied cell systems [Birringer et al., 2003; Miller et al., 2003; Sparrow et al., 2003]. However, the preventive mechanisms underlying diverse ROS reactions have not been elucidated yet.

As a cell-death prevention model, we used the non-tumorigenic, spontaneously immortalized human keratinocyte cell line HaCaT [Boukamp et al., 1988] and examined its susceptibility to diverse oxidative stresses. Tert-butyl hydroperoxide (*t*-BuOOH) can induce the continuous stepwise productions of radical substances. The biologic oxidation of alkenes with *t*-BuOOH involves *tert*-butylperoxy and *tert*-butoxyl radicals [Gan et al., 2002]. In the present study, *t*-BuOOH was employed as oxidants to the keratinocytes. We attempted to elucidate the antioxidative role of ChrCrx against *t*-BuOOH-induced DNA damages and apoptosis-like cell death, and examined whether the antioxidative effect of ChrCrx was superior to that of its parent compound alpha-tocopherol. We found that ChrCrx protected HaCaT cells against *t*-BuOOH-induced cell injuries by decreasing intracellular ROS level.

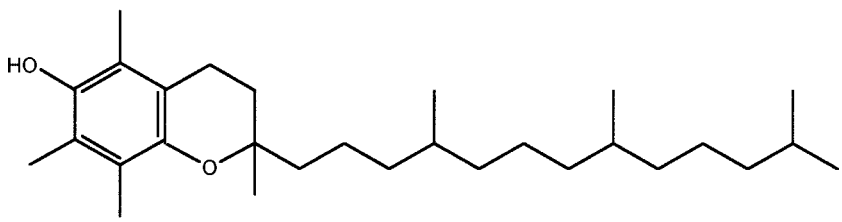
MATERIALS AND METHODS

Cell Culture

Human skin epidermal keratinocytes HaCaT were kindly provided by Dr. Fusening (German Cancer Research Center, Heidelberg, FRG) [Boukamp et al., 1988]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), penicillin (100 U/ml), streptomycin (100 μg/ml), and 4 mM L-glutamine at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Near-confluent cells were trypsinized and subcultivated in culture dishes at a density of 3×10^4/ml. The cells were cultured under standard conditions described above. At 24 h after seeding, the culture medium was replaced with fresh medium. For all the experiments, logarithmically growing HaCaT cells were used.



Croman carboxylic acid (ChrCrx)



alpha-Tocopherol (α -Toc)

Fig. 1. Chemical structure of ChrCrx (chroman carboxylic acid) and alpha-tocopherol.

t-BuOOH and Antioxidant Treatment

t-BuOOH and alpha-tocopherol were purchased from Sigma Chemical Company (St. Louis, MO). ChrCrx (Fig. 1) was manufactured and supplied by Kuraray Co., Ltd. (Tokyo, Japan). Various concentrations of *t*-BuOOH were dissolved in culture medium. ChrCrx and alpha-tocopherol were prepared in ethanol as stock solutions at a concentration 10^3-fold as high as in working solution. Respective controls were treated with an equal volume (0.1% v/v) of ethanol. The antioxidants were added to the culture plates before *t*-BuOOH treatment for indicated periods.

Cell Viability Assay

WST-1 assay was used to determine viable cell number. At different time points, the cells were rinsed with phenol red-free DMEM, then incubated for 3 h in phenol red-free DMEM medium containing 10% WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2-disulfophenyl)-2H-tetrazolium, monosodium salt; Dojn Laboratories Co., Kumamoto, Japan) at 37°C. Cell viability was determined based on mitochondrial conversion of WST-1 to yellowish formazan, being indicative of the number of viable cells [Ishiyama et al., 1996]. The absorbance at 450 nm was read with an absorbance multiplate reader (Bio-Rad, San Jose, CA, microplate photometer).

TUNEL Staining Assay

The detection of apoptotic nuclei using the terminal deoxybonucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method was performed using an in situ apoptosis detection kit according to the protocol specified by the manufacturer (TaKaRa, Kyoto, Japan). Briefly, subconfluent cells were treated with sham or *t*-BuOOH after the addition of ChrCrx or alpha-tocopherol. Cells were directly stained on chamber slides. After washing three times with phosphate buffer saline (PBS (−)), the cells were fixed in a freshly prepared solution of 4% paraformaldehyde in PBS (−) for 25 min at room temperature. Then the cells were further washed three times with PBS (−). Cells were treated with permeation buffer for 5 min on ice and washed three times with PBS (−). To label DNA strand cleavage terminals, cells were incubated with TUNEL reaction mixture containing TdT and fluorescent-dUTP for 90 min at 37°C in a humidified chamber. Thereafter, the slides were washed three times with PBS (−). Finally, the cells were mounted with coverslips and examined using a laser scanning confocal fluorescence microscope MRC-600 (Bio-Rad).

Measurement of Intracellular ROS

The cells were treated under the same condition as described above, and were washed with PBS (−). Then, the medium was replaced for 1 h by phenol red-free DMEM containing 20 μM 6-caboxy-2′, 7′-dichlorodihydrofluorescein diacetate (CDCFH) (Molecular Probes, Eugene, OR). The fluorescent intensity of the oxidative form of CDCFH was measured at 534 nm of an emission wavelength after excitation at 510 nm using a fluorescence microplate reader CytoFluor 2350 (Millipore, Bedford, MA). The methanol-killed cells were treated as the blank ones. On the other hand, cells were seeded on chamber slides and treated in the same manner as figure legends. Finally, the cells were examined using a laser scanning confocal fluorescence microscope MRC-600 (Bio-Rad).

