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# Roles of heme oxygenase-1 in curcumin-induced growth inhibition in rat smooth muscle cells

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Abbreviations: ARE, antioxidant response element; HASMCs, human aortic smooth muscle cells; HO-1, heme oxygenase-1; Nrf2, nuclear transcription factor E2-related factor-2; VSMCs, vascular smooth muscle cells

## Abstract

**In vascular smooth muscle cells (VSMCs), induction of the heme oxygenase-1 (HO-1) confers vascular protection against cellular proliferation mainly via its up-regulation of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ that is involved in negative regulation of cellular proliferation. In the present study, we investigated whether the phytochemical curcumin and its metabolite tetrahydrocurcumin could induce HO-1 expression and growth inhibition in rat VSMCs and, if so, whether their antiproliferative effect could be mediated via HO-1 expression. At non-toxic concentrations, curcumin possessing two Michael-reaction acceptors induced HO-1 expression by activating antioxidant response element (ARE) through translocation of the nuclear transcription factor E2-related factor-2 (Nrf2) into the nucleus and also inhibited VSMC growth triggered by 5% FBS in a dose-dependent manner. In contrast, tetrahydrocurcumin lacking Michael-reaction acceptor showed no effect on HO-1 expression, ARE activation and VSMC growth inhibition. The antiproliferative effect of curcumin in VSMCs was accompanied by the increased expression of $p21^{WAF1/CIP1}$. Inhibition of VSMC growth and expression of $p21^{WAF1/CIP1}$ by curcumin were partially, but not completely, abolished when the cells were co-incubated with the HO inhibitor tin protoporphyrin. In human aortic smooth muscle cells (HASMCs), curcumin also inhibited growth triggered by TNF-$\alpha$ and increased $p21^{WAF1/CIP1}$ expression via HO-1-dependent manner. Our findings suggest that curcumin has an ability to induce HO-1 expression, presumably through Nrf2-dependent ARE activation, in rat VSMCs and HASMCs, and provide evidence that the antiproliferative effect of curcumin is considerably linked to its ability to induce HO-1 expression.**

**Keywords:** carbon monoxide; cell proliferation; curcumin; heme oxygenase-1; NF-E2-related factor 2; muscle, smooth, vascular

## Introduction

Vascular smooth muscle cells (VSMCs) comprise the major cellular component of the normal arterial wall and the excessive growth of VSMCs is a key abnormality in the development of atherosclerosis lesions and restenosis after balloon angioplasty (Sata, 2006). Therefore, inhibition of VSMC proliferation represents an important therapeutic strategy for treatment of these diseases. Recently, it has been well established that heme oxygenase-1 (HO-1), the inducible enzyme that uses the pro-oxidant heme as a substrate to produce bilirubin/biliverdin and carbon monoxide (CO), confers vascular protection against abnormal proliferation (Durante, 2003). For example, VSMCs from HO-1-deficient mice display abnormal growth (Yet *et al*., 2003). Conversely, over-expression of HO-1 by either transfection of the HO-1 gene or exogenous administration of a certain HO-1 inducer reduces excessive VSMC proliferation (Juan *et al*., 2001). Thus, genetic approaches targeting HO-1 or pharmacological interventions using HO-1 inducers may offer a promising therapeutic modality in treating occlusive vascular disease.

The rhizome of *Curcuma longa* (turmeric) has been widely used as a spice and coloring agent in many foods. Consumption of turmeric has been associated with various beneficial effects on human health (Chainani-Wu, 2003). Turmeric has also been used as a traditional remedy for the treatment of inflammation and other diseases (Surh, 2002). Curcumin (chemical structure shown in Figure 1), as a major biologically active compound, is isolated from the turmeric (Surh, 2002). A wide range of biological and pharmacological activities of curcumin have been investigated (Maheshwari *et al*., 2006). The dietary pigment curcumin is rapidly metabolized during absorption from the intestine, yielding colorless tetrahydrocurcumin (chemical structure shown in Figure 1) (Sugiyama *et al*., 1996), which has shown the stronger antioxidant activity than curcumin (Huang *et al*., 1995). Several studies in experimental animals indicated that like curcumin, tetrahydrocurcumin is also effective in cancer prevention (Huang *et al*., 1995) and protection against inflammation (Ireson *et al*., 2001) and hepatotoxicity (Pari and Murugan, 2004). Thus, tetrahydrocurcumin was thought to be one of the metabolites with higher physiological and pharmacological activities than curcumin in the intestine.

Recently, it has been reported that curcumin is capable of inhibiting VSMC proliferation (Yang *et al*., 2006). However, a possible mechanism of antiproliferative action of curcumin is not fully understood. Curcumin can induce HO-1 expression in endothelial cells (Jeong *et al*., 2006), renal epithelial cells (Balogun *et al*., 2003), and astrocytes (Scapagnini *et al*., 2002), and HO-1 expression in these types of cells mediates pharmacological actions of curcumin. In this regard, it is questioned whether curcumin could induce HO-1 expression also in rat VSMCs and, if so, whether inhibition of VSMC proliferation by curcumin could be mediated via its HO-1 expression. It is also questioned whether the curcumin metabolite tetrahydrocurcumin may be also effective in HO-1 expression and growth inhibition in VSMCs.

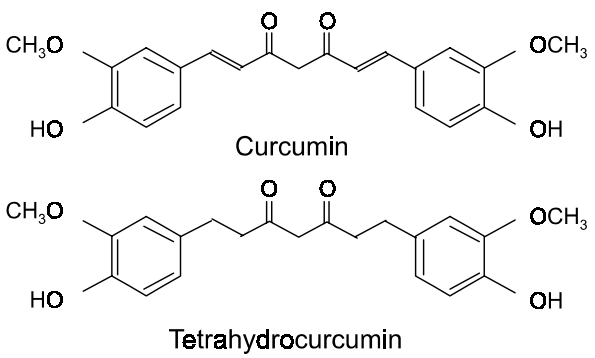


**Figure 1.** Chemical structures of curcumin and tetrahydrocurcumin.

The nuclear transcription factor E2-related factor 2 (Nrf2) has a highly conserved basic region-leucine-zipper structure and belongs to the cap-n-collar family together with Nrf1 and Nrf3. Nrf2 forms heterodimers with the small oncogene family proteins for the selective recognition of the antioxidant responsive element (ARE) on target genes, followed by the regulation of gene expression of phase II detoxifying enzymes including HO-1 (Srisook *et al*., 2005). From several lines of investigation, it is reported that curcumin stimulates HO-1 gene activity by promoting dissociation of the Nrf2-Keap1 complex, leading to increased Nrf2 binding to the resident HO-1 AREs (Balogun *et al*., 2003).

