Rebamipide Inhibits Neutrophil Adhesion to Hypoxia/Reoxygenation-Stimulated Endothelial Cells via Nuclear Factor-κB-Dependent Pathway

CHI DAE KIM, YONG KI KIM, SO HYUN LEE, and KI WHAN HONG

Departments of Pharmacology (C.D.K., S.H.L., K.W.H), Internal Medicine (Y.K.K.), and Research Center for Molecular Medicine (C.D.K.), College of Medicine, Pusan National University, Pusan, Korea

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ABSTRACT

This study was designed to determine whether rebamipide can inhibit neutrophil adhesion to human umbilical vein endothelial cells (HUVECs) stimulated with 1 h of hypoxia followed by 4 h of reoxygenation (H/R). Furthermore, to define the action mechanisms, we determined the effect of rebamipide on the surface expression of endothelial cell adhesion molecules E-selectin, P-selectin, and intercellular adhesion molecule-1 (ICAM-1) on H/R-stimulated HUVECs. Under resting conditions, both E-selectin and P-selectin were not expressed on the surface of HUVECs in contrast to ICAM-1, which was constitutively expressed. After stimulation with H/R, HUVECs showed an enhanced neutrophil adhesivity in association with an increased surface expression of E-selectin and P-selectin with a marginal increase in ICAM-1 expression. In parallel, the increased nuclear translocation of nuclear factor-κB in H/R-stimulated HUVECs was monitored by electrophoretic mobility shift assay (adjusted volume units, 11.9 ± 2.5 × 10^4 counts × mm² in unstimulated cells versus 24.2 ± 3.0 × 10^4 counts × mm² in H/R-stimulated cells). Rebamipide suppressed the surface expression of E-selectin and P-selectin with a subsequent inhibition of neutrophil adhesion to H/R-stimulated HUVECs. In line with these results, rebamipide (100, 300, and 1000 µM) inhibited H/R-induced nuclear translocation of nuclear factor-κB in a concentration-dependent manner. Taken together, this study demonstrates that rebamipide inhibits neutrophil adhesion to HUVECs by a mechanism involving inhibition of transcription-dependent surface expression of E-selectin and P-selectin in H/R-stimulated endothelial cells.

Challenging endothelial cells with hypoxia/reoxygenation (H/R) has been reported to increase oxidant production, induce endothelial cell adhesion molecules (ECAMs), and increase adhesivity to neutrophils (Kvietys and Granger, 1997). Adhesion of neutrophils to vascular endothelial cells is a critical step in recruitment and infiltration of leukocytes to the site of tissue injury and inflammation. These processes are mediated by a wide variety of ECAMs such as E-selectin, P-selectin, and intercellular adhesion molecule-1 (ICAM-1). ECAMs are significantly induced in response to proinflammatory mediators such as cytokines as well as oxidants (Collins et al., 1995). The modulation of ECAM expression is considered to be an important therapeutic target, as shown by the beneficial effects of monoclonal antibodies against ECAMs on the progression of inflammatory responses in several studies (Barton et al., 1989; Albelda et al., 1994).

The transcription factor nuclear factor-κB (NF-κB) regulates expression of a wide range of genes involved in immune response and inflammation as well as several viral genes (Baeuerle and Henkel, 1994). ECAM genes are known to contain NF-κB-binding sites that are critical for the expression of these proteins on endothelial cells (Collins et al., 1995; Pan and McEver, 1995). Deletion and point mutation studies have demonstrated that the interaction of transcription factor and these elements is necessary for the induction of ECAM expression by cytokines or mitogens (Collins, 1993; Sen and Packer, 1996). Despite the fact that these NF-κB-activating factors likely use a number of different signal transduction pathways, all induce phosphorylation of inhibitory protein IκB and its proteolytic degradation (Whiteside et al., 1995). Thus, the diversity of responsive genes and activating agents has implicated NF-κB as a pleiotropic mediator of inducible gene control.

Recently, we have demonstrated that rebamipide inhibits...
neutrophil adhesion to human umbilical vein endothelial cells (HUVECs) via down-regulation of surface expression of adhesion molecule (CD11b) on neutrophils (Kim et al., 1999). However, the exact molecular mechanisms by which rebamipide inhibits neutrophil adhesion to endothelial cells are unclear. Presently, little is known about the intracellular mediators and molecular determinants of the H/R-induced increase in the neutrophil adhesion to endothelial cells. Thus, in this study, to determine the molecular mechanisms responsible for the enhanced adhesion of neutrophils to H/R-stimulated HUVECs, both the expression of ECAMs and the activity of NF-κB were measured after reoxygenation of hypoxic endothelial cells. Thereafter, we determined the effect of rebamipide on the adhesion of neutrophils to HUVECs, the expression of ECAMs, and the activity of NF-κB on H/R-stimulated endothelial cells.

