JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2011, 62, 1, 21-28
www.jpp.krakow.pl

Original articles

X. TAN[1], E.M. POULOSE[1], V.V. RAVEENDRAN[1], B.-T. ZHU[2], D.J. STECHSCHULTE[1], K.N. DILEEPAN[1]

REGULATION OF THE EXPRESSION OF CYCLOOXYGENASES AND PRODUCTION OF PROSTAGLANDIN I_2 AND E_2 IN HUMAN CORONARY ARTERY ENDOTHELIAL CELLS BY CURCUMIN

[1]Division of Allergy, Clinical Immunology and Rheumatology, Department of Medicine, University of Kansas Medical Center, Kansas City, USA; [2]Department of Pharmacology and Toxicology, University of Kansas Medical Center, Kansas City, USA

Curcumin regulates prostaglandin (PG) synthesis in a variety of cells. PGE_2 and PGI_2 are generated from arachidonic acid (AA) by cyclooxygenases 1 and 2 (COX-1 and COX-2) and the synthase (PGES and PGI_2S) pathways. This study evaluates the *in vitro* effect of curcumin on the expression of COX-1, COX-2, PGI_2S and microsomal PGES-1 (mPGES-1), and the production of PGE_2 and PGI_2 in human coronary artery endothelial cells (HCAEC). HCAEC monolayers were incubated with curcumin and the expression of mRNA, protein and the production of PGI_2 and PGE_2 were quantified. Incubation of HCAEC with curcumin led to a time and concentration-dependent increases in COX-2 mRNA with a small but significant decrease in COX-1 mRNA expression. Curcumin also stimulated the expression of PGI_2S and mPGES-1 mRNA. Although curcumin stimulated COX-2, PGI_2S and mPGES-1 gene expression, it failed to increase PGI_2 or PGE_2 production. Interestingly, supplementation of the culture medium with AA increased prostanoid production by both quiescent and curcumin-treated cells. However, in comparison to the quiescent cells, the prostanoid production by curcumin-treated cells was markedly enhanced as AA concentrations in the medium were increased, and the enhanced prostanoid production was blocked by the presence of COX-2 specific inhibitor. Taken together, these results suggest that curcumin regulates prostanoid homeostasis in HCAEC by modulating multiple steps including the expression of COX-1, COX-2, PGI_2S and mPGES-1.

Key words: *curcumin, cyclooxygenase, arachidonic acid, endothelial cells, microsomal PGE synthase-1, prostaglandins, prostaglandin I_2 synthase*

INTRODUCTION

Curcumin is a hydrophobic polyphenol present in the 'yellow curry spice' turmeric (*Curcuma longa*). For centuries, turmeric has been used as a popular spice, coloring agent and preservative in food preparations in Southeast Asia. Furthermore, turmeric has been extensively used in traditional Chinese and Asian Indian therapeutics to treat infection, inflammatory diseases, skin lesions, wounds, arthritis, liver disease, cancer, and gastrointestinal disorders (1). In animal models, curcumin has been shown to exert potent antioxidant, anti-inflammatory and anti-tumor properties. Some of the anti-inflammatory effects of curcumin have been attributed to its ability to decrease the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin in endothelial cells (2), and inhibit the production of interlukin-1β (IL-1β), IL-6, IL-8, and TNF-α in many cell types (3). Several *in vitro* studies have shown that curcumin has the ability to inhibit the expression of cyclooxygenase 2 (COX-2) and production of prostanoids in human cells. These include breast epithelial cells (4), colon cancer colo205 cells (5), bladder cancer T24 cells (6), lung epithelial cells (7) and HaCaT cells (8) as well as in human intestinal microvascular endothelial cells (9). Curcumin was also noted to inhibit lipopolysarccharide (LPS) induced COX-2 expression in the mouse macrophage cell line, Raw 264.7 (10). The fact that curcumin is able to induce pharmacological effects in different cell types with a wide array of therapeutic effects and be active in animal models, indicates that this herbal product is biologically active and of potential therapeutic interest.

The COX pathways play a critical role in the normal maintenance of cellular functions as well as in the pathobiology of inflammation. COX-1 and COX-2 belong to a family of enzymes that catalyze the oxygenation of AA to prostaglandin G_2 (PGG_2) and PGH_2. PGH_2 is utilized by specific prostaglandin synthases to generate PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TXA_2) (11, 12), which then act through specific receptors to initiate physiological responses. COX-1 is expressed constitutively in most tissues and mediates 'housekeeping' functions, whereas COX-2 is expressed acutely at sites of inflammation (13, 14). Interest is now focused on the role of COX-2 and prostanoid homeostasis because of their involvement in normal cardiovascular function and in the pathogenesis of atherosclerosis (14-16). Prostaglandin I_2 (prostacyclin, PGI_2) and PGE_2 are potent modulators of inflammation. Increasing evidence indicates that inflammation plays a major role in the progression of atherosclerosis. PGI_2 is a potent vasodilator and an inhibitor of leukocyte adhesion and

platelet aggregation (17). Hence, PGI_2 is thought to play a protective role in atherothrombosis. COX-2 contributes significantly to systemic PGI_2 synthesis in humans (18, 19), and COX-1 also contributes to vascular PGI_2 synthesis (15, 20). Recently, curcumin was shown to decrease progression of atherosclerosis in the apoE/LDL receptor knockout mouse model without altering serum lipid profiles (21). Since atherosclerosis is considered to be an inflammatory disease, and COX-2 expression in the endothelium is likely to contribute to prostanoid homeostasis, we evaluated the direct effect of curcumin on the expression of COX-1 and COX-2 in human coronary artery endothelial cells. This report demonstrates that curcumin stimulates the expression of COX-2 mRNA and protein in human coronary artery endothelial cells (HCAEC), with a small, but significant decrease in COX-1 mRNA expression. Curcumin also increased the expression of PGI_2 and PGE_2 synthase mRNA. Although curcumin-stimulated HCAEC did not generate higher amounts of PGI_2 or PGE_2 despite the increased expression of COX-2, PGI_2S and mPGES-1, they generated markedly higher amounts of prostanoids than quiescent cells when adequate amounts of AA were provided exogenously.

