Toxicology Letters 190 (2009) 172–178

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Cholesterol-3-beta, 5-alpha, 6-beta-triol induced PI_3K-Akt-eNOS-dependent cyclooxygenase-2 expression in endothelial cells

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ARTICLE INFO

Article history:
Received 5 June 2009
Received in revised form 6 July 2009
Accepted 8 July 2009
Available online 16 July 2009

Keywords:
Atherosclerosis
Cholesterol-3-beta, 5-alpha, 6-beta-triol
Cyclooxygenase-2
Nitric oxide

ABSTRACT

Oxidized cholesterols belong to a subgroup of oxLDLs which play major roles in atherosclerosis. In order to investigate the contribution of oxysterols from oxLDLs in atherosclerosis, cholesterol-3-beta, 5-alpha, 6-beta-triol (α-Triol) was studied in human umbilical vein endothelial cells. We found that α-Triol concentration- and time-dependently enhanced COX-2 protein expression and mRNA production followed by PGE_2 generation in human umbilical vein endothelial cells. In addition, α-Triol upregulated $peNOS^{1177}$ protein phosphorylation and concentration-dependently increased nitric oxide production. $eNOS^{1177}$ phosphorylation was abrogated by the PI3K inhibitor, LY294002. In studying the mechanisms involved in α-Triol-induced COX-2/PGE_2 production, inhibitors of NOS, PI3K, p38, and NF-κB, effectively attenuated COX-2 protein induction and mRNA expression, suggesting that the PI_3K-Akt-eNOS pathway, p38MAPK, and NF-κB are involved in α-Triol-induced COX-2 expression, and following increases in p38 and Akt phosphorylation, the concentration-dependent inhibition of COX-2 protein expression by L-NAME further suggested their involvement at the translation level. We concluded that α-Triol increases COX-2 mRNA and protein expression via coordination with the PI_3K-Akt-eNOS pathway and NF-κB. Moreover, COX-2 gene expression might be regulated by activated p38 MAPK in another unknown regulation pathway. Our findings also suggested that α-Triol might contribute to the effect of induced atherosclerosis in humans through COX-2 production in endothelial cells.

1. Introduction

Oxysterols are oxygenated cholesterol derivatives and constitute a family of compounds with various biological activities (Guardiola et al., 1996). They have been ascribed a number of important roles in connection with atherosclerosis, apoptosis, necrosis, inflammation, immunosuppression, cholesterol turnover, and carcinogenesis (Brown and Jessup, 1999; Schroepfer, 2000; Wang and Afdhal, 2001; Yoon et al., 2004). They are generated by enzymatic mechanisms in cells as well as by nonezymatic mechanisms in various kinds of food during processing or storage for long periods (van de Bovenkamp et al., 1988). Oxysterols accumulate in the subendothelial level of the arterial wall during atherogenic processes (Berliner and Heinecke, 1996) and are believed to play important roles in the development of atherosclerosis (Witztum and Steinberg, 2001). They are also a major component of oxLDLs, which are some of the most notorious atherogenic factors, suggesting that oxysterols might be responsible for the toxicity of oxLDLs (Hubbard et al., 1989; Imai et al., 1980).

COX-2, an essential enzyme involved in inflammatory and other pathogenetic processes (Kuwano et al., 2004), is detectable only in certain types of tissues, and is the inducible form of a biphasic enzyme responsible for catalyzing the conversion of arachidonic acid to PGH_2. PGH_2 is then subsequently catalyzed to other prostanoids, including PGE_2. Prostanoids are potent mediators of inflammatory responses and increase vascular permeability. COX-2 is found in macrophages, vascular endothelial cells, and vascular smooth muscle cells (LaPointe et al., 2004). Since atherosclerosis is a chronic inflammatory condition (Li, 2001), it is possible that COX-2 is involved in the formation of atherosclerotic plaques. Endothelial cells are known to possess both COX isoforms, and their induction has been demonstrated to occur in response to different proinflammatory cytokines, such as interleukin IL-1α and β, and TNF-α (Caughey et al., 2001; Eligini et al., 2001). Therefore, the induction of COX-2 in endothelial cells might result from an inflammatory response.

Nitric oxide (NO) produced in the endothelium was considered as an endothelium-derived relaxing factor (Furchgott and Zawadzki, 1980). Three isoforms of NOS have been identified: two constitutive NOSs, endothelial (e)NOS and neuronal (n)NOS, which are regulated by Ca^{2+}, and one inducible (i)NOS, which is independent of Ca^{2+} regulation (Marletta, 1993). NO is now recognized

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doi:10.1016/j.toxlet.2009.07.012

to play key roles in several inflammatory diseases. Elevated levels of NO have been detected in a variety of pathophysiological processes, including circulatory shock (Szabo, 1995), inflammation (MacMicking et al., 1997), and carcinogenesis (Ohshima and Bartsch, 1994). Several studies suggested an important link between the NOS and COX pathways, although the precise mechanisms underlying such an interaction remain poorly understood (Di Rosa et al., 1996).

