Curcumin Attenuates Nuclear Factor-κB, c-Jun N-Terminal Kinase and p38 in Tumor Necrosis Factor-α-Stimulated Endothelial Cells

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ABSTRACT

Background and Objectives: Curcumin, a yellow pigment of turmeric in curry, has been reported to interfere with nuclear factor (NF)-κB. This study was designed to investigate the underlying pathway of the anti-inflammatory effect of curcumin on endothelial cells. Materials and Methods: Human umbilical vein endothelial cells (HUVECs) were stimulated with tumor necrosis factor (TNF)-α (10 ng/mL). The levels of intracellular reactive oxygen species (ROS) were examined using a fluorescent dye DCFH-DA, and the adhesion of U-937 monocytes to the HUVECs was then examined. Nuclear factor kappa B (NF-κB) activation was determined by the NF-κB p65 translocation to the nucleus via immunocytochemistry. The expression of the NF-κB dependent pro-inflammatory molecules were measured by RT-PCR and ELISA. The phosphorylations of c-Jun N-terminal protein kinase (JNK), p38 and STAT-3 (signal transducer and activator of transcription-3) were measured by Western blotting. Results: Curcumin blocked the activation of NF-κB by TNF-α, and it also reduced the ROS, monocyte adhesion and the phosphorylation of JNK, p38 and STAT-3 in the TNF-α-stimulated HUVECs. The expression of intracellular cell adhesion molecule (ICAM)-1, monocyte chemoattractant protein (MCP)-1, and interleukin (IL)-8 were attenuated by curcumin at both the transcription and translation levels. Conclusion: We suggest that curcumin could contribute to protection against the adverse vascular effects of the pro-inflammatory response through the modulation of NF-κB, JNK, p38 and STAT-3, and this is in addition to its antioxidant effect in endothelial cells. (Korean Circulation J 2006;36:482-489)

KEY WORDS: Curcumin; Nuclear factor-κB; Tumor necrosis factor-α; Inflammation; Endothelial cell.

Introduction

Curcumin [1, 7-bis-(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione] is the yellow pigment of turmeric in curry. It is derived from the rhizome of the plant Curcuma longa, a widely used spice and food coloring agent that has anti-inflammatory and anticancer properties. In addition, curcumin has been reported to inhibit ROS production, nitric oxide synthase activity and the lipoxigenase and cyclooxygenase activities that are involved in the inflammatory pathways.12)

Accumulated evidence suggests that curcumin is a potential chemopreventive agent that could suppress tumor initiation, promotion and metastasis.13) Consumption of turmeric and curcumin has been associated with beneficial effects on human health with the most prominent among them being anti-inflammatory and cancer chemopreventive activities. Some recent studies have shown that curcumin is a potent inhibitor of protein kinase C and I kappa B (IκB) kinase.14)

Much evidence has demonstrated that inflammation and endothelial dysfunction are key initiating events in atherosclerosis.35) Atherosclerotic lesions that are prone to rupture are characterized by the increased expression of pro-inflammatory cytokines like tumor necrosis factor(TNF)-α, and so this promotes an activated immune response.36) TNF-α is one of the major inflammatory cytokines that mediates a wide range of biological responses including inflammation, infection,
injury and apoptosis. The effects of TNF-α are initiated by this cytokine binding to its receptors, which causes activation of two major transcription factors, AP-1 and nuclear factor kappa B (NF-κB); these in turn induce genes that are involved in inflammatory responses and apoptosis. NF-κB is a pivotal transcription factor that’s been implicated in the regulation of many genes, and particularly those genes of the inflammatory and immune responses and the regulation of both the cytoprotective and cell death pathways.

Cytokine-derived signaling is amplified by several pathways, including mitogen-activated protein kinase (MAPK). MAPKs that contain extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 are well known stress-activated kinases. ERK is involved in cell survival and proliferation, while JNK and p38 are activated by various extracellular stimulations such as reactive oxygen species (ROS), ultraviolet irradiation and cytotoxins. The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway have recently been shown to be activated in the remodeling of post-myocardial infarctions. It could be proposed that STAT proteins play an important role in the maintenance of cardiac function; however, their molecular mechanisms remain to be fully elucidated and especially under pathological conditions in vivo. Thus, gaining control of the cytokine signaling molecules may play a critical role in protecting against inflammatory conditions.