Microscopy and Photography

HaCaT cells were observed using a Nikon microscope DiaPhot and photographed using a Nikon digital camera E 450. These images were formatted as images in Adobe Photoshop. AquaCosmos software was used in TUNEL staining analysis and CDCFH method including pseudo-color images and the equivalent numerical values for intensity of signal. Composite figures were made using a Microsoft software PowerPoint.

RESULTS

Preventive Effects of ChrCrx on *t*-BuOOH-Induced Cell Death of Keratinocytes

To examine the cytoprotective effects of ChrCrx and alpha-tocopherol, we analyzed the cell viability of human epidermal keratinocytes HaCaT that were previously administered with or without varying concentrations of ChrCrx or alpha-tocopherol, and 2 h after treated with *t*-BuOOH, a model compound of lipid hydroperoxide by photometric bioassay using a mitochondrial dehydrogenase-based formazan-forming dye WST-1. After the previous administration, the keratinocytes were treated with *t*-BuOOH of 200 μM for 3 h and then incubated for

additional 48 h in fresh medium containing ChrCrx or alpha-tocopherol in the absence of *t*-BuOOH. Cell viability of the *t*-BuOOH-treated keratinocytes was decreased, but restored with ChrCrx in a manner dependent on concentrations (Fig. 2A). The most marked cytoprotection was executed with ChrCrx at 300 μM out of diverse concentrations, whereas