In the present study, we demonstrated that curcumin, but not tetrahydrocurcumin, induced HO-1 expression by activating ARE through Nrf2 nuclear translocation and growth inhibition in rat VSMCs. In addition, we provide evidence that the antiproliferative effect of curcumin is mediated considerably through its HO-1 expression not only in rat VSMCs but also in human aortic smooth muscle cells (HASMCs).

## Materials and Methods

### Reagents

Curcumin ($>$ 95% pure) was isolated from the rhizomes of turmeric, as described earlier (Jeong *et al*., 2006). Tetrahydrocurcumin ($>$ 95% pure) was prepared from curcumin by hydrogenating the two double bonds conjugated to the $\beta$-diketone (Figure 1), as described previously (Uehara *et al*., 1987). DMEM and FBS were obtained from Gibco/Invitrogen (Carlsbad, CA). Bilirubin, biliverdin, ferrous citrate, HEPES, 4,6-Diamidino-2-phenylindole (DAPI), hemin, trypsin, and monoclonal antibody against smooth muscle $\alpha$-actin were purchased from the Sigma- Aldrich (St. Louis, MO). Antibodies for HO-1, Nrf2, $p27^{KIP1}$ and $p21^{WAF/CIP1}$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [$^3$H]thymidine was purchased from Dupont/New England Nuclear (Boston, MA). Cupper protoporphyrin (CuPP) and tin protoporphyrin (SnPP) were from Porphyrin Products (Logan, UT). Recombinant TNF-$\alpha$ was purchased from Sigma-Aldrich. All other chemicals were of the highest purity commercially available.

### Rat VSMC isolation and culture

Rat VSMCs were isolated by enzymatic dispersion, as previously described (Kim *et al*., 2002). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 8

mM HEPES and 2 mM L-glutamine at 37°C in a humidified 5% $CO_2$ incubator. The purity of VSMCs cultures was confirmed by the immunocytochemical localization of α-smooth muscle actin. VSMCs from between passages 3 and 6 were used in this experiment. This study was performed via a protocol approved by the Institutional Animal Care and Use Committee of Wonkwang University in accordance with the Guide for the Care and Use of Laboratory Animals.

Human aortic smooth muscle cells (HASMC) were purchased from Bio-Whittaker (San Diego, CA) and cultured in smooth muscle cell growth medium containing 10% FBS, 2 ng/ml of human basic fibroblast growth factor, 0.5 ng/ml of human EGF, 50 μg/ml of gentamycin, 50 μl/ml of amphotericin-B, and 5 μg/ml of bovine insulin.

### Plasmids, transfections and luciferase assays

To construct ARE-Luciferase vector, tandem repeats of double-stranded oligonucleotides spanning the Nrf2 binding site 5'-TGACTCAGCA-3' (Lee *et al*., 2006) were introduced into the restriction sites of the pGL2 promoter plasmid (Madison, WI). The human $p21^{WAF1/CIP1}$ promoter construct was a gift from Dr. Bert Vogelstein (el-Deiry *et al*., 1993). All transfection experiments were performed using lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For luciferase assays, the cell lysate was first mixed with the luciferase substrate solution (Promega), and luciferase activity was measured using a luminometer. For each experiment, luciferase activity was determined in triplicate and normalized for each sample using β-galactosidase activity.

### Cell proliferation and [$^3$H]thymidine incorporation assays

For all experiments, cells were made quiescent by serum-free medium for 48 h before adding mitogenic agents or test compounds to the medium. VSMCs, grown to near confluence in 12-well tissue culture plates, were made quiescent and treated with curcumin, as indicated. Cells were stimulated with 5% FBS (for rat VSMCs) or 100 ng/ml TNF-α (for HASMCs), trypsinized with trypsin-EDTA, and counted using a hemocytometer. To assess the count of viable cells, an equal volume of trypan blue dye solution (0.1% w/v) was added briefly to stain a portion of the cells.

For [$^3$H]thymidine incorporation experiments, cells were seeded in 24-well culture plates under the above-mentioned conditions, and 2 μCi/ml of [$^3$H]-thymidine was added to medium for 4 h. Reactions were terminated by aspirating medium and washing cultures with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v). The acid-insoluble [$^3$H]thymidine was extracted into 250 μl of 0.5 M NaOH/well, and this solution was then mixed with 3 ml of scintillation cocktail (Ultimagold, Packard Bioscience, CT), and quantified using a liquid scintillation counter (LS3801, Beckman, Düsseldorf, Germany). Protein contents were determined in 50 μl aliquots of residual solutions using BCA Protein Assay Reagent Kits (Pierce Biotechnology, Rockford, IL).

### Western blotting analysis for protein expression

After treatment, cells ($3 \times 10^6$ cells/3 ml in 60 mm dish) were collected and washed with PBS. After centrifugation, cell lysis was carried out at 4°C by vigorous shaking for 15 min in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM $Na_3VO_4$ and protease inhibitors]. After centrifugation at 14,800 × *g* for 15 min, the supernatant was separated and stored at -70°C until use. The protein concentration was determined by using the BCA Protein Assay Reagent Kit (Pierce Biotechnology). After addition of sample loading buffer, protein samples were electrophoresed on a 12.5% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride blots at 300 mA for 6 h. The blots were blocked for 1 h at room temperature in fresh blocking buffer (0.1% Tween 20 in Tris-buffered saline, pH 7.4, containing 5% non-fat dried milk). Dilutions (1:1,000) of primary antibodies were made in PBS with 3% non-fat dried milk. Following three washes with PBST (PBS and 0.1% Tween 20), the blots were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) in PBS with 3% non-fat dried milk for 1 h at room temperature. The blots were washed again three times in PBST buffer, and transferred proteins were incubated with ECL substrate solution (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 min according to the manufacturer's instructions followed by visualization with X-ray film.