Expressions of NOS and COX-2 genes are regulated by NF-κB. NF-κB, one of the most ubiquitous transcription factors, regulates the expressions of genes involved in cellular proliferation, inflammatory responses, cell adhesion, and is activated in response to various extracellular stimuli, including interferon (INF)-γ, lipopolysaccharide (LPS), and oxidative stress. NF-κB sites have been identified in the promoter region of the COX-2 genes (Appleby et al., 1994).

Numerous studies have suggested that inflammation mediates eNOS and COX-2 expressions through the MAPK signaling pathway (Li et al., 2007; Yadav et al., 2003). Three well-defined MAPKs, extracellular signal-regulated kinase (ERK), p38 MAP kinase (p38), and c-Jun NH_2-terminal kinase (JNK), have been implicated in the transcriptional regulation of NOS and COX-2 genes (Chen and Wang, 1999). In addition, several studies have implicated MAPKs in LPS-induced NF-κB activation (Carter et al., 1999).

α-Triol is the most toxic member of the oxysterols (Peng et al., 1985), and has been demonstrated to cause endothelial cell death in vitro (Ramasamy et al., 1992) and in vivo (Peng et al., 1985); however, endothelial cells are particularly vulnerable to its deleterious effects, suggesting that it may participate in atherogenic events. Although several reports suggested the involvement of oxysterols in atherosclerosis, the detailed mechanisms are still not clear. In the present study, we demonstrate that α-Triol increased COX-2 mRNA and protein expressions via coordination with the PI3K-Akt-eNOS pathway, and that NF-κB also plays an important role in transducing the signal.

2. Materials and methods

2.1. Chemicals and antibodies

Cholestane-3β, 5α, 6β-triol (α-Triol), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), L-NAME, SC-791 (a COX-2 inhibitor), aprotinin, leupeptin, sulfanilamide, and trypan blue were obtained from Sigma Chemical (St. Louis, MO, USA). Fetal bovine serum (FBS), M199 medium, collagenase (type I), were obtained from Gibco BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), endothelial cell growth supplement (ECGS) was obtained from Upstate Biotechnology (Lake Plaeid, NY, USA).

SB203580, SP600125, LY294002, and Bay were obtained from Calbiochem (San Diego, CA, USA). The phospho-JNK antibody (Ab), phospho-ERK Ab, phospho-p38 Ab, JNK Ab, ERK Ab, p38 Ab, phospho-Akt Ab, and Akt Ab were obtained from Cell Signaling (Beverly, MA, USA). The β-actin Ab was obtained from Sigma. The COX-2 Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The phospho-eNOS (S1177) Ab was obtained from BD Transduction (Erembodegemm, France). The horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (IgG) Ab was obtained from Amersham Biosciences (Sunnyrale, CA, USA). Prostaglandin E2 EIA kit-monoclonal was obtained from Cayman Chemical (Ann Arbor, MI, USA). A NO quantitative kit was obtained from ACTIVE MOTIF (Carlsbad, CA, USA).

2.2. Cell culture

Human umbilical cords were obtained from National Taiwan University Hospital, Taipei, Taiwan. Human umbilical vein endothelial cells were isolated by collagenase digestion from umbilical cord veins and cultured (Rosenkranz-Weiss et al., 1994). After 15 min of incubation at 37 °C, vein segments were perfused with 30 ml of medium 199 containing 10 U/ml penicillin and 100 μg/ml streptomycin to collect the cells. After centrifugation for 8 min at 900 × *g*, the cell pellet was resuspended in the same medium supplemented with 20% heat-inactivated FBS, 15 μg/ml ECGS, and 90 μg/ml heparin. Human umbilical vein endothelial cells from passages 3 to 6 were used in the present study. To analyze the effects of the inhibitors, cells were preincubated for 30 min with selective inhibitors LY294002 (20 μM); L-NAME (100 μM); Bay (10 μM); SP600125 (20 μM); SB203580 (20 μM), and then stimulated with α-Triol for 24 h. After the appropriate treatment, cells were lysed for the Western blot analysis or processed for PGE_2 and NO measurements by an enzyme immunoassay (EIA) system as described below. The authors have read and approved of the Declaration of Helsinki for medical research involving human material.

2.3. Cell viability determination

Viability of cells was assessed using the MTT assay by measuring mitochondrial dehydrogenase activity (Carmichael et al., 1987). Cells were treated with different concentrations of α-Triol for 22 h. After incubation, 50 μl MTT (0.5 mg/ml) was added and incubated at 37 °C for 2 h, and then the medium was gently removed. Cells and dye crystals were dissolved in 100 μl DMSO, and the absorption was measured at 570 nm in an enzyme-linked immunosorbent assay (ELISA) reader (MRX-TC; Dynex Technology, Chantilly, VA, USA).

