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EVODIAMINE REPRESSES HYPOXIA-INDUCED INFLAMMATORY PROTEINS EXPRESSION AND HYPOXIA-INDUCIBLE FACTOR 1α ACCUMULATION IN RAW264.7

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ABSTRACT—Inflammation and low oxygen diffusion are recognized characteristics of cardiovascular diseases such as atherosclerosis. Evodiamine, extracted from the traditional Chinese herb, *Evodia rutaecarpa*, is a bioactive anti-inflammatory alkaloid. The objective of this study was to investigate whether evodiamine could repress hypoxia-induced inflammatory response. We showed that evodiamine repressed not only COX-2 and iNOS expression but also prostaglandin E_2 release in a concentration-dependent manner under hypoxic conditions. Furthermore, our studies indicated that *COX-2* mRNA was inhibited by evodiamine, implying that transcriptional activity is involved in the mechanistic pathway. It is striking that hypoxia-inducible factor 1α (HIF-1α) inhibitor, camptothecin, suppressed hypoxia-induced COX-2 expression rather than pyrrolidine dithiocarbamate, a nuclear factor κB inhibitor. In addition, our studies have confirmed that evodiamine inhibited HIF-1α, which accounted for the transcriptional activity of COX-2, rather than nuclear factor κB in RAW264.7 cells. Finally, evodiamine did not affect either the level of *HIF-1α* mRNA or the degradation rate of HIF-1α protein, but it regulated the translational process of HIF-1α. We found that hypoxia-evoked phosphorylation of Akt and p70S6K was blocked after evodiamine treatment, in addition to the inhibition of phosphorylation of 4E-BP. These results suggest that the mechanism of repression of hypoxia-induced COX-2 expression by evodiamine is through the inhibition of HIF-1α at the translational level and is primarily mediated via dephosphorylation of Akt and p70S6K. Therefore, evodiamine could be an effective therapeutic agent against inflammatory diseases involving hypoxia.

KEYWORDS—Macrophage, translation, p70S6K

INTRODUCTION

Microenvironmental conditions found in injured tissues are characterized low level of oxygen and by infiltration of monocytes/macrophages (1–3). Now, the presence of hypoxia is a characteristic of various inflamed, diseased tissues, including tumors, atherosclerosis, rheumatoid arthritis, and bacteria infection (4). And, a wide range of inflammatory cells traits, for example, cell surface markers expression, viability, phagocytosis, metabolic activity, adhesion, migration, proteolytic enzymes, and release of cytokines by macrophages, has been reported to be profoundly influenced by hypoxic conditions (4–6). It is reported that hypoxia-inducible factor 1 (HIF-1) activity in inflammation was upregulated under hypoxia through the disrupted interaction between HIF-1 and pVHL (von Hippel-Lindau) (7, 8). Furthermore, it was also demonstrated that HIF-1α is essential for myeloid cell–mediated inflammation in transgenic knockout mice, and hypoxia upregulated a lot of inflammatory genes and activated transcription factor, nuclear factor κB (NF-κB) (7, 9, 10).

Hypoxia-inducible factor 1 is a heterodimeric protein composed of an oxygen-labile HIF-1α subunit and a constitutively expressed HIF-1β subunit. Under hypoxic conditions, the HIF-1α subunit accumulates because of a decrease in the rate of proteolytic degradation. After hydroxylation at specific proline residues by hydroxylase (proline hydroxylase domain [PHD]) and binding to the pVHL tumor suppressor protein, HIF-1α is ubiquitinated and degraded under normoxic conditions (11, 12). In contrast, pVHL cannot recognize HIF-1α protein under hypoxic conditions because it is not hydroxylated at the proline residues (because of the inhibition of PHD activity) under those conditions. Finally, HIF-1α translocates into nuclei and binds to the hypoxic response element of target genes, thus activating transcription of these genes (11, 12). Briefly, because of great consumption of oxygen by aggregated immune cells in inflammatory tissue, PHD is unable to hydroxylate HIF-1α under hypoxia, which resulting HIF-1α cannot be recognized by VHL and degraded by proteasome.

On the other hand, synthesis of HIF-1α protein is modulated by transcription and translation through receptor-mediated signals in an oxygen-independent pathway (13). Initiation of translation in eukaryotic cells is regulated by eukaryotic translation initiation factor 4E (eIF4E)–mediated, cap-dependent process and p70S6K, which phosphorylates 40S ribosomal protein S6 (14, 15). The regulation of eIF4E activity is known to occur via two characterized mechanisms, namely, mitogen-activated protein kinase (MAPK)/4E-BP and phosphoinositide 3-kinase (PI3K)/Akt/4E-BP kinase cascades (13, 16). Interaction with hypophosphorylated 4E-BP (eIF4E-binding protein) disrupts the incorporation of eIF4E into the "cap" structure of mRNA, but hyperphosphorylation of 4E-BP leads to disassociation from eIF4E, which initiates cap-dependent translation (13,

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16–18). It has been reported that upstream effector, the PI3K, phosphorylates both 4E-BP and p70S6K to regulate the translational process, and the other kinase, mammalian target of rapamycin (mTOR), is the alternate signaling pathway (13). These pathways have been implicated in most eukaryotic proteins translation, and HIF-1α is no exception (19).