### HO activity assay

Cells ($2.5 \times 10^6$ cells) were treated with vehicle, hemin (positive control) or curcumin for 6 h. Cell pellets were re-suspended in magnesium supplemented potassium phosphate solution [MgCl-PBS; 100 mM potassium phosphate and 2 mM magnesium chloride; pH 7.4] and subjected to three freeze-thaw cycles for release of cytoplasmic HO-1 proteins. HO-1 enzymatic assay used a reaction mixture containing 100 mM PBS, 2 mM $MgCl_2$, 3 mg of rat liver cytosol, 0.8 mM NADPH, 2 mM glucose-6-phosphate (Sigma), 0.2 U of glucose-6-phosphate

dehydrogenase and 20 μM enzyme substrate hemin (Porphyrin Products) plus 400 μl of sample. The reaction was made up to a final volume of 1 ml for each sample and was incubated at 37°C for 1 h in the dark. Chloroform was added to terminate the reaction, and bilirubin was extracted following centrifugation, and measured by spectrophotometry reading the difference in absorbance between 464 and 530 nm ($\varepsilon$=40 $mM^{-1}$ $cm^{-1}$). The protein concentration in each sample was determined with the BCA Protein Assay Reagent Kit (Pierce Biotechnology), and the HO-1 activities were expressed in picomoles of bilirubin formed per milligram of protein per hour.

### Nrf2 Localization and immunofluorescence

Quiescent VSMCs were treated with curcumin for 6 h and then cultured with DMEM containing 5% FBS for 24 h. The cells were then washed in PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and washed again in PBS. To visualize the nuclei, cells were then treated with 1 μg/ml of DAPI for 30 min, washed again with PBS for 5 min, and treated with 50 μl of VectaShield (Vector Laboratories, Burlingame, CA). For localization of Nrf2, rat VSMCs were grown on Lab-Tek II chamber slides and treated as described in figure legends. The cells were treated with 20 μM curcumin for 1 h. Cells were then fixed in formalin and permeabilized with cold acetone. The cells were probed with Nrf2 antibody followed by FITC-labeled secondary antibody (Santa Cruz Biotechnology). Stained cells were visualized and photographed using a Zeiss fluorescence microscope (Provis AX70; Olympus Optical Co., Tokyo, Japan). Apoptotic cells were morphologically defined by nuclear shrinkage and by chromatin condensation or fragmentation.

## Results

### Effects of curcumin and tetrahydrocurcumin on HO-1 expression and heme oxygenase activity in rat VSMCs

Curcumin has previously been reported to increase

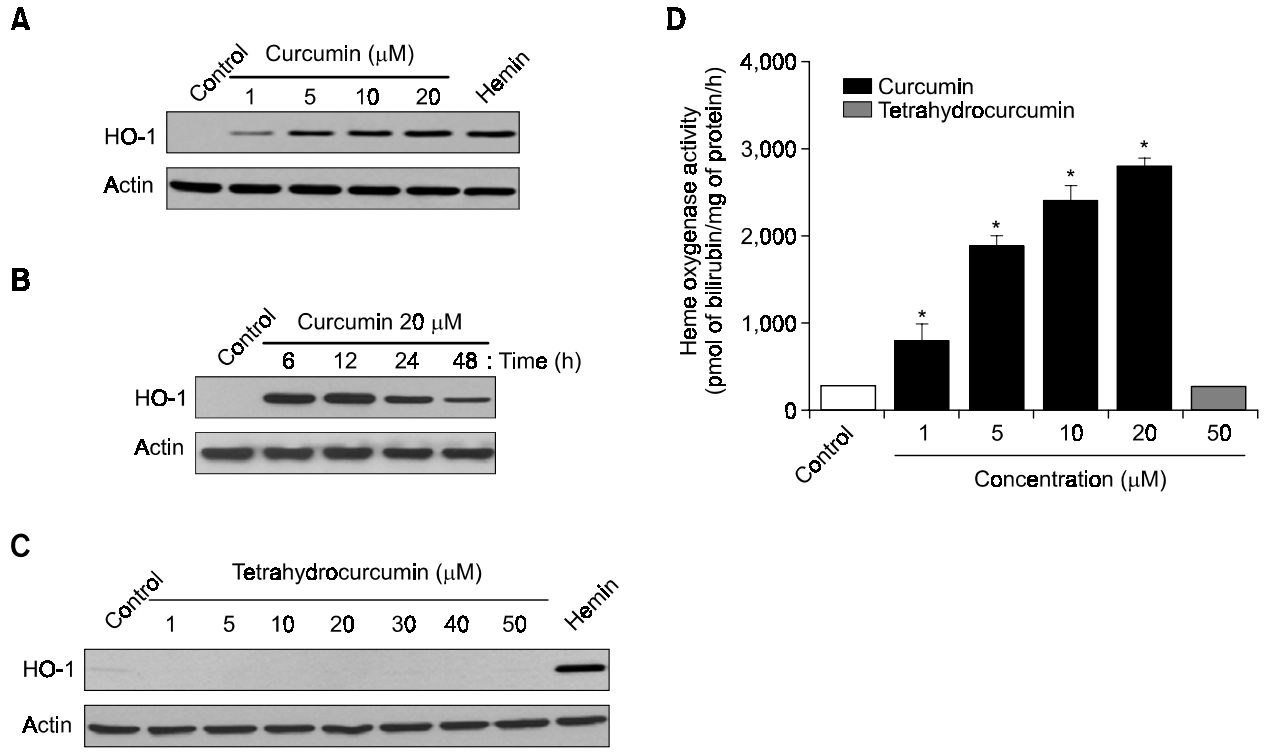


**Figure 2** Effects of curcumin and tetrahydrocurcumin on HO-1 expression and HO activity in rat VSMCs. Quiescent cells were incubated for 6 h with indicated concentrations of curcumin (A and D) or tetrahydrocurcumin (C and D). (B) Quiescent cells were incubated for indicated periods with 20 μM of curcumin. Expression of HO-1 (A, B and C) was determined by Western blot analysis described under Materials and Methods, and representative blots of 3 independent experiments are shown. Heme oxygenase activity (D) was determined via bilirubin formation as described under Materials and Methods. Data shown are mean ± SEM of 6 independent observations in separate cell culture wells. *$P < 0.05$, treatment vs. control, one-way ANOVA and Bonferroni's multiple comparison test.