MATERIALS AND METHODS

Materials

HCAEC, endothelial cell growth medium (EGM-2 MV), trypsin-EDTA, and trypsin neutralizing solution were purchased from Lonza. Enzyme immunoassay (EIA) kits for 6-keto $PGF_{1\alpha}$ and PGE_2, anti-COX-2 and goat anti-rabbit IgG HRP antibodies, as well as selective inhibitors of COX-2 (NS-398) were obtained from Cayman Chemical Co. Anti-actin, rabbit anti-goat IgG antibodies, and ECL chemiluminescence reagent were purchased from Santa Cruz Biotechnology. Curumin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent were purchased from Sigma-Aldrich. RNeasy mini kit was purchased from Qiagen. High-capacity cDNA Achieve kit, SYBR Green PCR Master Mix kit, and all the gene-specific primers were purchased from Applied Biosystems. The cell lysis solution, radioimmunoprecipitation assay (RIPA) buffer, is a product of Pierce Biotechnology.

Culture of human coronary artery endothelial cells (HCAEC)

HCAEC were grown in EGM-2 MV containing 1 µg/ml hydrocortisone acetate, 50 ng/ml gentamycin, and the recommended concentrations of human epidermal growth factor, vascular endothelial growth factor, human fibroblast growth factor-B, recombinant insulin-like growth factor-1, ascorbic acid and 5% fetal bovine serum. The cells used in all experiments were between three and six passages.

Cell viability assays

HCAEC (1.5×10^5) were plated on to each of the wells of a 12-well cell culture plate and allowed to adhere for 16 to 18 hours. Following adherence, selected concentrations of curcumin or medium (containing 0.1% DMSO as vehicle) were added and incubated for 24 hours in a final volume of 1.0 ml at 37°C in 5% humidified CO_2. After incubation, cells were subjected to either microscopic phase contrast imaging or MTT assay. For MTT assay, the cells were washed with RPMI-1640 (without phenol red) twice and then incubated with MTT working solution for 3 hours. At the end of incubation period, the incubation solution was removed, and 0.5 ml of 2-isopropanol was added to the wells. Absorbance of the converted dye was measured at a wavelength of 570 nm with background substraction at 650 nm.

Real-time RT-PCR analyses of cyclooxygenase-1, cyclooxygenase-2, prostaglandin I_2 synthase and microsomal PGE synthase-1

After incubating HCAEC monolayers with medium (containing 0.1% DMSO as vehicle) or indicated concentration of curcumin for the indicated time periods, supernatant was removed from the cell culture dish, total RNA isolated using RNeasy mini kit, and real-time RT-PCR was carried out as described previously (22). Briefly, total RNA was reverse transcribed into first-strand cDNA using high-capacity cDNA Achieve kit; real-time RT-PCR was performed on ABI 7500 real-time PCR system (Applied Biosystems) equipped with a 96-well optical reaction plate. The amplification reactions were performed in 25 µl total volume containing SYBR Green PCR Master Mix, gene specific primers and cDNA of each sample. The sequences of primers used in the real-time PCR analyses of various genes are as follows: COX-1, forward, 5'-GCCAGTGAATCCCTGTTGTTACT-3' and reverse, 5'-GGCCGAAGCGGACACA-3'; COX-2, forward, 5'-AGGGTTGCTGGTGGTAGGAA-3' and reverse, 5'-GGTCAATGGAAGCCTGTGATACT-3'; β-actin, forward, 5'-CCAGCTCACCATGGATGATG-3' and reverse, 5'-ATGCCGGAGCCGTTGTC-3'; PGI_2 synthase, forward, 5'-GCCACATAGCTCATAAGCTGTAGAAC-3' and reverse, 5'-AGTTGCTCATCCAGCATTTGC-3'; mPGES-1, forward, 5'-CCTGGGCTTCGTCTACTCCTT-3' and reverse, 5'-AGTGCATCCAGGCGACAAA-3'. All real-time RT-PCR experiments were run in triplicate, and mean values were used for the determination of mRNA levels. Messenger RNA levels of tested genes were normalized to the corresponding amount of β-actin mRNA levels.

Western blot analysis

Confluent HCAEC monolayers were incubated with medium (containing 0.1% DMSO as vehicle) or indicated concentrations of curcumin for 8 hours at 37°C in a 5% CO_2 incubator. The cells were washed twice with ice-cold PBS and then solubilized in RIPA buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS, supplied with 1% (v/v) protease inhibitor mixture at 4°C. Cell debris was removed by centrifugation of the lysate at 13,000 x *g* for 15 min at 4°C. Aliquots of supernatants were mixed with equal volumes of 2x SDS sample buffer and heated to 100°C for 5 min. The above samples were fractionated by 10% SDS-PAGE and then electrophoretically transferred to a PVDF membrane. After blocking in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk overnight, membranes were washed thrice in TBST and incubated with primary antibody for 1 h in blocking solution at room temperature. After washing thrice, membranes were incubated with HRP-conjugated secondary antibodies for 1 hour and washed three times, and the signals were detected using ECL reagents.

Enzyme immunoassay (EIA) determinations of the production of prostaglandin I_2 and prostaglandin E_2

HCAEC (1.5×10^5) were plated on to each of the wells of a 12-well cell culture plate and allowed to adhere for 16 to 18 hours. Following adherence, selected concentrations of curcumin or medium (containing 0.1% DMSO as vehicle) were

added and incubated for the indicated time periods in a final volume of 1.0 ml at 37°C in 5% humidified CO_2. After the indicated incubation period, culture supernatants were harvested, appropriately diluted, and assayed for PGE_2 and 6-keto $PGF_{1\alpha}$ (PGI_2) levels by EIA.

Statistical analysis

All analyses were performed using SAS version 9.1. To test for homogeneity among treatment groups, Levene's test was applied. A one-factor analysis of variance was used for overall comparisons among treatment groups. Follow-up post-hoc analyses were performed using the Student-Newman-Keuls procedure. To test for a synergy effect for the combined use of two agents, a cell-means model approach to the synergy hypothesis was used, requiring the use of the IML procedure in SAS to derive test statistics from the hypothesis and model matrices. Results are reported as mean ±S E M. For all analyses the comparison was considered significant if $p < 0.05$.

RESULTS

Effect of curcumin on the viability of human coronary artery endothelial cells (HCAEC)

Curcumin has been reported to be toxic to certain cell types at concentrations higher than 10 μM (23). Therefore, to determine the optimal concentration of curcumin to be used in our studies, HCAEC were incubated with selected concentrations of curcumin or medium for 24 hours. After incubation, cells were examined for their viability by phase contrast microscopy and MTT assay. As shown in *Fig. 1*, curcumin concentration up to 5 μM did not affect cell viability. However, higher than 10 μM concentrations, curcumin led to morphological changes (A) and significantly decreased MTT reduction (B). In view of the relatively low levels of curcumin in the systemic circulation (0.4-1.77 μM) in individuals taking up to 8 g of curcumin per day (24) and concentrations higher than 5 μM were found to be toxic to HCAEC *in vitro,* concentrations greater than 5 μM were not tested in most of the subsequent studies.