The purpose of this study was to demonstrate the protective effects of curcumin on TNF-α-stimulated human endothelial cells. The data from this study provides a mechanism for how curcumin is involved in TNF-α-induced inflammatory pathways, as well as providing a possible strategy for preventing cytokine-induced endothelial dysfunction.

Materials and Methods

Materials
Curcumin, dichlorofluorescein diacetate (DCFH-DA), 2’, 7’-Bis (2-carboxyethyl)-5 (6) - carboxyfluorescein, acetoxymethyl ester (BCECF-AM), and bovine serum albumin (BSA) were purchased from Sigma (MO, USA). TNF-α and the Duoset ELISA kit were obtained from R & D Systems (Minneapolis, MN). Fetal bovine serum (FBS) and the antibiotics/antimycotics were obtained from Gibco-BRL (Grand Island, NY). Endothelial basal medium (EBM)-2 and Single Quot were obtained from Cambrex (East Rutherford, NJ). Vascular cell adhesion molecule (VCAM)-1, β-actin, mouse-horse radish peroxidase conjugate, rabbit-horse radish peroxidase conjugate and WesternBreeze were obtained from Santa Cruz. The images were obtained from R & D Systems. Serum albumin were obtained from Santa Cruz. The images were obtained from Molecular Probes (CA, USA), and the TNF-αB p65 antibody was obtained from SantaCruz (CA, USA). MMLV reverse transcriptase and SuperScript reverse transcriptase were purchased from Invitrogen (Carlsbad, CA).

Cell culture
The human umbilical vein endothelial cells (HUVECs) were purchased from Modern Tissue Technologies (Seoul, Korea) and they were cultured in EBM-2 supplemented with a Single Quot kit. The HUVECs were grown to 70-80% confluence and they were used at the 7th passage.

Determination of the intracellular reactive oxygen species (ROS)
The cells were cultured on a 96-well plate and they were pretreated with curcumin. After 1 hour, cells were preloaded with 10 μM DCFH-DA for 30 minutes at 37°C; this was followed by incubation with TNF-α (10 ng/mL). The fluorescence intensity was analyzed by a fluorescence reader (Fluoroscan Ascent FL, Labsystems, Finland) with using a 485 nm excitation filter and a 538 nm emission filter.

U937 Adhesion assay
The U937 cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and they were maintained in RPMI-1640 supplemented with 10% FBS. The U937 cells were labeled with BCECF-AM (10 μg/mL) for 30 minutes at 37°C, and then they were washed and resuspended in serum-free media. The HUVECs were cultured and incubated with reagents on a 24-well culture plate; they were then co-cultured with the BCECF-AM-labeled U937 cells (106 cells/well) for 30 minutes at 37°C. The non-adhering U937 cells were removed by gentle aspiration and the wells were washed with PBS. The cells were lysed in 0.1% Triton X-100 in 0.1 M Tris-HCl, pH 7.4 to evaluate the U937 cells’ adhesion to the HUVECs. The fluorescence was measured with a microplate fluorescence reader using an excitation wavelength of 510 nm and an emission wavelength of 531 nm. Each adhesion assay was performed 12 hours after treating the HUVECs with TNF-α. The effect of curcumin on U937 cellular adhesion was assessed by pre-incubating curcumin with the HUVECs for 1 hour before adding the TNF-α.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)
The total RNA of the HUVECs was extracted with
using Trizol reagent (Invitrogen) according to the manufacturer’s instruction. 1 μg of the total RNA was used as a template for cDNA synthesis under the conditions of 65°C for 15 minutes, 25°C for 10 minutes, 42°C for 60 minutes and 95°C for 10 minutes with using SuperScript reverse transcriptase; the products were then stored at 4°C. The first-strand cDNA was amplified by PCR with using specific primers. The amplified PCR products were fractionated by 1.5% agarose gel electrophoresis, and the amplified products were then visualized by ultraviolet fluorescence after being stained with ethidium bromide.

