

Different characteristics of reactive oxygen species production by human keratinocyte cell line cells in response to allergens and irritants

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Abstract: Keratinocytes mount immune responses through the secretion of a variety of inflammatory cytokines, soluble proteins and reactive oxygen species (ROS). However, the role of ROS in keratinocytes in response to allergens and irritants has not yet been elucidated. In this study, we investigated the (i) ROS production; (ii) potential sites of ROS production; (iii) expression of cell surface molecules; (iv) secretion of cytokines; and (v) ROS-dependent protein carbonylation in chemical-treated human keratinocyte cell line (HaCaT) cells. Treatment of HaCaT cells with 2,4-dinitrochlorobenzene (DNCB) and benzalkonium chloride (BKC) increased ROS levels in a time- and dose-dependent manner, as determined with dichlorodihydrofluorescein diacetate (CM-H₂DCFDA), without reducing cell viability. Potential sources of ROS production were evaluated with pretreatment of diphenylene iodonium (DPI), an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; rotenone, an inhibitor of the mitochondrial electron transport

chain complex or allopurinol, a xanthine oxidase inhibitor. The DNCB-induced ROS was related to both NADPH oxidase and mitochondrial electron transport chain complex. Conversely, BKC-induced ROS was related to NADPH oxidase only. Western blotting using an anti-DNP antibody revealed ROS-dependent protein carbonylation in response to DNCB but not BKC. Both DNCB and BKC increased the secretion of IL-1 α from HaCaT cells; however, ROS production as well as other changes, except DNCB-induced secretion of IL-1 α , was not inhibited by antioxidants. Although the role of ROS in keratinocytes in response to chemicals was inconclusive, our results suggest that the characteristics of ROS produced by keratinocytes in response to chemicals might differ.

Key words: allergen – contact dermatitis – human keratinocyte cell line cells – irritant – reactive oxygen species

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Introduction

Ultraviolet (UV) is a major well-characterized generator of reactive oxygen species (ROS), which mediate molecular damage in skin (1,2). The ROS are believed to activate proliferative and cell survival signaling that can alter apoptotic pathways, which in turn may be involved in the pathogenesis of a number of skin disorders and some types of cutaneous malignancy (3). The skin has numerous intracellular mechanisms to attenuate oxidative stress from ROS originating from the environment and the skin itself. However, increased or prolonged oxidative stress can overwhelm ROS defense mechanisms, contributing to the development of several cutaneous diseases (4).

Contact dermatitis, originating from both allergic and irritant etiologies, is the most common eczematous disease. However, distinguishing between allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) by clinical and histological examination alone can be difficult. Contact allergens can elicit inflammatory response in the skin, which is a crucial step in the development of ACD (5). Although both forms of contact dermatitis involve an inflammatory pathway, the skin reaction of ICD is non-immunologic and results from direct epidermal keratinocyte damage after contact with a stimulus. Unlike ICD, ACD exhibits a delayed-type hypersensitivity and affects only sus-

ceptible individuals who have been previously sensitized by allergen exposure (6).

Recently, there have been several reports supporting the participation of ROS in the pathogenesis of contact dermatitis. In antigen-presenting cells, type 1 helper T cell (Th1) polarization is readily transformed to Th2 dominance through depletion of intracellular glutathione (GSH) (7). Positive patch tests in patients who exhibit allergic responses after skin contact with nickel manifest increased tissue iron and an elevated oxidized/reduced GSH ratio, both of which characterize oxidative stress in skin (8). The ROS are generated in dendritic cells (DCs) during antigen presentation, suggesting that ROS-dependent regulation of DC–T cell communication during Ag presentation can be blocked by antioxidants (9). Previously, we investigated whether allergens and irritants induce ROS in DCs, and found that allergen-induced ROS are likely to increase immune response (10,11).

In contrast to DCs, the roles of ROS produced by keratinocytes, which represent the majority of epidermal cells and can produce pro-inflammatory cytokines, have not yet been elucidated. In the present study, we confirmed that various chemicals could produce ROS in the human keratinocyte cell line (HaCaT) cells. Potential sites of ROS production and ROS-dependent protein carbonylation were found to differ between 2,4-dinitrochlorobenzene

(DNCB) and benzalkonium chloride (BKC)-treated HaCaT cells. These results suggest that the characteristics of ROS produced by keratinocytes in response to chemicals are heterogeneous.

Materials and methods

Reagents

Chemicals, including DNCB, 2,4-dinitrofluorobenzene (DNFB), 2,4,6-trinitrobenzene sulfonic acid (TNBS), nickel sulfate (NiSO_4), cobalt chloride (CoCl_2), thimerosal, hydroquinone, sodium dodecyl sulfate (SDS), BKC, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antioxidants, including glutathione, *N*-acetyl cysteine (NAC) and vitamin-E were purchased from Sigma. Inhibitors of ROS-generating sources, including diphenylene iodonium (DPI), rotenone and allopurinol were purchased from Sigma. Mouse monoclonal fluorescence-conjugated antibodies (mAbs) against HLA-DR, intercellular adhesion molecule 1 (ICAM-1), E-cadherin and the appropriate isotype controls were purchased from BD Pharmingen (San Diego, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1 α , IL-1 β and TNF- α were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Cells culture

The human keratinocyte cell line was maintained in RPMI-1640 (Gibco; BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) (Sigma) at 37°C and used throughout this study.