In a previous study, evodiamine, extracted from *Evodia rutaecarpa*, exhibited anti-inflammation activity through inhibition of reactive oxygen species production and iNOS expression after LPS treatment in activated inflammatory cells (20, 21). Hence, because of the critical role of HIF-1α in inflammation, Ogasawara et al. (22) showed that evodiamine could inhibit metastasis of colon tumor cells mediated by HIF-1α and its downstream genes *in vivo* and *in vitro* (12, 23). In the present study, we explored the mechanism of evodiamine on the inhibition of hypoxia-induced inflammatory response in murine macrophage RAW264.7 cells.

MATERIALS AND METHODS

Materials

Dulbecco modified Eagle medium, fetal bovine serum, antibiotic, and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). Leupeptin, dithiothreitol (DTT), dimethyl sulfoxide, phenylmethylsulfonyl fluoride (PMSF), cycloheximide, pyrrolidine dithiocarbamate (PDTC), and Trizma base were ordered from Sigma Chemical (St Louis, Mo). Evodiamine was purchased from Matsuura Yakugyo Co Ltd (Nagoya, Japan). TRIzol reagent was from Invitrogen (Carlsbad, Calif); random primer and M-MLV RT were from Promega (Madison, Wis); Pro Taq was from Protech (Taipei, Taiwan). Prostaglandin E_2 (PGE_2) assay kit was from R&D Systems (Minneapolis, Minn). Anti–HIF-1β antibody was purchased from BD Biosciences (Franklin Lakes, NJ). Antibodies against iNOS, COX-2, and HIF-1α were purchased from Novus Biologicals (Littleton, Colo). Antibodies against phospho-ERK, phospho-Akt, phospho-4EBP, phospho-eIF4E, and phospho-p70S6K were purchased from Cell Signaling Technology (Beverly, Mass). Nuclear factor κB (p65 subunit), actin, and horseradish peroxidase–conjugated anti-mouse, anti-rat, and anti-rabbit IgG antibodies were ordered from Santa Cruz Biotechnology (Santa Cruz, Calif). Nucleolin antibody was purchased from Sigma (St Louis, Mo).

Cell culture condition

RAW264.7 (murine macrophage cell line) was grown in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin 100 U/mL and streptomycin 100 U/mL) at 37°C in a 5%/95% air atmosphere. For hypoxic exposure, cells were placed in an airtight chamber (BioSpherix, Redfield, NY) flushed with a mixture of 1% O_2, 5% CO_2, and 94% N_2 to maintain O_2 concentration at 1% with ProOxmodel 110 O_2 regulator (BioSpherix).

Western blot and preparation of nuclear fraction

For analysis of protein expression, cells were treated with the indicated agents for different time periods and then harvested in ice-cold lysis buffer. Nuclear proteins were extracted as the following. Briefly, phosphate-buffered saline (PBS)–washed cells were resuspended in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM PMSF, and 0.5 mM DTT). After incubation on ice for 15 min, cells were centrifuged at 3,000 rpm for 5 min, and then pellets were resuspended in buffer C (20 mM HEPES, 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF). After incubation on ice for 20 min, cells were centrifuged at 13,000 rpm for 10 min. The blots were electrophoretically transferred to polyvinylidene difluoride membranes and incubated with antibody in PBS overnight at 4°C. Signal was detected with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, England).

PGE_2 kit assay

RAW264.7 cells were seeded in 96-well plate and incubated overnight. After incubation, the cells were treated with evodiamine of indicated concentration under hypoxia. The PGE_2 assay was determined by ELISA kit (R&D Systems).

Sulforhodamine B assay

For cellular viability assay of RAW264.7, the cells were fixed *in situ* with 10% trichloroacetic acid after cell inoculation, to represent a measurement of the cell population at the time of drug addition. The plates were incubated for the indicated time after drug addition, and the assay was terminated by 10% trichloroacetic acid. Sulforhodamine B (SRB) dye at 0.4% (wt/vol) in 1% acetic acid was added to stain cellular proteins. Unbound dye was removed by repeated washing with 1% acetic acid, and the plates were air-dried. Bound stain was subsequently soluble with 10 mM Trizma base, and the absorbance was read on a microplate reader at a wavelength of 515 nm.