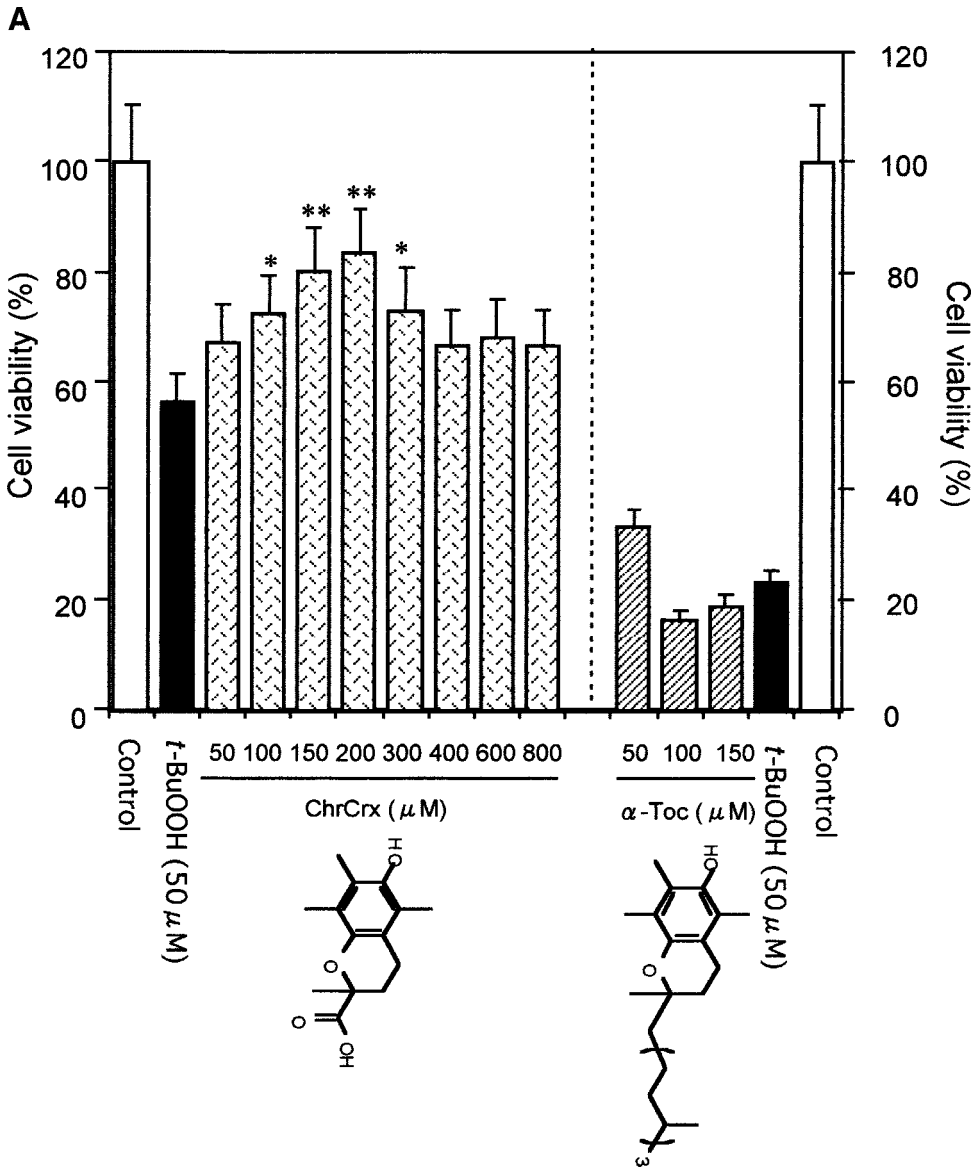


Fig. 2. **A**: Preventive effects of the water-soluble chroman derivative ChrCrx on higher concentrations of *t*-BuOOH induced acute cytotoxicity of human skin keratinocytes HaCaT. Cells were seeded on 24-well plates at densities of 12,000~15,000 cells/well, and cultivated for 18 h in DMEM-10% FBS. Cells were administered with ChrCrx contained in fresh medium for 30 min, and then added with *t*-BuOOH at a final concentration of 200 μM. After 2 h treatment, the medium was removed, rinsed twice and replaced by DMEM-10% FBS containing ChrCrx. Cells were then further cultivated for 48 h, and assayed for cell viability by mitochondrial dehydrogenase-based photometric method using formazan-forming dye WST-1 at 450 nm. The data shown are typical of four independent experiments, each of which contained well in triplicate in microplates. The bar represents SD of each group. (*, $P < 0.05$; **, $P < 0.01$). **B**: Preventive effects of ChrCrx on lower concentrations of t-BuOOH induced subacute cytotoxicity of HaCaT keratinocytes. HaCaT cells were seeded, cultivated, and administered with ChrCrx in the same manner as in Figure 1. After 2 h incubation cells were given with *t*-BuOOH of a concentration 20-fold higher than a final concentration of 50 μM (4-fold lower than in A) at a 1:20 volume ratio, and further treated for 48 h (24-fold longer than in A). Cells were similarly assayed by WST-1 method. The data shown are typical of four independent experiments, each of which contained well in triplicate in microplates. The bar represents SD of each group. (*, $P < 0.05$; **, $P < 0.01$). **C**: Preventive effects of ChrCrx on *t*-BuOOH induced degeneration of human skin keratinocytes HaCaT. Cells were treated in the same manner as in A and photographed under a phase-contrast microscope with Hoffman interference. The bar indicates 50 μm.

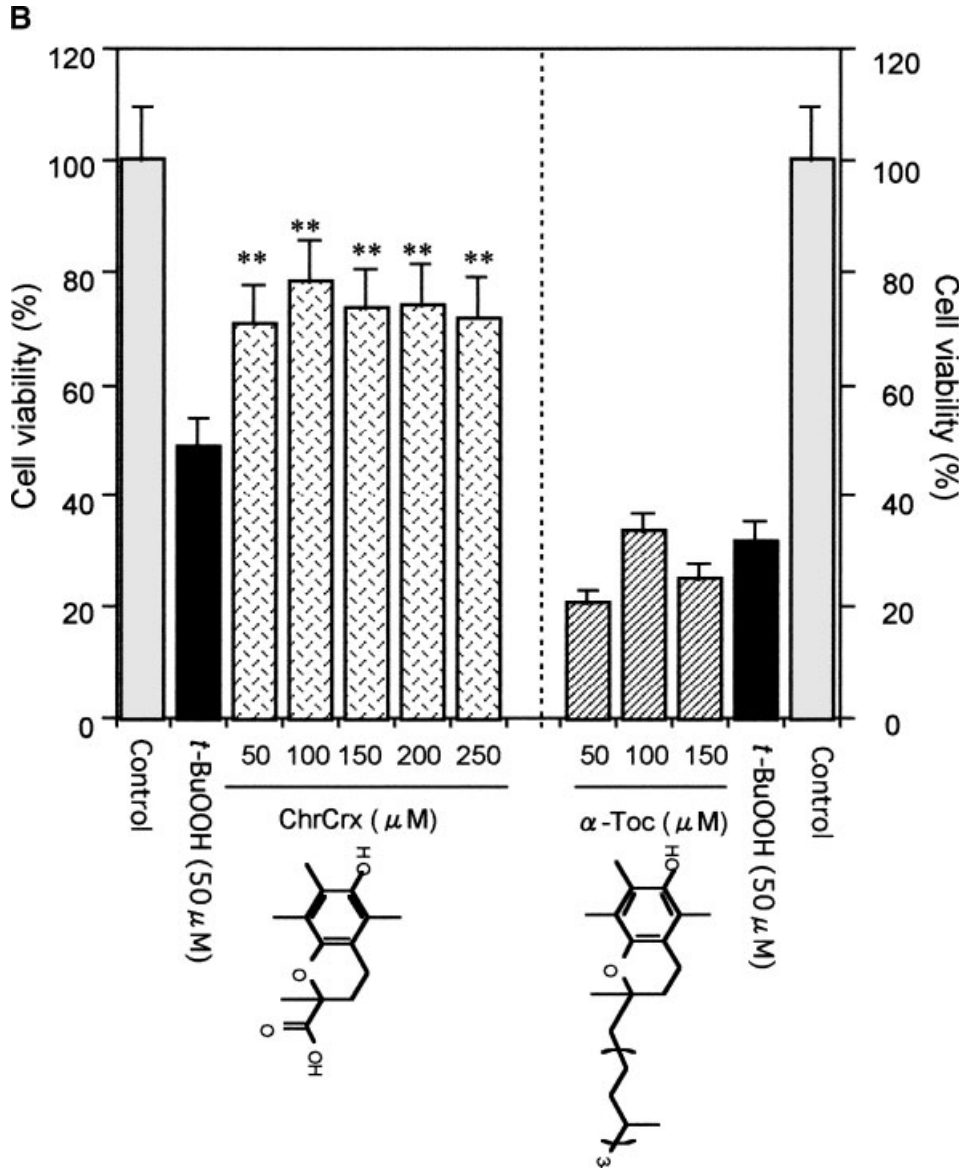


Fig. 2. (*Continued*)

alpha-tocopherol did not prevent the cell death at any concentrations examined.

In contrast to the above-described "acute cytotoxicity," the more moderately cytotoxic effect designated as "subacute cytotoxocity" was also examined in the keratinocytes which were treated with lower concentrations of *t*-BuOOH for longer periods. Cell viability of the keratinocytes was assayed after similar previous administration with various concentrations of ChrCrx or alpha-tocopherol for 2 h and the subsequent treatment with *t*-BuOOH at a concentration of 50 μM for 48 h. ChrCrx also prevented the keratinocytes from being subjected to subacute cytotoxicity with *t*-BuOOH (Fig. 2B). In contrast, any examined concentrations of alpha-tocopherol did not increase the cell survival.