ROS detection using dichlorodihydrofluorescein diacetate (CM-H₂DCFDA)

After 1-h treatment with chemicals, HaCaT cells were harvested, washed with warm PBS and resuspended in 50 μl PBS. Cells were stained with 3 μM CM-H₂DCFDA at 37°C for 15 min and washed twice with PBS. Before analysis, 10- μl propidium iodide (PI) (100 $\mu\text{g}/\text{ml}$) was added to exclude dead cells and to control the toxicity of chemicals. Samples were analysed by flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, CA, USA) for fluorescence signals within the PI⁺ populations. To rule out the influence of HaCaT cell itself on ROS production, we checked whether chemicals convert DCFDA to fluorescent DCF or not in a cell-free media. The DCF fluorescence intensity was measured by a fluorescence plate reader (Perkin Elmer LS50B, Shelton, CT, USA) at excitation wave length 485 and emission wave length 530 nm with excitation split of 10.

Determination of potential sources of ROS production

To determine potential sources of ROS, we used three major ROS generating enzyme blockers. The HaCaT cells were preincubated with 10- μM DPI, rotenone and 100 μM allopurinol for 30 min and treated with both 20- μM BKC and DNCB. Afterwards, cells were harvested and ROS levels were detected by flow cytometry using CM-H₂DCFDA.

Flow cytometric analysis of cell surface molecules

After treatment with chemicals for 24 and 48 h, cells were harvested, washed and stained with mouse monoclonal anti-HLA-DR, ICAM-1 or E-cadherin antibodies and the appropriate isotype controls. Changes of cell surface molecules on HaCaT cells were analysed by flow cytometry.

Detection of cytokine secretion from HaCaT cells

Presence of the cytokines IL-1 α , IL-1 β and TNF- α in cell culture supernatants after treatment with chemicals was analysed by ELISA according to the manufacturer's protocol.

Western blotting

Protein carbonylation was determined in HaCaT cells treated with 20- μM DNCB and 10- μM BKC for 3, 6 and 12 h. Protein concentrations were determined using BCA assay, after which 30 μg of total protein was loaded by 10% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA, USA). The membranes were incubated with an anti-DNP antibody at 4°C overnight. They were then washed, incubated further with HRP-conjugated anti-rabbit IgG (1/1000 dilution) and detected using an ECL detection system (Amersham Biosciences, Piscataway, NJ, USA). An anti-actin antibody (Cell Signaling Technology, Danvers, MA, USA) was used as a loading control (1:5000 dilution).

Statistical analysis

Production of ROS and expression of surface molecules were determined as the mean fluorescence intensity using flow cytometry. Results are expressed as mean $\pm$ standard deviation. Data were analysed using one way ANOVA and Mann-Whitney *U*-tests. Values of *P* < 0.05 were considered to be significant.

Results

Treatment of HaCaT cells with DNCB and BKC generate ROS in a time- and dose-dependent manner

In preliminary experiments, the concentrations of compounds at which live cells (PI-/Annexin V-) exceeded 90%, were chosen for further experiments. Increases in ROS levels were detected after treatment of HaCaT cells with DNCB, DNFB, TNBS, CoCl_2 , thimerosal, SDS and BKC (Fig. S1a). Among the treatments, DNCB and BKC were selected as representatives of allergen and irritant compounds, respectively. The DNCB and BKC increased ROS levels at concentrations ranging from 5 to 40 μM in a time-dependent manner (1–24 h) without reducing cell viability (Fig. S1b). In addition, we measured DCF fluorescence in a cell-free system to exclude the possibility that the increased DCF fluorescence observed herein is due to somewhat cell-independent phenomenon. The BKC and DNCB itself did not convert DCFDA into fluorescent DCF. There were no differences between the lowest and highest doses of DNCB and BKC (data not shown).

Sources of DNCB- and BKC-induced ROS in HaCaT cells

Potential sites of ROS production were evaluated by pretreatment with (i) DPI, an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; (ii) rotenone, an inhibitor of the mitochondrial electron transport chain complex or (iii) allopurinol, a xanthine oxidase inhibitor prior to treatment of DNCB and BKC. The DNCB-induced ROS was partially blocked by both rotenone and DPI, but not allopurinol. The BKC-induced ROS was completely blocked by DPI, but not rotenone and allopurinol. Therefore, DNCB-induced ROS might be related to both NADPH oxidase and mitochondrial electron transport chain complex, whereas BKC-induced ROS might be related to NADPH oxidase pathway (Fig. S2a,b).