2.4. PGE_2 determination

A PGE_2 ELISA kit was used according to the manufacturer's instructions. Briefly, cells were incubated for different concentrations of α-Triol for 24 h. The medium was collected and centrifuged at 2500 rpm for 5 min. The centrifuged medium was added directly to the ELISA plate as directed. Data were analyzed using a standard curve generated by plotting the percentage bound divided by the maximum binding vs. the log of the PGE_2 concentration.

2.5. NO determination

Cells were incubated with different concentrations of α-Triol for 24 h. The medium was collected and centrifuged at 2500 rpm for 5 min, and nitrite in the medium was measured using a NO quantitative kit (Active Motif), which is an enhanced Griess reagent-based method. $NaNO_2$ was used to generate a standard curve, and NO production was determined by measuring the optical density (OD) at 550 nm with a microplate reader (Spectra Max, Molecular Devices, Sunnyvale, CA, USA).

2.6. Western blot analysis

Cells were collected by centrifugation and washed twice with PBS. The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, and 0.5 mM sodium orthovanadate) containing 5 μg/ml each of leupetin and aprotinin and then incubated at 4 °C for 20 min. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The concentration of cell lysates was determined using a Bio-Rad protein assay kit (Bio-Red, Richmond, CA, USA) according to the manufacturer's instructions. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% polyacrylamide gels and then transferred onto polyvinylidene difluoride (PVDF) membranes (PerkinElmer Life Sciences, Boston, MA, USA) by the Panther™ Semidry Electroblotter (Owl Scientific, Portsmouth, NH, USA). The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4 °C, and then incubated with primary antibodies that recognize COX-2 (BD Transduction), β-actin (Sigma), peNOS^{1177}, pAkt, and MAPKs (ERK, JNK, p38, pERK, pJNK, and pp38) (Santa Cruz). After washing with TBST, HRP-conjugated secondary antibodies (Amersham, Piscataway, NJ, USA) (1:5000 dilution in TBST) were applied, and blots were developed on an enhanced chemiluminescence (ECL) detection system (PerkinElmer). In the concentration-dependent experiments, human umbilical vein endothelial cells were stimulated with 0.1–10 μM α-Triol for 24 h.

2.7. RNA extraction and RT-PCR

Total cellular RNA from treated cells was isolated using an NE-PERTM kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. From each sample, 3 μg of RNA was reverse-transcribed (RT) for 1 h at 37 °C in a reaction mixture containing 5 U RNase inhibitor (Invitrogen), 0.5 mM dNTP (Boeringer Mannheim, Indianapolis, IN, USA), 2 mM hexamer (Promega, Madison, WI, USA), 1× RT buffer, and 5 U reverse transcriptase. A PCR analysis was then performed on aliquots of the complementary (c)DNA preparations to detect COX-2 and β-actin gene expressions using a PTC-150 Minicycler MJ (MJ Research, Watertown, MA, USA). The reaction was carried out in a volume of 25 mL containing 1 U of Taq DNA polymerase, 0.2 mM dNTP, 10× reaction buffer, and 0.2 μmol each of the 5′- and 3′-primers. After initial denaturation for 5 min at 95 °C, 30 amplification cycles were performed consisting of 1 min of denaturation at 95 °C, 1 min of annealing at 56 (for COX-2) or 53 °C (for β-actin), and a 2-min extension at 72 °C. The PCR primers used in this study were as follows: two specific probes, COX-2 (5′-AAG-CCT-TCT-CTA-ACC-TCT-CC/3′-TAA-GCA-CAT-CGC-ATA-CTC-TG) and β-actin (5′-TCA-TGA-GGT-AGT-CAG-TCA-GG/3′-TGA-CCC-AGA-TCA-TGT-TTG-AG), were used, and the PCR products were separated by 2% agarose gel electrophoresis with ethidium bromide (EtBr) staining. The conditions of reverse transcription were based on protocols provided by the manufacturer. Analysis of the resulting PCR products on 1% agarose gels showed single-band amplification products with the expected sizes.