In terms of cell morphology, after *t*-BuOOH addition, HaCaT keratinocytes appeared unhealthy and underwent degenerative symptoms such as shrinkage of cytoplasm and pycnosis-like condensation of nucleus resulting in cell detachment from the plate substratum. The addition of increasing concentrations of ChrCrx to the culture medium allowed the cells to progressively recover the normal morphology that is indistinguishable from that of sham-treated cells (Fig. 2C). In contrast alpha-tocopherol could not appreciably prevent the cell damage.

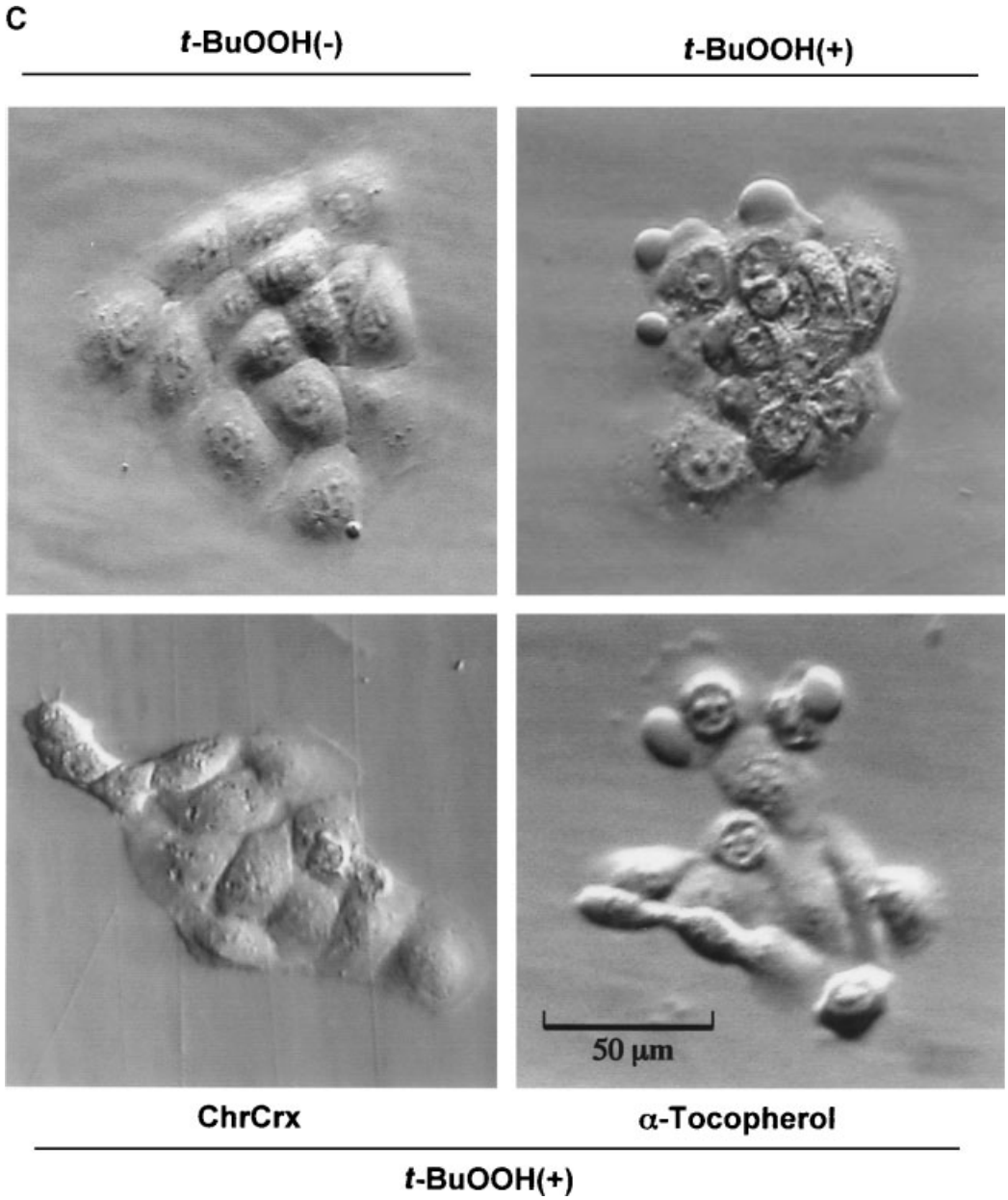


Fig. 2. (*Continued*)

Inhibitory Effects of ChrCrx on *t*-BuOOH-Induced DNA Strand Cleavages in HaCaT Keratinocytes

The effects of ChrCrx and alpha-tocopherol on *t*-BuOOH-induced apoptosis in HaCaT cells were examined by TUNEL assay. The DNA 3′-OH cleavage terminals characteristic of apoptosis were detected in most of the cells after treatment with 200 μM *t*-BuOOH for graded times. Cells subjected to the most extensive DNA cleavages were shown at 3 h after *t*-BuOOH addition (data not shown). The cells were previously administered with 300 μM of ChrCrx or 300 μM of alpha-tocopherol for 2 h, and then treated with 200 μM *t*-BuOOH for 3 h followed by TUNEL stain. Reductions of both DNA strand cleavages and apoptosis-like cells were achieved by administration with ChrCrx but not with alpha-tocopherol. After 3 h treatment with *t*-BuOOH, DNA cleavages in the ChrCrx-administered cells was less numerous than those in alpha-tocophenol-administered cells (Fig. 3). Taken together, it was suggested

