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Intracellular redox regulation by a cystine derivative suppresses UV-induced NF-κB activation

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Abstract Nuclear factor (NF)-κB pathways are influenced by the intracellular reduction–oxidation (redox) balance. While NF-κB is activated through inhibitor (I)-κB degradation by oxidative stress, its DNA binding is accelerated in the reduced state. We found that *N*,*N*′-diacetyl-L-cystine dimethylester (DACDM) suppressed the UVB-induced NF-κB binding activity at a much lower concentration (50–100 μM) than *N*-acetyl-L-cysteine (NAC, 10–30 mM). While NAC suppressed the I-κB degradation but not the DNA binding, DACDM prevented the activated NF-κB from binding DNA, without influencing the I-κB degradation. These properties of DACDM make it possible to effectively regulate the intracellular redox balance. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cystine; Inflammation; Free radicals; Nuclear factor-κB; Oxidative stress; Redox

1. Introduction

Exposure of mammalian cells to UV light and other oxidative stresses stimulates signal transduction pathways that activate transcriptional factors, such as nuclear factor-κB (NF-κB). Fisher et al. showed that UVB irradiation induced NF-κB activation in human skin [1]. NF-κB regulates the expression of inflammatory cytokine genes, such as interleukin (IL)-1β and tumor necrosis factor-α [2,3]. Several reports have suggested that IL-1α production is also mediated through NF-κB activation [4,5]. Therefore, NF-κB is considered to be a crucial regulator of the inflammatory response in human tissues [6].

NF-κB activation occurs upon its dissociation from the inhibitory protein, inhibitor-κB (I-κB), in the cytosol and its subsequent nuclear translocation [7]. When a variety of stimuli, such as UV light and inflammatory cytokines, act as signals that activate I-κB kinase (IKK), I-κB is phosphorylated by IKK and is degraded and dissociated from NF-κB [7,8]. Numerous studies have indicated that reactive oxygen species (ROS), caused by a diverse range of stimuli, serve as common intracellular agents that contribute to I-κB degradation [9]. In addition, the effect of antioxidants, such as *N*-acetyl-L-cysteine (NAC) and lipoate/dihydrolipoate, on NF-κB activation is mediated by the suppression of the I-κB degradation [9,10]. The suppression by a wide range of antioxidants suggests that NF-κB activation is controlled by the intracellular reduction–oxidation (redox) balance [7,9,11]. NAC is considered to be an important antioxidant for regulating this balance. This cysteine derivative is a glutathione precursor and a potent scavenger of ROS [12]. These properties seem to be advantageous to shift the redox balance into the reduced state, thus preventing the initiation of the IKK cascades.

Although NF-κB activation through the I-κB degradation occurs under oxidized conditions, its nuclear translocation and DNA binding processes are accelerated in the reduced state [13,14]. Indeed, antioxidants increase the DNA binding of activated NF-κB [15]. Thus, the biphasic property of the NF-κB pathway can be regulated by oxidants as well as antioxidants. In this regard, we focused our attention on the cystine derivative, which is an oxidized form of NAC.

Here we report the influence of a cystine derivative, *N*,*N*′-diacetyl-L-cystine dimethylester (DACDM), on the UVB-induced NF-κB binding activity in a human keratinocyte cell line (HaCaT cells) in comparison with NAC. Furthermore, we describe how the compound suppressed the NF-κB pathway through redox regulation via the oxidized state.

2. Materials and methods

2.1. Materials

All reagents were used without purification. NAC was purchased from Sigma Chemical (St. Louis, MO, USA). DACDM was from Bachem (Bubendorf, Switzerland). Dulbecco's modified Eagle's medium (DMEM) was from Nikken Bio Medical Laboratory (Kyoto, Japan). Fetal bovine serum (FBS), phosphate-buffered saline, and minimum essential medium (MEM) were from Gibco BRL (Rockville, MD, USA). Other chemicals were obtained from Sigma Chemical or Nacalai Tesque (Kyoto, Japan).