Cytokine analysis via enzyme-linked immunosorbent assay (ELISA)

The HUVECs were seeded onto a 96-well plate at 3 × 10^4 cells/well, and they were pretreated with curcumin for 1 hour; this was followed by the addition of TNF-α. After stimulation with TNF-α for 12 hours, the cell culture medium was collected for measurement. The concentrations of ICAM-1, MCP-1 and IL-8 were quantified using a commercially available ELISA development system according to the manufacturer’s protocols.

Immunocytochemistry

The HUVECs were pretreated with curcumin for 1 hour and then they were stimulated with TNF-α for 1 hour. The cells were fixed for 10 minutes with 2% paraformaldehyde at room temperature and then washed three times with PBS. They were then permeabilized for 10 minutes with 0.5% Triton X-100 in PBS, washed three times with PBS and incubated for 10 minutes in 1% BSA in PBS to block the non-specific binding sites before labeling with the NF-κB p65 antibody. The primary antibodies were applied for 1 hour at room temperature, and this was followed by incubation with Alexa Fluor 488 goat anti-rabbit antibody. The HUVECs’ nuclei were counterstained with DAPI. Recording and analysis of the fluorescence signals were performed using ImagePro software 5.0 (Media Cybernetics, Inc. MD, USA).

Statistical analysis

Each experiment was performed at least three times. The data are presented as means ± SDs. Differences were analyzed by ANOVA testing. p<0.05 were deemed statistically significant.
Results

Curcumin reduced the ROS production and U937 monocyte adhesion induced by TNF-α

The cytotoxicity of curcumin on the HUVECs was examined by performing MTT assay. The cell viability was not different from the control cells with treatment of curcumin at concentrations under 50 μM for 24 hours (p>0.05) (Fig. 1A). The intracellular ROS level was increased 1.7 ± 0.1-fold by the TNF-α. Curcumin at concentrations of 3 μM, 10 μM and 30 μM reduced the intracellular ROS level by 3.0 ± 0.3%, 47.8 ± 5.3% and 62.7 ± 6.0%, respectively (Fig. 1B). U937 monocyte adhesion to the HUVECs was increased 2.4 ± 0.5-fold by stimulation with TNF-α treatment (10 ng/mL) for 12 hours. Pretreatment with curcumin at 3 μM, 10 μM and 30 μM showed inhibitory effects of 21.8 ± 1.1%, 50.7 ± 2.7% and 98.9 ± 8.7%, respectively (Fig. 1C).

Curcumin inhibited the NF-κB activation induced by TNF-α

As shown in Fig. 2, unstimulated HUVECs have NF-κB (green) only in the cytoplasm (red), and not the nuclear area (blue). TNF-α (10 ng/mL) activated the NF-κB, leading to its translocation to the nucleus in 30 minutes. Pretreatment of curcumin (10 μM) significantly attenuated NF-κB translocation and it remained in the cytosol to a significant degree. BAY 11-7082 (10 μM), a commercially available inhibitor of NF-κB, impaired this nuclear translocation.

Curcumin suppressed the TNF-α induced expressions of CAM, MCP-1 and IL-8

The effects of curcumin on the TNF-α induced expressions of adhesion molecules and cytokines at the transcription level in the HUVECs were assessed by RT-PCR. After being incubated with curcumin for 1 hour, the cells were treated for 3 hours with TNF-α. ICAM-1, MCP-1 and IL-8 mRNAs were expressed at very low levels in the HUVECs and their expressions were drastically induced by TNF-α. Pretreatment with curcumin decreased the TNF-induced expression of ICAM-1, MCP-1 and IL-8 mRNAs (Fig. 3A). In addition to transcriptional analysis, the effect of curcumin at the translational level was assessed by ELISA. The ICAM-1, MCP-1 and IL-8 proteins were also dramatically increased by TNF to 6.6 units (0.4-fold), 6.5 units (0.3-fold), and 55.0 units (2.0-fold), respectively, compared to the control. After treatment with curcumin at 10M, the ICAM-1, MCP-1 and IL-8 were decreased by 68.0%, 62.8% and 27.8%, respectively (Fig. 3B).