Secretion of cytokines in response to DNCB and BKC in HaCaT cells

Cytokine secretion (IL-1 α , IL-1 β and TNF α) in HaCaT cells treated with BKC or DNCB was assessed by ELISA. The IL-1 α secretion in BKC- and DNCB-treated cells was increased by 2.2- and 2.4-fold respectively compared to the control, which was statistically significant (Fig. 1a). Other cytokines (IL-1 β , TNF α) were not increased significantly (data not shown). When HaCaT cells cul-

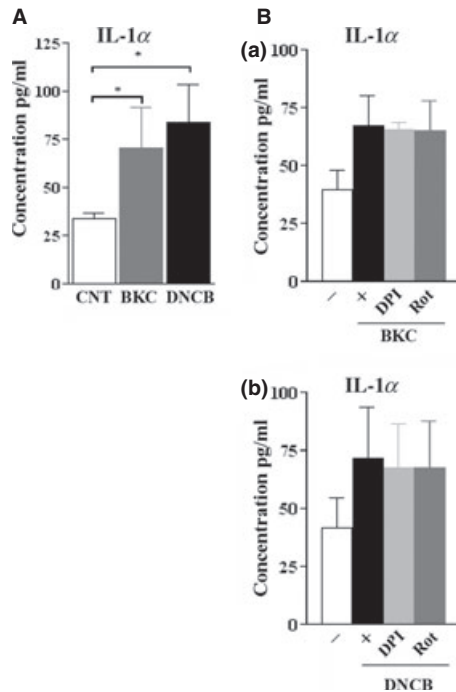


Figure 1. Human keratinocyte cell line (HaCaT) cells produce IL-1 α in response to both benzalkonium chloride (BKC) and 2,4-dinitrochlorobenzene (DNCB). (A) Cytokine secretion was measured in 5- μ M BKC- and 5- μ M DNCB-treated supernatants of HaCaT cells. IL-1 α secretion in BKC- and DNCB-treated HaCaT cells were increased by 2.2- and 2.4-fold respectively compared to the controls, the differences of which were statistically significant (P values < 0.05). (B) HaCaT cells treated with BKC (a) and DNCB (b) in the presence of diphenylene iodonium (DPI) and rotenone. Only DNCB-induced secretion of IL-1 α was reduced, the difference of which was not statistically significant.

tured with DNCB in the presence of DPI or rotenone, the increased levels of IL-1 α were decreased slightly, although the difference was not statistically significant. Conversely, pretreatment with DPI or rotenone did not alter the increased levels of IL-1 α by BKC (Fig. 1b).

Changes in surface molecules after treatment of HaCaT cells with either BKC or DNCB

To examine whether BKC- and DNCB-treated cells exhibit enhanced expressions of ICAM-1 and HLA-DR, we used flow cytometry analysis. The ICAM-1 expression in HaCaT cells cultured with BKC and DNCB was increased 1.6- and 1.2-fold respectively (Fig. 2a). When we checked for expression of ICAM-1 on BKC- and DNCB-treated HaCaT cells in the presence of DPI and rotenone, BKC-induced ICAM-1 expression was slightly decreased by DPI whereas DNCB-induced ICAM-1 expression was slightly decreased by rotenone; however, neither of these decreases was statistically significant (Fig. 2b). In addition, HLA-DR expression was increased slightly by both BKC and DNCB compared to that of the control, although this difference was not statistically significant.

ROS induced by DNCB, but not BKC, results in protein carbonylation

The ROS can cause a number of protein modifications, such as protein carbonylation and modification of protein side chains. Carbonylation results from direct oxidation of amino acids side chains. Although both DNCB and BKC induced ROS similarly,

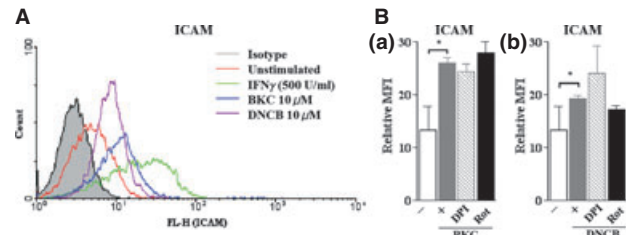


Figure 2. Augmentation of intercellular adhesion molecule 1 (ICAM-1) expression in human keratinocyte cell line (HaCaT) cells by BKC and 2,4-dinitrochlorobenzene (DNCB). HaCaT cells were treated with 5- μ M BKC and 5- μ M DNCB for 24 h and then analysed by flow cytometry. (A) Filled lines indicate untreated cells; red lines indicate stained cells with ICAM-1; green lines indicate positive control (500 U/ml IFN γ) and blue and purple lines indicate ICAM-1 expression on HaCaT cells after treatment with BKC and DNCB, respectively. (B) Bar graphs showing the relative increase of ICAM-1 as determined by MFI after treatment of BKC (a) or DNCB (b) and pretreatment with diphenylene iodonium (DPI) and rotenone respectively. BKC-induced ICAM-1 was decreased slightly by DPI whereas DNCB-induced ICAM-1 was decreased slightly by rotenone, although none of the differences was statistically significant.

only DNCB-induced ROS resulted in protein carbonylation as determined by Western blot analysis (Fig. 3a). The DNCB-induced protein carbonylation was inhibited by DPI, but not rotenone (Fig. 3b).