2.2. Cell culture and treatment

The HaCaT cell line [16] was maintained in DMEM supplemented with 10% FBS at 37°C and in a 5% CO_2 atmosphere. The cells were seeded into 96-well microplates (2.0×10^5 cells/well) and were used for cytotoxicity assays or enzyme-linked immunosorbent assays (ELISA). One day after seeding, the cells were cultured with the test compounds in the medium for 18 h. For use in Western blotting analyses, electrophoretic mobility shift assays (EMSA), or glutathione measurements, the cells were seeded into 6-well plates (4.5×10^5 cells/well). Three days after seeding, the cells were cultured with the test compounds for 18 h. The medium with NAC was adjusted to pH 7.4.

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Abbreviations: NF-κB, nuclear factor-κB; I-κB, inhibitor-κB; IKK, I-κB kinase; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; Redox, reduction–oxidation; GSSG, oxidized glutathione; DACDM, *N*,*N*′-diacetyl-L-cystine dimethylester

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2.3. Cytotoxicity assays

Cytotoxicity was determined by the MTT assay as previously described [17].

2.4. UVB irradiation

UVB irradiation was performed 18 h after the addition of the test compound. The medium was removed and the cells were rinsed with MEM. The cells were exposed to UVB using a bank of six sun lamps (Toshiba Medical Supply, Tokyo, Japan) emitting 0.15 mW/cm^2. The UVB dose was 50 mJ/cm^2 (exposure time 333 s). During the UVB or sham exposure, the cells were covered with MEM. The MEM was replaced by the maintenance medium immediately after the irradiation. The cells were subsequently incubated for the specified times.

2.5. Cytosolic and nuclear extraction

Extractions from HaCaT cells were carried out 4–8 h after UVB irradiation, as described by Suzuki et al. [10]. The cytosolic and nuclear protein extracts were stored at −80°C for later use in the Western blotting analysis and EMSA, respectively. The protein concentration was measured using the Protein Assay I (Bio-Rad, Richmond, CA, USA).

2.6. EMSA

EMSAs were performed as previously described [18]. Nuclear proteins (7.5 μg) were incubated with a ^{32}P-labeled probe including the NF-κB-specific binding site along with poly(dI-dC)·poly(dI-dC) (Amersham Bioscience, Piscataway, NJ, USA). The samples were loaded and run on a 6% polyacrylamide gel. Visualization and quantification of radioactive bands were carried out by using an imaging analyzer (Fuji Photo Film, Tokyo, Japan).

2.7. Western blotting analysis

Western blotting analysis was performed as described by Spiecker and Liao [19] with slight modifications. Cytosolic proteins (6.0 μg) were separated by 10% SDS–polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride membranes. After incubations with an anti-IκB-α rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by horseradish peroxidase-coupled anti-rabbit IgG (Amersham Bioscience), the I-κB was visualized using the ECL–Western Blot System (Amersham Bioscience).

2.8. ELISA

Culture supernatants were collected 24 h after UVB irradiation, and the concentrations of IL-1α were measured by an ELISA based on the use of an anchored monoclonal mouse anti-IL-1α antibody, a polyclonal rabbit anti-IL-1α antibody (Genzyme, Framingham, MA, USA), and a goat anti-rabbit IgG conjugated with alkaline phosphatase (Biosource International, Camarillo, CA, USA).

2.9. Glutathione measurement

After the incubation with the test compounds, the cells were harvested and lysed with 0.1% Triton X-100. The lysate was deproteinized by 5% metaphosphoric acid. The supernatant was stored at −80°C until analysis. One plate was kept for the protein determination. The oxidized glutathione (GSSG) measurement was performed as described by Anderson [20].

2.10. Statistical analysis

Significance of differences was calculated by the Student's *t*-test using Microsoft Excel. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Cytotoxicity

The viability of HaCaT cells without UVB irradiation was determined with different concentrations of NAC and DACDM. NAC caused a significant decrease in cell viability at 50 mM as compared to the vehicle treatment. The cytotoxicity of DACDM (10 mM) was slightly higher than that of NAC. No enhancement of the cytotoxicity at the tested concentrations by the UVB irradiation (photocytotoxicity) was observed. The present study described below was carried out using concentrations at which no cytotoxicity was observed.

3.2. Suppression of UVB-induced NF-κB binding activity

Previously, it was shown that the NF-κB binding activity in HaCaT cells was significantly increased by exposure to UV light (290–400 nm), and the UVB range (290–320 nm) was considered to be solely responsible for the increase [18,21]. In this study, a significant increase in NF-κB binding activity was detected with a UVB dose of 30–100 mJ/cm^2, without photocytotoxicity. Therefore, the assay was performed with a 50 mJ/cm^2 dose.