HO-1 expression (Scapagnini *et al*., 2002; Balogun *et al*., 2003; Jeong *et al*., 2006). However, this effect has not been reported in rat VSMCs to date. In rat VSMCs, curcumin dose-dependently increased HO-1 expression (Figure 2A) and heme oxygenase activity (Figure 2D). A marked increase in HO-1 levels was first observed at 6 h after treatment, with subsequent decrease thereafter (Figure 2B). Despite displaying a similar basic chemical structure (see chemical structure shown in Figure 1), the metabolite tetrahydrocurcumin did not induce HO-1 expression even at higher concentrations (Figure 2C). It should be noted that HO-1 expression and heme oxygenase activity were analyzed in non-cytotoxic concentrations of each tested compound. At 40 μM, curcumin, but not tetrahydrocurcumin, induced apoptosis of VSMCs (see Figure 4C), and curcumin concentrations ranging 1-20 μM were, therefore, selected for further studies.

### Effects of curcumin and tetrahydrocurcumin on Nrf2 nuclear localization and ARE activation in rat VSMCs

Exposure of cells to the naturally occurring antioxidants possessing Michael-reaction acceptor disrupts the Keap1-Nrf2 complex, allowing Nrf2 to translocate into the nucleus, where it binds to ARE and activates transcription (Balogun *et al*., 2003). Fluorescence microscopy of rat VSMCs revealed the nuclear localization of Nrf2 in response to curcumin possessing two Michael-reaction acceptors (Figure 3A). Western blot analysis of the nuclear fractions of curcumin-treated cells showed a gradual increase in Nrf2 levels with time, while they declined concomitantly in the cytoplasm (Figure 3B).

Rat VSMCs transiently transfected with ARE-luciferase plasmid were exposed to either curcumin or tetrahydrocurcumin, and changes in luciferase activity were used as a measure of ARE activation. Figure 3C shows that curcumin dose-dependently increased ARE-driven luciferase activity; this ARE activation was strongly associated with an increase in heme oxygenase activity (Figure 2D). Unlike curcumin, tetrahydrocurcumin lacking Michael-reaction acceptor had no significant effect on ARE activation (Figure 3C).

### Effects of curcumin and tetrahydrocurcumin on proliferation and $p21^{WAF1/CIP1}$ expression in rat VSMCs

It has been reported that curcumin has an ability to inhibit VSMC proliferation (Yang *et al*., 2006). We