Materials and Methods

Reagents. Rebamipide (Otsuka Pharmaceutical, Tokushima, Japan) was dissolved in 10 mM NaOH solution at a concentration of 10 mM. Formyl-methionyl-leucyl-phenylalanine (fMLP) and anti-human IgG (Fab-specific) were purchased from Sigma Chemical Co. (St. Louis, MO). Na125iCrO4 was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). The anti-human monoclonal antibodies directed against E-selectin (mouse IgG type), P-selectin (mouse IgG type), and ICAM-1 (mouse IgG type) were obtained from R&D Systems (Minneapolis, MN).

Culture of Endothelial Cells. HUVECs (American Type Culture Collection HB-11608) were cultured in medium 199 (Life Tech, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum, heparin sodium (90 μg/ml), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and endothelial cell growth supplement (150 μg/ml; Sigma Chemical Co.). Cells were grown to confluence at 37°C on 0.5% gelatin-coated cell culture plates and incubated at 37°C in a 5% CO2 incubator.

Culture of Neutrophils. Neutrophils were isolated from freshly drawn human blood by standard dextran sedimentation and gradient separation on Histopaque 1077 (Sigma Chemical Co.). The procedure yielded a polymorphonuclear leukocyte population that was 98% viable (estimated by trypan blue exclusion assay and measurement of lactate dehydrogenase activity in the supernatants).

Isolation of Neutrophils and Adhesion Assay. Human neutrophils were isolated from venous blood of healthy adults with standard dextran sedimentation and gradient separation on Histopaque 1077 (Sigma Chemical Co.). This procedure yielded a polymorphonuclear leukocyte population that was 98% viable (estimated by trypan blue exclusion assay) and 97% pure (by acetic acid-cystic crystal staining).

Isolated neutrophils (2 × 106 cells/ml) were suspended in PBS and radiolabeled with 30 μCi of Na125iCrO4/ml at 37°C for 60 min. The cells were washed twice with 4°C PBS at 250g for 8 min to remove unincorporated radioactivity and then resuspended in plasma-free PBS. The radiolabeled neutrophils (2 × 106 cells/ml) were added to HUVEC monolayers at a neutrophil-to-endothelial cell ratio of 10:1. After coincubation for 30 min at 37°C, the endothelial monolayers were washed, and then the remaining cells were lysed. The 51Cr activities of the supernatant, wash fluid, and lysate were assessed in a gamma counter (Wallac 11470 Wizard). The percentage of added neutrophils that adhered to the HUVEC monolayers was determined as follows: neutrophil adherence (%) = lysate (cpm) × 100/supernatant (cpm) + wash (cpm) + lysate (cpm).

In experiments with rebamipide (100, 300, and 1000 μM) and anti-human monoclonal antibodies directed against E-selectin, P-selectin, and ICAM-1 (3 μg/ml each), HUVECs were pretreated with either rebamipide or antibodies 30 min before H/R procedure. After H/R stimulation rebamipide or antibodies added were washed away before adding the leukocytes.

Measurements of ECAM Expression. Surface expression of E-selectin, P-selectin, and ICAM-1 on HUVECs was determined with enzymelinked immunosorbent assay kits (R&D Systems). Endothelial cell monolayers on 48-well tissue culture plates were previously treated with rebamipide 30 min before H/R challenge. Cells were fixed in 1% paraformaldehyde for 15 min and then blocked with 2% BSA overnight at 4°C. The endothelial monolayers were incubated with monoclonal antibody against E-selectin, P-selectin, and ICAM-1 (10 μg/ml each) for 2 h at 37°C, washed three times with PBS containing 0.05% Tween 20, and once more washed with PBS. Thereafter, cells were further incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma Chemical Co.). After additional washing with PBS, p-nitrophenyl phosphate (100 μl of 100 μM) was added as a substrate, and optical density was read at 450 nm. Each measurement was performed in duplicate or triplicate and the specific antibody binding was estimated by subtracting the mean negative control value from each test.

Electrophoretic Mobility Shift Assay (EMSA). After incubation with test agents, HUVECs were harvested and washed twice with ice-cold PBS, and then nuclear extracts were prepared as follows. Endothelial cells (~5 × 106) were lysed in 800 μl of hypotonic buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride] on ice for 15 min, after which 50 μl of 10% Nonidet P-40 solution was added, and the mixture was vortexed vigorously for 15 s and centrifuged for 30 s at 12,000 rpm. The pelleted nuclei were resuspended in 50 μl of buffer B [50 mM HEPES (pH 7.9), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol] and incubated on ice for 20 min with intermittent mixing. The tubes were centrifuged for 5 min at 12,000 rpm, and the supernatant containing nuclear extracts was collected. The protein concentration was determined with a Bio-Rad protein concentration assay kit (Bio-Rad Laboratories, Richmond, CA).

