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The Role of Reactive Oxygen Species (ROS) in the Expression of Endothelial Adhesion Molecules in Allergic Inflammation

Yoo Jean Choi, Jai Youl Ro*, and Kwang Hoon Lee

Department of Dermatology, Yonsei University College of Medicine, Seoul,
**Department of Pharmacology, Sungkyunkwan University School of Medicine, Suwon, Korea*

In the pathogenesis of allergic diseases, such as atopic dermatitis, the expressions of adhesion molecules and subsequent adhesion of inflammatory cells to endothelial cells are necessary. It is known that reactive oxygen species (ROS) are important second messengers in this process. We studied the effect of allergic reaction-related cytokines on the production of ROS as well as the role of ROS in the expressions of adhesion molecules in the human dermal microvascular endothelial cells (HDMECs). The generation and expressions of ROS and adhesion molecules were determined by FACstar and ELISA, respectively after stimulations with H_2O_2, IL-1α, TNF-α, IL-4, IL-13, and H_2O_2 combined with cytokines and cytokines combined with antioxidants. The results are:

1) Stimulations of HDMECs with IL-1α, TNF-α, IL-4 and IL-13 increased production of ROS at early time points from 15 min to 30 min after incubation. The expressions of ICAM-1, VCAM-1 and E-selectin were upregulated or induced by IL-1α and TNF-α while the expression of VCAM-1 was induced by IL-4, IL-13.
2) The stimulation of HDMECs with H_2O_2 upregulated or induced the expressions of ICAM-1, VCAM-1 and E-selectin but no synergistic or additive effect was found between cytokines and H_2O_2.
3) The expressions of the adhesion molecules upregulated or induced by cytokines were variably inhibited by antioxidants.

These findings suggest that ROS play an important role in the expressions of the ICAM-1, VCAM-1 and E-selectin induced by allergic reaction-related cytokines and that pharmaceutical approaches manipulating reduction-oxidation mechanism could lead to a new therapeutic approach for allergic diseases.

Key words: Reactive oxygen species, Human dermal microvascular endothelial cells, Cytokines, Adhesion molecules

Abbreviations used

AD : atopic dermatitis
ROS : reactive oxygen species
HDMECs : human dermal microvascular endothelial cells
HUVECs : human umbilical vein endothelial cells
NAC : N-acetyl-L-cysteine
PDTC : pyrrolidine dithiocarbamate
DMSO : dimethyl sulfoxide
SOD : superoxide dismutase
ICAM-1 : intercellular cell adhesion molecule-1
VCAM-1 : vascular cell adhesion molecule-1

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Corresponding author: Kwang Hoon Lee, M.D., Department of Dermatology, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea. Tel. 82-2) 361-5720 / Fax. 82-2) 393-9157, E-mail: kwanglee@yumc.yonsei.ac.kr

INTRODUCTION

Endothelial cells lining the postcapillary venules and microcirculation elaborate leukocyte-specific adhesion molecules both constitutively and in response to a wide range of inflammatory mediators. Circulating leukocytes routinely display receptors for these inducible endothelial cell adhesion molecules[1,2]. It is known that adhesion molecules such as ICAM-1, VCAM-1, and E-selectin mediate T lymphocytes' adherence to endothelial cells[3-8]. The degree of adherence of inflammatory cells to endothelial cells varies following the stimulation with cytokines and it may be directly associated with the change in the expressions of adhesion molecules induced by cytokines[3,4].

Reactive oxygen species (ROS) which include superoxide anion (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) are produced by various cell types[9]. Production of ROS is enhanced by stimuli such as tissue injury, UV irradiation, and bacterial infection[10], while the overproduction of ROS causes oxidative injury on genes, lipids, and proteins. Recently it has been reported that ROS serve as messengers for the gene regulation[11].

It is known that cytokine-induced expressions of adhesion molecules are dependent, at least in part, on the nuclear factor-κ B (NF-κ B) -like transcriptional factor, and that ROS mediate as messengers in the activation of NF-κ B[11-14].

Although there have been reports that ROS play a role in the cytokine-induced expressions of adhesion molecules[15,16], there has been no report of the role of ROS in the human dermal microvascular endothelial cells (HDMECs) in allergic inflammation. Therefore, an experimental approach to determine the role of ROS in the cytokine-induced expressions of adhesion molecules on HDMECs would be helpful to determine the pathogenesis of cutaneous allergic inflammation and to develop new treatment modalities.