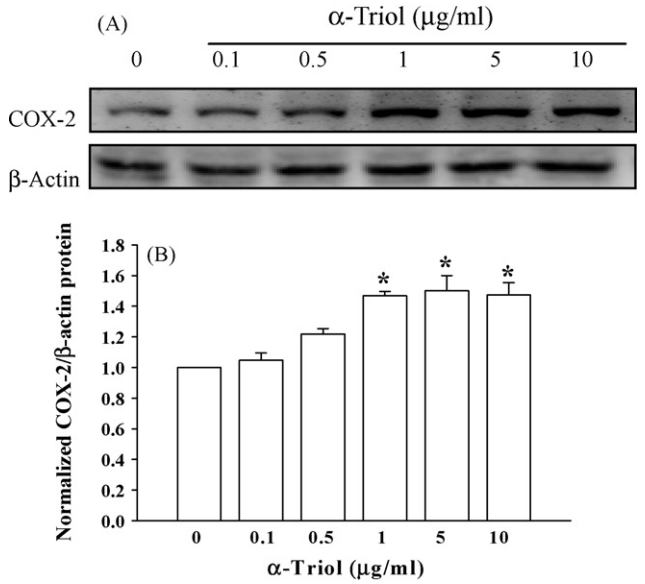

Fig. 1. α-Triol induced COX-2 protein expression in human umbilical vein endothelial cells. (A) Cells were treated with various concentrations of α-Triol for 24 h and cell lysate proteins (50 μg/lane) were separated by 10% SDS-PAGE, and immunoblotted with a mouse anti-COX-2 antibody. β-Actin was used as the internal control. Each blot is representative of at least three separate experiments. (B) Densitometric analysis of COX-2 protein induction in human umbilical vein endothelial cells. Data are expressed as the mean ± S.E. $^*p < 0.05$, compared to the control by Student's *t*-test ($n > 3$).

2.8. Statistical analysis

Results are expressed as the mean ± SE of at least three experiments performed using different *in vitro* cell preparations. Statistically significant values were compared using Student's *t*-test followed by Dunn's test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Cell viability induced by α-Triol in human umbilical vein endothelial cells

When cells were incubated with various concentrations (0.05–20 μg/ml) of α-Triol for 24 h, we found that the mitochondrial activity decreased starting with 20 μg/ml (data not shown). α-Triol had no effect on the viability of human umbilical vein endothelial cells in a concentrations range of 0–10 μg/ml. Also, the morphology did not change after 24 h of treatment with α-Triol at up to 10 μg/ml. Our data indicated that at concentrations of <10 μg/ml for human umbilical vein endothelial cells, α-Triol did not exert a toxic effect, and so these concentrations were used for the following experiments.

3.2. α-Triol induced COX-2 expression in human umbilical vein endothelial cells

The expression of COX-2 protein was low or undetectable in non-stimulated cells. Treatment with α-Triol significantly enhanced the expression of COX-2 protein in human umbilical vein endothelial cells (Fig. 1A). Densitometric analysis showed that α-Triol induced a concentration-dependent increase in COX-2 expression in human umbilical vein endothelial cells (1–10 μg/ml; $p < 0.05$) (Fig. 1B). The maximum increase was 1.5-fold at 5 μg/ml α-Triol treatment.

3.3. α-Triol concentration-dependently induced mRNA transcriptional activation in human umbilical vein endothelial cells

The level of COX-2 mRNA was determined by RT-PCR using a specific COX-2 primer, and β-actin was used as an internal control in these experiments. α-Triol (1 μg/ml) significantly enhanced the expression of COX-2 mRNA in human umbilical vein endothelial cells (Fig. 2A). Densitometric analysis showed that α-Triol (1 μg/ml) induced a time-dependent increase in COX-2 mRNA expression (1–6 h; $p < 0.05$) (Fig. 2B), and the maximum increases were 1.52 ± 0.09-fold at 4 h in human umbilical vein endothelial cells.

3.4. α-Triol caused PGE_2 production in human umbilical vein endothelial cells, which was blocked by a COX-2 inhibitor

Corresponding to the effect of α-Triol on COX-2 protein expression, the ELISA results from cell culture supernatants demonstrated that α-Triol stimulation for 24 h significantly increased PGE_2 production in human umbilical vein endothelial cells (1–10 μg/ml) (Fig. 3A). The maximum increase was 199.24 ± 6.14 pg/ml at 10 μg/ml α-Triol in the cells. TNF-α (10–30 ng/ml) was taken as positive control. Co-treatment with TNF-α and α-Triol did not cause a synergistic effect on PGE_2 release (Fig. 3A). Pretreatment with a highly selective COX-2 inhibitor (SC-791) concentration-dependently abrogated α-Triol-induced PGE_2 production in human umbilical vein endothelial cells as shown in Fig. 3B, confirming that α-Triol-induced PGE_2 production occurs through a COX-2-dependent pathway in this study.

3.5. α-Triol regulated peNOS protein levels in human umbilical vein endothelial cells

The effects of α-Triol on endothelial NO production were investigated in human umbilical vein endothelial cells. $eNOS^{1177}$ serine phosphorylation was induced in a concentration-dependent manner by α-Triol (0.5–10 μg/ml) in human umbilical vein endothelial