The effects of antioxidants on ROS production, cytokine secretions and upregulation of ICAM-1 in HaCaT cells in response to DNCB and BKC

ROS production by HaCaT cells in response to DNCB and BKC is not decreased in the presence of various antioxidants

After demonstrating the production of ROS from HaCaT cells cultured with BKC and DNCB, the effect of various antioxidants on ROS production was examined. Pretreatment of HaCaT cells with NAC, glutathione or vitamin E did not reduce ROS production (Fig. S3a).

Increased secretion of IL-1 α by DNCB but not BKC is decreased in the presence of antioxidants

The IL-1 α is constitutively produced by epidermal keratinocytes as a 33-kDa pro-form, that is proteolytically processed into a 17-kDa form by calpain. This cleavage can be triggered by ROS similar to pro-IL-1 β and IL-18. Therefore, we next examined IL-1 α secretion

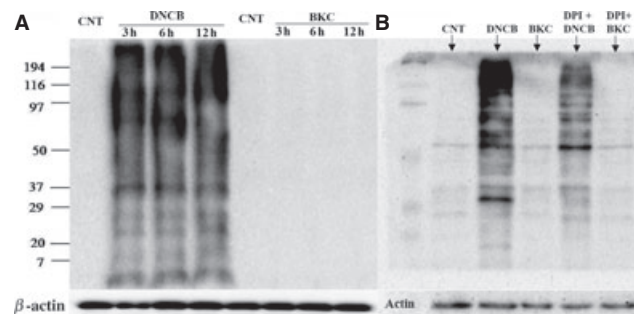


Figure 3. Contact allergen 2,4-dinitrochlorobenzene (DNCB), but not irritant BKC, induces protein carbonylation. (A) Human keratinocyte cell line (HaCaT) cells were incubated with 20- μ M DNCB or 10- μ M BKC for 6 h. Carbonylated groups were detected by immunoblotting with an anti-DNP antibody. Cells treated with DNCB, but not BKC, exhibited increased carbonylation compared to controls. Actin is shown as a loading control. (B) DNCB-induced protein carbonylation was decreased by pretreatment with diphenylene iodonium (DPI).

by ELISA in HaCaT cells treated with BKC and DNCB in the presence of antioxidants, such as NAC and glutathione. Whereas BKC-triggered secretion of IL-1 α was not diminished by antioxidants, DNCB-induced secretion of IL-1 α was decreased by pretreatment with glutathione and NAC (Fig. S3b).

Up-regulation of ICAM-1 on HaCaT cells by DNCB and BKC is not decreased by antioxidants

To verify that BKC- and DNCB-induced up-regulation of ICAM-1 was related to ROS production, we examined these parameters upon the addition of antioxidants. The HaCaT cells were treated with BKC and DNCB in the presence or absence of antioxidants and cell surface molecules were analysed using flow cytometry. Augmentation of ICAM-1 by BKC and DNCB was not reversed by the addition of antioxidants, as described in Materials and Methods (Fig. S3c).

Discussion

While previous studies regarding increased ROS as a result of chemical-induced contact dermatitis have focused mostly on DCs, the role of ROS in keratinocytes remains uncertain (10). Therefore, we aimed to investigate whether ROS are produced by chemicals in keratinocytes as well as the effect of ROS inhibition in chemical-treated keratinocytes.

In this study, increased ROS levels were detected using the fluorogenic probe CM-H₂DCFDA. Changes in ROS levels were detected after treatment for 30 min with allergens, including DNCB, DNFB, TNBS, CoCl₂, thimerosal and irritants, including BKC and SDS. The DNCB and BKC were found to produce ROS at low concentrations compared to other ROS-producing chemicals. Specifically, NiSO₄ and hydroquinone did not produce ROS within concentrations, non-toxic to HaCaT cells. In addition, despite being the most common allergen associated with ACD, nickel failed to produce ROS not only in HaCaT cells but also in DCs (data not shown). These results imply that cellular oxidative stress after contact with chemicals might depend on the specific nature of the chemicals themselves. Thus, we hypothesized that the pathogenesis of chemical-induced contact dermatitis may be partly dependent on oxidative stress; however, not all chemicals, including nickel, may exert such an effect. Thus, as not all types of oxidative stress were assessed, further studies verifying oxidative stress-dependent or oxidative stress-independent mechanisms should be addressed.