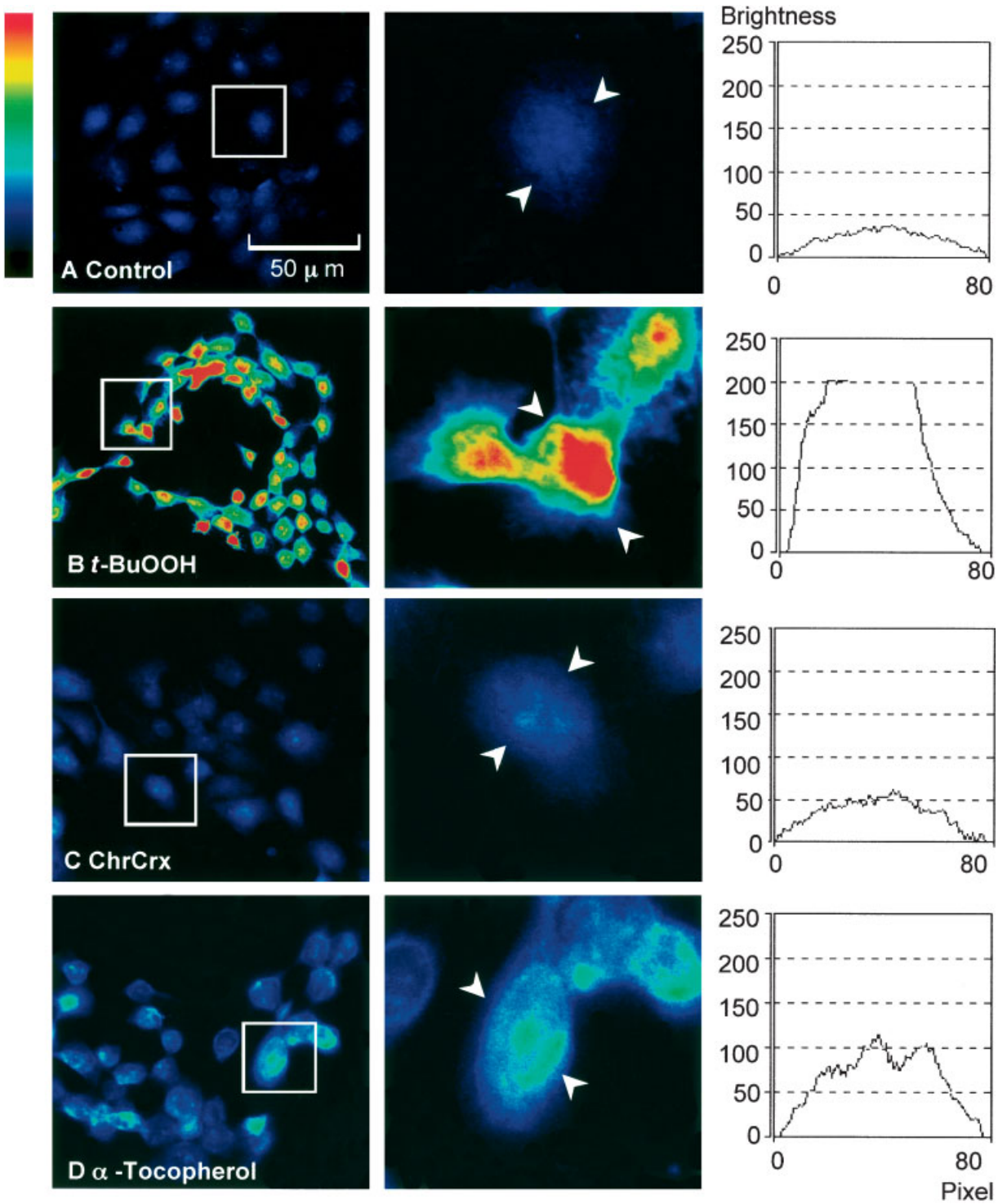


Fig. 3. Preventive effect of ChrCrx on nuclear DNA fragmentation in *t*-BuOOH-treated HaCaT cells. Cells were seeded on a chamber slide and cultivated for 18 h and administered with DMEM-10% FBS containing ChrCrx for 2 h. Then the medium was removed and rinsed. Cells were treated with *t*-BuOOH of 200 μM for 3 h, and underwent the fixation and staining for the cellular DNA cleavage terminals by fluorescent dye-based TUNEL method. The fluorescence intensity corresponding to extents of DNA cleavages was processed by an AquaCosmos software, and is expressed in a rainbow-colored gauge from red (scarce DNA cleavages) to purple (abundant DNA cleavages). The intracellular linear scanning from one end to another end of a cell shows the histogram of DNA cleavage extents. The data shown are typical of three independent experiments, each of which contained well in duplicate in chamber slides.

that ChrCrx may exert protective effects on *t*-BuOOH-induced apoptosis-like cell death in the keratinocytes.

Preventive Effects of ChrCrx on *t*-BuOOH-Induced Elevation of Intracellular Oxidative Stress

To examine whether ChrCrx could influence ROS (reactive oxygen species) level in human keratinocytes HaCaT treated with *t*-BuOOH, we quantified the intracellular ROS by fluorometry using the fluorescein derivative CDCFH, a redox indicator. After taken up into the cells, CDCFH is esterolyzed and resultantly becomes membrane-impermeable, the dye is oxidized to highly fluorescent CDCF primarily by H_2O_2, hydroxyl radicals, and diverse peroxides [Szejda et al., 1984]. A significant and time-dependent accumulation of intracellular ROS was observed at 150 min after 200 μM *t*-BuOOH treatment. In the cells pretreated with 300 μM ChrCrx, the ROS level was significantly reduced after treatment with *t*-BuOOH for 150, 180, and 210 min (Fig. 4A). Treatment with *t*-BuOOH induced marked morphological abnormalities in the keratinocytes which became notably fluorescent and bright after loading with CDCFH. The ROS-enhancing effects coupled with cell degeneration were prevented by addition of 300 μM ChrCrx (Fig. 4B).