A 22 mer NF-κB consensus oligonucleotide (5-AGTGGAGG-GACTTTCTCCAGGGC-3’; Promega, Madison, WI) was end-labeled with γ-32P]-ATP (Amersham Pharmacia Biotech) with T4 polynucleotide kinase (Promega). Five micrograms of nuclear extract proteins were preincubated in 5 μl of a binding solution (4% (v/v) glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)) for 10 min at room temperature. After addition of the 32P-labeled oligonucleotide probe, the incubation was continued for 20 min, and DNA-protein complex formed was separated on a 5% nondenaturing polyacrylamide gel electrophoresis. The gel was then dried and analyzed with a Molecular Imager system (Bio-Rad GS-525). The results are expressed as an adjusted volume units (counts × mm2).

Statistics. All results are expressed as mean ± S.E. Multiple comparisons were performed with an ANOVA and statistical differences between groups were determined by Student’s t test. P values <.05 were considered to be significant.

Results

Effect of H/R on Surface Expression of ECAMs. To measure time course effect of reoxygenation on the surface expression of ECAMs on endothelial cells, HUVECs were
subjected to various times of reoxygenation after 1 h of hypoxia. Under resting condition, ICAM-1 was constitutively expressed but E-selectin and P-selectin were not. As shown in Fig. 1, the optical densities of both E-selectin and P-selectin levels were gradually increased with the peak responses at 3 to 4 h after reoxygenation. Thereafter, the magnitudes of E-selectin and P-selectin gradually declined and returned to basal values by 24 h. In this experiment, the surface expression of ICAM-1 on HUVECs was gradually increased up to 24 h of reoxygenation, but the peak expression of ICAM-1 was comparatively low in degree, unlike E-selectin and P-selectin. Thus, 1 h of hypoxia followed by 4 h of reoxygenation was chosen in this experiment. Under this protocol, endothelial cells were intact without loss of viability.

**Effect of H/R on Neutrophil Adhesion to HUVECs.** To study the effect of H/R on the adhesion of neutrophils to HUVECs, the percentage of neutrophils adhering to H/R-stimulated HUVECs was assessed (Fig. 2). The adhesion of unstimulated neutrophils to HUVECs was 5.1 ± 0.3% under resting condition. When HUVECs were subjected to 1 h of hypoxia followed by 4 h of reoxygenation, the adhesion of unstimulated neutrophils to HUVECs was significantly increased (8.0 ± 1.0%) but the magnitude of increased adhesion was modest to gain access to the effect of rebamipide on the neutrophil adhesion to endothelial cells. Thus, in this experiment, neutrophil adhesion to H/R-stimulated HUVECs was further increased (14.5 ± 2.7%) by activating neutrophils with fMLP (1 nM).

**Effect of Monoclonal Antibodies on Neutrophil Adhesion to HUVECs.** To determine whether ECAMs induced by H/R are involved in neutrophil adhesion, HUVECs were subjected to H/R after 30 min of incubation with each monoclonal antibody (3 μg/ml), and then adhesion to neutrophils was assayed. The increased adhesion of neutrophils (13.1 ± 2.1%, P < .01) to HUVECs was significantly suppressed by the above-mentioned neutralizing antibodies (P < .01 for each), respectively, whereas control anti-human IgG (1:400 in dilution) had no effect (Fig. 3). These results suggest that the ECAMs expressed on HUVECs, including E-selectin, P-selectin, and ICAM-1, are importantly implicated in the neutrophil adhesion to H/R-stimulated endothelial cells.

**Effect of Rebamipide on Neutrophil Adhesion to HUVECs.** To examine the effect of rebamipide on neutrophil adhesion to H/R-stimulated HUVECs, rebamipide was included in the endothelial cell culture medium 30 min before H/R procedure. As shown in Fig. 4, neutrophil adhesion to HUVECs was significantly increased when HUVECs were stimulated with H/R. The increased adhesion (14.9 ± 2.4%) of neutrophils to H/R-stimulated HUVECs was inhibited by

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**Fig. 1.** Time course effect of reoxygenation on the expression of adhesion molecules (A, E-selectin; B, P-selectin; C, ICAM-1) on HUVECs after 1 h of hypoxia. Basal represents the expression of adhesion molecules on endothelial cells that were not subjected to hypoxia. All values are expressed as mean ± S.E. from six separate experiments with triplicate measurements.