Reverse transcriptase–polymerase chain reaction

RNA was extracted with Trizol reagent by a standard protocol (Invitrogen). Reverse transcription was performed with 5 μg mRNA and random primer at 65°C for 5 min, then mixed with M-MLV RT to react at 37°C for 1 h to obtain cDNA. Gene amplification was followed with reverse transcriptase–polymerase chain reaction (RT-PCR). Primer sequence was as described: *COX-2* sense, 5′-GGAGAGACTATCAAGATAGT-3′; *COX-2* antisense, 5′-ATGGTCAGTAGACTTTTACA-3′; *HIF-1α* sense, 5′-GTCTGGGTTGAAAC TCAAGCAACTG-3′; *HIF-1α* antisense, 5′-GGTTTGAGCACAGATTCTGT TTGTT-3′; *GAPDH* sense, 5′-TGATGACATCAAGAAGGTGGTGAAG-3′;

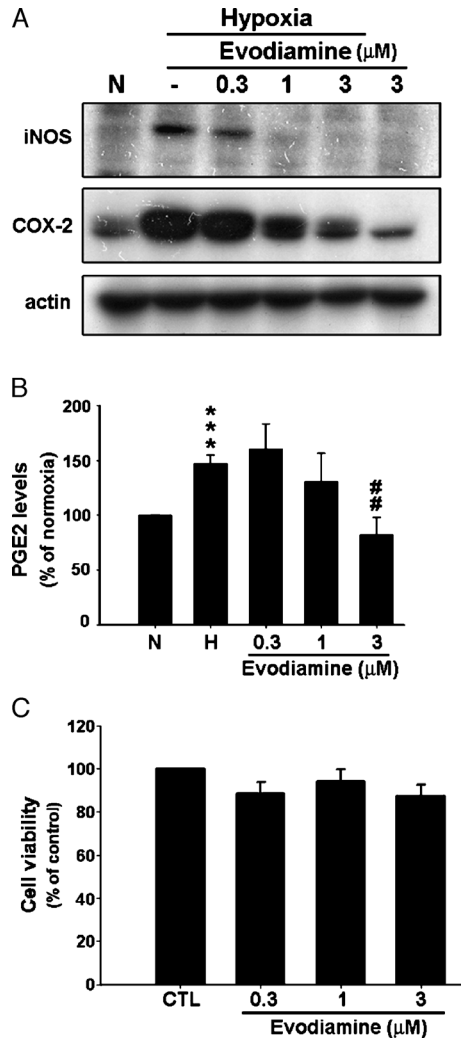

FIG. 1. **Effect of evodiamine on hypoxia-induced inflammatory response**. RAW264.7 cells were incubated in the presence or absence of indicated concentration of evodiamine for 1 h and then incubated in normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. A, Equal amount of total proteins was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis to detect COX-2, iNOS, and actin expressions by Western blotting analysis. B, The culture medium was assayed by PGE_2 ELISA kit. C, Cellular viability was assayed by SRB assay. N indicates normoxia; H, hypoxia, respectively. $^{***}P < 0.001$ compared with normoxic cells, and $^{\#\#}P < 0.01$ compared with hypoxic cells. The representative result of three separate experiments is shown.