DISCUSSION

In the present study, we demonstrated that the water-soluble chroman derivative ChrCrx protected human keratinocytes HaCaT against *t*-BuOOH-induced cell death by the evidences of repressions for mitochondrial dysfunction, cell morphological degeneration, and DNA stand cleavages in the keratinocytes. Mitochondria play an important role in cellular metabolism and proliferation. The mitochondrial dysfunction results in both deficiency in cellular energy supply and a disordered cell cycle. The cell morphology is closely related to cellular activity and metabolism, and was exemplified by the *t*-BuOOH-treated HaCaT keratinocytes that were deformed, appeared unhealthy, and underwent degenerative symptoms such as shrinkage of cytoplasm and pycnosis-like nuclear condensation resulting in cell detachment from the plate substratum upon either acute or subacute cytotoxicity. The superiority of ChrCrx to alpha-tocopherol (Fig. 2A,B) in terms of repressions of both mitochondrial disfunction and the cellular morphological abnormalities suggests that introduction of a carboxyl group into chroman moiety of alpha-tocopherol may promote the transmembrane permeability of ChrCrx into the intracellular space through enhanced endocytosis due to improvement of its molecular lipophilicity-hydrophlicity balance. The similar degrees in cytoprotection against acute and subacute cytotoxicities with *t*-BuOOH (Fig. 2A,B) suggest that the intracellular antioxidant pool ensuing from ChrCrx administration was excellent in terms of both instant antioxidant degrees and time-retentive duration.

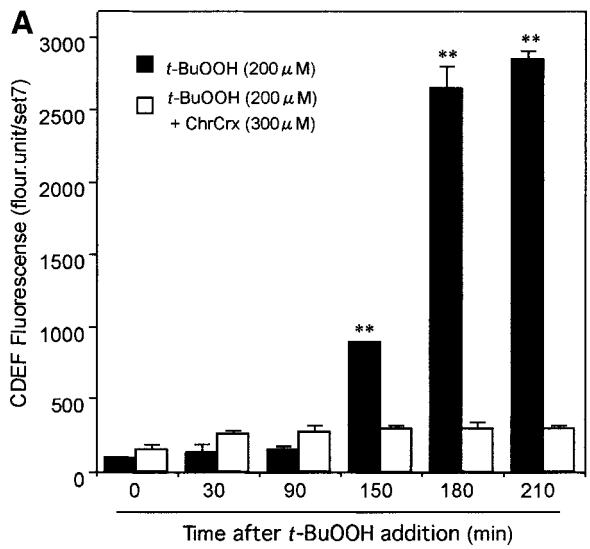


Fig. 4. Repressive effects of ChrCrx on intracellular oxidative stress in HaCaT cells treated with *t*-BuOOH. **A**: Cells were seeded on 24-well plates at a density of 30,000 cell/well and cultivated for 18 h. Then cells were administered with DMEM-10% FBS containing ChrCrx for 2 h. Then the medium was removed, rinsed and replaced by phenol red-free DMEM containing CDCFH-DA. After 30 min, cells were then further cultivated for 0∼210 min, and assayed by fluorescence plate reader (em: 458 nm; ex: 530 nm). The data shown are typical of three independent experiments, each of which contained wells in duplicate in microplates. The bar represents SD of each group. (*, $P < 0.05$; **, $P < 0.01$). **B**: Cells were seeded on a chamber slide and cells were treated in the same manner as in A, after addition with CDCFH-DA, cells were then further cultivated for 180 min. The fluorescence intensity corresponding to extents of ROS-generation was processed with an AquaCosmos software, and is expressed in a rainbow-colored gauge from red to purple. The intracellular linear scanning from one end to another end of a cell shows the histogram of ROS-enhancing. The data shown are typical of three independent experiments, each of which contained well in duplicate in chamber slides.