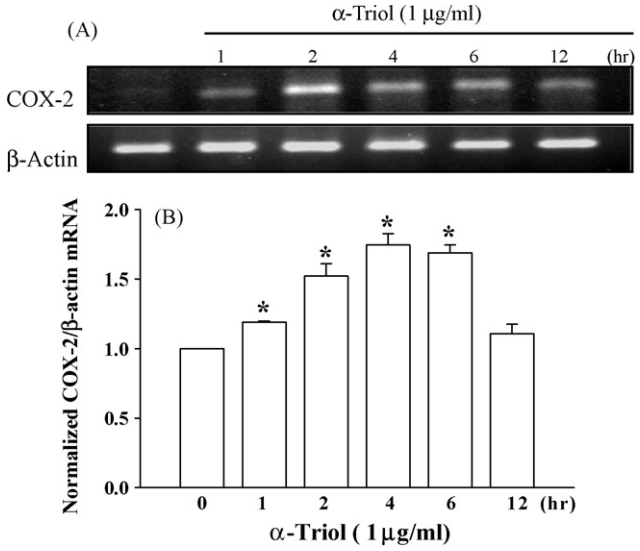

Fig. 2. Effect of α-Triol-induced COX-2 mRNA expression in human umbilical vein endothelial cells. (A) Cells were treated for different times with 1 μg/mL α-Triol and then lysed in RNA extraction buffer. Total RNA then underwent RT-PCR analysis. β-Actin was used as the internal control. Each graph is representative of at least three separate experiments. (B) Densitometric analysis of COX-2 mRNA induction of human umbilical vein endothelial cells. Data are expressed as the mean ± S.E. $^*p < 0.05$, compared to the control by Student's *t*-test ($n > 3$).

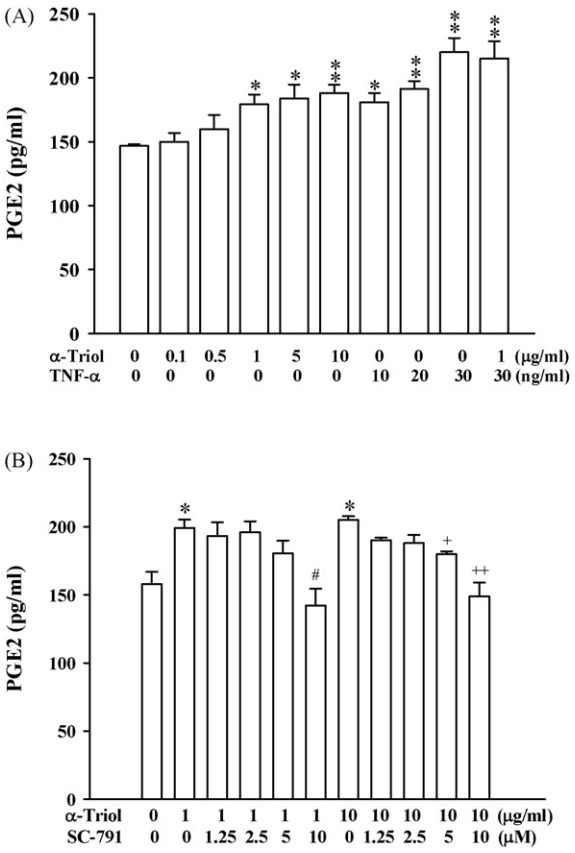

Fig. 3. Effect of α-Triol-induced PGE_2 production in human umbilical vein endothelial cells. Amounts of PGE_2 were measured using a PGE_2 enzyme immunoassay kit (Cayman). Results showed that α-Triol significantly increased PGE_2 synthesis. (A) Cells were treated with various concentrations of α-Triol for 24 h as indicated. Tumor necrosis factor (TNF)-α (10–30 ng/ml) was used as the positive control. (B) COX-2 inhibitor (SC-791) inhibited α-Triol-induced PGE_2 production in human umbilical vein endothelial cells. Data are expressed as the mean ± S.E. from four independent experiments. Ethanol was used as the solvent control. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. the control. $^{\#}p < 0.05$ vs. Triol (1 μg/ml). $^{+}p < 0.05$; $^{++}p < 0.01$ vs. Triol (10 μg/ml).

