Original Article

Curcumin Attenuates Oxidative Stress-Induced Effects on TGF-β Expression and NF-κB Signaling in Bovine Aortic Endothelial Cells

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ABSTRACT

Objectives: The endothelium constitutes a significant part of the vasculature. Oxidative stress, an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense system, contributes to the impairment of endothelial function. ROS regulate several signaling pathways, such as transforming growth factor β (TGF-β) and nuclear factor kappa B (NF-κB). This study aimed to investigate the effects of curcumin, a polyphenol with antioxidant properties, on these two molecules in endothelial cells treated with hydrogen peroxide (H_2O_2).

Methods: Cultured Bovine aortic endothelial cells (BAECs) were treated with different concentrations of H_2O_2 (20 µM, 40 µM, and 200µM) for 1 and 24 hours in the absence and presence of curcumin. TGF-β expression was detected by quantitative real-time PCR analysis, and phosphorylation of NF-κB-p65 was examined by western blot.

Results: H_2O_2 up-regulates TGF-β mRNA expression and induces the phosphorylation of the p65 subunit of NF-kB in a dose-dependent manner in BAECs. Curcumin inhibited both H_2O_2-stimulated TGF-β expression and phosphorylation of NF-κB-p65 in these cells.

Conclusion: The findings demonstrate that curcumin reduces TGF-β mRNA expression and inhibits NFκB-p65 phosphorylation in endothelial cells.

Keywords: Curcumin, Transforming growth factor-β, Antioxidants, Oxidative stress, nuclear factor κB

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Introduction

The endothelium, the inner layer of the coronary artery, acts as a gatekeeper of vascular function, while endothelial dysfunction leads to vascular diseases such as atherosclerosis (1). Endothelial dysfunction is described as an imbalance between the production of endothelial-derived relaxing and contracting factors (2). Oxidative stress, an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense system (3, 4), can lead to endothelial dysfunction, an early marker in the pathogenesis of many cardiovascular diseases including atherosclerosis (5, 6).

During endothelial dysfunction, some adhesion molecules and chemokines are expressed at high levels, promoting the recruitment of monocytes and other inflammatory cells into the intima of the artery wall (4). Overexpression of cytokines such as interleukin (IL)-1, IL-4, IL-6, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, monocyte chemo-attractant protein (MCP)-1 and transforming growth factor-β (TGF-β) (7) has been identified at sites of arterial wall injury. TGF-β, one of the major cytokines that mediates endothelial dysfunction, is a secreted homodimer and multifunctional protein that regulates cellular proliferation, differentiation, adhesion, apoptosis, and production of proteoglycan (8). In smooth muscle cells, TGF-β stimulates the synthesis of proteoglycans especially biglycan-caused hyperelongation of glycosaminoglycan (GAG) on proteoglycan (9). The hyperelongation GAG chain leads to increased binding of proteoglycans to low-density lipoprotein (LDL) (10). This connection increases retention and keeping LDL in the vessel wall resulting in progress of atherosclerosis (11). Another factor that activates in endothelial dysfunction is nuclear factor κB (NF-κB) (12). NF-κB is a transcription factor that plays a major role in physiological and pathological signaling pathways (13). Several activators such as cytokines and growth factors and oxidative stress can stimulate this factor (14). NF-κB can control various aspects of immune responses such as pro-inflammatory genes expression (15). Five genes encode the members of the NF-κB family; the gene products are p50, p65, p52, relB and relC. P65 is a key effector in the NF-κB signaling pathway (16). NF-κB is involved in all stages of atherosclerosis from the formation of plaques to the disconnection of plaques (17).

Dietary polyphenols have been shown to exert a powerful antioxidant action for protection against oxidative stress-related pathological conditions (18, 19). In this context, curcumin, a polyphenol compound present in the rhizomes of turmeric plants (Curcuma spp.), has been reported to have several beneficial effects such as anti-inflammatory, anti-microbial, anti-mutagenic, and anti-cancer activities (20-22). Importantly, curcumin has been suggested to act as a powerful antioxidant by enhancing the effect of superoxide dismutase, glutathione, and catalase, thereby reducing mitochondrial oxidative stress. The aim of this study was to investigate whether curcumin could inhibit TGF-β mRNA expression and NF-κB-p65 phosphorylation in oxidative stress conditions induced by H2O2 in endothelial cells.