**Fig. 2.** Effect of H/R on neutrophil adhesion to HUVECs. Both unstimulated neutrophils (PMN) and fMLP (1 nM)-stimulated neutrophils (aPMN) were allowed to adhere to either unstimulated or H/R-stimulated HUVECs. All values are expressed as mean ± S.E. from five separate experiments with duplicate measurements. *P < .05; **P < .01 versus PMN adhesion to endothelial cells that were not challenged with H/R.

**Fig. 3.** Effect of neutralizing antibodies against E-selectin (E-sel ab), P-selectin (P-sel ab), and ICAM-1 (ICAM-1 ab), and IgG on the neutrophil adhesion to HUVECs challenged with 1 h of hypoxia followed by 4 h of reoxygenation (H/R). Basal represents the neutrophil adhesion to endothelial cells that were not subjected to H/R. Vehicle represents the results obtained in the absence of antibodies. All values are expressed as mean ± S.E. from five separate experiments with duplicate measurements. ##P < .01 versus basal; **P < .01 versus vehicle.

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pretreatment with rebamipide (100, 300, and 1000 μM) in a concentration-dependent manner. However, the basal value of neutrophil adhesion to HUVECs was not affected by rebamipide (1000 μM). In this experiment, the viability of HUVECs and neutrophils was not affected by either addition of 1000 μM concentration of rebamipide or H/R procedure.

**Effect of Rebamipide on Surface Expression of ECAMs.** We examined the effects of rebamipide on the H/R-dependent expression of E-selectin and P-selectin. As shown in Fig. 5, the increased levels of E-selectin and P-selectin were significantly suppressed by pretreatment with rebamipide (100, 300, and 1000 μM) in a concentration-dependent manner. The basal expression of both E-selectin and P-selectin on HUVECs, unless stimulated, was not affected by rebamipide (1000 μM).

**EMSA.** The effect of rebamipide on the nuclear translocation of NF-κB was further monitored with EMSA that allows the measurement of nuclear, hence active, NF-κB in response to H/R stimulation. As shown in Fig. 6, HUVECs were found to contain significant levels of constitutive NF-κB activity as evidenced by the presence of a NF-κB complex in the resting cells. Stimulation of endothelial cells with H/R resulted in 2.1-fold increases in NF-κB complex in the nuclear extract (adjusted volume units in unstimulated cells are 11.9 ± 2.5 × 10⁴ counts × mm² versus 24.2 ± 3.0 × 10⁴ counts × mm² in H/R-stimulated cells). Pretreatment of HUVECs with rebamipide (100, 300, and 1000 μM) inhibited nuclear translocation of NF-κB as demonstrated by a decrease in nuclear NF-κB protein. The effect was concentration-dependent and most evident at 1000 μM concentration of rebamipide, at which 42% inhibition of stimulated binding reaction was shown from 24.2 ± 3.0 × 10⁴ counts × mm² in vehicle to 13.7 ± 2.3 × 10⁴ counts × mm² in rebamipide-treated cells (Fig. 6). However, in the absence of H/R stimulation, rebamipide showed no effect on the NF-κB activity.

**Discussion**

Neutrophil adhesion to the vascular endothelial cells is an early and important event after reperfusion of ischemic tissues, in that ECAMs are involved. The adhesion molecules, including E-selectin, P-selectin, and ICAM-1, are up-regulated on the surface of endothelial cells after exposure to inflammatory mediators such as oxygen-derived free radicals and cytokines (Lorant et al., 1991; Patel et al., 1992). Exposure of endothelial cells to H/R was demonstrated to cause an enhanced expression of ECAMs with subsequent increase in the neutrophil adhesivity to them (Ichikawa et al., 1997). Our results clearly show that the expression of E-selectin and P-selectin was significantly increased after reoxygenation of hypoxic HUVECs in association with an increased adhesion of neutrophils to endothelial cells. The maximal expression of E-selectin and P-selectin occurred 3 to 4 h after reoxygenation of the hypoxic HUVECs, and then gradually decreased to the basal level 24 h after reoxygenation. The expression of ICAM-1, however, was gradually increased up to 24 h of reoxygenation but the magnitude of increased expression was not dramatic as those of E-selectin and P-selectin. Considering other reports (Barton et al., 1989; Albelda et al., 1994; Ichikawa et al., 1997) with our results that demonstrate the inhibitory effect of monoclonal antibodies against ECAMs on neutrophil adhesion to HUVECs, adhesion molecules on endothelial cells play a critical role in the neutrophil adhesion to endothelial cells.