Curcumin suppressed the TNF-α induced phosphorylation of JNK, p38 and STAT-3

The signaling molecules JNK, p38 and STAT-3 that were activated by TNF-α were examined by Western blotting. The HUVECs were pretreated with curcumin (10 μM) for 1 hour, and this was followed by incubation with TNF-α for 30 minutes, 1 hour, or 2 hours. TNF-α increased the phosphorylation of JNK and p38 in 1 hour, and STAT-3 was increased in 2 hours; however, pretreatment of curcumin significantly blocked the phosphorylations of JNK, p38 and STAT-3 (Fig. 4A). To investigate which phosphorylation residue was inhibited by curcumin, western blotting was performed with using two different antibodies: anti-Ser phosphorylation and anti-Thr phosphorylation of JNK and p38 in 1 hour. Pretreatment of curcumin decreased the phosphorylations of JNK and p38, while STAT-3 phosphorylation was decreased only by anti-Ser phosphorylation antibodies. Pretreatment of curcumin significantly blocked phosphorylation of JNK and p38, while STAT-3 phosphorylation was decreased only by anti-Ser phosphorylation antibodies.

![Fig. 2.](image.png) Immunochemical analysis of NF-κB p65 localization. HUVECs were incubated with TNF-α (10 ng/mL) for 30 minutes in the absence (C, D) or presence of curcumin (10 μM, E, F) and BAY 11-7082 (10 μM, G, H), and the cells were subjected to immunochemistry as described in Materials and Methods. NF-κB p65 was stained green and the nuclei were counter-stained blue. Nuc: nucleus, Cyto: cytoplasm, NF-κB: nuclear factor-kappa B, HUVECs: human umbilical vein endothelial cells, TNF-α: tumor necrosis factor-α.
orylated STAT-3 and anti-Tyr phosphorylated STAT-3. As seen in Fig. 4B, the Ser residue stayed phosphorylated to some extent and was not induced by TNF-α. On the other hand, phosphorylation of the Tyr residue was induced by TNF-α and this was inhibited by curcumin.

The effects of AG490, BAY 11-7082 and NAC on the TNF-α in the HUVECs

Curcumin attenuated the intracellular ROS level, NF-κB activation and STAT-3 activation during the stimulation with TNF-α. To examine the effects of NF-κB, STAT-3 and oxidative stress on the TNF-α-stimulated HUVECs, AG490 (50 μM), BAY 11-7082 (10 μM), and N-acetylcysteine (NAC, 10 mM) were used as a NF-κB inhibitor, a STAT-3 inhibitor, and an antioxidant, respectively. The U937 adhesion was significantly inhibited by curcumin (53.3%), AG490 (20.0%), BAY11-7082 (46.7%), and NAC (40.0%) significantly (Fig. 5A). The protein expressions of ICAM-1 was also significantly inhibited by curcumin (69.4%), AG490 (60.5%), BAY11-7082 (65.8%), and NAC (47.4%) significantly (Fig. 5B). The release of MCP-1 protein release was significantly reduced by pretreatment withof curcumin (66.7%), AG490 (44.6%), BAY11-7082 (53.2%), with statistical significance but not by NAC (10.6%, p>0.05) (Fig. 5B). In addition, the phosphorylation of JNK, p38, and STAT-3 was examined by immunoblotting (Fig. 5C). The phosphorylation of JNK was inhibited by curcumin and NAC, and the phosphorylation of p38 was only inhibited by curcumin. On the other hand, STAT-3 phosphorylation was inhibited by curcumin

Fig. 3. The effects of curcumin on the ICAM-1, MCP-1 and IL-8 levels in the HUVECs stimulated with TNF-α. A: the levels of mRNA were determined by RT-PCR. Curcumin was pretreated and then TNF-α (10 ng/mL) was added. After 3 hour incubation, the mRNA was extracted and subjected to RT-PCR. The cells were incubated with TNF-α for 3 hours before treatment of curcumin at the indicated dosage for 1 hour. The representative RT-PCR is presented. In the graph, the expressions of ICAM-1, MCP-1 and IL-8 were normalized to that of β-actin and they were expressed as fold increases: the control ratio was defined as 1.0. B: the protein levels were quantitated by ELISA. Curcumin was pretreated onto the cells and TNF-α was then added. After 12 hours, the cell culture medium was collected to determine the protein level. *: p<0.05 compared with the TNF-α-treated cells. ICAM-1: intracellular adhesion molecule, MCP-1: monocyte chemoattractant protein, IL-8: interleukin, HUVECs: human umbilical vein endothelial cells, TNF-α: tumor necrosis factor-α, mRNA: messenger ribonucleic acid, RT-PCR: reverse transcriptase-polymerase chain reaction, ELISA: enzyme-linked immunosorbent assay.
cumin, AG490, BAY11-7082 and NAC.