The most noteworthy results of this study were the differences in potential sites of ROS production by HaCaT cells in response to DNCB and BKC. Importantly, there are several known sources of cellular ROS, including NADPH oxidase pathway, which was initially observed as a response to microbial infection in phagocytes, but is now known to exist in almost all cell types, including epidermal keratinocytes (12,13). Another main pathway of cellular ROS production is the mitochondrial respiratory cycle. Specifically, the mitochondrial respiratory chain leaks approximately 2 percent of its yield of reactive (intermediate) molecules during normal cell respiration (14). A third pathway involves xanthine oxidase, which catalyses the oxidation of xanthine to uric acid, generating superoxide, a major ROS (15). Together, these main ROS generating pathways in keratinocytes can be altered by environmental invaders. However, the characteristics and sources of ROS produced by keratinocytes, which represent the majority of

epidermal cells and can produce proinflammatory cytokines, has not yet been elucidated.

In the present study, the DNCB-induced ROS were inhibited by DPI and rotenone but not allopurinol, which can be interpreted that DNCB-induced ROS might be related to either mitochondrial ROS generation system or NADPH oxidase pathway as DPI is non-specific inhibitor of NADPH oxidase. Meanwhile, BKC-induced ROS is not related to mitochondrial ROS generation system and xanthine oxidase system, but related to the NADPH oxidase pathway only as it was completely inhibited by DPI, but not rotenone and allopurinol. To clarify the role of NADPH oxidase in ROS production by HaCaT cells in response to DNCB and BKC, it is necessary to demonstrate the translocation of the cytosolic components, p47phox and p67phox, to the plasma membrane of NADPH oxidase (16).

Besides the differences of potential site of ROS production, protein carbonylation as an oxidative stress marker exhibits a marked difference between DNCB and BKC. In Western blotting using an anti-DNP antibody, ROS-dependent protein carbonylation in response to DNCB was increased, whereas BKC was not.

The expressions of ICAM-1 are up-regulated in keratinocytes and other types of cells in response of oxidative stress (17,18). In this study, the level of ICAM-1 expression in HaCaT cells treated with BKC and DNCB was increased 1.6- and 1.2-fold, which was slightly decreased by DPI and rotenone, respectively. However, increased ICAM-1 level was not decreased by antioxidants.

Antioxidants, including glutathione, selenium, NAC, Vit A, Vit E and catalase were utilized to inhibit the production of ROS in chemical-treated HaCaT cells; however, none inhibited the production of ROS in DNCB- and BKC-treated HaCaT cells. Glutathione is a classic antioxidant that removes ROS directly by acting on hydroxyl radical (OH), peroxy radical (ROO), alkoxy radical (RO) and hypochlorous acid (HClO) (19). The NAC is a precursor for the synthesis of glutathione and increases the concentration of intracellular glutathione (20). Likewise, selenium inhibits the apoptosis of keratinocytes induced by exposure to ultraviolet light irradiation (21), whereas Vit E inhibits lipid peroxidation and removes ROO (9) and catalase removes hydrogen peroxide (H₂O₂). The reasons why antioxidants do not inhibit ROS are fairly elusive. On the basis of the possibility that the conditions for antioxidant production were limited in our assays, we increased the concentration of catalase from 50 to 500 U/ml; however, similar results were obtained even under increased concentrations of catalase. On the other hand, DNCB-induced ROS were not completely reduced by antioxidants, and DNCB-induced IL-1 α secretion was diminished by antioxidants, such as GSH and NAC, whereas BKC-induced IL-1 α secretion was not reduced by pretreatment with antioxidants. Antioxidants are expected to be beneficial for the treatment of inflammatory skin diseases. However, ROS production by HaCaT cells in response to DNCB and BKC was not inhibited by a variety of antioxidants in this study. It is needed to distinguish whether chemicals lead to an increase in the ROS production or rather simply decrease the cell's antioxidant status (total antioxidant capacity/ability). Relationships between oxidative stress and antioxidant status in HaCaT cells in response to chemicals might be important regarding the effects of antioxidants on keratinocytes (20,22,23). We used specific blockers of ROS generating pathway and thiol-containing antioxidants.

Observing the result, we thought that both allergen and irritant-related ROS are relevant to the increased ROS generating system because specific blockers of ROS generating pathway prevented ROS production, and pretreatment of antioxidants did not diminish ROS production.

Mehrotra et al. (24). studied free radicals (reactive oxygen and nitrogen species) by contact allergens (NiSO₄, K₂Cr₂O₇, DNFB, CoCl₂ and paraphenylene diamine) and irritants (sodium lauryl sulfate, acetic acid, NaOH, sorbitol, sodium lauryl ethyl sulfate, α -olefin sulfonate, oleic acid, capsaicin and UVA+B) in human keratinocyte cell line A431. While irritants induce release of ROS even at noncytotoxic concentration, allergens produced ROS at the toxic concentration. Differing from our results, the antioxidants prevented irritant-induced cell death in their study. Based on our observations, there can be both ROS-dependent and ROS-independent changes in allergen- and irritant-treated HaCaT cells. With respect to this observation, DNCB-induced changes, including increased expression of ICAM-1, IL-1 α secretion and protein carbonylation, were dependent on ROS because they were reduced when we used ROS blockers. Further, the most significant changes induced by BKC were not reversed by antioxidants and ROS blockers, and as such, the effects of BKC can be attributed as ROS-independent effects.