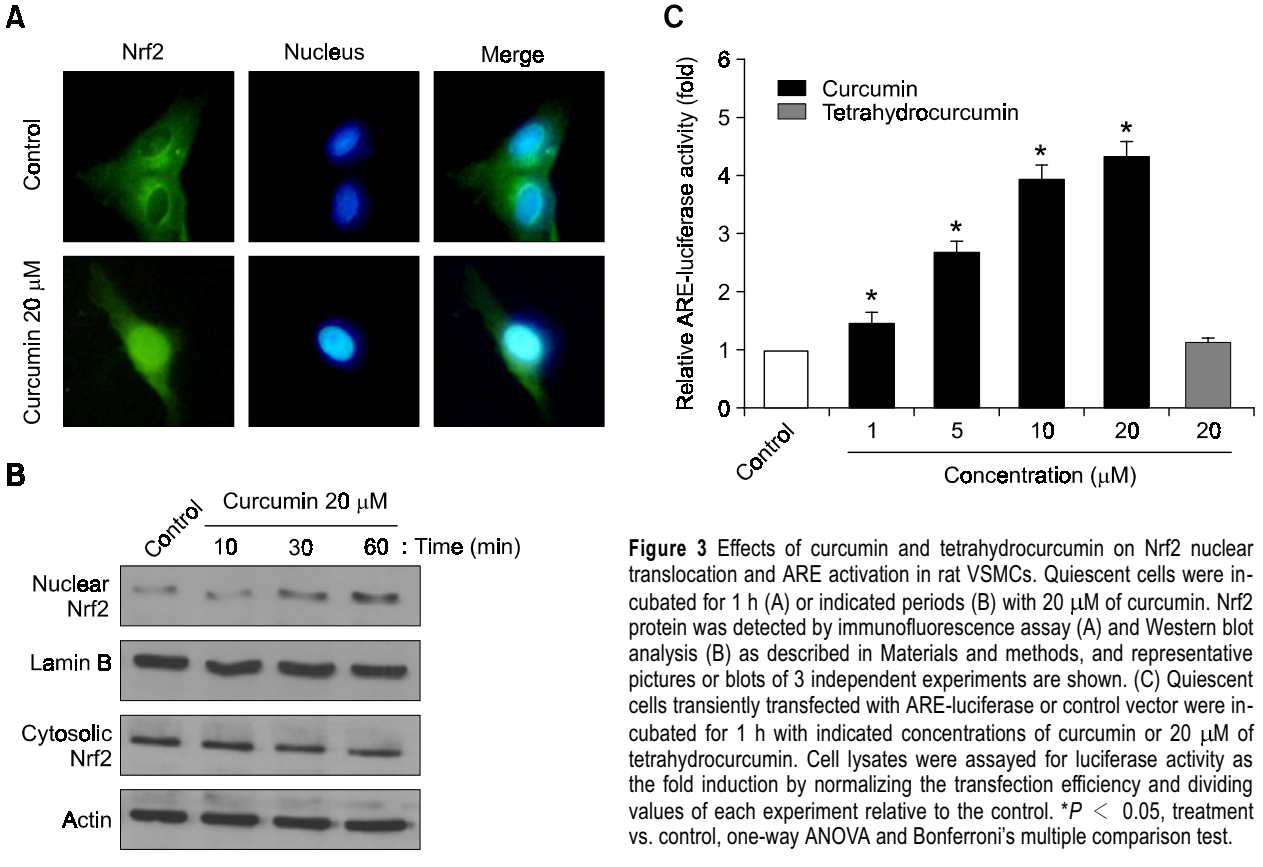


**Figure 3** Effects of curcumin and tetrahydrocurcumin on Nrf2 nuclear translocation and ARE activation in rat VSMCs. Quiescent cells were incubated for 1 h (A) or indicated periods (B) with 20 μM of curcumin. Nrf2 protein was detected by immunofluorescence assay (A) and Western blot analysis (B) as described in Materials and methods, and representative pictures or blots of 3 independent experiments are shown. (C) Quiescent cells transiently transfected with ARE-luciferase or control vector were incubated for 1 h with indicated concentrations of curcumin or 20 μM of tetrahydrocurcumin. Cell lysates were assayed for luciferase activity as the fold induction by normalizing the transfection efficiency and dividing values of each experiment relative to the control. *$P < 0.05$, treatment vs. control, one-way ANOVA and Bonferroni's multiple comparison test.

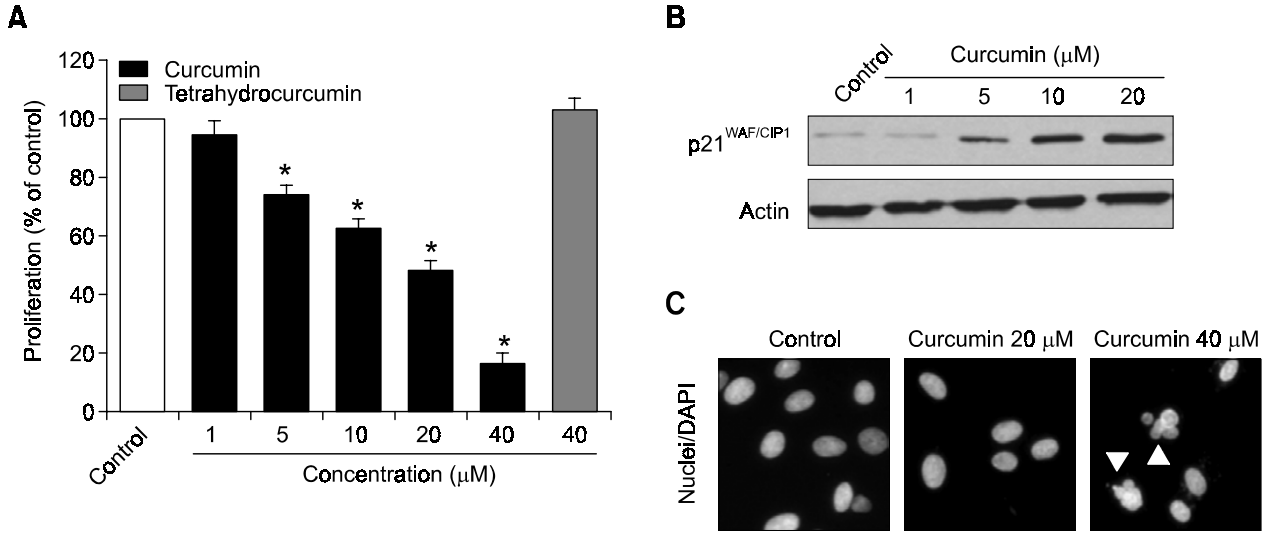


**Figure 4** Effects of curcumin and tetrahydrocurcumin on cellular proliferation, $p21^{WAF/CIP1}$ expression and apoptosis in rat VSMCs. (A) Quiescent cells were treated for 6 h with indicated concentrations of curcumin or 40 μM of tetrahydrocurcumin and then stimulated for 24 h with 5% FBS. The cells were labelled with [$^3$H]-thymidine for 2 h at the end of the incubation. Cellular proliferation was assessed by measuring [$^3$H]thymidine incorporation as described under Materials and Methods. Data shown are mean ± SEM of 6 independent observations in separate cell culture wells. *$P < 0.05$, treatment vs. control, one-way ANOVA and Bonferroni's multiple comparison test. (B) Quiescent cells were incubated for 12 h with indicated concentrations of curcumin. Expression of $p21^{WAF/CIP1}$ was determined by Western blot analysis described under Materials and Methods, and representative blots of 3 independent experiments are shown. (C) Quiescent cells were incubated for 6 h with indicated concentrations of curcumin and then stimulated for 24 h with 5% FBS. Apoptotic nuclei (▶) were analyzed under fluorescence microscope after DAPI staining as described under Materials and Methods, and representative pictures of 3 independent experiments are shown.

thus examined whether tetrahydrocurcumin might also inhibit VSMC proliferation. Surprisingly, tetrahydrocurcumin was not effective in inhibition of VSMC proliferation (Figure 4A). As expected, curcumin inhibited cellular proliferation triggered by 5% serum in a dose-dependent manner (Figure 4A). The antiproliferative effect of curcumin in VSMCs was accompanied by the increased expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (Figure 4B). It should be noted that curcumin at 40 μM caused apoptosis of VSMCs, which was analyzed by morphological examination of apoptotic nuclei stained with DAPI (Figure 4C) and could explain why cellular proliferation was abnormally dropped at this concentration (Figure 4A).

## Roles of HO-1 in curcumin-mediated inhibition of proliferation in rat VSMCs

Because HO-1 expression in VSMCs is antiproliferative (Durante, 2003), we tested whether curcumin-induced HO-1 expression could facilitate its antiproliferative effect. VSMCs were initially pretreated with curcumin at 20 μM for 6 h in the absence or presence of SnPP, an inhibitor of heme oxygenase activity. The cells were then stimulated with 5% serum for 48 h followed by an assessment of cell proliferation. As shown in Figure 5A, SnPP partially reversed the antiproliferative effect of curcumin. In addition, inhibition of VSMC proliferation was also observed when the cells were pre-incubated with hemin, a HO-1 inducer (Figure 5A). CuPP without HO-1-inducing potency was used as a hemin control.