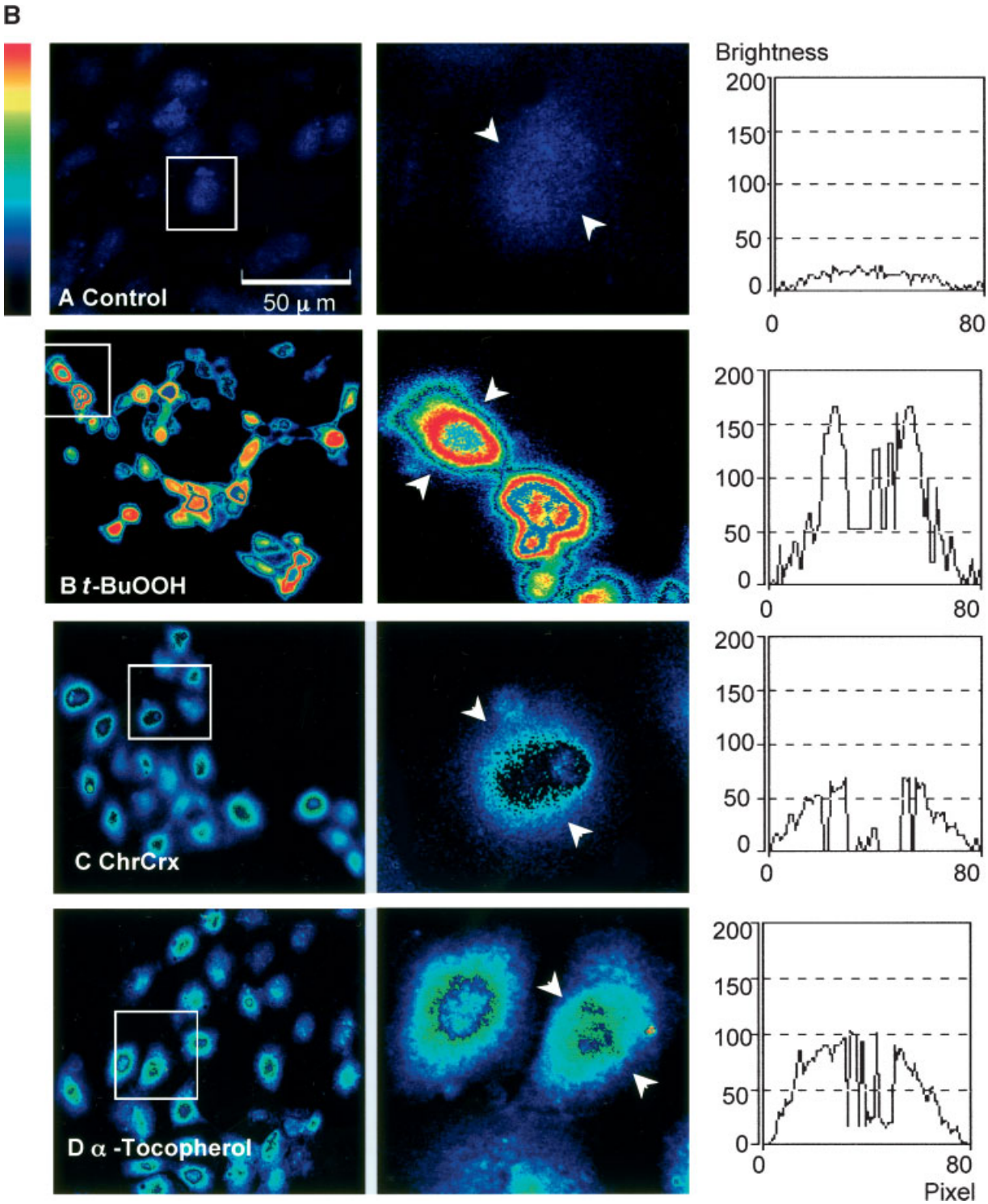


Fig. 4. (*Continued*)

The DNA 3′-OH terminals in nuclei as a typical symptom of apoptosis were shown to be increased in a manner dependent on periods of treatment with the prooxidant *t*-BuOOH [Adams et al., 1996; Cregan et al., 1999; Cai et al., 2000; Barak et al., 2001; Haidara et al., 2002] and restored down nearly to the initial level by ChrCrx, suggesting that preventive effects on apoptotic cell death of the keratinocytes may result from sufficient enlargement of

an intracellular antioxidant pool necessary to be accomplished prior to extracellular attack due to the hydroperoxide.

The *t*-BuOOH-induced cell damage is known to be at least partly mediated by ROS [O'Donovan et al., 1999; Hara et al., 2001]. The present study showed that the *t*-BuOOH-caused increase in ROS generation (Fig. 4A) preceded the onset of DNA strand cleavages and cell death, suggesting ROS serving as a trigger to *t*-BuOOH-induced cell injuries which can be suppressed by ChrCrx through alleviation of intracellular oxidative stress. These results indicate that ChrCrx interrupted the chain reaction during lipid peroxidation that may occur sequentially after *t*-BuOOH-induced ROS generation at a stage as early as 150–210 min after the hydroperoxide addition (Fig. 3). Taken together, these data suggest that ChrCrx protects the keratinocytes from *t*-BuOOH-induced apoptosis-like cell death via mitochondrial membrane retention and the repressed ROS level. Localization of more abundant ROS preferentially in the cytoplasm over in the nucleus of *t*-BuOOH-treated keratinocyte (Fig. 4B) suggests that, after entry into the intracellular space, ChrCrx may be accumulated in enriched state in the cytoplasm and can efficiently scavenge *t*-BuOOH-derived ROS before reaching of *t*-BuOOH to the vicinity of DNA, resulting in DNA protection (Fig. 3).

Another vitamin E analog lacking in all the methyl groups on the chromanol ring was somewhat more effective than alpha-tocopherol (by Huang et al., data not shown). Furthermore, modifications of different functional moieties of alpha-tocopherol can enhance the biological activity [Pentland and Morrison, 1992; Suzuki and Packer, 1993; Sen et al., 2000; Birringer et al., 2003]. Our data show that ChrCrx, one of the analogues of alpha-tocopherol, with lower number of methyl groups and replacement of the aliphatic side-chain by a carboxyl group, greatly enhanced the antioxidant and anti-apoptotic activity relative to its parent compound.

Further studies are needed to elucidate the molecular mechanisms underlying the cytoprotection of ChrCrx. The oxidative stress-responding transcriptional factor NF-κB and some apoptosis-related genes such as *c-myc*, *p53*, and *p21* remain to be involved in the cytoprotection. The lower hydrophobicity and smaller molecular size of ChrCrx due to deletion of its side-chain phytyl moiety may occur the shift and localization of the molecule preferably into the cytoplasm and the more exterior portion within lipid bilayer of the cell membrane, which may be governed by the different physicochemical characteristics and lipophilicity-hydrophilicity balance. The molecular modification of vitamin E and ChrCrx may result in influencing a common biological outcome, and is important in future drug design by defining the structure-activity relationship at the cellular and molecular levels.