In conclusion, in the present study, we demonstrated the production of ROS by chemical-treated HaCaT cells, as well as the

different characteristics of DNCB and BKC on ROS production in HaCaT cells. However, the role of ROS produced by hapten-treated HaCaT cells is not well understood yet, because several antioxidants do not inhibit production of ROS by DNCB- and BKC-treated HaCaT cells. Future studies should investigate the roles of antioxidants and additional inhibitors of ROS production in inhibiting the production of ROS by chemical-treated HaCaT cells or primary keratinocytes.

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Conflict of interest

The authors have declared no conflict of interest.

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Supporting Information

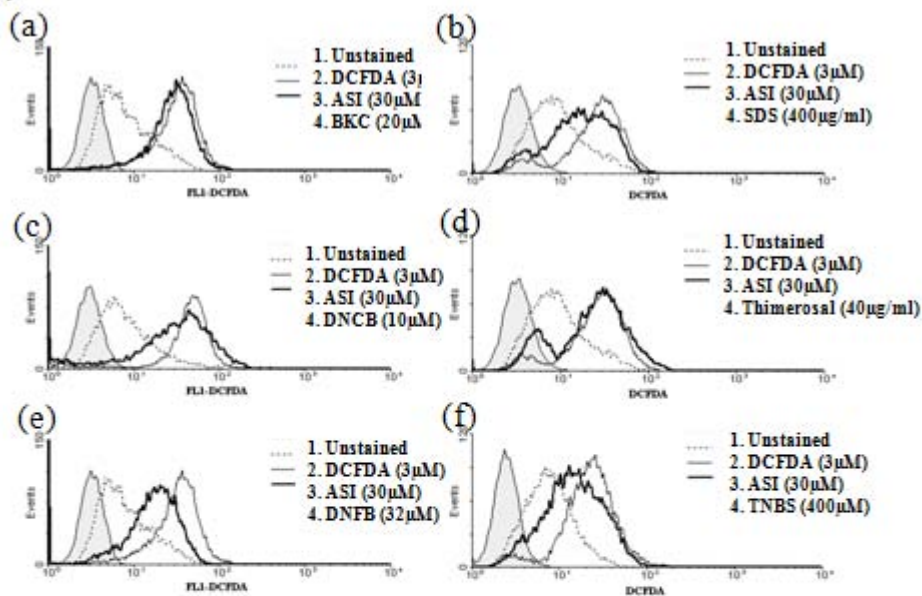
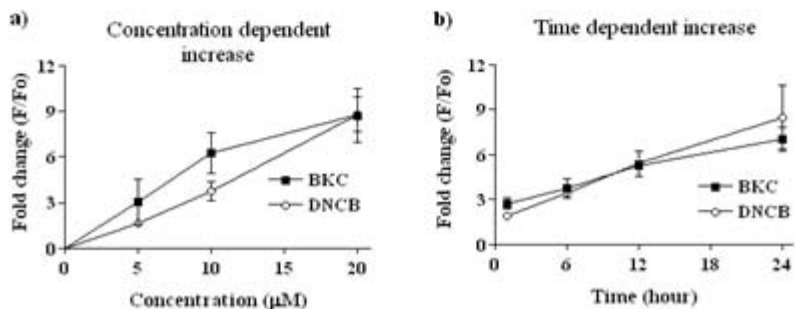
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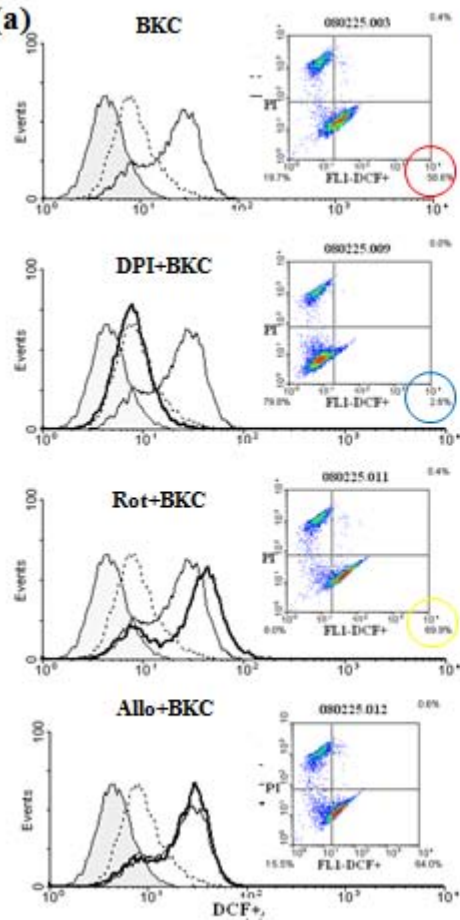
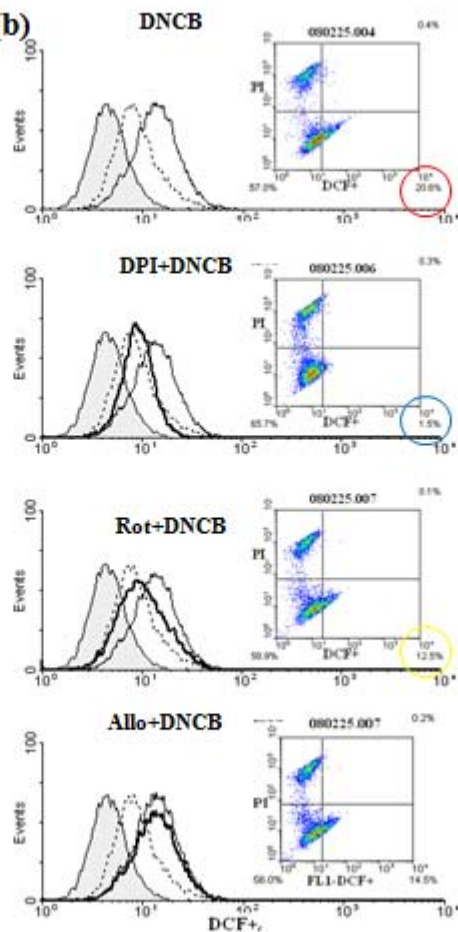
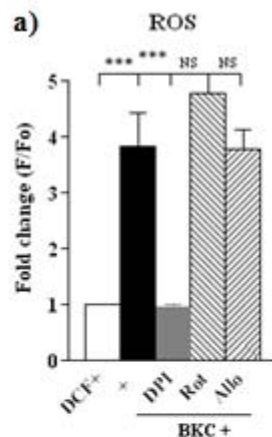
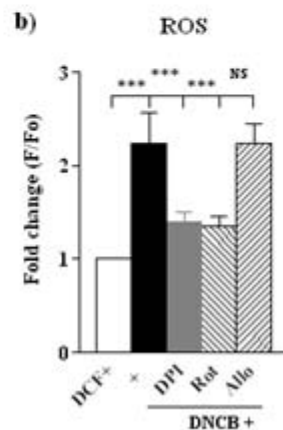
Figure S1. Human keratinocyte cell line (HaCaT) cells produce reactive oxygen species (ROS) after incubation with various chemicals, including allergens and irritants. HaCaT cells were incubated for 1 h with sublethal concentrations of irritants, including (a) benzalkonium chloride (BKC) and (b) sodium dodecyl sulfate (SDS); and allergens, such as (c) dinitrochlorobenzene (DNCB), (d) thimerosal (Thim), (e) dinitrofluorobenzene 2,4-dinitrofluorobenzene (DNFB) and (f) trinitrobenzene sulfonic acid (TNBS). ROS production was detected by CM-H₂DCFDA assay. (a) Filled lines represent control cells without CM-H₂DCFDA staining; dotted lines indicate physiologic ROS level in the cells (DCFDA staining only) without treatment; solid lines indicate ATP synthase inhibitor-treated cells as a positive control; solid thick lines indicate ROS levels induced by chemicals. (b) Plots showing dose- (a) and time (b)-dependent increase in ROS of HaCaT cells after treatment with BKC and DNCB. Plot values were obtained from FACS analysis. DCF fluorescence intensity is expressed as F/F₀, where F represents the MFI of samples and F₀ represents that of untreated but stained cells with DCFDA.