The antiproliferative effects of curcumin and HO-1 are mainly due to their up-regulation of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ that is involved in negative regulation of cellular proliferation (Durante, 2003; Notoya *et al*., 2006). We thus investigated whether HO-1 expression could be necessary for the $p21^{WAF1/CIP1}$ up-regulation by curcumin. Treatment of VSMCs with curcumin increased the levels of $p21^{WAF1/CIP1}$ expression, which was partially abolished by SnPP treatment (Figure 5B). Moreover, $p21^{WAF1/CIP1}$ expression was also observed when the cells were incubated with hemin (Figure 5B). Unlike their effects on $p21^{WAF1/CIP1}$ expression, curcumin and hemin had no effect on the expression of $p27^{KIP1}$ that is another cyclin-dependent kinase inhibitor. It should be noted that tetrahydrocurcumin showed no significant effect on both $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ expressions (data not shown). Our results suggest that, by activating $p21^{WAF1/CIP1}$ expression, HO-1 expression is necessary for the antiproliferative effects of curcumin in rat VSMCs.

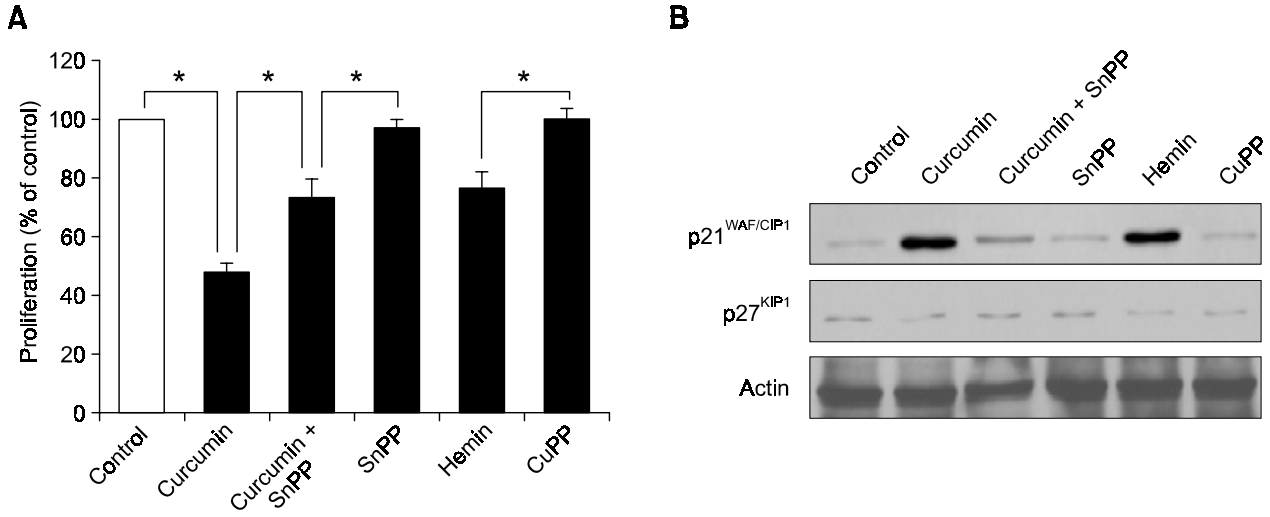


**Figure 5** Roles of curcumin-induced HO-1 in cellular proliferation and $p21^{WAF/CIP1}$ expression in rat VSMCs. (A) Quiescent cells were treated for 6 h with 20 μM of curcumin, curcumin plus 20 μM of SnPP, 20 μM of hemin, or 20 μM of CuPP and then stimulated for 24 h with 5% FBS. The cells were labelled with [$^3$H]thymidine for 2 h at the end of the incubation. Cellular proliferation was assessed by measuring [$^3$H]thymidine incorporation as described under Materials and Methods. Data shown are mean ± SEM of 6 independent observations in separate cell culture wells. *$P < 0.05$, one-way ANOVA and Bonferroni's multiple comparison test. (B) Quiescent cells were incubated for 12 h, as indicated in panel A. Expression of $p21^{WAF/CIP1}$ was determined by Western blot analysis described under Materials and Methods, and representative blots of 3 independent experiments are shown.

### Effects of curcumin on HO-1 expression, TNF-α-induced proliferation, and $p21^{WAF1/CIP1}$ expression in HASMCs

HASMCs were exposed to increasing concentrations of curcumin using the same protocol as for rat VSMCs. HO-1 expression was significantly detectable at 10 μM and 20 μM of curcumin, but not at 20 μM of tetrahydrocurcumin (Figure 6A). A significant increase in HO-1 activity occurred at 20 μM of curcumin (data not shown). In accordance with the results obtained in rat VSMCs, curcumin inhibited cellular proliferation triggered by TNF-α (Figure 6B). This effect was abolished in the presence of SnPP (Figure 6B). In addition, curcumin increased the levels of $p21^{WAF1/CIP1}$ expression in HASMCs (Figure 6C), as observed in the case of rat VSMCs. Moreover, $p21^{WAF1/CIP1}$ promoter activity was also increased as the result of curcumin treatment, which was also abolished in the presence of SnPP (Figure 6D). These results show that curcumin-mediated HO-1 expression and antiproliferation are not restricted to rat VSMCs but occur also in HASMCs.