Regulation of the expression of ECAM gene has been related to oxidative stress through specific reduction-oxidation-sensitive transcriptional or post-transcriptional mechanisms (Marui et al., 1993; Ikeda et al., 1994). It is well known that NF-κB plays a key role in the regulation of expression of
ECAMs such as ICAM-1 and vascular cell adhesion molecule (Collins, 1993). Recent evidence shows that reactive oxygen intermediates are involved in NF-κB activation and binding of activated NF-κB to its cognate DNA site (Sen and Packer, 1996). Furthermore, antioxidants, such as N-acetylcysteine, dimethylsulfoxide, and α-lipoic acid, have been reported to inhibit NF-κB activation in a wide range of cell types (Sen and Packer, 1996). In agreement with other reports (Ichikawa et al., 1997), our results clearly showed that the increase in NF-κB activity was associated with enhanced expression of ECAMs on H/R-stimulated HUVECs. Thus, the increased nuclear translocation of NF-κB in H/R-stimulated HUVECs is considered to be implicated in the enhanced transcription-dependent expression of ECAMs because the promoter regions of the genes for ECAMs contain NF-κB-binding sites that are critical for the expression of these proteins on endothelial cells (Collins et al., 1995; Pan and McEver, 1995).

Recently, rebamipide has been reported to exert an oxygen free radical-scavenging effect (Yoshikawa et al., 1993) and to prevent gastric ulcers induced by indomethacin and diethylthiocarbamate (Ogino et al., 1992; Murakami et al., 1997). Kim and Hong (1995) documented that rebamipide prevented gastric lesions induced by ischemia/reperfusion, in that both myeloperoxidase activity (an index of neutrophil infiltration) and products of lipid peroxidation were reduced in association with inhibition of the production of superoxide anion from activated neutrophils. Our most recent experiment also showed that rebamipide inhibited neutrophil adhesion to cultured endothelial cells through inhibition of surface expression of adhesion molecule (CD11b) on neutrophils (Kim et al., 1999). In this experiment, exposure of endothelial cells to H/R caused an increased adhesivity of endothelial cells to neutrophils in association with enhanced expression of ECAMs E-selectin and P-selectin. Furthermore, rebamipide inhibited ECAM expression in association with suppression of nuclear translocation of NF-κB in H/R-stimulated HUVECs. Our findings that rebamipide suppresses H/R-mediated activation of NF-κB in the same dose range that is effective in down-regulating the H/R-induced ECAM expression suggest that rebamipide suppresses the expression of these adhesion molecules via suppressing the H/R-induced activation of NF-κB. At present, it is not easy to analyze the exact mechanism(s) by which rebamipide inhibits the nuclear translocation of NF-κB in H/R-stimulated HUVECs. Considering the fact that rebamipide has potent antioxidant properties (Ogino et al., 1992; Yoshikawa et al., 1993; Kim and Hong, 1995), it can be inferred that the inhibitory effect of rebamipide on NF-κB activation in H/R-stimulated HUVECs may be related to its antioxidant activity. Provided the action of rebamipide is ascribed to its antioxidant properties, there may be a reduction-oxidation-sensitive site around IκB kinase in the signaling pathway, thereby leading to the activation of NF-κB (DiDonato et al., 1997). At present, we do not know whether rebamipide acts at multiple sites inside the cells.

In summary, our results provided convincing evidence for a novel mechanism of the rebamipide-mediated inhibition of neutrophil adhesion to H/R-stimulated HUVECs. This study showed that rebamipide inhibited the H/R-induced NF-κB activation with a subsequent down-regulation of the expression of E-selectin and P-selectin on H/R-stimulated HUVECs. Thus, it is suggested that the inhibitory effect of rebamipide on neutrophil adhesion to HUVECs, in part, represents a mechanism that contributes to the antiagruccic ulcer effect of rebamipide.

Acknowledgments

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Fig. 6. A, representative EMSAs showing effects of rebamipide on NF-κB activation induced by 1 h of hypoxia followed by 4 h of reoxygenation (H/R). Positions of the NF-κB complex are indicated to the left of the blot. Nuclear extracts were prepared from HUVECs treated with various concentrations of rebamipide (100, 300, and 1000 μM) for 30 min before H/R stimulation. B, densitometric analysis of EMSA for NF-κB complex in nuclear extracts from endothelial cells. The experimental conditions are the same as those described in A. Values represent averages of six independent experiments. All values are expressed as mean ± S.E. from six separate experiments. R100, R300, and R1000 indicate 100, 300, and 1000 μM rebamipide, respectively. *P < .01 versus basal; **P < .05; ***P < .01 versus vehicle.

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