REFERENCES

Adams JD, Mukherjee SK, Klaidman LK, Chang ML, Yasharel R. 1996. Apoptosis and oxidative stress in the aging brain. Ann NY Acad Sci 786:135–151.

Barak A, Morse LS, Goldkorn T. 2001. Ceramide: A potential mediator of apoptosis in human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 42:247–254.

Birringer M, Eytina JH, Salvatore BA, Neuzil J. 2003. Vitamin E analogues as inducers of apoptosis stracture-function relation. Br J Cancer 88:1948–2955.

Birringer M, EyTina JH, Salvatore BA, Neuzil J. 2003. Vitamin E analogues as inducers of apoptosis: Structure–function relation. Br J Cancer 88:1948–1955.

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106:761–771.

Cai J, Nelson KC, Wu M, Sternberg P, Jr., Jones DP. 2000. Oxidative damage and protection of the RPE. Prog Retin Eye Res 19:205–221.

Cregan SP, Smith BP, Brown DL, Mitchel RE. 1999. Two pathways for the induction of apoptosis in human lymphocytes. Int J Radiat Biol 75:1069–1086.

Gan L, Huang S, Zhang X, Zhang A, Cheng B, Cheng H, Li X, Shang G. 2002. Fullerenes as a tert-butylperoxy radical trap, metal catalyzed reaction of tert-butyl hydroperoxide with fullerenes, and formation of the first fullerene mixed peroxides C(60)(O)(OO(t)Bu)(4) and C(70)(OO(Bu)(10). J Am Chem Soc 124:13384–13385.

Haidara K, Morel I, Abalea V, Gascon Barre M, Denizeau F. 2002. Mechanism of tert-butylhydroperoxide induced apoptosis in rat hepatocytes: Involvement of mitochondria and endoplasmic reticulum. Biochim Biophys Acta 1542:173–185.

Hara S, Shoji Y, Sakurai A, Yuasa K, Himeno S, Imura N. 2001. Effects of seleniumdeficiency on expression of selenoproteins in bovine arterial endothelial cells. Biol Pharm Bull 24:754–759.

Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y, Ueno K. 1996. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol Pharm Bull 19:1518–1520.

Marques M, Pei Y, Southall MD, Johnston JM, Arai H, Aoki J, Inoue T, Seltmann H. 2002. Identification of platelet-activating factor acetylhydrolase II in human skin. J Invest Dermatol 119:913–919.

Miller MW, Miller WM, Battaglia LF. 2003. Biological and environmental factors affecting ultrasound-induced

hemolysis in vitro: 3. Antioxidant(Trolox) inclusion. Ultrasound Med Biol 29:103–112.

Nachbar F, Korting HC. 1995. The role of vitamin E in normal and damaged skin. J Mol Med 73:7–17.

O'Donovan DJ, Katkin DJP, Tamura T, Husser R, Xu XD, Smith CV, Welty SE. 1999. Gene transfer of mitochondrially targeted glutathione reductasw protects H441 cell from t-Butyl hydroperoxide-induced oxidant stresses. Am J Resplr Cell Mol Bio 20:256–263.

Pentland AP, Morrison AR. 1992. Tocopherol analogs suppress arachidonic acid matabolism via phospholipase inhibition. J Biol Chem 267:15578–15584.

Saitoh Y, Ouchida R, Kayasuga A, Miwa N. 2003. Anti-apoptotic defense of *bcl-2* gene against hydroperoxide-induced cytotoxicity together with suppressed lipid peroxidation, enhanced ascorbate uptake, and upregulated Bcl-2 protein. J Cell Biochem 89:321–334.

Sen CK, Khanna S, Roy S, Packer L. 2000. Molecular basis of vitamin E action. Tocotrienol potently inhibits glutamate-induced pp60(c-Src) kinase activation and death of HT4 neuronal cells. J Biol Chem 275:13049–13055.

Serbinova E, Kagan V, Han D, Packer L. 1991. Free radical recycling and intramembrane mobility in the antioxidant properties of alpha-tocopherol and alpha-tocotrienol. Free Radic Biol Med 10:263–275.

Singh RP, Agarwal R. 2002. Flavonoid antioxidant silymarin and skin cancer. Antioxid Redox Signal 4:655–663.

Sparrow JR, Vollmer-Snarr HR, Zhou J, Jang YP, Jockusch S, Itagaki Y, Nakanishi K. 2003. A2E-epoxides damage DNA in retinal pigment epithelial cells. Vitamin E and other antioxidants inhibit A2E-epoxide formation. J Biol Chem 278:18207–18213.

Suzuki YJ, Packer L. 1993. Inhibition of NF-kappa B activation by vitamin E derivatives. Biochem Biophys Res Commun 193:277–283.

Szejda P, Parce JW, Seeds MS, Bass DA. 1984. Flow cytometric quantitation of oxidative product formation by polymorphonuclear leukocytes during phagocytosis. J Immunol 133:3303–3307.

Trouba KJ, Hamadeh HK, Amin RP, Germolec DR. 2002. Oxidative stress and its role in skin disease. Antioxid Redox Signal 4:665–673.

Virgili F, Santini MP, Canali R, Polakowska RR, Haake A, Perozzi G. 1998. Bcl-2 overexpression in the HaCaT cell line is associated with a different membrane fatty acid composition and sensitivity to oxidative stress. Free Radic Biol Med 24:93–101.