Curcumin stimulates cyclooxygenase-2 expression

To examine whether curcumin regulates the expression COX-2 expression in HCAEC, the time course and concentration dependency of the effect of curcumin on the mRNA expression was monitored using real-time RT-PCR. Curcumin (5 μM) was used to evaluate the time course of COX-2 mRNA expression in HCAEC after incubating for 1, 2, 4, 8, and 24 hours. As shown in *Fig. 2A*, incubation of HCAEC with curcumin led to a 5-fold increase in COX-2 mRNA expression within the first hour of treatment, and a 9-fold increase by 2 hours. The curcumin-induced COX-2 mRNA expression remained steady up to 4 hours, decreased progressively with time and reached the basal level by 24 hours. Curcumin-stimulated COX-2 expression was concentration-dependent between 1.25 μM and 5 μM when HCAEC were treated for 4 hours, and a significant increase in the mRNA expression was evident even at 1.25 μM concentration (*Fig. 2B*). However, when the curcumin concentration was increased to 10 μM, a decline in the level of induction of COX-2 mRNA expression was noted.

To determine whether curcumin-stimulated COX-2 mRNA expression is associated with increased expression of protein, Western blot analyses were carried out after incubating HCAEC for 8 hours with 1.25 μM to 20 μM curcumin. As shown in *Fig. 2C*, only low-level expression of COX-2 protein was noted in quiescent HCAEC (medium-treated cells). In consistent with the curcumin-induced COX-2 gene expression profile, a concentration-dependent increase in COX-2 protein expression was noted when HCAEC were incubated with 1.25 μM to 5 μM curcumin. However, when the concentrations were increased to

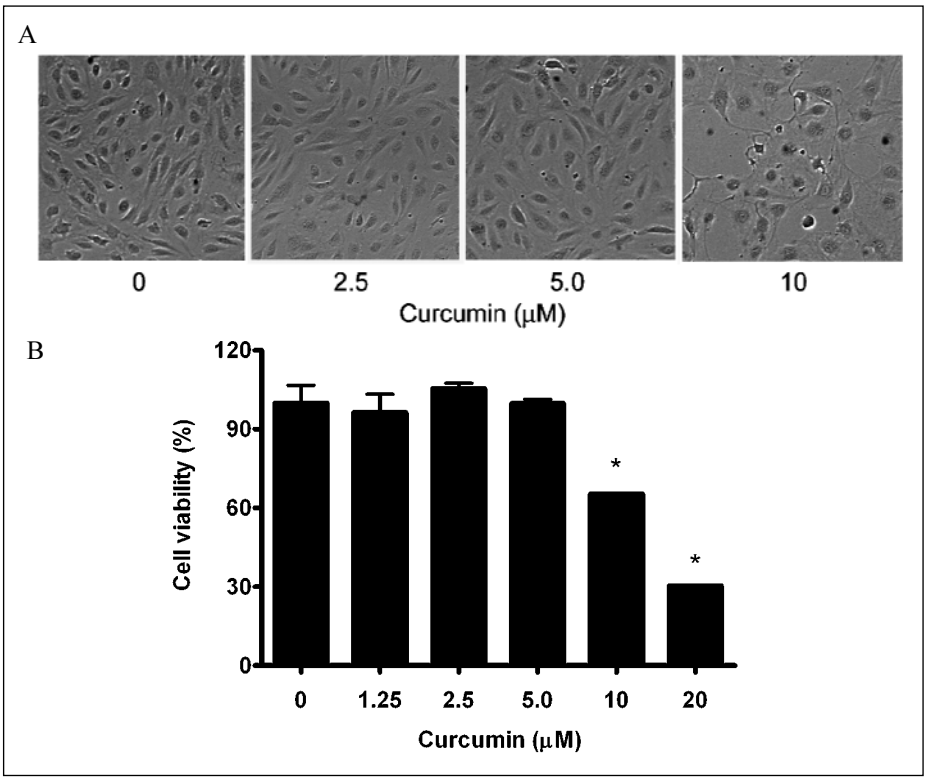

Fig. 1. Effect of curcumin on the viability of HCAEC. HCAEC monolayers were incubated with curcumin (0-20 μM) for 24 hours. After incubation, cell viability was determined by morphological evaluation under phase contrast microscopy (A) or by MTT assay (B). The results presented are representative of two experiments with similar results. Each value presented for MTT assay is the mean ±S.E.M. of triplicate determinations. *, indicates $p<0.05$ when compared with medium-treated cells.

10 μM and 20 μM, a progressive reduction in the level of COX-2 protein expression was noted. The reduction in the level of expression of both COX-2 mRNA and protein in the cells treated with curcumin concentration higher than 5 μM maybe attributed to the decreased cell viability described in *Fig. 1*.