MATERIALS AND METHODS

1. Isolation and culture of HDMECs

HDMECs were isolated from human neonatal foreskins as described previously[17,18]. Cells were cultured in endothelial basal medium with epidermal growth factor 5 ng/ml, hydrocortisone acetate 1 μg/ml, dibutyryl cyclic AMP 5×10^{-5}M, penicillin 100 U/ml, streptomycin 100 mg/ml, amphotericin B 250 mg/ml, and 2% fetal bovine serum. The resulting cell cultures were free of contaminating fibroblasts as assessed by morphologic and immunologic criteria. Experiments were conducted at passages of 3-4.

2. Determination of the oxidative activity in HDMECs induced by cytokines

HDMECs were plated in 24-well plates and allowed to grow to confluence over 24 hours at a concentration of 4×10^4 cells per well. To determine the oxidative activity induced by cytokines in HDMECs, endothelial monolayers were incubated with 100 U/ml of recombinant human IL-1 α, 100 U/ml of TNF-α, 100 U/ml of IL-4, and 10 U/ml of IL-13 (Strathmann Biochem., Hannover, Germany) at 37℃ for 15 and 30 minutes, 1, 4 and 24 hours. For positive and negative control, 100 μM of H_2O_2 (Merck, Darmstadt, Germany) and culture medium were added to HDMECs for the same durations, respectively. HDMECs were incubated with 50 μM of dihydrorhodamine 1,2,3 (Molecular Probes, Inc., Eugene, OR, USA) for 5 minutes, washed and resuspended in PBS (pH 7.2) with 0.5% BSA. 100 μg/ml of propidium iodide (Sigma chemical Co., St. Louis, MO, USA) was added immediately before flow cytometric analysis to identify dead cells. Flourescence was examined on a flourescence activating cell sorter (FACStar, Becton-Dickinson, Lincoln, NJ, USA).

Superoxide rapidly dismutates to H_2O_2 which oxidize nonflourescent dihydrorhodamine 1,2,3 to rhodamine 1,2,3. Rhodamine 1,2,3 cannot cross the plasma membrane and therefore retained within the cell resulting in an increase in its fluorescence[19].

Leary et al noted that the fluorescence of a single cell, represented by the error bars for each fluorescent point on the histogram which represents the biological variability of ROS on a cell-by cell basis, is far too small to be plotted. Therefore, even slightly shifted histograms, while partially overlapping, are statistically significant[20]. Therefore, ROS level is considered to be significantly higher than the control ($p < 0.05$) when the value is two standard deviations above the geometric mean of the control.

3. Expressions of adhesion molecules on HDMECs induced by cytokines, H_2O_2 and cytokines combined with H_2O_2

a. Incubation of HDMECs with cytokines, H_2O_2 and cytokines combined with H_2O_2

HDMECs were plated in 96-well plates and allowed to grow to confluence over 24 hours at a concentration of 4×10^4 cells per well. Cultured endothelial monolayers were incubated with 100 U/ml of recombinant human IL-1α 100 U/ml of TNF-α, 100 U/ml of IL-4, and 10 U/ml of IL-13, 100 μM of H_2O_2 alone and the indicated concentrations of each cytokine combined with 100 μM of H_2O_2 at 37℃ for 1 hour, 4 hours, 16 hours and 24 hours.

b. Enzyme-linked immunosorbent assay (ELISA)

Each mouse monoclonal antibody (mAb) against human ICAM-1 (BBIG-L1(11C81): R&D, Minneapolis, MN, USA), human VCAM-1 (BBIG-V1(4B2): R&D, Minneapolis, MN, USA) and human E-selectin (1.2B6: R&D, Minneapolis, MN, USA) were diluted to 1:1000 and a total of 100 μl of each mAb were added to each well and the plates were incubated at 37℃ for 1 hour. After washing with Hank's balanced salt solution (HBSS, GibcoBRL, Gathersberg, MD, USA) for 3 times cells were incubated with 100 μl of peroxidase- conjugated goat anti-mouse IgG (Biosource international., Camarillo, CA, USA), which is diluted to 1 : 1000 for 1 hour and washed with HBSS as above. 100 μl of 100mg tetramethylbenzidene (TMB, Sigma chemical Co., St. Louis, MO, USA) in 10 ml acetone was mixed with 1ml distilled water and 1ml of 30% H_2O_2. Then, 100 μl of mixed solution was added.