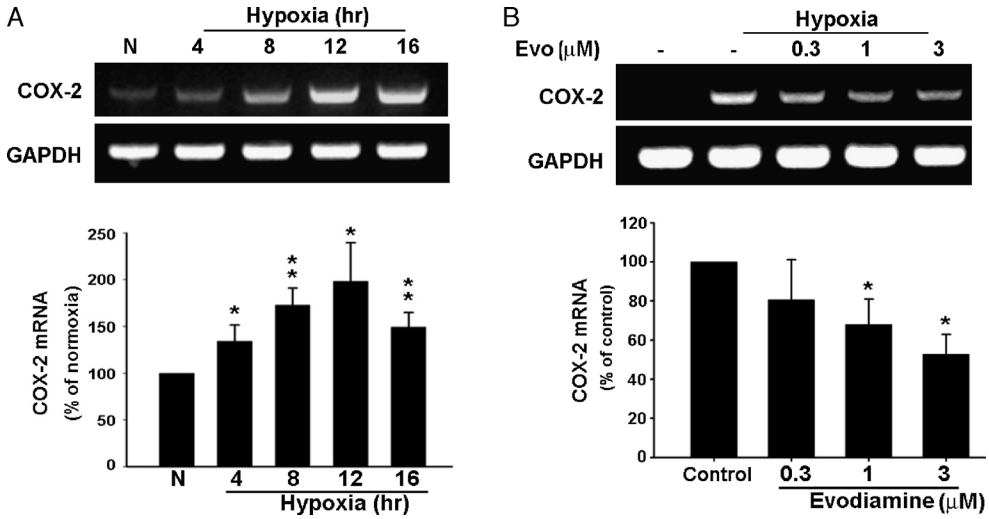

FIG. 2. **Effect of evodiamine on hypoxia-induced *COX-2* mRNA**. A, RAW264.7 cells were incubated in the indicated time for *COX-2* mRNA expression by RT-PCR analysis. B, RAW264.7 cells were incubated in the presence or absence of indicated concentration of evodiamine for 1 h and then incubated in normoxia (21% O_2) or hypoxia (1% O_2) for 8 h. The expression of *COX-2* mRNA was detected by RT-PCR, and GAPDH was an internal control. N indicates normoxia. $^*P < 0.05$ compared with vehicle-treated hypoxic cells. The representative result of three separate experiments is shown.

GAPDH antisense, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′. Reaction cycles for *COX-2*, *HIF-1α*, and *GAPDH* includes 35 cycles of 94°C for 30 s, 55°C for 30 s (50°C for *HIF-1α*), 72°C for 1 min, and a final incubation at 72°C for 10 min. Polymerase chain reaction products were analyzed on 1.5% agarose gel in the presence of 1 μg/mL of ethidium bromide.

Statistical analysis

All data are represented as mean ± SEM. An unpaired Student *t* test was used to compare same data. $P < 0.05$ was considered statistically significant for all comparisons.

RESULTS

Inhibition of hypoxia-induced COX-2 expression in RAW 264.7

Both COX-2 and iNOS are known to play key roles in physiological and pathological actions, such as inflammation. It was reported that hypoxia could result in tissue inflammation and activation of iNOS transcription (24); hence, we investigated whether evodiamine would repress hypoxia-induced COX-2 and iNOS expression in RAW 264.7 cells. Our experiments indicate that, as compared with normoxia (21% O_2), hypoxia (1% O_2) significantly induced COX-2 and iNOS expressions, and evodiamine inhibited COX-2 and iNOS expression in a concentration-dependent manner (A). In addition, it also repressed the PGE_2 level (Fig. 1B). To exclude the cytotoxicity of evodiamine, we conducted the SRB assay to evaluate cellular viability after treatment with evodiamine. We found that the cellular viability was not significantly affected by evodiamine (Fig. 1C). However, it seemed that hypoxia was unable to increase NO release (see Figure, Supplemental Digital Content 1, http://links.lww.com/SHK/A11).

Furthermore, we found a significant induction of *COX-2* mRNA at the fourth-hour period of hypoxia, which peaked between the 8th- and 12th-hour period (Fig. 2A), and the level of *COX-2* mRNA decreased after the treatment with evodiamine (Fig. 2B).

Effect of evodiamine on HIF-1α and NF-κB

Nuclear factor κB is a critical transcriptional factor involved in the activation of COX-2 transcription, and evodiamine has been implicated in inhibiting constitutive and 12-myristate 13-acetate (PMA)-induced NF-κB nuclear translocation (25). As demonstrated in our previous study (26), we noted that hypoxia