Materials and Reagents

The Bovine Aortic Endothelial Cells (BAECs) were generously provided by Prof. Peter J. Little from RMIT University, Melbourne, Australia. Hydrogen peroxide (H_2O_2) was obtained from Merck Chemicals. Curcumin, fetal calf serum (FBS), and Dulbecco's Modified Eagle Medium were purchased from Sigma (Saint Louis, MO, USA). Trizol reagent for total RNA isolation was from Invitrogen (Paisley, UK). For cDNA synthesis, the PrimeScript® RT-PCR Kit and SYBR Green QPCR Master Mix were used (Takara, Shiga, Japan). The polyclonal antibody anti-phospho NF-κB-p65 was purchased from Santa Cruz Technology. Anti-GAPDH was obtained from Abcam (Cambridge, MA, USA).

Cell culture

The Bovine Aortic Endothelial Cells (BAECs) were cultured in low-glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin), and maintained at 37°C with 95% humidity and 5% CO_2. For each experiment, the cells were seeded into 6-well plates at a density of 6×10^5 cells/well and maintained until confluent. The cells were then serum deprived by culturing in low glucose DMEM with 0.5% FBS for 24 hours prior to experimentation.
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Real-time quantitative RT-PCR

The mRNA level of TGF-β was determined by quantitative real-time polymerase chain reaction (RT-PCR). Total RNA was extracted from 6 ×105 cells using Trizol reagent according to the manufacturer's protocol, and the RNA concentration and quality were assessed with a Nano Drop 2000 spectrophotometer (Thermo scientific, USA). cDNA was synthesized from 1 µg DNase-treated RNA using a cDNA synthesis kit (Takara, Japan). Quantitative RT-PCR was performed using the SYBR® Green PCR Master Mix kit (Takara, Japan) according to the manufacturer's protocol. The primers had the following sequences: TGF-β Forward: 5'-ACTACTACGCCAAGGAGGTCAC- 3', Reverse 5'- GCCCGAGAGAGCAACACAGG-3'; 18S rRANA Forward: 5-'GGACACGGACAGGATTGACAG-3', Reverse: 5'- ATCGCTCCACCAACTAAGAACG-3'. Data were normalized to the ribosomal 18S rRNA housekeeping gene. All experiments were performed at least three times.

Western Blot for detection of p-p65

Cell lysate was prepared from treated cells. Then, 50µg of total protein was separated on a 10% SDS-PAGE gel under reducing conditions. After transferring onto a PVDF membrane, blocking was carried out with 3% skim milk powder in TBST at room temperature for 1 hour. After washing, the membrane was incubated with an anti-p65 rabbit monoclonal antibody (1:100) and an anti-GAPDH polyclonal antibody (1:5000) at 4°C overnight, then with horseradish peroxidase–anti–rabbit IgG (1:10000) for 1 hour at room temperature. The bands were detected with an enhanced chemiluminescent (ECL) western blot detection kit.

Statistical analysis

All statistical analyses were performed using SPSS statistical software. Differences among groups were analyzed by one-way analysis of variance followed by the Tukey test. Differences were considered significant at p values less than 0.05. Data are reported as mean ± SEM.

Results

H_2O_2 induces TGF-β gene expression in bovine aortic endothelial cells

To investigate the effect of H2O2 on TGF-β expression, the cells were treated with 20 and 40 µM H_2O_2 at the 24-hour time point. The results showed that TGF-β expression was stimulated by H_2O_2 at both concentrations. As shown in Fig 1, 20 µM and 40 µM H_2O_2 increased the mRNA expression by 2.9 and 5-fold, respectively.

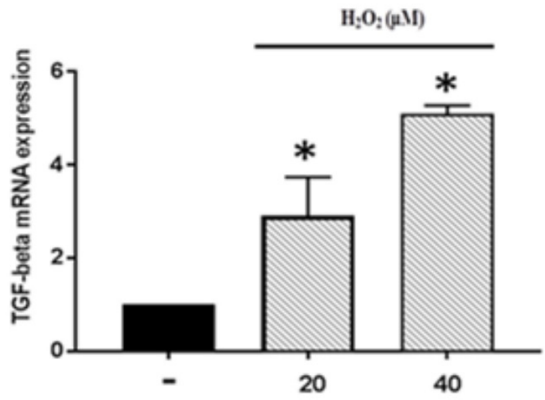

Figure 1: Effect of H_2O_2 on the expression of TGF-β. The expression of TGF-β was induced at 20 and 40 µM H_2O_2. Represented data are from three independent experiments and are means ± SEM. * Indicates significant difference the H_2O_2 treatment group with control group. $p < 0.05$.