The results are shown in Fig. 1 and Table 1. The inhibition of NF-κB binding activity by NAC has been reported in various systems [9,10,18]. In the present study, NAC diminished the UVB-induced NF-κB binding activity at a concentration of 30 mM. However, no inhibition was observed at a 1 mM concentration. Since NAC showed cytotoxicity at 50 mM, the inhibitory effects were found at a concentration close to that causing the cytotoxicity. In contrast, DACDM suppressed the activity at concentrations ranging from 50 to 100 μM, which are much lower than the concentration causing the cytotoxicity (10 mM).

3.3. Suppression of UVB-induced IL-1α production

The IL-1α level in HaCaT cells is increased by UV light [21,22]. In this study, the release of IL-1α from the cells into the culture medium for 24 h after UVB irradiation was measured. Since the IL-1α release was significantly increased by UVB doses ranging from 50 to 100 mJ/cm^2, the assay was performed using a UVB dose of 50 mJ/cm^2.

The results are shown in Fig. 2. UVB caused a significant increase in the release of IL-1α. The ability of the two compounds to suppress the IL-1α production paralleled that against the NF-κB binding activity. The inhibitory effects by NAC were evident at 10–30 mM concentrations. In contrast, DACDM significantly suppressed the IL-1α production in the micromolar range (50–100 μM), which was much lower than that of NAC.

3.4. Effect on UVB-induced I-κB degradation

Since the activation of NF-κB involves I-κB degradation in

Table 1
Inhibition rates of NAC and DACDM on the UVB-induced NF-κB binding activity

Compound	Conc. (mM)	Inhibition (%)
–	0	0
NAC	1	0.2 ± 3.0
	10	54.0 ± 28.6
	30	72.5 ± 27.5
DACDM	0.01	29.0 ± 5.0
	0.05	56.9 ± 13.7
	0.1	97.8 ± 2.6

The cells were incubated with the tested compounds for 18 h before sham or UVB (50 mJ/cm^2) irradiation. Nuclear extracts were prepared and the NF-κB binding activity was analyzed by EMSA. The inhibition rate (%) is given as follows: [(NF-κB band intensity from cells with vehicle exposed to UVB)−(NF-κB band intensity from the cells with test compounds exposed to UVB)]/[(NF-κB band intensity from cells with vehicle exposed to UVB irradiation)−(NF-κB band intensity from the cells with vehicle exposed to sham)]×100. The results are the means ± S.D. of three experiments. Conc., concentration.

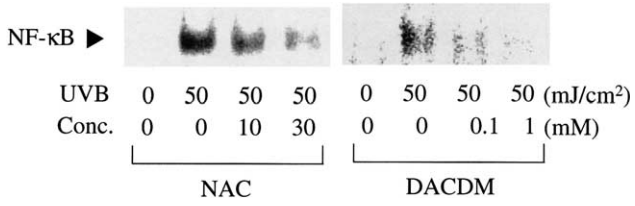

Fig. 1. Effects of NAC and DACDM on UVB-induced NF-κB binding activity in HaCaT cells. The cells were incubated with the tested compounds for 18 h before sham or UVB (50 mJ/cm^2) irradiation. Nuclear extracts were prepared and the NF-κB binding activity was analyzed by EMSA.

the cytosol, we assessed whether the suppressive effects on the NF-κB binding activity result from the degradation, using Western blotting analyses.

The I-κB was almost undetectable at a UVB dose of 50 mJ/cm^2, without photocytotoxicity. The UVB-induced I-κB degradation was not observed when the cells were pretreated with NAC, as shown in Fig. 3. In contrast, DACDM did not suppress I-κB degradation. Although DACDM showed significant suppression of the UVB-induced NF-κB binding activity at 50–100 μM concentrations, it had no effect on the I-κB degradation within these concentration ranges (Fig. 3). The I-κB degradation process is often activated by oxidants. However, no indications of further I-κB degradation by DACDM were detected. The results indicate that DACDM suppressed the UVB-induced NF-κB binding activity, regardless of the I-κB degradation process.