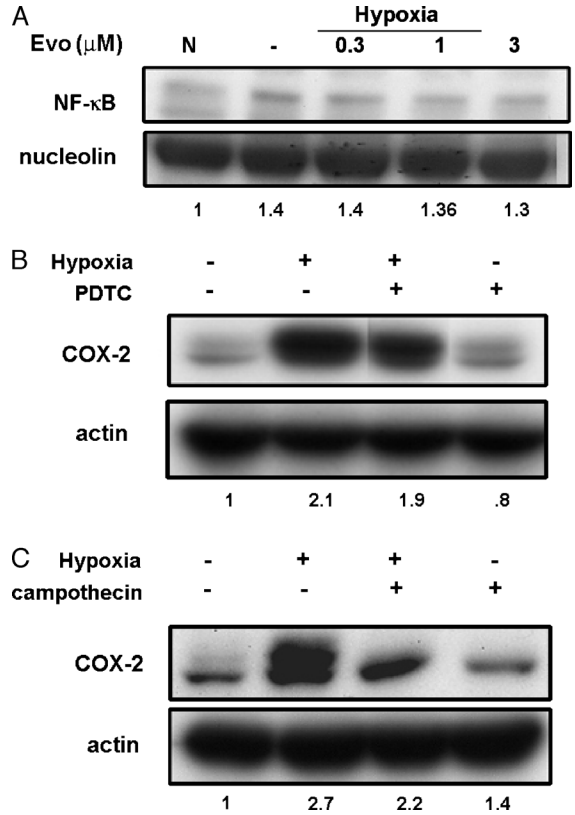

FIG. 3. **The role of NF-κB and HIF-1α on COX-2 expression**. A, RAW264.7 cells were incubated in the presence or absence of indicated concentration of evodiamine for 1 h and then incubated in normoxia (21% O_2) or hypoxia (1% O_2) for 6 h. Nuclear extracts were collected to detect nuclear translocation of NF-κB and analyzed by Western blotting analysis. Nucleolin was used as the loading control. Effect of 20 μM PDTC (B) and 1 μM campothecin (C) on hypoxia-induced COX-2 expression. Cells were incubated in the presence or absence of indicated concentration of evodiamine for 1 h and then incubated in normoxia or hypoxia for 24 h. Protein expressions were evaluated using Western blot analysis. The representative result of three separate experiments is shown.

moderately promoted the nuclear translocation of NF-κB, but its reversal by evodiamine was not significant in RAW264.7 cells (Fig. 3A). Pyrrolidine dithiocarbamate, a known NF-κB inhibitor, was used in the study to exclude the involvement of NF-κB. Consistent with previous results, we did not observe the significant reversal after 20 μM PDTC treatment (Fig. 3B). It has been reported recently that both LPS and hypoxia could induce expressions of many similar genes (10), and HIF-1α is postulated to play a critical role in the signaling pathway. Hence, we assessed the effect of HIF-1α on the COX-2 expression in our study. Interestingly, HIF-1α inhibitor, camptothecin, repressed hypoxia-induced COX-2 expression (Fig. 3C). This led us to conclude that the inhibition of hypoxia-induced COX-2 expression by evodiamine was dependent on decreasing HIF-1α level rather than NF-κB.

Inhibition of hypoxia-induced HIF-1α expression

The results obtained in this study demonstrated that HIF-1α accumulation increased in the second hour after hypoxia, and evodiamine had a greater inhibition on HIF-1α at the eighth hour (see Figure, Supplemental Digital Content 2, http://links.lww.com/SHK/A12), and it also significantly decreased hypoxia-induced HIF-1α expression in a concentration-dependent manner (Fig. 4A). The expression of HIF-1α as well as in the case of most proteins is strictly regulated by protein biosynthesis and degradation. Hence, we investigated the effect of evodiamine on the rate of degradation of HIF-1α protein. A proteasome inhibitor, MG132, was used to block proteasome-mediated degradation in the experiment. However, we found that MG132 did not reverse evodiamine-mediated decline of HIF-1α protein under hypoxia (Fig. 4B). Therefore, the repression of HIF-1α by evodiamine is independent of proteasome-mediated degradation. Because HIF- 1α is an oxygen-labile protein with a short half-life, we investigated whether evodiamine could enhance the degradation rate of HIF-1α after treatment with cycloheximide, a protein translation inhibitor. Our data indicated that the degradation rate of HIF-1α protein was not promoted by evodiamine (Fig. 4D). Next, we then investigated whether the subunit of E3 ubiquitin ligase protein, pVHL, was affected by evodiamine treatment. Our results showed that pVHL expression was not increased after evodiamine treatment (Fig. 4C). Based on the aforementioned results, we can conclude that evodiamine does not promote degradation of HIF-1α in this study. In addition, our data showed that the mRNA level of *HIF-1α* was not affected by evodiamine, leading to the conclusion that the transcription process was not involved in the hypoxia-induced HIF-1α expression (Fig. 4E).

Translation machinery involved in the inhibition of HIF-1α expression by evodiamine

The results obtained showed that neither the degradation nor the transcription process was involved in the inhibition of hypoxia-induced HIF-1α expression by evodiamine. The next logical step was to clarify whether translational processes play a role in it. To determine whether downregulation of HIF-1α expression found in RAW264.7 cells exposed to evodiamine was due to alterations in the translation, we tested the translation regulatory proteins, eIF4E, 4E-BP, and p70S6K. Using specific phospho-p70S6K antibody, we observed that hypoxia-evoked phosphorylation developed at the threonine 389 residue after 30 min under hypoxia (Fig. 5A). As shown in Figure 5A, it is obvious that evodiamine prevented hypoxia-induced phosphorylation of p70S6K (Thr389) at each indicated time point. In addition, the translational repressor protein, 4E-BP1, was slightly dephosphorylated during the 4- to 8-h period after evodiamine treatment under hypoxic conditions (Fig. 5B). Furthermore, we also observed that evodiamine did not have a significant effect on the phosphorylation status of eIF4E (Ser 209)