**Discussion**

The goal of the present study was to examine the mechanism by which curcumin exert its inhibitory effects on the proinflammatory response of human endothelial cells.

In the previous studies, curcumin has been reported to have a ROS scavenging property in addition to its anti-inflammatory and anti-cancer effects. Curcumin inhibited the NF-κB activation that may participate in the reaction to various stimuli such as oxidative stress, cytokines and hypoxic injury. Extensive research has recently shown that curcumin can prevent inflammation and cellular injury via NF-κB inhibition in steatohepatitis mice, intestine epithelial cells and microglial cells. However, the molecular mechanisms of curcumin for reducing endothelial inflammation have not been clearly elucidated.

We examined the effect of curcumin on HUVECs viability to exclude the possible cytotoxic effects. Curcumin was noted to relatively preserve the HUVECs’ viability and so it is regarded as safe when it is used at 10 μM, which inhibited the ROS elevation and U937 adhesion induced by TNF-α within a non-toxic range.

NF-κB is a well-known transcription factor that is critical for the pro-inflammatory gene regulation related to cancer, atherosclerosis, myocardial infarction, diabetes, arthritis and etc. We confirmed the activation of NF-κB by immunocytochemistry (ICC) and the representative ICC pictures are illustrated in Fig. 2. NF-κB activation was significantly increased in the HUVECs subjected to TNF-α treatment, which indicated a rapid activation process, while curcumin and BAY11-7082, an inhibitor of NF-κB, inhibited NF-κB p65 nuclear translocation.

NF-κB has been known to induce cytokines and adhesive molecules, and we examined whether curcumin inhibits the NF-κB-dependent pro-inflammatory molecules. In the TNF-α-stimulated HUVECs, the ICAM-1, MCP-1 and IL-8 expressions were atte-

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**Fig. 4.** The effects of curcumin on JNK, p38 and STAT-3 activation. A: the HUVECs were treated with TNF-α for the indicated time in the presence or absence of curcumin pretreatment. Cells were harvested and then subjected to Western blotting against phosphorylated JNK, p38 and STAT-3. B: the phosphorylation residue that was inhibited by curcumin was confirmed by immunoblotting with using phosphoserine-STAT-3 antibody or phospho-tyrosine-STAT-3 antibody. JNK: c-Jun N-terminal kinase, STAT-3: signal transducer and activator of transcription-3, HUVECs: human umbilical vein endothelial cells, TNF-α: tumor necrosis factor-α.
nated by curcumin at both the transcriptional and translational levels. The up-regulation of adhesion molecules, including ICAM-1, on the surface of the endothelium is required for the firm adhesion of rolling monocytes. Curcumin inhibited the TNF-α-induced ICAM-1 and MCP-1 expressions, which indicated that the mechanism of anti-adhesion, at least in part, was related to the down-regulation of these proteins. These results suggest that suppressing the surface proteins and their mRNA expression was one of the pathways of curcumin inhibiting the adhesion of U937 to TNF-α-stimulated HUVECs.

Of all the signaling molecules involved in TNF-α-mediated pathways, we detected the activation of JNK, p38 and STAT-3 by Western blotting. JNK and p38 are members of the MAPK family, which is a family of proteins that are known to be involved in cellular damage or cell death. They are activated by extracellular stresses such as ROS, UV and cytokines, and their activation leads to cellular death. STAT factors are a family of cytoplasmic transcription factors that mediate the intracellular signaling that’s initiated at the cytokine cell surface receptors and is then transmitted to the nucleus. STAT-3 is a key molecule down-stream of gp130, and is activated under various stressful conditions such as pressure-overload and myocardial infarction. Previous research has demonstrated that STAT-3 may be a survival factor in the heart that is able to protect the myocardium following ischemic injury. In malignant cells, however, curcumin suppressed JAK-STAT signaling and so it suppressed tumor cell growth in brain microglia, multiple myeloma cells and T lymphocytes. Thus, STAT-3 seems to mediate either a pro-apoptotic or anti-apoptotic effect depending on the cell or tissue type.