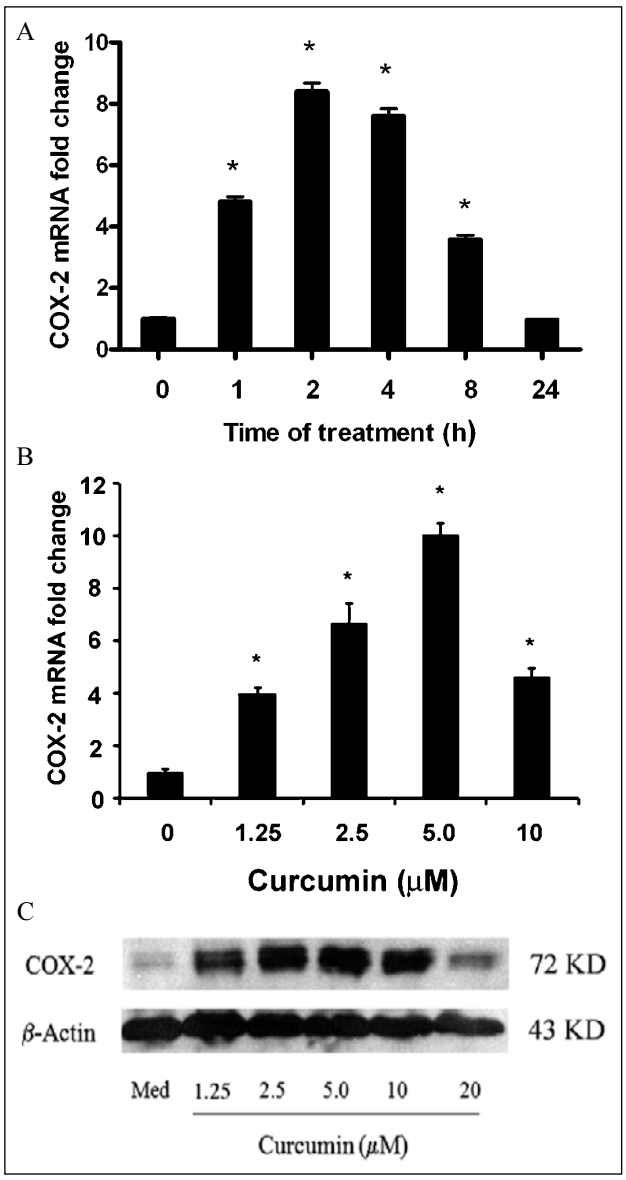

Fig. 2. Curcumin induces COX-2 mRNA and protein expression in HCAEC. HCAEC monolayers were incubated with curcumin (5 μM) for 1–24 h, (A); or the cells were treated with various concentration of curcumin (0-10 μM) for 4 hours (B). After the incubation, total RNA was extracted, reverse transcribed, and analyzed by real-time RT-PCR using specific primers, as described in 'Materials and Methods'. The amplicons were normalized for β-actin and assigned a value of 1 for medium-treated cells. The magnitude of changes of mRNA expression in curcumin-treated cells was calculated by comparing with the message in medium-treated HCAEC. The results presented are representative of three experiments with similar results. Each value presented is the mean ±S.E.M. of triplicate determinations. *, indicates $p<0.05$ when compared with medium-treated cells. For monitoring protein expression, HCAEC monolayers were incubated with curcumin (0 to 20 μM) for 8 hours. After washing with phosphate-buffered saline, total cell lysates were prepared and subjected to Western blot analyses (C) as described in 'Materials and Methods'.

The effect of curcumin on cyclooxygenase-1 expression

In contrast to the expression of COX-2, the expression of COX-1 mRNA was significantly inhibited when HCAEC were treated with 5 μM curcumin for 2 to 8 hours (*Fig. 3A*). As in the case of the induction of COX-2 expression, the effect on the inhibition of COX-1 mRNA expression was transient and returned to basal level by 24 hours treatment. However, treatment of HCAEC with 5 μM curcumin for 4 to 24 hours did not alter COX-1 protein expression (*Fig. 3B*).

Curcumin decreases prostaglandin E_2 and prostaglandin I_2 production in human coronary artery endothelial cells

Since curcumin was found to induce the expression of both COX-2 mRNA and protein, we tested whether curcumin-stimulated HCAEC would produce higher amounts of prostaglandins. It is well-established that COX-2 mediates the enzymatic conversion of arachidonic acid to PGH_2, which in turn is rapidly converted to PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 by the terminal synthases. In this study, we measured the production of PGE_2 and PGI_2, the two prostanoids that are directly associated with immunomodulatory and vasoactive functions. Accordingly, HCAEC monolayers were incubated with 1.25, 2.5, or 5.0 μM curcumin for 24 hours and the culture supernatants were analyzed for the levels of PGI_2 and PGE_2 by EIA. It is noteworthy that, in spite of the increase in the expression of COX-2 mRNA and protein, a trend towards

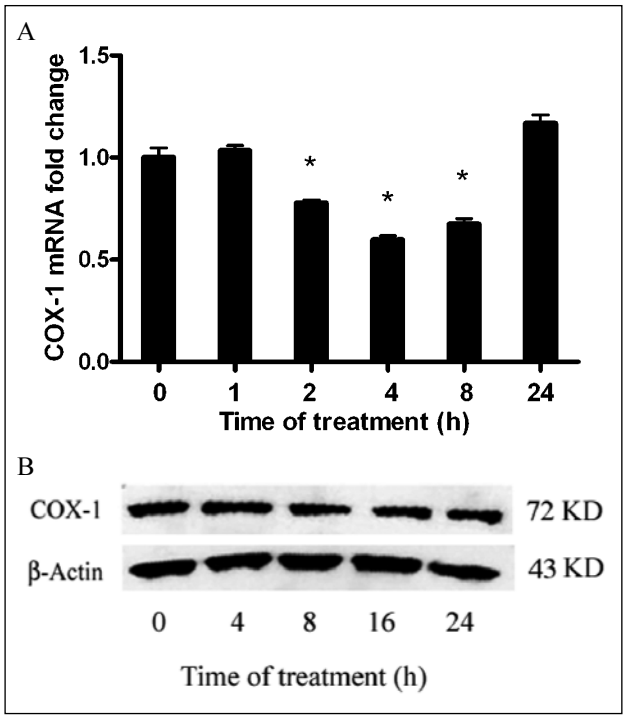

Fig. 3. Effect of curcumin on COX-1 mRNA and protein expression. HCAEC monolayers were incubated with curcumin (5 μM) for 0 to 24 hours. After the incubation, the expression of COX-1 mRNA (A) or protein (B) was determined by real-time RT-PCR or western blot analysis, respectively, as described in 'Materials and Methods'. The data presented are representative of three experiments with similar results.

decreased production of PGI_2 and PGE_2 by curcumin-treated HCAEC was evident (*Fig. 4A*).

To test whether curcumin might alter the production of prostanoids earlier than 24 hours after treatment, a kinetic study on the production of PGI_2 by HCAEC monolayers incubated with 5 μM curcumin for 2, 4, 8, and 24 hours was carried out. As shown in *Fig. 4B*, quiescent HCAEC generated PGI_2 in a time-dependent fashion during the first 8 hours of culture, which remained almost at a steady state until up to 24 hours. In contrast, curcumin-treated HCAEC released less PGI_2 beginning at 4 h and this difference continued for 24 hours.

Considering the possibility that curcumin might increase the production of other prostanoids, such as PGD_2, $PGF_{2\alpha}$, or TXA_2, while inhibiting PGE_2 and PGI_2 production, we tested the total PG production in cell culture supernatants using prostaglandin screening EIA kit. The antiserum used in this assay exhibits high cross-reactivity for most prostaglandins which allows an estimation of the total prostaglandin level in a given sample. The incubation of HCAEC with 1.25 to 5.0 μM curcumin for up to 24 hours led to an overall decrease in PG production, suggesting that the synthesis of prostanoids other than PGE_2 and PGI_2 also might be reduced in the absence of exogenous AA (data not shown).

To rule out the possibility that the lower amounts of prostanoids detected in the supernatants of curcumin-treated cells is not due to interference by curcumin in the EIA, known concentrations of 6-keto $PGF_{1\alpha}$ or PGE_2 standards were mixed with 5 μM curcumin and their readings were compared with the standards run concurrently in the absence of curcumin. The results revealed that the presence of curcumin up to 5 μM does not affect EIA analyses of 6-keto $PGF_{1\alpha}$ or PGE_2 (data not shown).