## Discussion

Curcumin derived from turmeric has been shown to suppress proliferation (Notoya *et al*., 2006; Yang *et al*., 2006) and induce apoptosis (Chen and Huang, 1998) in a wide variety of cells. It also down-regulates the transcription factors, NF-κB (Dorai and Aggarwal, 2004) and AP-1 (Park *et al*., 1999). Thereby, this phytochemical displays antioxidant and anti-inflammatory properties and found to suppress tumor initiation, promotion, and metastasis (Dorai and Aggarwal, 2004). Recently, curcumin has been reported to induce the expression of cytoprotective HO-1 in endothelial cells (Jeong *et al*., 2006), renal epithelial cells (Balogun *et al*., 2003), and astrocytes (Scapagnini *et al*., 2002). To our best knowledge, there is so far no available information as to whether curcumin could also induce HO-1 expression in rat VSMCs. In the present study, we found that curcumin induced HO-1 expression and HO activity in a dose-dependent manner (Figure 2). In contrast, the curcumin metabolite tetrahydrocurcumin lacking Michael-reaction acceptor (chemical structure shown in Figure 1) did not induce HO-1 expression even at higher concentrations. This indicates that two Michael-reaction acceptors (α,β-unsaturated carbonyl group) in curcumin molecule may play a pivotal role in HO-1 expression in rat VSMCs. It is interesting to note that curcumin was also effective in inhibition of VSMC proliferation, but its metabolite was not (Figure 3). Thus, the effects of curcumin on both HO-1 expression and growth inhibition were determined by the same structural feature. It, therefore, appears that the structural feature of curcumin that is essential for its effect on HO-1 expression is also the

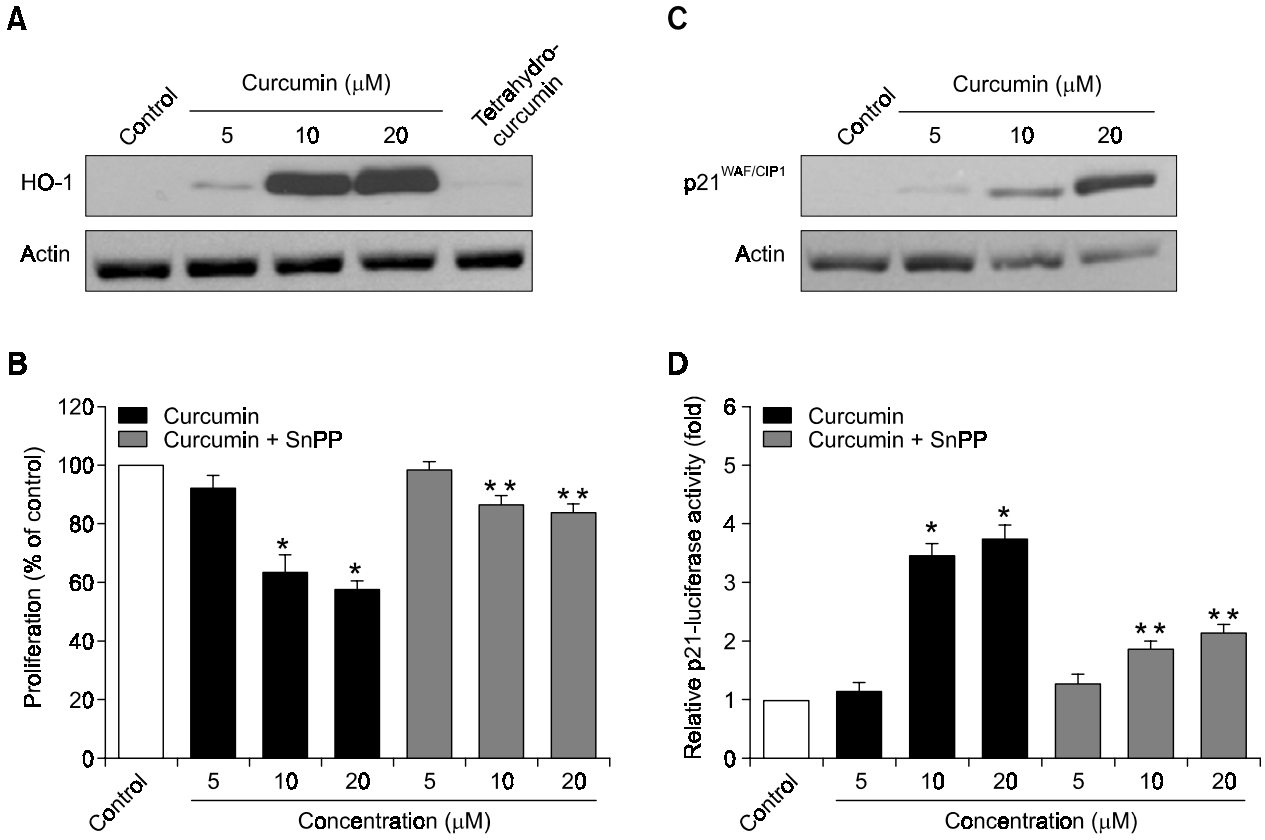


**Figure 6** Effects of curcumin on HO-1 expression, proliferation and $p21^{WAP1/CIP1}$ expression in HASMCs. (A) Quiescent cells were incubated for 6 h with indicated concentrations of curcumin and 20 μM of tetrahydrocurcumin. (B) Quiescent cells were incubated with indicated concentration of curcumin in the presence or absence of 20 μM of SnPP and then stimulated for 24 h with 100 ng/ml of TNF-α. (C) Quiescent cells were incubated for 12 h with indicated concentrations of curcumin. (D) Quiescent cells transiently transfected with $p21^{WAF1/CIP1}$-luciferase or control vector were incubated for 12 h with indicated concentrations of curcumin. Expressions of HO-1 (A) and $p21^{WAF1/CIP1}$ (C) were determined by Western blot analysis described under Materials and Methods, and representative blots of 3 independent experiments are shown. Cellular proliferation (B) was assessed by measuring [3H]thymidine incorporation as described under Materials and Methods. Data shown are mean ± SEM of 6 independent observations in separate cell culture wells. $^{*}P < 0.05$ with respect to control group and $^{**}P < 0.05$ with respect to the group treated with curcumin at each concentration, one-way ANOVA and Bonferroni's multiple comparison test.

one that is important for its antiproliferative activity. This raises an important question of whether the antiproliferative action of curcumin could be mediated via its HO-1 expression. To test this, we employed the HO inhibitor SnPP to block HO activity resulted from curcumin-mediated HO-1 expression. Our results showed that inhibition of VSMC proliferation by curcumin was partially, but not completely, reversed in the presence of SnPP (Figure 5). Moreover, the HO-1 inducer hemin significantly inhibited VSMC proliferation (Figure 5). Our findings, therefore, suggest that HO-1 expression may contribute, to a certain extent, to the antiproliferative effect of curcumin, and are consistent with the recent findings that HO-1 expression inhibits VSMC proliferation (Juan *et al*., 2001).