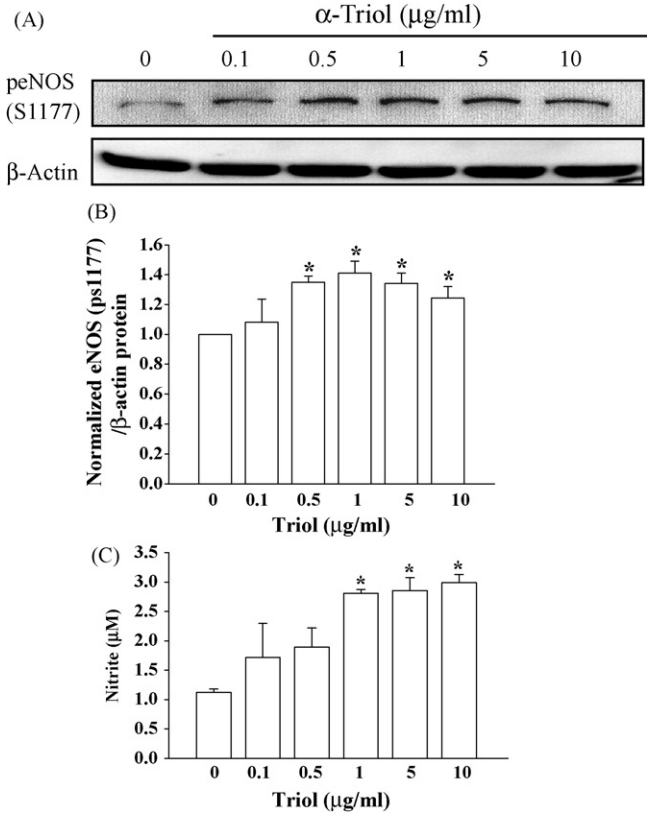

Fig. 4. α-Triol regulated endothelial nitric oxide synthase serine 1177 (peNOS)^{1177} protein phosphorylation in human umbilical vein endothelial cells. (A) Western blot analysis showed the concentration-dependent effects of α-Triol on peNOS^{1177} protein phosphorylation in human umbilical vein endothelial cells. Cells were treated with various concentrations of α-Triol for 24 h and immunoblotted with a specific antibody against peNOS (pS1177). β-Actin was used as the internal control. Each blot is representative of at least three separate experiments. (B) Densitometric analysis of peNOS (pS1177) induction in the cells (C). α-Triol concentration-dependently increased nitric oxide production in human umbilical vein endothelial cells. Data are expressed as the mean ± S.E.M. from three independent experiments. $^{*}p < 0.05$, compared to the control by Student's *t*-test ($n > 3$). Ethanol was used as the solvent control.

cells (Fig. 4A). Densitometric analyses of peNOS (pS1177) induction in the cells are shown in Fig. 4B. Corresponding to the effect of α-Triol on eNOS^{1177} protein phosphorylation, the ELISA results from cell culture supernatant demonstrated that α-Triol significantly increased NO production in human umbilical vein endothelial cells (1–10 μg/ml) (Fig. 4C). The maximum increase was 2.99 ± 0.13 μM at 10 μg/ml α-Triol in the cells. These results suggest that α-Triol can promote eNOS activation through phosphorylation of serine 1177 and NO formation in human endothelial cells.

3.6. *MAPK and Akt phosphorylation were induced in human umbilical vein endothelial cells by α-Triol*

Activation of JNK, p38 MAPK, and Akt phosphorylation was assessed by an immunoblot analysis. Incubation of human umbilical vein endothelial cells with 1 μg/ml α-Triol resulted in the phosphorylation of p38 MAPK and Akt in time-dependent manners (Fig. 5). The activation of p38 MAPK peaked at 120 min after α-Triol addition and could still be clearly detected after 240 min (data not shown), while Akt was maximally phosphorylated 30 min after α-Triol addition, with activation lasting for up to 45 min and then slowly decreasing.

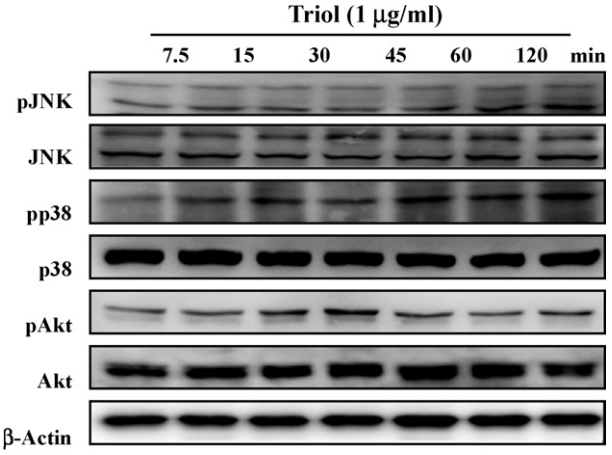

Fig. 5. α-Triol activates Akt and both p38 and JNK MAPK phosphorylation in human umbilical vein endothelial cells. Cells were treated with α-Triol (1 μg/ml) and immunoblotted by using six specific antibodies against p-JNK, p-p38 and p-Akt; JNK, p38 and Akt. Each blot is representative of at least three separate experiments.