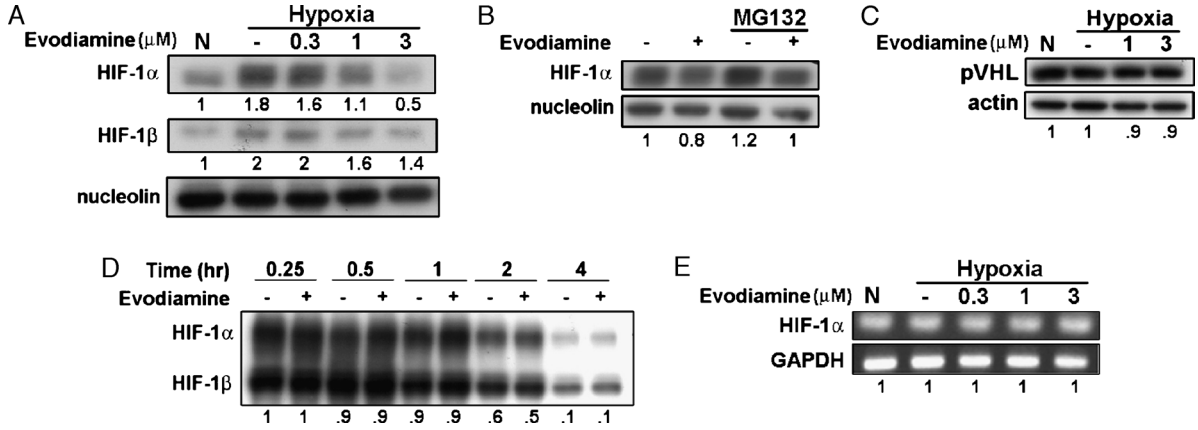

FIG. 4. **Effects of evodiamine on degradation and transcription of HIF-1α protein**. A, Cells were incubated in the presence or absence of indicated concentration of evodiamine for 1 h and then incubated in normoxia (21% O_2) or hypoxia (1% O_2) for 8 h. B, Cells were incubated in the presence or absence of evodiamine (3 μM) and MG132 (1 μM) for 1 h and then incubated under hypoxia. C, Cells were incubated in the presence or absence of indicated concentration of evodiamine for 1 h and then incubated in normoxia or hypoxia for 6 h. Total proteins were analyzed for pVHL expression. D, Cells were treated with 100 μM $CoCl_2$ overnight and followed by treatment with cycloheximide (30 μg/mL) and evodiamine (3 μM) simultaneously for evaluation of protein stability. Nuclear extracts were collected at the indicated time and analyzed using Western blotting analysis. Nucleolin and actin were used as the loading control. E, RAW264.7 cells were incubated in the presence or absence of indicated concentration of evodiamine for 1 h and then incubated in normoxia or hypoxia for 8 h. The expression of *HIF-1α* mRNA was detected by RT-PCR, and GAPDH was an internal control. N indicates normoxia. The representative result of three separate experiments is shown.

(Fig. 5B). Hence, we concluded that the repression of phosphorylation of p70S6K by evodiamine under hypoxia contributed to the downregulation of HIF-1α.

Delineation of the role of PI3K/Akt pathway and MAPK in translational regulation

It was known that most eukaryotic translational processes are mediated through MAPK and PI3K/Akt signaling pathways; thus, we investigated the possibility that p44/p42 MAPK (ERK1/2) and protein kinase B (Akt) could be involved in these pathways. Our data indicated that ERK1/2 was constitutively phosphorylated under normoxia (21% O_2), but the level of phospho-ERK1/2 was not affected under hypoxia (Fig. 6A). Also, we did not observe any changes at each indicated time after evodiamine treatment, implying that the MAPK signaling pathway did not play a critical role in the regulation of HIF-1α by evodiamine. However, we found that Akt was phosphorylated at serine 473 residues under hypoxia, and it was repressed in the presence of evodiamine (Fig. 6A). Recently, it was reported that inhibition of PI3K/Akt would promote the degradation of HIF-1α by diminishing the expression of chaperone (27). However, evodiamine failed to reduce the expression of the molecular chaperones heat shock protein (Hsp) 70 and Hsp90 under hypoxia (Fig. 6B), which corresponds with aforementioned data (Fig. 4D). Furthermore, we evaluated whether the PI3K inhibitor, LY294002, was able to blunt HIF-1α and COX-2 expression. As shown, inhibition of PI3K/Akt resulted in the reduction of HIF-1α accumulation and COX-2 expression under hypoxia (Fig. 6, C and D).