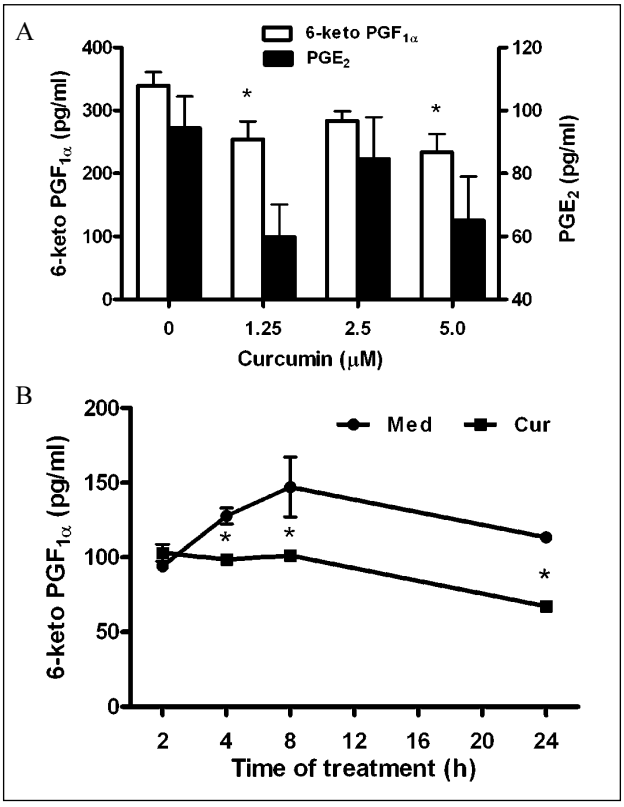

Fig. 4. Curcumin decreases PGE_2 and PGI_2 production despite increased COX-2 expression in HCAEC. HCAEC monolayers were incubated with curcumin (0–5.0 μM) for 24 hours (A), or with curcumin (5.0 μM) for various of time periods (B). After incubation, the culture supernatants were collected and analyzed for PGI_2 (6-keto $PGF_{1\alpha}$ or PGE_2 by EIA. Data presented are mean ±S.E.M. of triplicate determinations of 2 representative experiments. *, indicates $p<0.05$ when compared to the medium-treated cells.

Curcumin induces the mRNA expression of microsomal PGE synthase-1 and PGI_2 synthase

In order to examine whether the inability of curcumin-treated HCAEC to generate PGE_2 and PGI_2 was due to reduced expression of mPGES-1 and PGI_2S, the level of expression of their mRNA was evaluated by real-time RT-PCR. As depicted in *Fig. 5*, incubation of HCAEC with 2.5 or 5.0 μM curcumin for 4 hours resulted in significant increases in the expression of mPGES-1 and PGI_2S mRNA.

Supplementation of the culture media with arachidonic acid (AA) enhances prostanoid production by curcumin-treated human coronary artery endothelial cells (HCAEC)

AA is derived from the membrane phospholipids released by phopholipase A_2 (PLA_2). Curcumin is known to inhibit the phosphorylation of cytosolic phospholipase A_2 (c-PLA_2) and inhibit the activation process of the enzyme in Raw 264.7 cells (10). Therefore, the inhibition of PGE_2 and PGI_2 production in curcumin-treated HCAEC, despite the increased COX-2, mPGES-1 and PGI_2S expression, may be the consequence of reduced availability of AA. To examine whether exogenous supplementation of AA can restore prostanoid synthesis by the newly expressed enzymes, HCAEC were pretreated with 5 μM curcumin for 6 hours, washed, and incubated for 30 minutes in culture medium containing 10, 20 and 40 μM of AA, and the production of PGE_2 and PGI_2 in the supernatants were monitored. As shown in *Fig. 6*, supplementation of the culture medium with exogenous AA markedly increased PGE_2 (*Fig. 6A*) and PGI_2 (*Fig.*

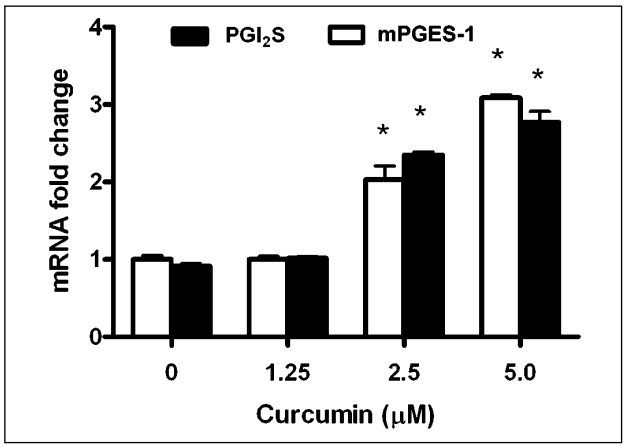

Fig. 5. Curcumin induces PGI_2S and mPGES-1 gene expression in HCAEC. HCAEC monolayers were incubated with curcumin (1.25-5 μM) for 4 hours. Total RNA was extracted, reverse transcribed and analyzed by real-time PCR using specific primers, as described in 'Materials and Methods'. The amplicons were normalized for β-actin and assigned a value of 1 for medium-treated cells. The magnitude of changes of mRNA expression in curcumin -treated cells was calculated by comparing with the message in medium-treated HCAEC. The results presented are representative of three experiments with similar results. Each value presented is the mean ±S.E.M. of triplicate determinations. *, indicates $p<0.05$ when compared with medium-treated cells.

6B) production by both quiescent cells and curcumin-treated cells. Interestingly, the production of PGE_2 and PGI_2 by curcumin-treated cells was significantly higher than. quiescent cells when the AA concentrations were raised to 20 to 40 μM.

Curcumin-induced enhancement in prostaglandin E_2 and prostaglandin I_2 production is inhibited by cyclooxygenase-2 specific inhibitor

In view of our finding that incubation of HCAEC with curcumin led to increased expression of COX-2, we wanted to determine whether the curcumin-induced enhancement of the prostanoid production is COX-2-mediated. To test this, HCAEC monolayers were pre-treated with 5 μM curcumin for 6 hours in the presence or absence of the COX-2 inhibitor NS-398 (10 μM) during the last 30 minutes of incubation. After the incubation, the cells were washed to remove curcumin, and were incubated for an additional 30 minutes in medium containing 40 μM AA in the presence or absence of NS-398 (10 μM). The culture supernatants were then collected and analyzed for PGE_2 and PGI_2. As shown in *Fig. 7*, the production of PGE_2 (A) and PGI_2 (B) by quiescent and curcumin-treated HCAEC were increased by AA supplementation. In agreement with the increased expression of COX-2, mPGES-1 and PGI_2S, curcumin-treated HCAEC produced significantly higher amounts of PGE_2 and PGI_2 than controls when AA was supplemented. When quiescent HCAEC were incubated with COX-2-specific inhibitor (NS-398), 75 to 80% inhibition of PGE_2 and PGI_2 production was noted. COX-2 inhibitor also inhibited PGE_2 and PGI_2 production by curcumin-treated HCAEC by 75% and 100%, respectively.