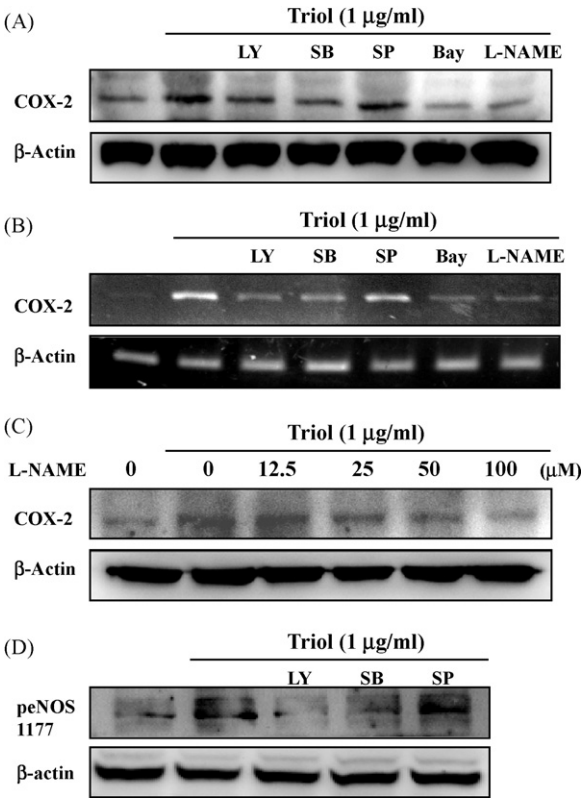

Fig. 6. Effects of kinase inhibitors on Triol-induced COX-2 expression and eNOS^{1177} phosphorylation in human umbilical vein endothelial cells. (A) Cells were treated with α-Triol (1 μg/ml) for 24 h, before pretreated with various concentrations of the selective inhibitors (20 μM LY-294002, 20 μM SB203580, 20 μM SP600125, 10 μM Bay, and 100 μM L-NAME) for 30 min and immunoblotted with a mouse anti-COX-2 antibody. (B) RT-PCR analysis showed that the selective pharmacologic inhibitors effectively attenuated COX-2 mRNA in the cells. (C) Western blot analysis suggested that L-NAME could concentration-dependently reduce Triol-induced COX-2 protein in the cells. (D) Western blots analysis showing the LY294002 effectively attenuated eNOS^{1177} phosphorylation in the cells. β-Actin was used as the internal control. Data are expressed as the mean ± S.E. from three independent experiments.

3.7. p38MAPK, eNOS, AKT, and NF-κB inhibitors abrogate α-Triol-induced COX-2 protein and mRNA expressions in human umbilical vein endothelial cells

To further investigate the specificity of α-Triol-induced kinase activation, we used selective inhibitors of these kinases, including SB202190 (a p38 inhibitor), SP600125 (a JNK inhibitor), and LY (a PI3K inhibitor). When human umbilical vein endothelial cells were treated with SB, SP, and LY, the α-Triol-induced increases in COX-2 protein and mRNA induction were consistently inhibited by SB and LY but not SP (Fig. 6A and B). In addition, Bay (an NF-κB inhibitor) and L-NAME (NOS inhibitor) also significantly inhibited both COX-2 protein and mRNA induction (Fig. 6A–C). Results indicated that α-Triol induced COX-2 expression through upregulating the PI3K-Akt-eNOS and p38MAPK pathways, NF-κB also plays an important role in transcription level.

3.8. The PI3K kinase inhibitors abrogate the α-Triol-induced eNOS expression in human umbilical vein endothelial cells

To further investigate the involvement of PI3K-Akt-eNOS and MAPKs in eNOS^{1177} phosphorylation, pJNK, p38, and PI3K inhibitors were used to test their effects on the pathway in human umbilical vein endothelial cells. We found that only LY showed significant inhibition of α-Triol-induced peNOS^{1177} in the cells, but not SP and SB (Fig. 6D). These data indicate that JNK and p38 did not involve in α-Triol-induced COX-2 and eNOS^{1177} phosphorylation.

4. Discussion

Oxysterols are cholesterol oxidation products which exist in ox-LDLs. The concept that oxLDLs play an important role in the etiology of atherosclerosis is a well-established hypothesis. Recent research in this area has focused on the potential of oxidized lipid products to initiate specific signal transduction pathways in cells, which are relevant to the development of atherosclerosis, including macrophages, smooth muscle cells, and endothelial cells (Chisolm and Chai, 2000). At present, the component of oxLDL molecules which mediates the activation of atherosclerosis is not known. Many reports have proposed the possibility that oxysterols mediate the early events in atherosclerosis induced by oxLDLs, such as the production of various proinflammatory cytokines (Lemaire et al., 1998), the expression of adhesion molecules, and the cytotoxicity towards vascular smooth muscle cells, endothelial cells, macrophage/monocytes, and fibroblasts (Lizard et al., 1997). 7β-Hydroxycholesterol and 7-ketocholesterol are the most investigated oxysterols among the 60 oxysterols identified (Lizard et al., 1996), and the induction towards apoptosis is characterized by well-know events (Ares et al., 2000; Miguet et al., 2001).

α-Triol is the most toxic member of the oxysterols (Peng et al., 1985), and has been found to cause endothelial cell death in vitro (Ramasamy et al., 1992). In the present study, we found that α-Triol concentration-dependently induced COX-2 mRNA and protein expressions and PGE_2 production in human umbilical vein endothelial cells. COX-2 induction in human umbilical vein endothelial cells has been demonstrated to occur in response to different inflammatory cytokines, such as IL-1α and β or TNF-α (Caughey et al., 2001; Eligini et al., 2001). The expression of COX-2 has been suggested to play an important role in inflammation and facilitates the formation of atherosclerosis (Lim et al., 2008). Our data suggest that oxysterol might also act as an atherosclerotic factor through induction of COX-2 in endothelial cells.