Twenty-five μl of 8N H_2SO_4 was added to stop the reaction and the optical density was measured by an ELISA reader (Dynatech Laboratories, Inc., Alexandria, VA, USA) at 450 nm.

4. Measurements of the expressions of adhesion molecules after incubation with antioxidants

The antioxidants administered exogenously were 30 mM of N-acetyl-L cysteine (NAC, Aldrich chem Co., Milwaukee, WI, USA), 100 μM of pyrrolidine dithiocarbamate (PDTC, Sigma chemical Co., St. Louis, MO, USA), 30 mM of dimethyl sulfoxide (DMSO, Sigma chemical Co., St. Louis, MO, USA), 100 U/ml of superoxide dismutase (SOD, Sigma chemical Co., St. Louis, MO, USA). The preliminary experiments were done with lower concentrations of each antioxidants (1:10 and 1:100 dilutions). There were maximal effects of antioxidants at the indicated concentrations above (data not shown). To evaluate the effect of each antioxidant on the cell viability, HDMECs were incubated with indicated concentrations of each antioxidant for 15 and 30 minutes, and the viability was determined by FACStar.

HDMECs were incubated with indicated concentrations of antioxidants for 30 minutes before cytokines were added. Each cytokines (100 U/ml of recombinant human IL-1α 100 U/ml of TNF-α, 100 U/ml of IL-4, and 10 U/ml of IL-13) were added directly to the medium containing each antioxidant and the plates were incubated for 4 and 16 hours. For comparison, HDMECs were incubated with medium alone, antioxidants alone and cytokines alone. The expressions of adhesion molecules in HDMECs were analyzed by ELISA in the same method as described above.

5. Statistical analysis

Apparent differences between normally distributed means were evaluated by paired t-test and t-test. $P < 0.05$ was considered to indicate statistical significance. Values were expressed as means $\pm$ S.D.

RESULTS

1. Effects of cytokines on the generation of ROS in HDMECs

Increased fluorescence indicating upregulated ROS activities was observed after the treatment of HDMECs with cytokines.

ROS activity was not increased at 16 hours after incubation with media but they were generated after 24 hours. In contrast, incubation with each cytokines and H_2O_2 generated ROS at 15 to 30 minutes. H_2O_2 and all cytokines except IL-4 showed another increase in ROS after 24 hours (Fig. 1).