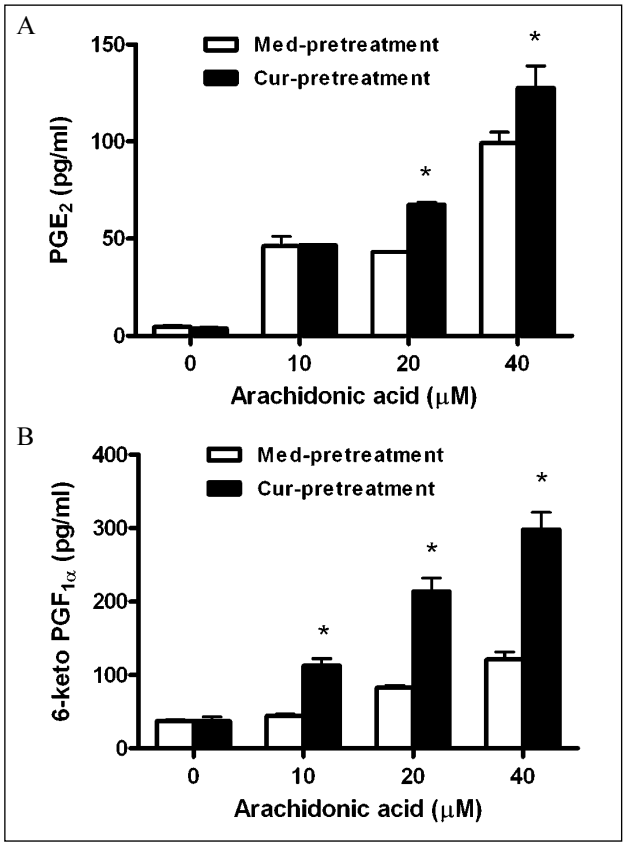

Fig. 6. Exogenous arachidonic acid at higher concentrations further increases prostanoid production in curcumin treated cells. HCAEC monolayers were pre-incubated with medium or curcumin (5 μM) for 6 hours. Cells were washed twice with HBSS followed by incubation with various concentration of AA (10 μM to 40 μM). Culture supernatants were collected and analyzed for PGE_2 (A) and 6-keto $PGF_{1\alpha}$ (B) by EIA. Data presented are mean ±S.E.M. of triplicate determinations of 2 representative experiments. *, indicates $p<0.05$ when compared with medium-pretreated cells with same concentration of AA treatment.

DISCUSSION

Curcumin has gained significant attention in recent years as a potent chemopreventive and anti-inflammatory agent that modulates cellular functions including, but not limited to, the regulation of transcription factors, protein kinases, cytokines, enzymes, apoptosis, cell differentiation and angiogenesis (25, 26). Considerable interest has also been focused on the potential involvement of COX-2 and the biosynthesis of prostanoids in the pathogenesis of coronary heart disease and atherosclerosis (16). Previous studies have suggested that curcumin has the ability to inhibit the expression of COX-2 and subsequently reduce the production of prostanoids in a variety of cell types including

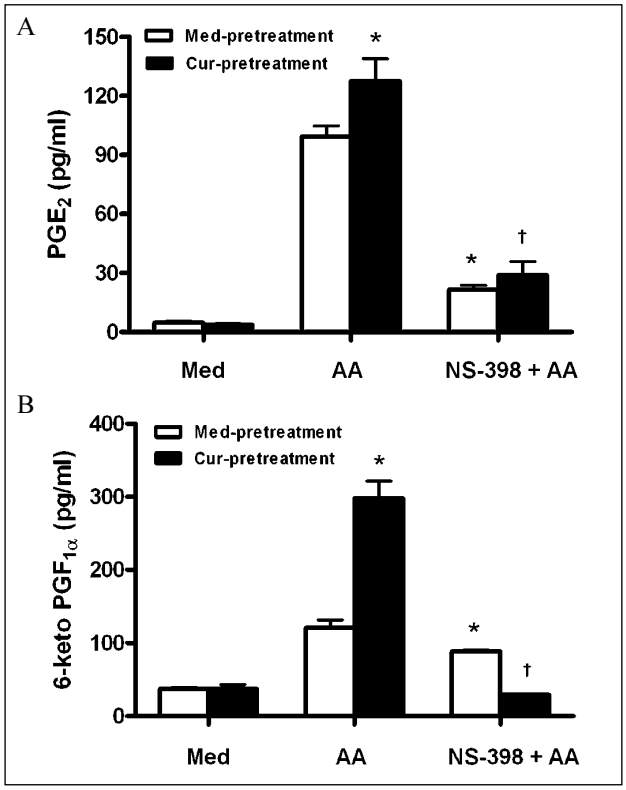

Fig. 7. Exogenous arachidonic acid enhanced prostanoid production was inhibited by COX-2 specific inhibitor. HCAEC monolayers were pre-treated with 5 μM curcumin for 6 hours with the presence or absence COX-2 inhibitor NS-398 during the last 30 minutes of incubation. After the incubations the cells were washed, fresh medium, AA (40 μM) in the presence or absence of NS-398 (10 μM) was added, and incubated for an additional 30 minutes. The culture supernatants were then harvested and analyzed for PGE_2 (A) and 6-keto $PGF_{1\alpha}$ (B) production by EIA. The results presented are representative of two experiments with similar results. Each value presented is the mean ±S.E.M. of triplicate determinations. *, indicates $p<0.05$ when compared with AA alone treated cells in the medium pretreatment group. †, indicates $p<0.05$ when compared with AA alone treated cells in the curcumin pretreatment group.

macrophages, gastric epithelial cells, and malignant cells (3). Although the importance of COX-2 and prostanoid homeostasis in vascular inflammation and atherosclerosis is well-recognized (14-16), and curcumin has been shown to reduce atherosclerotic progression in apoE/LDLR-double knockout mice (21), the effect of curcumin on the expression and function of COX-2, mPGES-1 and PGI_2S, and the potential impact on prostanoid synthesis by HCAEC has not been studied. In the present report, we demonstrate that curcumin has the ability to stimulate the expression of COX-2, mPGES-1 and PGI_2S gene expression while concurrently inhibiting COX-1 gene expression. We also present evidence showing that the curcumin-induced COX-2, mPGES-1 and PGI_2S are functionally active as documented by the increased ability of curcumin-treated HCAEC to produce PGE_2 and PGI_2 than quiescent cells when adequate concentration of AA is available. These findings are in agreement with previous reports demonstrating induction of these enzymes in response to inflammatory stimuli (27, 28). However, it should be emphasized that curcumin also has been reported to inhibit PGE_2 production by blocking mPGES-1 in certain carcinoma cell lines (29).