3.5. Influence on DNA binding of NF-κB

Since NF-κB activated by I-κB degradation translocates from the cytosol into the nucleus, and then binds to DNA, we assessed the influence of the compounds on the DNA binding of activated NF-κB. Activated NF-κB that was capable of binding to a DNA probe was present in the nuclear extracts prepared from HaCaT cells exposed to UVB, as shown above. Hence, NAC and DACDM were added to the nuclear extracts and were assessed for their ability to

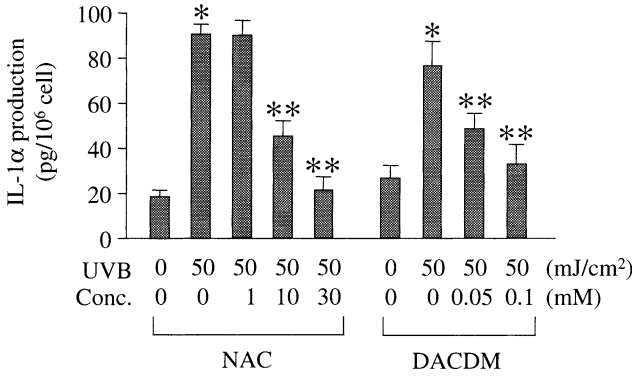

Fig. 2. Effects of NAC and DACDM on UVB-induced IL-1α production in HaCaT cells. The cells were incubated with the tested compounds for 18 h before sham or UVB (50 mJ/cm^2) irradiation. The concentration of IL-1α released from the cells was measured by ELISA. The results are the means ± S.D. of three experiments. *$P < 0.05$ as compared to the control value (sham-irradiation samples). **$P < 0.05$ as compared to the vehicle value (0 mM and UVB-irradiation samples).

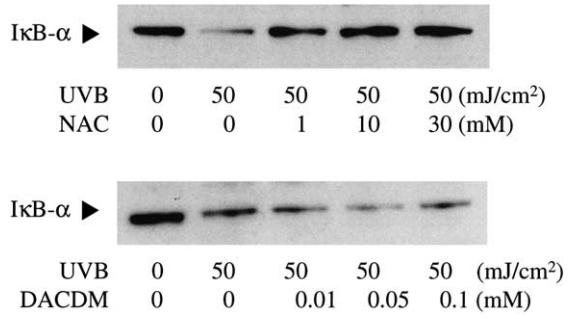

Fig. 3. Effects of NAC and DACDM on UVB-induced I-κB degradation in HaCaT cells. The cells were incubated with the tested compounds for 18 h before sham or UVB (50 mJ/cm^2) irradiation. Cytosol extracts were prepared and I-κB was detected by a Western blotting analysis.

suppress the binding of NF-κB to DNA by EMSA. The addition of NAC from 1 to 30 mM did not reduce the intensity of the detected NF-κB (Fig. 4). NAC had no influence on the binding of NF-κB onto a labeled probe including the NF-κB-specific binding site. In contrast, DACDM suppressed the binding in the concentration range from 0.05 to 0.1 mM (Fig. 4). These ranges were parallel to those causing the suppression of the UVB-induced NF-κB binding activity.

3.6. Modulation of intracellular glutathione

The NF-κB binding activity is known to be regulated by the intracellular redox balance. The redox balance is often described by the variation of the intracellular glutathione content. The GSSG concentrations in the HaCaT cells treated with NAC and DACDM are shown in Table 2. There was no variation of the GSSG content by treatment with 10–30 mM concentrations of NAC. In contrast, DACDM caused a significant enhancement in the GSSG content, at 0.05–0.1 mM concentrations. The results show that the cystine derivative effectively shifted the redox balance to the oxidized state.

4. Discussion

DACDM as well as NAC suppressed the UVB-induced NF-κB binding activity and IL-1α production in HaCaT cells. However, the influences of DACDM on the I-κB degradation and the DNA binding of activated NF-κB were contrary to those of NAC. While NAC suppressed the I-κB degradation but not the DNA binding of activated NF-κB, DACDM pre-

Table 2
Effects of NAC and DACDM on the GSSG content in HaCaT cells

Compound	Conc. (mM)	GSSG (mmol/g protein)
–	0	9.8 ± 1.6
NAC	10	10.7 ± 1.5
	30	10.0 ± 2.0
DACDM	0.05	17.6 ± 1.2*
	0.1	20.3 ± 4.4*

After an 18 h incubation period in the presence of the tested compounds, the intracellular GSSG concentration (conc.) was measured as described by Anderson [20]. The results are the means ± S.D. of three experiments. *$P < 0.05$ as compared to the control value (0 mM samples).