Oxysterol-induced COX-2 protein expression was first reported by Yoon et al. (2004) in human cholangiocarcinoma cell lines, which showed that 22(R)-hydroxycholesterol induced COX-2 expression through stabilizing COX-2 mRNA via a p38 MAPK-dependent mechanism, and this enhanced COX-2 protein expression by oxysterol which may participate in the genesis and progression of a cholangiocarcinoma (Yoon et al., 2004). In this study, we also showed that α-Triol activated p38 MAPKs; however, we found COX-2 protein and mRNA expression were inhibited by the p38 inhibitor, SB202190. These data suggest that p38 also plays an important role in α-Triol's induction of COX-2 expression in endothelial cells. It is also known that transcriptional regulation of COX-2 gene expression can occur via MAPK activation (Guan et al., 1998). Exposure to oxLDL has been reported to activate both ERK and JNK through independent signal transduction pathways (Go et al., 2001), suggesting this involvement of MAPK in α-Triol induced COX-2 induction.

The COX-2 promoter is subjected to a tight regulatory network involving nuclear factors (NF-κB), which can be activated by complex kinase pathways centered around p38 and ERK1/2 MAPK (Chun and Surh, 2004; N'Guessan et al., 2006, 2007b). NF-κB mediates multiple aspects of a host's response to bacterial infection (N'Guessan et al., 2007a; Schmeck et al., 2007, 2004) and activation of transcription factor NF-κB is considered to significantly contribute to COX-2 expression and PGE_2 liberation (N'Guessan et al., 2006, 2007b). Our data showed that α-Triol-induced COX-2 mRNA expression was significantly inhibited by pretreatment with Bay (an NF-κB inhibitor) and SB (a p38 MAPK inhibitor), further suggesting that both p38 MAPK and NF-κB involved in α-Triol-induced COX-2 expression at the transcriptional level.

A key role of eNOS activation through a phosphatidylinositol-3-kinase-dependent mechanism leading to phosphorylation of eNOS was demonstrated with ox-LDL-dependent activation of JNK (Go et al., 2001). Previous studies showed that phosphorylation of eNOS is PI3K kinase dependent, and that Akt may be the upstream kinase which directly phosphorylates Ser^{1179} in response to shear stress (Dimmeler et al., 1999). We report here for the first time that oxysterol might have a similar effect on eNOS. α-Triol concentration-dependently induced Akt and $eNOS^{1177}$ protein phosphorylation and enhancement of NO production. In addition to p38 MAPK, we found that pretreatment with the PI3K inhibitor, LY294002, inhibited eNOS phosphorylation, COX-2 protein induction and mRNA expression. We also found that α-Triol not only induced the phosphorylation of eNOS at $serine^{1177}$ but also enhanced the production of NO. NO then induced the expression of COX-2 through an unknown pathway, which is supported by both COX-2 mRNA and protein expression being inhibited by prior treatment with L-NAME, However, we do not know, at this moment, the exact mechanisms by which α-Triol induces PI3K activation. Interestingly, we also found that although inhibiting p38 activation can inhibit COX-2 expression, it might not be involved in PI3K pathway activation or eNOS phosphorylation. Lim et al. (2008) suggested the tumor initiation and maintenance can be inhibited by blocking phosphorylation of the Akt substrate, eNOS1177. Whether NOS phosphorylation induced by α-Triol contributes to the carcinogenesis process awaits more investigation. Our previous study indicated α-Triol exerted slightly mutagenic effect on bacterial reversion assay and chromosome aberration test by induced ROS production (Cheng et al., 2005) further supported this possibility.

It has been reported that ox-LDL induced COX-2 expression in monocytes/macrophages (Pontsler et al., 2002; Taketa et al., 2008). However, the mechanisms of ox-LDL-induced COX-2 expression are not clearly understood. Previously, Yoon et al. (2004) showed that the oxysterol, 22(R)-hydroxycholesterol, induced COX-2 expression in human cholangiocarcinoma cells, and we showed that another oxysterol, α-Triol, also enhanced the expression of COX-2 mRNA and protein. Whether these oxysterols play crucial roles in the effect seen with ox-LDL remains to be further investigated.

In conclusion, we found that α-Triol stimulated COX-2 gene expression leading to PGE_2 synthesis through activation PI3K-Akt-eNOS pathway in endothelial cells. Moreover, COX-2 gene expression might be regulated by activated p38 MAPK in another unknown regulation pathway. Thus, understanding the mechanisms underlying oxysterol-induced COX-2 expression and production of PGE_2 and NO would help in exploring the mechanisms of the atherosclerosis related vascular disease.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was supported by the grants NSC95-2320-B-038-030-MY2 from National Science Council, Taiwan.

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