It is known that there are two phosphorylation sites in Stat-3: tyrosine 705 and serine 727. These two phosphorylation sites appear to be induced by distinct signaling. Phosphorylation of both sites is necessary for the maximal activation of transcription tyrosine 705 phosphorylation, which is required for STAT-3 dimerization, nuclear translocation and gene activation. Serine 727 phosphorylation is necessary for maximal transcription efficiency, although it was not known why. In our study, curcumin attenuated the tyrosine phosphorylation of STAT-3 as well as the phosphorylation of JNK and p38 in the TNF-α-stimulated HUVECs. Further, curcumin inhibited the phosphorylation of JNK, p38 and STAT-3.

Curcumin inhibited the TNF-α-induced intracellular ROS production (Fig. 1B), and we thought that curcumin’s antioxidative activity could partly contribute to its inhibitory effects. Several research groups have reported that oxidative stress might contribute to the NF-κB-dependent inflammatory responses. Rocksen et al. have reported that dexamethasone treatment resulted in reduced ROS production and the reduced expression of TNF-α, IL-1α, IL-6 and IL-12 in lipopolysaccharide (LPS)-stimulated mice. Hayashi et al. have reported that the anti-oxidant NAC suppressed vascular NF-κB activation and this inhibition reduced the pathological thickening of the arterial wall. NF-κB has been shown to be ROS-sensitive. Therefore, we think that the anti-oxidant property of curcumin inhibits the activation of NF-κB. In our work, TNF-α produced a significant increase in ROS after 1 hour of exposure. Therefore, we examined whether treatment with an anti-oxidant will inhibit the TNF-α-induced phosphorylation of JNK and p38.

To determine what property of curcumin contributes to its anti-inflammatory effects, we used the chemicals AG490, BAY11-7082 and NAC. AG490 is an inhibitor of Jak2, an upstream kinase of STAT-3. BAY11-7082 is a NF-κB inhibitor and NAC is a well-known antioxidant.

AG490 and BAY 11-7082 inhibited the expression of ICAM-1 and MCP-1 in the TNF-α-stimulated HUVECs. It also suppressed U937 adhesion and STAT-3 activation, and these events were considered to be dependent on NF-κB. On the other hand, the activations of JNK, p38 and intracellular ROS (unpublished data) were inhibited by curcumin, but not by BAY 11-7082. These data revealed that the activation of JNK and p38 by TNF-α was an independent event on NF-κB activation. NAC, an anti-oxidative reagent, blocked the activation of JNK and STAT-3 that was induced by TNF-α, but NAC did not block the activation of p38. Although AG490, BAY 11-7082 and NAC all showed inhibitory effects on TNF-α, the extent of their inhibitory effect was less than that of curcumin.

In this work, we studied whether the vascular inflammation could be influenced by curcumin, and we checked the possible molecules that had important roles. STAT-3 has been reported to be a survival factor and it is constitutively activated in some type of cancers. We did not address the physiological meaning of STAT-3 activation by TNF-α as well as its inhibition by curcumin, but we presumed STAT-3 activation was a compensatory response, and inhibition by curcumin counteracted the TNF-α.

The MAPK and STAT3 pathways have been implicated in the responses to pro-inflammatory stress in endothelial cells, and curcumin may block these responses by counteraction. Our data indicate that curcumin suppresses the expression of gene products involved in inflammation via NF-κB-dependent and independent pathways. Other pathways are possibly involved in the capacities of curcumin to block the MAPK and STAT-3 pathways and to scavenging ROS. In TNF-α-stimulated endothelial cells, curcumin ap-
pears to function as a repressor in this model of acute inflammation.

We propose that curcumin could be a potential therapeutic agent for achieving endothelial protection against the pro-inflammatory cytokine-induced cytotoxicity that’s been observed in several pathological conditions.

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tial anti-inflammatory and anti-oxidative effects of dexamethasone and N-acetylcysteine in endotoxin-induced lung inflamma