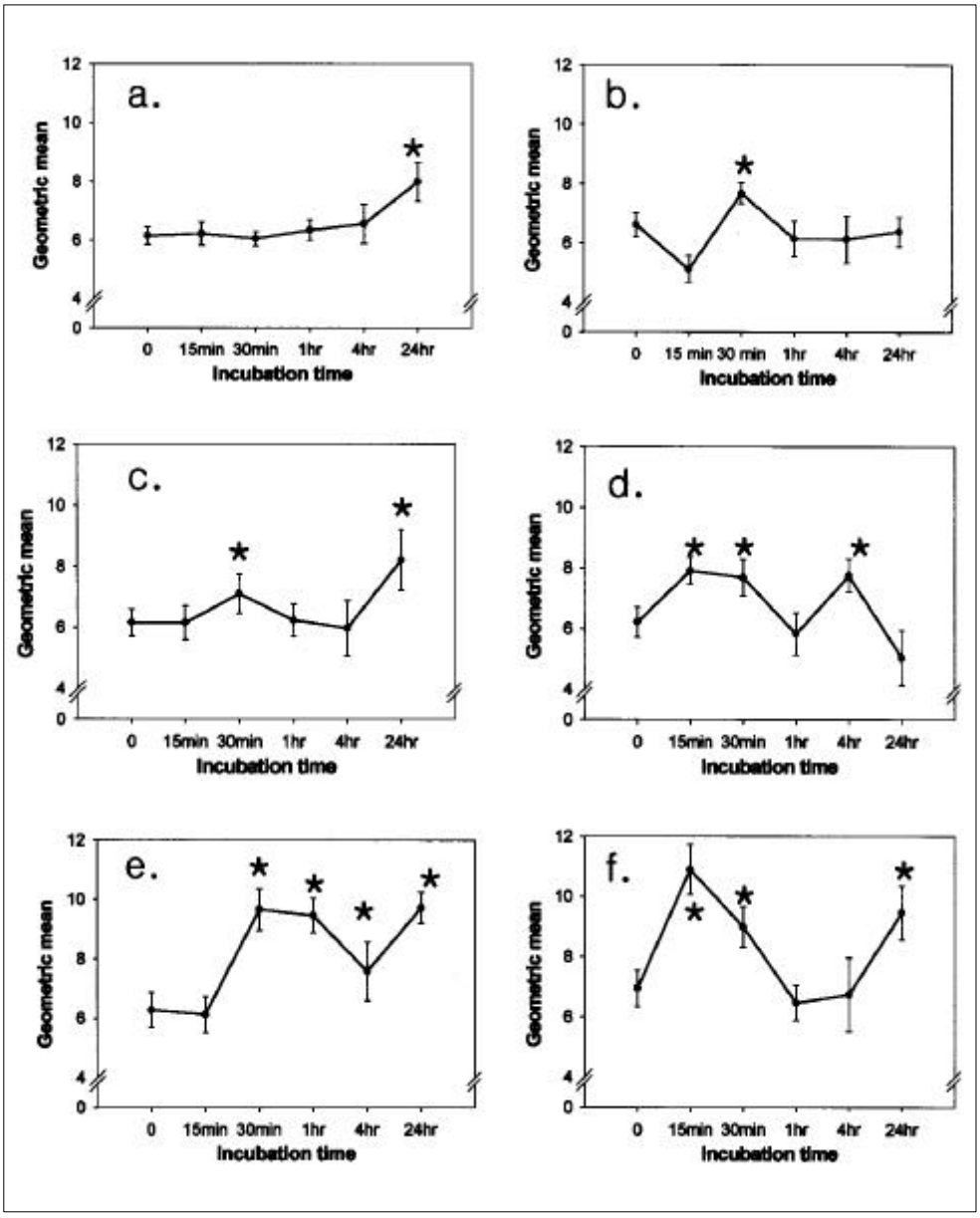

Fig. 1. The flow cytometric analysis of ROS generation of HDMECs after stimulation with cytokines. HDMECs were cultured with a, media; b, IL-1 α 100 U/ml; c, TNF-α 100 U/ml; d, IL-4 100U/ml; e, IL-13 10 U/ml; f, H_2O_2 100 μM for 15, 30 minutes, 1, 4 and 24 hours and incubated with dihydrorhodamine 1,2,3 before analysis with FACStar. *ROS is considered to be significantly higher than the control ($p < 0.05$) when the value is two standard deviations above the geometric mean of the control. The data are from one of the two similar experiments

2. Effects of cytokines and ROS on the expressions of adhesion molecules in HDMECs

a. Effects of cytokines

After incubation with IL-1α, the expression of ICAM-1 and E-selectin was significantly increased or induced, while the expression of VCAM-1 was mildly induced. The expression of ICAM-1, VCAM-1 and E-selectin was upregulated or induced significantly by TNF-α. After incubation with IL-4 and IL-13, the expression of VCAM-1 was mildly induced (data not shown).

b. Effects of ROS on the expression of adhesion molecules on HDMECs

Incubation with H_2O_2 increased or induced the expressions of ICAM-1, VCAM-1 and E-selectin (Fig. 2).

However, the expressions of adhesion molecules after incubation with H_2O_2 combined with each cytokine were not significantly different from the result after incubation with H_2O_2 alone in all groups (Fig. 3).

3. Effects of antioxidants on the expression of adhesion molecules on HDMECs induced by cytokines

All antioxidants showed no significant differences in the cell viability between after incubation with culture media and antioxidants.

The expressions of ICAM-1, VCAM-1 and E-selectin induced by IL-1 α and TNF- α and also the expressions of VCAM-1 induced by IL-4 and IL-13 were inhibited after preincubation with NAC (Fig. 4). Preincubation with PDTC inhibited the IL-1 α-induced expression of E-selectin and the TNF- α-induced expression of VCAM-1 and E-selectin. After preincubation with DMSO and SOD, the ICAM-1 expressions induced by IL-1 α and TNF- α were inhibited (data not shown).