It is well recognized that cyclooxygenases utilize the intracellular pool of AA to synthesize prostaglandins. The cellular concentration of AA is precisely regulated and is dependent on the release from the membrane phospholipids by the action of PLA_2 as well as its uptake by the cells from the extracellular milieu. Curcumin is reported to inhibit the phosphorylation of c-PLA_2, the activation process of the enzyme (10). It was of interest to note that, despite the enhanced expression of COX-2, PGI_2S and mPGES-1, curcumin-treated HCAEC failed to generate increased amount of PGI_2 or PGE_2, but in fact tended to decrease the generation of these prostanoids in the absence of exogenous AA. Therefore, if the blockade in prostanoid synthesis in curcumin-treated HCAEC is due to the deficiency of AA, supplementation of the culture medium with AA should rescue the prostanoid synthetic pathway. As shown in *Fig. 6*, supplementation of the culture medium with exogenous AA markedly increased PGE_2 and PGI_2 production by both quiescent cells and curcumin-treated cells. Furthermore, the production of PGE_2 and PGI_2 by curcumin-treated cells was markedly higher than the medium-treated cells when the AA concentrations were raised. These results suggest that, although there is an inherent deficiency of intracellular AA pool in HCAEC to meet the substrate demand of COX enzymes, adequate supply of exogenous AA can increase prostanoid production to full potential by the constitutively expressed COX enzymes as well by the newly expressed COX-2, mPGES-1 and PGI_2S in curcumin-treated cells. The fact that supplementation of the culture media with AA enhanced the prostanoid production by both quiescent and curcumin treated cells would suggest that there is an overall deficiency of the substrate in HCAEC for the COX-2 enzyme to function optimally. This deficiency of AA could further be magnified in curcumin-treated HCAEC if PLA_2 is inhibited and uptake of AA from the extracellular supply is diminished. However, the ability of curcumin-treated HCAEC to produce greater amounts of PGE_2 and PGI_2 than the quiescent cells when AA concentration was increased indicates that the newly expressed COX-2 and the synthases are catalytically active and can generate higher amounts of prostanoids. These results demonstrate for the first time that curcumin is capable of stimulating the expression of COX-2, PGI_2S, and mPGES-1 and generating elevated levels of prostanoids if an adequate amount of AA is available. It is of interest to note that curcumin treatment resulted in a 30-40% reduction in the expression COX-1 mRNA which was also transient in nature. In spite of the decrease in mRNA expression, curcumin treatment did not result in decreased COX-1 protein expression. There is evidence which suggests that an inhibition in COX-1 expression or its catalytic activity might modulate the activity of COX-2 and affect prostanoid production (26). Since curcumin was able to enhance the expression of COX-2 with concurrent decrease in the expression of COX-1, the enhanced production of prostanoids might be derived from the catalytic activity of the newly expressed COX-2 in the presence of exogenously supplied AA. Treatment of quiescent HCAEC with COX-2-specific inhibitor NS-398 caused 75 to 80% inhibition of PGE_2 and PGI_2 production (*Fig. 7*), which suggested that COX-2 contributes to the basal production of prostanoids in the presence of exogenous of AA. Although the COX-2 inhibitor also inhibited 75% of the PGE_2 production by curcumin-treated HCAEC as in the case of control cells, it blocked 100% of PGI_2 production suggesting that most of newly expressed COX-2/PGI_2S synthesizes PGI_2. Since the prostanoid production determined in this experiment was only for the initial 30 minute immediately following the addition of AA and neither COX-1 nor COX-2 gene expression was found to be altered within this short period (data not shown), it is reasonable to believe that the increased biosynthesis of prostanoids are by the COX enzymes which were expressed prior to AA supplementation.

Curcumin has been shown to inhibit the expression of COX-2 and prostanoid production in a variety of cell types including human cancer cell lines and non-cancerous cells (4-9). There are several possibilities for the disparity between our findings and the previous reports. First, malignant and transformed cells may respond differently to curcumin treatment compared to primary cells, such as HCAEC. In agreement with this contention, curcumin has been shown to induce apoptosis of lung fibroblasts from scleroderma patients but not of normal lung fibroblasts (30). Second, in the above cited studies (4-10), curcumin concentrations used were higher than 10 μM (10 μM to 30 μM). In our hands, although curcumin at concentrations higher than 10 μM led to lower COX-2 expression compared to 5 μM curcumin treated cells (*Fig. 2B* and *2C*), the morphology and viability of high concentration (>10 μM) curcumin-treated cells were found to be markedly affected (*Fig. 1*). Considering the low bioavailability of curcumin and its reported serum levels in individuals ingesting 8 g of curcumin/day to be <2 μM and its *in vitro* toxicity to endothelium at greater than 10 μM, we believe that the curcumin concentration used in this study (1.25 to 5 μM) may correctly define curcumin's direct effect on arachidonic acid/cyclooxygenase pathways in the HCAEC. The curcumin-induced enhancement in the expression of COX-2 in HCAEC is consistent with the findings of Hong *et al*., who reported increased expression of COX-2 protein in curcumin-treated Raw 264.7 cells (10). *In vivo* studies on the effect of curcumin on the production of prostanoids and determination of prostanoid metabolites in the urine will provide a better understanding of the pharmacological actions of curcumin.

In summary, the present study demonstrates that incubation of HCAEC with curcumin leads to increased expression of COX-2, mPGES-1 and PGI_2S with resultant enhancement of the production of PGE_2 and PGI_2 when adequate amounts of AA are present. It is also of interest to note that curcumin transiently inhibited COX-1 gene expression. We also present evidence that, most of the PGE_2 and PGI_2 generated by curcumin-treated HCAEC are derived from COX-2, which confirms that the newly expressed enzyme is functionally active.

Acknowledgments: This study was supported by National Institutes of Health Grants, R01HL070101 and 3R01HL070101-04S1, and Joseph and Elizabeth Carey Arthritis Fund from the Kansas University Endowment Association. We are grateful to Dr. Jo Wick in the Center for Biostatistics and Advanced informatics at the University of Kansas Medical Center for contributing to the statistical analyses.