It is presently unclear how curcumin can induce HO-1 expression in rat VSMCs. The nuclear factor Nrf2 has recently emerged as a key player in the activation of ARE-mediated gene expression. Nrf2 resides in the cytosol, bound to an inhibitor Keap-1. Upon cell stimulation, Nrf2 is released from Keap-1 and enters the nucleus where it binds to the ARE in the promoters of target genes (Srisook *et al*., 2005). HO-1 and several other antioxidant genes contain ARE or ARE-like sequences in their promoters (Srisook *et al*., 2005). It has been reported that curcumin disrupts the Nrf2-Keap1 complex, leading to increased Nrf2 binding to ARE (Banning *et al*., 2005). In porcine renal epithelial cells, curcumin stimulated nuclear translocation of Nrf2 by inactivating the Nrf2-Keap1 complex, which was associated with a significant increase in the activity and expression of HO-1 (Balogun *et al*., 2003). In another study, curcumin increased the nuclear translocation of Nrf2, ARE/DNA binding activity (Andreadi *et al*.,

2006). It is notable that curcumin and other HO-1-inducing compounds possess an $\alpha$, $\beta$-unsaturated carbonyl moiety, and can, therefore, act as Michael-reaction acceptors that are able to modify cysteine thiols present in Keap1 (Surh *et al*., 2005). Based on our findings that tetrahydrocurcumin lacking Michael-reaction acceptors was not able to induce HO-1 expression (Figure 2) and ARE activation (Figure 3), and curcumin possessing two Michael-reaction acceptors induced HO-1 expression, Nrf2 nuclear translocation and ARE activation in rat VSMCs (Figure 3), it is tempting to speculate that HO-1 expression by curcumin might occur via Nrf2-dependent ARE activation in rat VSMCs.

The molecular basis of the antiproliferative action of HO-1 in rat VSMCs remains to be fully elucidated, but the product of heme degradation, such as CO, seems to be responsible for the antiproliferative effects of HO-1. CO interacts with various components of the cell cycle machinery. The exogenous treatment with this gaseous molecule arrests VSMC in the $G_1$/S transition phase of the cell cycle (Duckers *et al*., 2001). The inhibition of cell cycle progression by CO is accompanied with a marked decrease in the phosphorylation of retinoblastoma, a critical event required for S-phase entry and DNA synthesis (Peyton *et al*., 2002). CO also selectively suppresses the expression of cyclin A, while having no effect on the expression of cyclin D1 and E (Peyton *et al*., 2002). In addition, inhibition of cyclin A expression by CO results in inhibition of both cyclin A-associated kinase activity and cyclin-dependent kinase-2 (cdk-2) activity, independent of any changes in the level of cdk-2 protein (Peyton *et al*., 2002). Because cdk2 is a key regulator of both G1 and S phase cell progression (Braun-Dullaeus *et al*., 1998), the ability of CO to block cdk2 activity may provide a potent mechanism by which CO can inhibit VSMC proliferation. Interestingly, CO induces the expression of the cdk inhibitor, $p21^{WAF1/CIP1}$, suggesting that CO may block cdk2 activity via multiple mechanisms (Duckers *et al*., 2001). A functional association between HO-1 and $p21^{WAF1/CIP1}$ in growth regulation is supported by the finding that the antiproliferative action of HO-1 is significantly reduced in VSMCs obtained from $p21^{WAF1/CIP1}$ null mice (Duckers *et al*., 2001). Moreover, VSMCs obtained from HO-1-deficient animals that exhibit excessive proliferation have a corresponding reduction in $p21^{WAF1/CIP1}$ levels compared to wild type mice (Duckers *et al*., 2001). In our study, we found that curcumin, but not tetrahydrocurcumin, increased $p21^{WAF1/CIP1}$ expression in a HO-1-dependent manner (Figure 4B and 5B), suggesting that the antiproliferative effect of curcumin is mediated, at least in part, via HO-1 expression.

Of importance in our study is the finding that HO-1 expression by curcumin was not restricted to rat VSMCs but also occurred in HASMCs. Similar to our results obtained in rat VSMCs, curcumin-induced HO-1 expression led to $p21^{WAF1/CIP1}$ expression and growth inhibition in human smooth muscle cells (Figure 6), as well. These findings may, in part, explain the therapeutic benefits of curcumin for atherosclerosis or restenosis. It should be noted that the anti-restenosis of curcumin was confirmed by a previous *in vivo* evaluation using pig coronary artery stent model (Nguyen *et al*., 2004).

Although we have shown that HO-1 expression by curcumin was involved in inhibition of VSMC proliferation, we do not exclude the possibility that curcumin may also induce the expression of other antiproliferative enzyme(s) and that the observed antiproliferative activity of curcumin may be achieved via the concerted actions of the multiple pathways that may be activated and/or inactivated by this phytochemical. In fact, curcumin has been demonstrated to inactivate NF-$\kappa$B (Dorai and Aggarwal, 2004), AP-1 (Park *et al*., 1999), extracellular signal-regulated kinase (Masamune *et al*., 2006) and PKC (Gopalakrishna and Gundimeda, 2002), all of which are involved in the regulation of VSMC proliferation. In other words, the multifarious effects of curcumin may help explain why inhibition of VSMC proliferation by curcumin was partially mediated via its HO-1 expression.

In summary, our findings suggest that curcumin at non-cytotoxic doses is capable of inducing both Nrf2-dependent HO-1 expression and growth inhibition in rat VSMCs. Neither HO-1 expression nor antiproliferative activity was observed when rat VSMCs were treated with tetrahydrocurcumin, indicating that the conjugated double bonds of the central seven-carbon chain of curcumin may play an important role in its biological activity. In addition, we provided evidence showing that the antiproliferative effect of curcumin is considerably linked to its ability to induce HO-1 expression in rat and human smooth muscle cells. Therefore, curcumin as a vascular HO-1 inducer would be considered as a promising pharmacological agent in the development of therapeutic approaches for the prevention or treatment of vascular obstructive diseases associated with excessive VSMC proliferation.

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