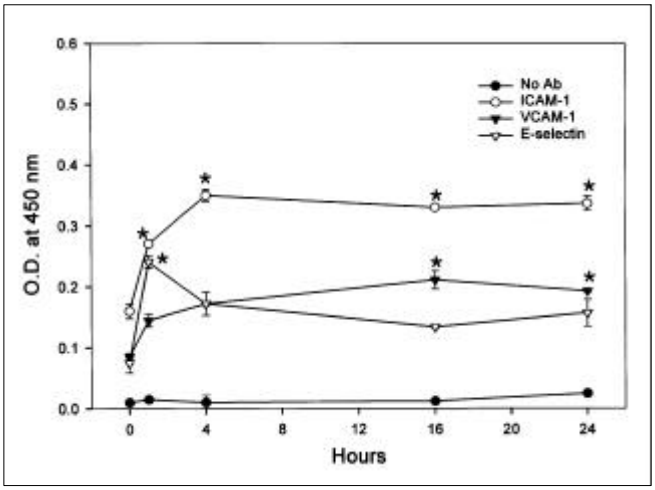

Fig. 2. The effects of H_2O_2 on the expression of adhesion molecules. Cultured HDMECs were incubated with H_2O_2 with for 1, 4, 16 and 24 hours. The expressions of ICAM-1, VCAM-1 and E-selectin were determined by ELISA at optical density of 450 nm. Results are representative of 3 separate experiments. *p < 0.05, comparison with unstimulated HDMECs

DISCUSSION

In allergic diseases, vascular endothelial cells are the major target of tissue injury induced by ROS and the recruitment of inflammatory cells may amplify such oxidative tissue injury. The vascular endothelium is equipped with antioxidants, catalase, and glutathione that serve as the primary scavengers against H_2O_2 and these antioxidants act as defense mechanism to oxidative stimuli so that tissue injury is minimized[21]. H_2O_2 induces neutrophil adhesion to the endothelium and the concentration of catalase in endothelial cells is important in this process[21].

Opioids suppress the inflammatory skin reactions induced by ROS[22]. It has also been reported that incubation with ROS induced granule membrane protein (GMP) - 140[23] and ICAM-1[16] on cultured human umbilical vein endothelial cells (HUVECs). Oxidized low density protein can induce the

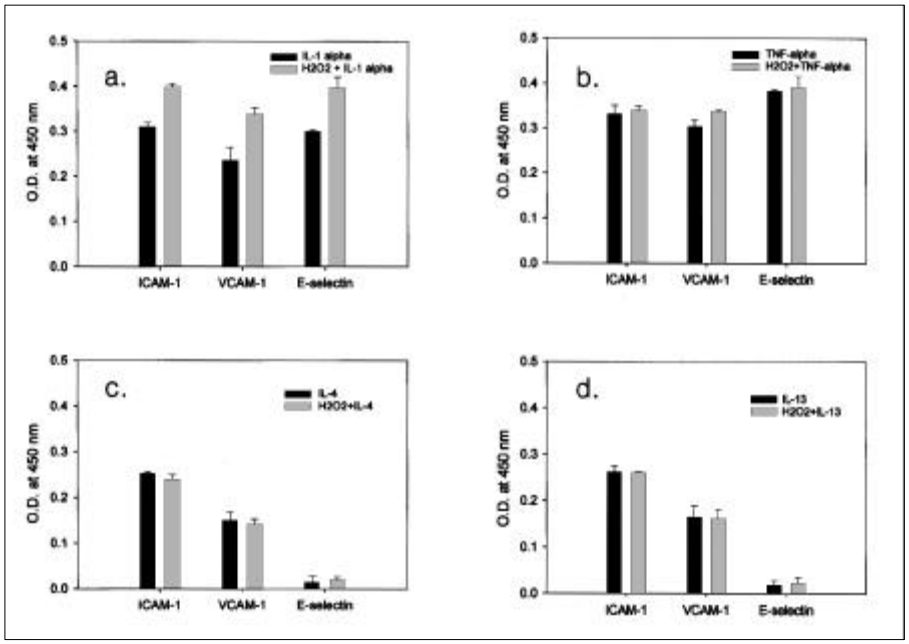

Fig. 3. The comparison of the expressions of adhesion molecules between incubation with H_2O_2 alone and H_2O_2 combined with cytokines. Cultured HDMECs were incubated with H_2O_2 alone and H_2O_2 combined with each cytokines (a,IL-1 α ; b,TNF- α c,IL-4 and d,IL-13). The expressions of ICAM-1, VCAM-1 and E-selectin were determined by ELISA at optical density of 450 nm. Results are representative of 3 separate experiments. *p < 0.05, comparison with unstimulated HDMECs