DISCUSSION

Inflammation and hypoxia are characteristic conditions of chronic vascular diseases, such as atherosclerosis, secondary to a dramatic accumulation of macrophage and a rapid consumption of oxygen. Hypoxia-inducible factor 1α accumulation under hypoxia is recognized as the driving force for inflammatory cytokine release, and the process is significantly reduced after functional knockout of HIF-1α (2, 7). Recently, evodiamine, one of the bioactive constituents of *E. rutaecarpa*, has been reported to have a profound anti-inflammatory effect on LPS-induced NO production via repression of NF-κB activation in RAW264.7 cells (21, 25). In this study, we demonstrated that evodiamine repressed hypoxia-induced inflammatory protein expressions (COX-2, iNOS, and PGE_2 via decreasing COX-2 transcription. Furthermore, we also observed camptothecin, as well as evodiamine, repressed hypoxia-induced COX-2 expression instead of PDTC, which indicated that HIF-1 should play a prominent role on hypoxia-induced inflammatory protein expression. In addition, we found that evodiamine did not affect the levels of *HIF-1α* mRNA

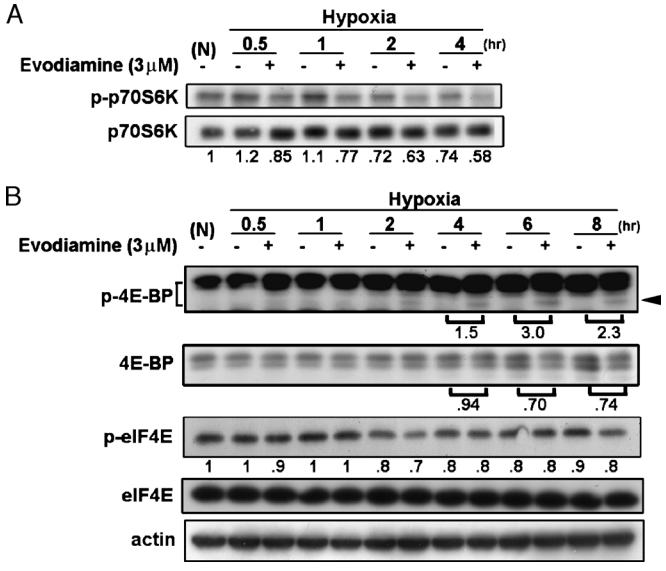

FIG. 5. **Effect of evodiamine on translational mediators**. RAW264.7 cells were incubated in the presence or absence of 3 μM evodiamine for the indicated times under hypoxia. Protein expressions for p70S6K (A), 4E-BP, and eIF-4E (B) were evaluated using Western blotting analysis. N indicates normoxia. Arrowhead indicates the hypophosphorylation of 4E-BP. The representative result of three separate experiments is shown.