Conflict of interests: None declared.

REFERENCES

1. Singh. From exotic spice to modern drug. *Cell* 2007; 130: 765-768.
2. Gupta B, Ghosh B. Curcuma longa inhibits TNF-alpha induced expression of adhesion molecules on human umbilical vein endothelial cells. *Int J Immunopharmacol* 1999; 21: 745-757.
3. Jagetia GC, Aggarwal B.B. "Spicing up" of the immune system by curcumin. *J Clin Immunol* 2007; 27: 19-35.
4. Lee KW, Kim JH, Lee HJ, Surh YJ. Curcumin inhibits phorbol ester-induced up-regulation of cyclooxygenase-2 and matrix metalloproteinase-9 by blocking ERK1/2 phosphorylation and NF-kappaB transcriptional activity in MCF10A human breast epithelial cells. *Antioxid Redox Signal* 2005; 7: 1612-1620.
5. Su CC, Chen GW, Lin JG, Wu LT, Chung JG. Curcumin inhibits cell migration of human colon cancer colo 205 cells through the inhibition of nuclear factor kappa B /p65 and down-regulates cyclooxygenase-2 and matrix metalloproteinase-2 expressions. *Anticancer Res* 2006; 26: 1281-1288.
6. Park C, Kim GY, Kim GD, Choi BT, Park YM, Choi YH. Induction of G2/M arrest and inhibition of cyclooxygenase-2 activity by curcumin in human bladder cancer T24 cells. *Oncol Rep* 2006; 15: 1225-1231.
7. Shishodia S, Potdar P, Gairola CG, Aggarwal BB. Curcumin (diferuloylmethane) down-regulates cigarette smoke-induced NF-kappaB activation through inhibition of NFkappaBalpha kinase in human lung epithelial cells: correlation with suppression of COX-2, MMP-9 and cyclin D1. *Carcinogenesis* 2003; 24: 1269-1279.
8. Cho JW, Park K, Kweon GR, *et al*. Curcumin inhibits the expression of COX-2 in UVB-irradiated human keratinocytes (HaCaT) by inhibiting activation of AP-1: p38 MAP kinase and JNK as potential upstream targets. *Exp Mol Med* 2005; 37: 186-192.
9. Binion DG, Otterson MF, Rafiee P. Curcumin inhibits VEGF-mediated angiogenesis in human intestinal microvascular endothelial cells through COX-2 and MAPK inhibition. *Gut* 2008; 57: 1509-1517.
10. Hong J, Bose M, Ju J, *et al*. Modulation of arachidonic acid metabolism by curcumin and related beta-diketone derivatives: effects on cytosolic phospholipase A_2, cyclooxygenases and 5-lipoxygenase. *Carcinogenesis* 2004; 25: 1671-1679.
11. Dubois RN, Abramson SB, Crofford L, *et al*. Cyclooxygenase in biology and disease. *FASEB J* 1998; 12: 1063-1073.
12. Smith WL, Marnett LJ. Prostaglandin endoperoxide synthase: structure and catalysis. *Biochim Biophys Acta* 1991; 1083: 1-17.
13. Wu KK. Cyclooxygenase-2 induction in congestive heart failure: friend or foe? *Circulation* 1998; 98: 95-96.
14. FitzGerald GA, Smith B, Pedersen AK, Brash AR. Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. *N Engl J Med* 1984; 310: 1065-1068.
15. Belton O, Byrne D, Kearney D, Leahy A, Fitzgerald DJ. Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. *Circulation* 2000; 102: 840-845.
16. Grosser T, Fries S, FitzGerald GA. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest* 2006; 116: 4-15.
17. Vane JR, Botting RM. Pharmacodynamic profile of prostacyclin. *Am J Cardiol* 1995; 75: 3A-10A.
18. McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. *Proc Natl Acad Sci USA* 1999; 96: 272-277.
19. Catella-Lawson F, McAdam B, Morrison BW, *et al*. Effects of specific inhibition of cyclooxygenase-2 on sodium balance, hemodynamics, and vasoactive eicosanoids. *J Pharmacol Exp Ther* 1999; 289: 735-741.
20. Wong E, Huang JQ, Tagari P, Riendeau D. Effects of COX-2 inhibitors on aortic prostacyclin production in cholesterol-fed rabbits. *Atherosclerosis* 2001; 157: 393-402.
21. Olszanecki R, Jawien J, Gajda M, *et al.* Effect of curcumin on atherosclerosis in apoE/LDLR-double knockout mice. *J Physiol Pharmacol* 2005; 56: 627-635.
22. Tan X, Essengue S, Talreja J, Reese J, Stechschulte DJ, Dileepan KN. Histamine directly and synergistically with lipopolysaccharide stimulates cyclooxygenase-2 expression and prostaglandin I_2 and E_2 production in human coronary artery endothelial cells. *J Immunol* 2007; 179: 7899-7906.
23. Wang Z, Zhang Y, Banerjee S, Li Y, Sarkar FH. Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells. *Cancer* 2006; 106: 2503-2513.
24. Cheng AL, Hsu CH, Lin JK, *et al*. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res* 2001; 21: 2895-2900.
25. Anand P, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB. Curcumin and cancer: an "old-age" disease with an "age-old" solution. *Cancer Lett* 2008; 267: 133-164.
26. Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. Curcumin: from ancient medicine to current clinical trials. *Cell Mol Life Sci* 2008; 65: 1631-1652.
27. Jakobsson PJ, Thoren S, Morgenstern R, Samuelsson B. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *PNAS* 1999; 96: 7220-7225.
28. Bulet L, Ouellet M, Bateman KP, *et al*. Deletion of microsomal prostaglandin E_2 (PGE_2) synthase-1 reduces inducible and basal PGE_2 production and alters the gastric prostanoid profile. *J Biol Chem* 2004; 279: 23229-23237.
29. Koeberle A, Northoff H, Werz O. Curcumin blocks prostaglandin E_2 biosynthesis through direct inhibition of the microsomal prostaglandin E_2 synthase-1. *Mol Cancer Ther* 2009; 8: 2348-2355.
30. Tourkina E, Gooz P, Oates JC, Ludwicka-Bradley A, Silver RM, Hoffman S. Curcumin-induced apoptosis in scleroderma lung fibroblasts: role of protein kinase cepsilon. *Am J Respir Cell Mol Biol* 2004; 31: 28-35.

Received: August 5, 2010
Accepted: February 25, 2011

Author's address: Dr. Kottarappat N. Dileepan, Department of Medicine, MS 2026, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160, USA; E-mail: kdileepan@kumc.edu