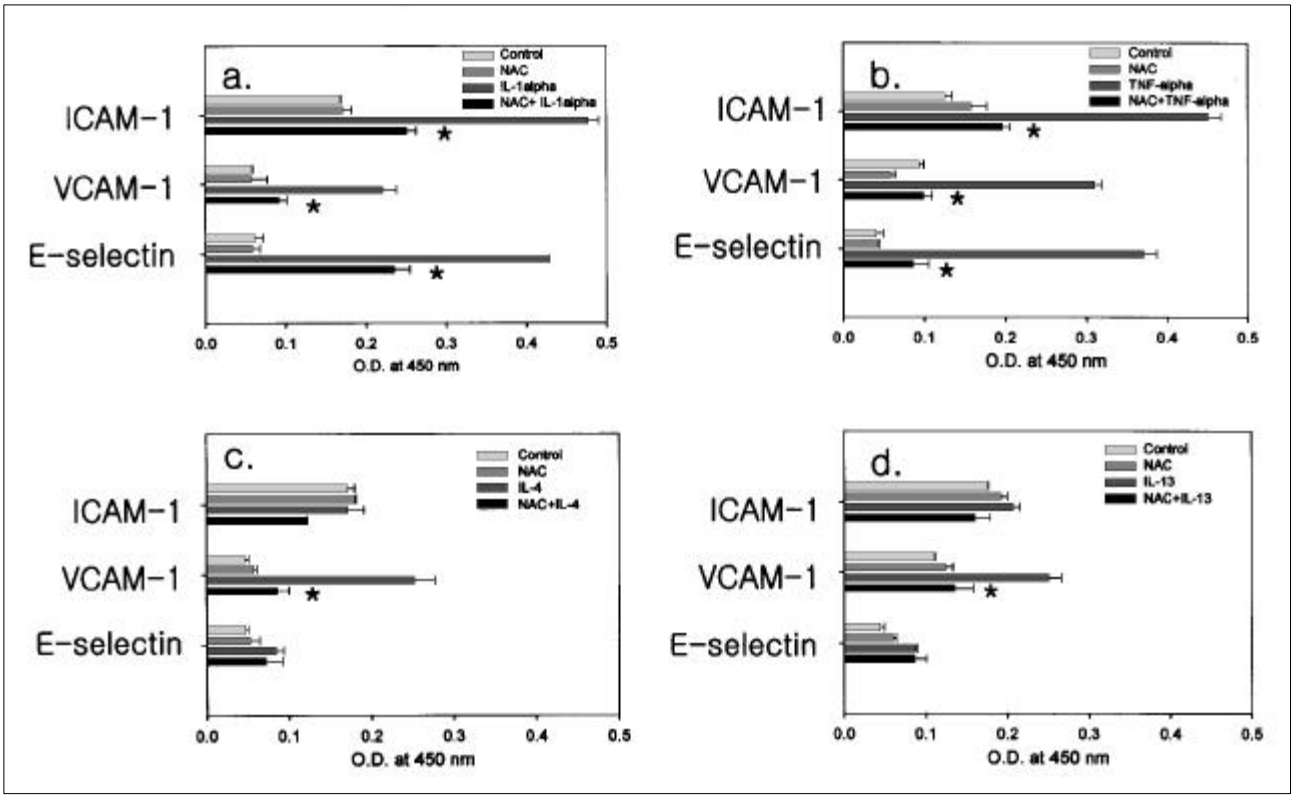

Fig. 4. The effect of NAC on the expressions of adhesion molecules induced by cytokines on HDMECs. Cultured HDMECs were incubated with media, NAC, cytokines (a, IL-1 α ; b, TNF- α c, IL-4 and d, IL-13) and cytokines preincubated with NAC for 30 min. The expressions of ICAM-1, VCAM-1 and E-selectin were determined by ELISA at optical density of 450nm. Results are representative of 3 separate experiments. *$p < 0.05$, comparison with unstimulated HDMECs (media only)

expression of different adhesion molecules on HUVECs and this induction can be prevented by pretreating either the low density protein or the cells with radical-scavenging antioxidants[24]. DeLano et al[25] reported that during blockade of experimental inflammation with an anti-P-selectin antibody, there was also attenuation of oxygen free radical formation and tissue injury. These results suggest that ROS plays an important role in inflammatory reaction.