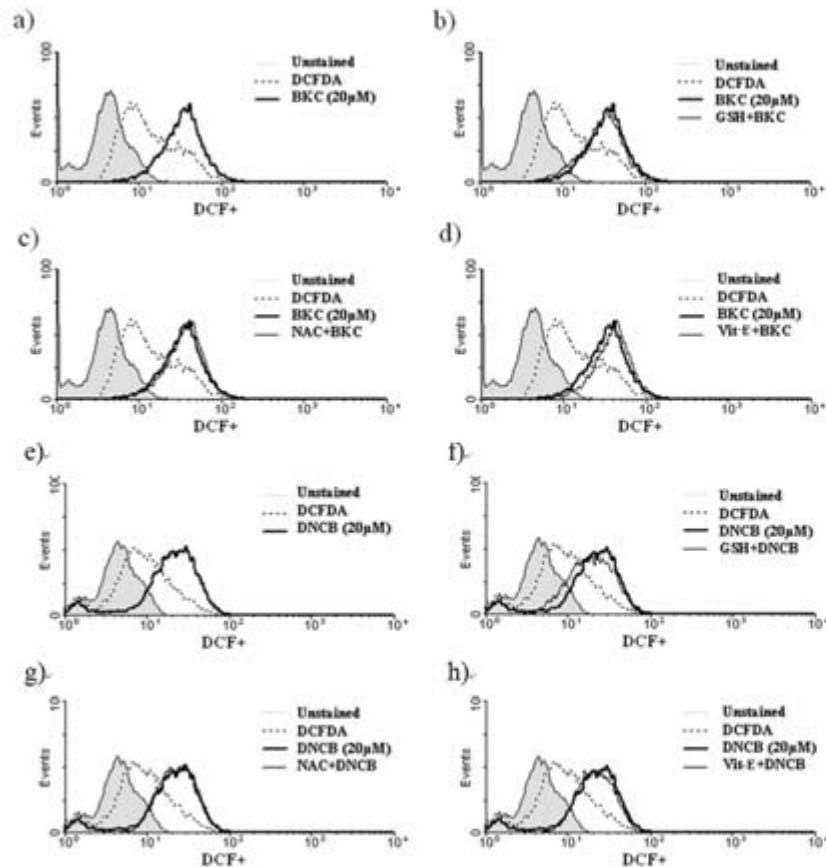
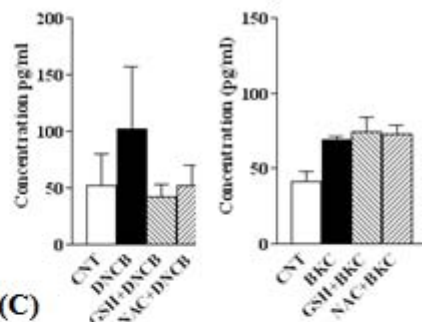
Figure S2. Inhibition of reactive oxygen species (ROS) generating pathways. (a) BKC-induced ROS were blocked by diphenylene iodonium (DPI) (a) whereas 2,4-dinitrochlorobenzene (DNCB)-induced ROS were blocked by both DPI and rotenone. (b) Likewise, allopurinol did not inhibit both DNCB- and BKC-induced ROS. Human keratinocyte cell line (HaCaT) cells were treated with 20- μ M BKC and 20- μ M DNCB for 1 h in the presence (thick solid line) or absence (solid narrow line) of 2- μ M DPI or 1- μ M rotenone. Filled lines indicate

no staining, whereas dotted lines indicate staining with CM-H₂DCFDA only. (b) Bar graph depicting the relative DCF fluorescence intensity (F/F₀) of CM-H₂DCFDA staining used to compare BKC- (a) and DNCB-treated (b) groups to ROS generation inhibitor pretreated treatment groups.

Figure S3. The effects of antioxidants on reactive oxygen species (ROS) production, cytokine secretion and increased intercellular adhesion molecule 1 (ICAM-1) expression in human keratinocyte cell line (HaCaT) cells treated with BKC and 2,4-dinitrochlorobenzene (DNCB). ROS production was detected by CM-H₂DCFDA assay. (a) Filled lines represent control cells without CM-H₂DCFDA staining; dotted lines indicate DCFDA staining only, without treatment; solid lines indicate increased ROS levels in BKC- and DNCB-treated cells; solid thick lines indicate chemical-induced ROS levels in the presence of antioxidants. ROS were generated by BKC and DNCB after incubation for 1 h in HaCaT cells (a) but were not decreased by glutathione (GSH) (b, f), N-acetyl cysteine (NAC) (c, g) or Vit-E (d, h). (b) DNCB- but not BKC-induced IL-1 α is decreased by antioxidants. The effects of antioxidants on cytokine secretion from HaCaT cells treated with DNCB and BKC. Cytokine secretion was measured in 5- μ M BKC- and 5- μ M DNCB-treated supernatants of HaCaT cells in the presence or absence of 3- μ M GSH and 5- μ M NAC. HaCaT cells produced IL-1 α in response to both BKC and DNCB; however, only DNCB-induced IL-1 α secretion was decreased by antioxidants, such as GSH and NAC. The decrease was statistically significant (*P* values < 0.05). (c) Augmentation of BKC-induced ICAM-1 molecules on HaCaT cells is not decreased by antioxidants. HaCaT cells treated with 5- μ M BKC and 5- μ M DNCB for 24 h in the presence of either GSH or NAC were analysed by flow cytometry. (a) Filled lines indicate untreated cells; dotted lines indicate ICAM-1-stained cells; solid lines indicate increased ICAM-1 by BKC; thick solid lines indicate ICAM-1 expression after antioxidant pretreatment followed by BKC treatment in HaCaT cells.

(A)**(B)**

(A)**(a)****(b)****(B)****a)****b)**

(A)**(B)****a) IL-1 alpha****b) IL-1 alpha****(C)**