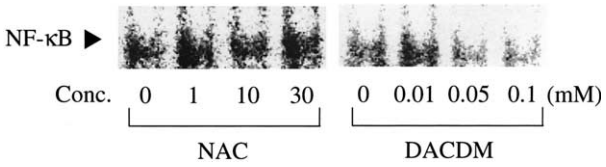

Fig. 4. Effects of NAC and DACDM on the DNA binding of activated NF-κB in nuclear extracts from HaCaT cells exposed to UVB. The cells were incubated with vehicle for 18 h and subsequently were exposed UVB irradiation (50 mJ/cm^2). Extracts were treated with the tested compounds for EMSA.

vented the DNA binding without influencing the I-κB degradation. These results suggest that the suppressive mechanism of DACDM is distinct from that of NAC. That is, DACDM does not seem to act as its reduced form in the cells.

In general, NF-κB activation is influenced and regulated by the intracellular redox balance. This balance is modulated by the reversible equilibrium between cysteine and cystine residues in peptides and proteins, such as glutathione and thioredoxin [23,24]. NAC, which is a cysteine derivative, is known to shift the redox balance to the reduced state by increasing the reduced glutathione (GSH) content [11,12]. However, since DACDM is a cystine derivative, it seems to shift the balance to the oxidized state. Actually, DACDM enhanced the intracellular GSSG content in the present study. Therefore, we expect that the characteristic influence of the cystine derivative on the redox balance resulted in a suppressive mechanism distinct from that of NAC.

The activation of NF-κB involves I-κB degradation, nuclear translocation, DNA binding, and transactivation processes that lead to the expression of various inflammatory cytokine genes [7]. An EMSA analysis provides information about the DNA binding, but not the transactivation process. In this study, DACDM as well as NAC suppressed the UVB-induced IL-1α production. IL-1α is a cytokine that mediates much of the inflammatory reaction and up-regulates the expression of other proinflammatory genes [25,26]. Its production is thought to be mediated through the NF-κB pathway [4,5]. Hence, we expect that the cystine derivative suppresses not only the DNA binding of activated NF-κB but also the ensuing transactivation process of inflammatory cytokines.

Since NAC seems to have only a limited interaction with cell membranes, due to its anionic property, only a portion of the NAC could penetrate through the membrane into the cells. As a result, a high concentration of NAC was required for the suppressive effects (10–30 mM). On the other hand, DACDM, in which the anionic carboxylate is esterified, showed highly suppressive effects (50–100 μM) on the UVB-induced damage. This suggests that the increase in the hydrophobicity by the esterification leads to an enhanced interaction between the molecules and the cell membranes. Actually, *N*,*N*′-diacetyl-L-cystine, which is the non-esterified cystine derivative corresponding to a dimer of NAC, showed less suppressive effects (10–30 mM) than DACDM.

The effectiveness of DACDM at a low concentration should lead to its potential therapeutic use. Moreover, DACDM clearly inhibited the DNA binding of activated NF-κB and increased the intracellular GSSG content, which is distinct from the NAC activity. These results confirmed the characteristic behavior of a cystine derivative in the NF-κB pathway. Recently, the immuno-modulatory effects of cystine derivatives in an in vivo assessment were reported [27,28]. The NF-κB regulation by the cystine derivatives is expected to yield favorable biological effects in vivo as well as in vitro.

We showed in this study that DACDM suppressed the UVB-induced NF-κB binding activity in a human keratinocyte cell line. Their suppressive mechanism seems to be derived from the shift of the intracellular redox balance into the oxidized state, indicating that DACDM worked as an oxidant. Oxidants may have harmful pro-oxidative effects by enhancing ROS activities. In this study, DACDM did not enhance either the UVB-induced cytotoxicity or the I-κB degradation. Rather, DACDM shifted the redox balance to the oxidized state without harmful pro-oxidative effects. In contrast, NAC acts as a typical compound for the regulation of the redox balance by shifting it into the reduced state. The present study shows that NAC and DACDM can be used as regulatory compounds for investigating how the intracellular redox balance affects signal transduction pathways. Moreover, since DACDM produces an oxidized state at a low concentration without harmful pro-oxidative effects, it may overcome the limitations of the therapeutic use of NAC.

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