Our results show that ROS play a role in the cytokine-induced expressions of ICAM-1, VCAM-1 and E-selectin. Cytokines generated ROS in HDMECs within 30 minutes and when exogenous ROS were added, the peak level of ROS was observed after 15 minutes on flow cytometry. The expressions of adhesion molecules after incubation with ROS followed a similar pattern to that after incubation with cytokines. However, the lack of a significant difference between the results after incubation with ROS combined with cytokines and those after incubation with cytokines alone suggests that endogenous and/or exogenous ROS exert inhibitory effects by a mechanism which remains uncertain. It may be further suggested that exogenous ROS activate a defense mechanism of endothelial cells to prevent excessive increase in net ROS generation. The pattern of ROS generation showed slight variation with different cytokines. It is noticeable that all cytokines except IL-4 showed second increase in ROS after 24 hours. This suggests that there is a biphasic reaction in the cytokine-induced generation of ROS, consisting of an immediate reaction which occurs within an hour and a delayed reaction which occurs in 24 hours. This pattern was also observed after incubation with culture medium alone or H_2O_2 alone so it is apparent that the reaction occurs due to the loss of defense mechanism against exogenous stimuli rather than due to being specific to the cytokines. The pattern of VCAM-1 expression induced by IL-4 and IL-13 was similar but that of ROS generation was different, which suggests that the

process of ROS generation and the expressions of adhesion molecule vary among different cytokines.

Swerlick et al[26] reported that VCAM-1 is expressed by IL-1 α and IL-4 on HUVECs but not on HDMECs, whereas our study showed mild expression of VCAM-1 by IL-1 α, IL-4 and IL-13. This result shows that the expression of adhesion molecules possibly varies with different experimental conditions.

In our study, the expressions of the adhesion molecules upregulated or induced by cytokines were inhibited variably by antioxidants. It has already been reported that the inhibitory effects on expressions of adhesion molecules in the endothelial ells induced by cytokines vary. The expression of ICAM-1 induced by TNF- α on HUVECs was inhibited by PDTC, NAC, DMSO and DMTU(dimethylthiourea) but not by SOD or catalase[15]. Also, the effects of an antioxidant vary on different kinds of endothelial cells and vascular endothelial adhesion molecules. Faruqi et al[27] reported that NAC did not inhibit IL-1 β-induced binding of NF- κ B to the E-selectin promoter in HUVECs but that it inhibited binding of NF- κ B to the VCAM-1 promoter, whereas Rahman et al[16] reported that NAC inhibited TNF- α-induced binding of NF- κ B to the E-selectin promoter in human pulmonary artery endothelial cells. Marui et al[28] reported that PDTC inhibited the expression of VCAM-1 but not those of ICAM-1 and E-selectin by TNF- α on HUVECs whereas Arai et al[15] reported that PDTC inhibited the ICAM-1 expression by TNF- α. These results suggest that the sensitivity to antioxidants in the regulation of adhesion molecules may vary according to different experimental conditions. And it has also been reported that the effect of an antioxidant could vary with time. PDTC initially inhibits NF- κ B activation and hence E-selectin expression, but this effect is masked at later time points because of the ability of PDTC to cooperate with TNF- α in inducing E-selectin expression through the activation of activating protein-1 (AP-1) and other transcription factors[16].

It is clear that ROS play an important role in the expressions of vascular endothelial adhesion molecules induced by cytokines and it is also apparent that the effects of ROS vary among different cytokines, types of endothelial cells, vascular endothelial adhesion molecules, antioxidants and experimental conditions. Therefore, further studies verifying the exact action mechanism of ROS are required in various experimental conditions. The role of other factors that regulate the cytokine-induced expressions of adhesion molecules and the interaction of ROS and other factors need to be verified. If the exact action mechanisms of ROS is known, pharmacologic manipulation of the redox equilibrium in the endothelial cells may allow new therapeutic approaches for allergic diseases.

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