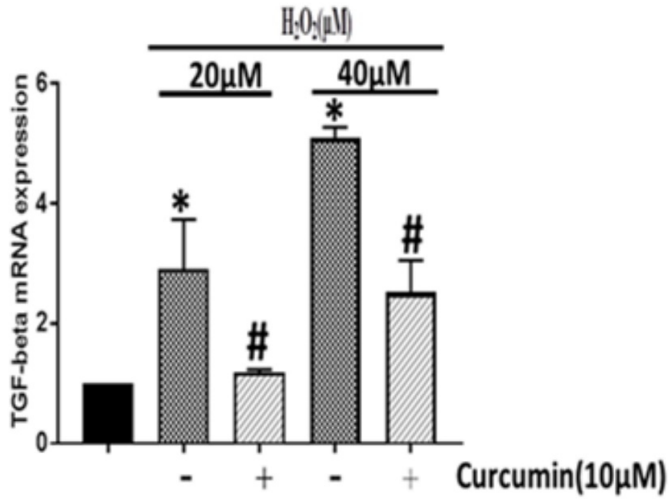

Figure 2: Effect of curcumin on the TGF-β expression in H_2O_2 treated cells. The expression of TGF-β was increased at 20µM and 40µM H_2O_2 and 10 µM curcumin could reduce TGF-β mRNA expression. Represented data are from three independent experiments and are means ± SEM. * Indicates significant difference the H_2O_2 treatment group with control group, $p < 0.05$. # indicates significant difference the H_2O_2 treatment group with curcumin group, $p < 0.05$.

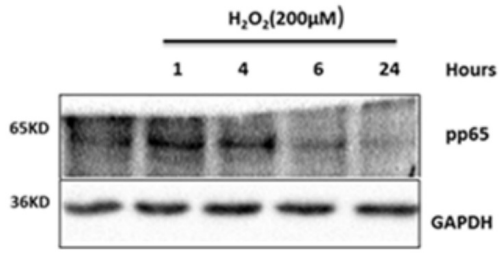

Figure 3: Effect of H_2O_2 on the phosphorylation of NF-κβ-p65. p-p65 was increased in presence 200μM H_2O_2 at 1 and 4 hours.

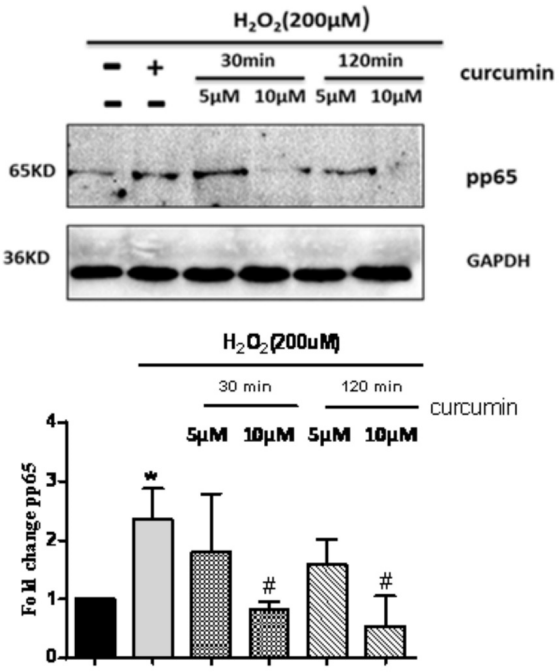

Figure 4: Effect of curcumin on the phosphorylation of NF-κB-p65 in BAECs treated with H_2O_2. The phosphorylation of p65 was increased at 200μM H_2O_2 and reduced by addition of 10 μM curcumin, whereas 5μM curcumin did not affect NF-κB-p65 phosphorylation. Represented data are from three independent experiments and are means ± SEM. * indicates significant difference the H_2O_2 treatment group with control group, $p < 0.05$. # indicates significant difference the H_2O_2 treatment group with curcumin group, $p < 0.05$.

Curcumin attenuated H_2O_2-inducede TGF-β mRNA expression in bovine aortic endothelial cells

In this experiment, the inhibitory effect of curcumin on TGF-β expression was evaluated. Treatment with curcumin (10 μM) for 24 hours effectively decreased TGF-β mRNA expression compared to the cells treated with H_2O_2 alone.

H_2O_2 induces phosphorylation of NF-κB-p65 in bovine aortic endothelial cells

The effect of H_2O_2 on the phosphorylation of p65 in BAECs was assessed. Quiescent cells were treated with H_2O2 for various times as indicated. BAECs were serum-starved for 24 hours, then incubated with H_2O_2 (200 μM) for 1, 4, 6, and 24 hours. The results showed that the maximum effect of H_2O_2 was at 1 and 4 hours after treatment, then declined to basal levels at 24 hours. These data suggest that H_2O_2 induces the phosphorylation of p65 in BAECs. Therefore, in subsequent experiments, the cells were treated with H_2O_2 at a concentration of 200 μM for 1 hour.

Curcumin reduces the phosphorylation of NF-κB-p65 induced by H_2O_2 in bovine aortic endothelial cells

In this experiment, the effect of curcumin on H2O2-stimulated phosphorylation of NF-κB-p65 was examined. Therefore, BAECs were treated with curcumin (5 μM, 10 μM) for 30 and 120 minutes prior to incubation with H2O2 (200 μM) for 1 hour. H_2O_2 increased the phosphorylation of p65, whereas curcumin at a concentration of 10 μM, but not 5 μM, inhibited the phosphorylation of p65 at both 30 and 120 minutes.

Discussion

In this study, it was demonstrated that curcumin, an active polyphenolic compound, has potent inhibitory effects on H_2O_2-stimulated TGF-β mRNA expression and NFκB-p65 phosphorylation in BAECs. These effects of curcumin may be mediated via its antioxidant activity. Reactive oxygen species (ROS) generated in the cells are important regulators of growth factor signal transduction pathways. Several studies have demonstrated that H2O2 produced from the vascular wall mediates diverse physiological functions such as cell proliferation, differentiation, and aging (23). TGF-β is one of the target genes regulated by oxidative stress in the vessel wall (24). TGF-β is a member of the TGF-β family that plays a key role in stimulating cell proliferation (25). Therefore, the first aim of this study was to evaluate TGF-β gene expression in BAECs stimulated with H_2O_2. The results revealed a linear and direct correlation between the concentration of exogenous oxidative stress and TGF-β mRNA expression. In this study, oxidative stress was modeled by H_2O_2 at different concentrations at the cellular levels. The findings show that at different concentrations of H2O2, TGF-β expression increased at 40 μM. Previous studies have also reported that H_2O_2 induces TGF-β synthesis in hair follicle dermal papilla cells (26).

The effects of oxidative stress on the NF-kB pathway were also investigated. NF-κB is a member of the transcription factors family that are involved in inflammatory responses in various diseases such as atherosclerosis. In response to ROS stimulation, Iκ-NF-κB becomes phosphorylated and then degraded, leading to NF-κB translocation from the cytoplasm into the nucleus, where it can transcriptionally activate the expression of many genes involved in inflammation (27). NF-κB can also induce the expression of genes involved in the process of atherosclerosis such as cell adhesion molecule (28). In this study, it was observed that H_2O_2 can activate the NF-κB transcription factor in BAECs. This finding is similar to a previous study (12). Antioxidants have been demonstrated to slow the development and progression of cardiovascular disease (21, 29). The second part of this study evaluated the antioxidant potency of curcumin in H_2O_2 treated cells. The experiments revealed that curcumin inhibited the oxidant effects of H2O2 through the inhibition of TGF-β expression and NF-κB-p65 phosphorylation in BAECs. At higher concentrations of H_2O_2 (40 and 80 μM H_2O_2), curcumin was able to reduce TGF-β expression. Consistent with these data, other studies have also identified that curcumin can reduce TGF-β expression in the liver of CCl4-treated rats, suggesting that down-regulation of TGF-β by curcumin may be a key mechanism of its anti-fibrogenic effects (30, 31). The stimulatory effect of H_2O_2 on phosphorylation of NF-κB-p65 was abolished by curcumin at 10 μM concentration, but it did not inhibit at 5 μM concentration. In agreement with these data, it has been reported that curcumin inhibits the activity of proteasome and thereby protein degradation inhibitor (Iκβ), resulting in preventing the transfer of NF-κB into the nucleus (32). In another study, Samuhasaneeto and colleagues evaluated the effects of curcumin on ethanol-induced liver injury in rats. Their results show that oxidative stress increases the NF-κB expression and lipid peroxidation, whereas curcumin reversed these effects leading to reducing liver injury in mice (33). In conclusion, these data suggest that curcumin is a potent antioxidant agent to neutralize hydrogen peroxide. H_2O_2 leads to increase the TGF-β expression and NF-κB phosphorylation. The treatment with curcumin reversed these effects. These findings suggest the potential application of curcumin in CVD treatment; however, further animal and human studies are required to confirm the results of this study

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Conflict of Interest

The authors have nothing to declare.

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