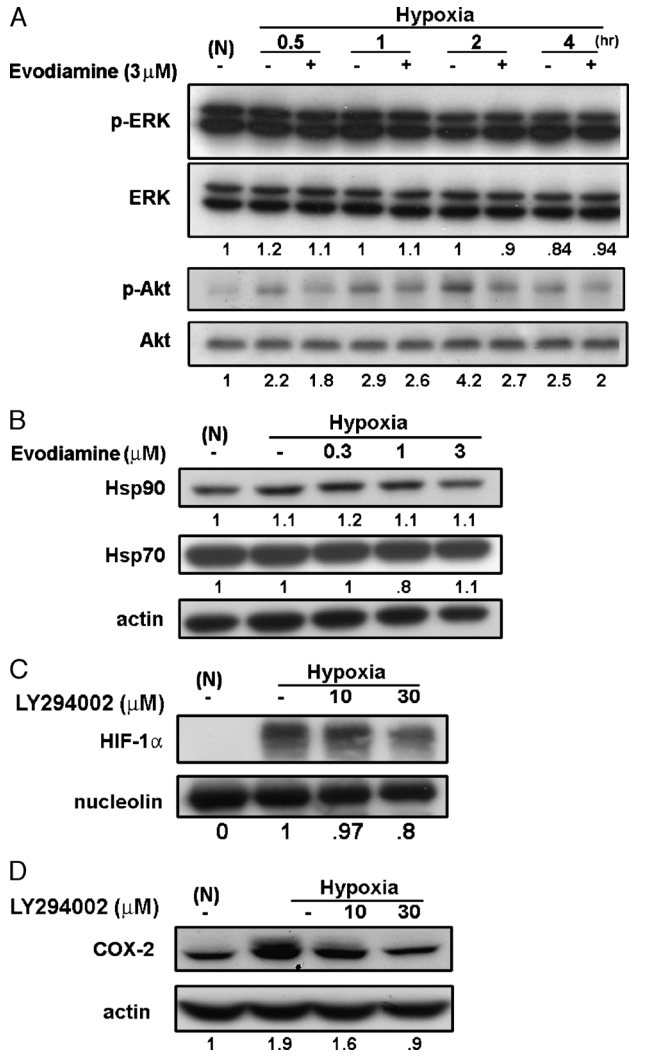

FIG. 6. **Effect of evodiamine on the activation Akt and ERK**. RAW264.7 cells were incubated in the presence or absence of 3 μM evodiamine for the indicated times (A) or 8 h (B) under hypoxia. Cells were pretreated with LY294002 (10 – 30 μM) for 1 h and then incubated for another 8 h for the detection of HIF-1α (C) or 24 h for the detection of COX-2 (D) under hypoxia. Protein expressions were evaluated using Western blotting analysis. N indicates normoxia. The representative result of three separate experiments is shown.

or its half-life, but it decreased HIF-1α accumulation through dephosphorylation of Akt and p70S6K, indicating that evodiamine inhibited HIF-1α via downregulating translational machinery.

It has been presented that NF-κB activation played an important role in TNF-α–induced HIF-1α accumulation through downregulation of HIF-1α degradation under normoxia in LLC-PK_1 cells (28). Furthermore, HIF-1α–evoked transcription of target genes did not increase in NF-κB–knockout PC-3 cells under hypoxia, which indicated that NF-κB participated in HIF-1α regulation, but the mechanism was unknown. Interestingly, Takada et al. (25) have demonstrated that evodiamine completely abolished constitutive and TNF-α–induced NF-κB activation at low concentration of 0.1 μM in KBM-5 cancer cells. However, we did not observe this significant effect on hypoxia-evoked NF-κB translocation after evodiamine treatment at concentrations of approximately 1 to 10 μM. The results indicated that NF-κB activation is not the principal reason for inducing COX-2 expression in RAW264.7 cells under hypoxia, and the regulation of HIF-1α by NF-κB activation would be cell-type specific.

Protein levels are generally regulated by protein synthesis and degradation. Degradation of HIF-1α protein was mainly manipulated by proteasome, and hydroxylation at proline 402 and 564 residues of HIF-1α is necessary for binding of pVHL to HIF-1α and subsequent proteasome-mediated degradation of HIF-1α (8, 11, 13). However, pretreatment of MG132 cannot reverse the repression of HIF-1α by evodiamine. In recent years, Hsp70 and Hsp90 expressions have been reported to regulate pVHL-independent degradation of HIF-1α through PI3K/Akt pathway in *pVHL*-deficient RCC4 renal carcinoma cells, which show constitutively expressed HIF-1α (27). Although the results constitutively demonstrated that evodiamine is capable of inhibiting hypoxia-induced phosphorylation of Akt, and HIF-1α expression is downregulated after treatment with PI3K/Akt inhibitor, the levels of Hsp70 and Hsp90, as well as pVHL, are not altered by evodiamine. It is known that pVHL-independent HIF-1α degradation mostly occurs in *pVHL*-deficient renal cancer cell lines, but not other cells. We examined whether *HIF-1α* mRNA was altered by evodiamine, and the result indicated that repression of HIF-1α protein by evodiamine was not through suppression of *HIF-1α* mRNA, which demonstrated that evodiamine does not interfere with transcription.

In the present study, neither HIF-1α protein half-life nor mRNA level was affected by evodiamine. These observations are consistent with the hypothesis that the evodiamine-dependent decline in HIF-1α accumulation is due to downregulation of *HIF-1α* mRNA translation. It is known that proliferating cells respond to growth factor–induced proliferative and survival signals, which, in turn, increase HIF-1α expression in most circumstances (12). Among these signaling pathways, MAPK and PI3K pathways are critically responsible for the HIF-1α translation in eukaryotes (12). Besides regulating HIF-1α translation, activation of p44/p42 MAPK is also required for HIF-1α transcriptional activity rather than HIF-1α stability (29–32). In our study, we found that the phosphorylated status of p44/p42 MAPK is not changed under hypoxia compared with normoxia, and evodiamine did not inhibit its phosphorylation either. Hence, it indicates that p44/p42 MAPK is not involved in the downregulation of HIF-1α by evodiamine.

It is well documented in the literature that PI3K/Akt signal plays an important role in LPS-induced inflammatory mediators and mediates the longitudinal PI3K/Akt/p70S6K signal (12, 33, 34). Several studies have demonstrated the role played by Akt in HIF-1α induction, linking Akt signal to an increased rate of HIF-1α protein synthesis (13, 26, 35). It is plausible that activated Akt could phosphorylate downstream signal molecules including p70S6K and 4E-BP, which, in turn, activate 40S ribosomal protein S6 and regulate the initiation of cap-dependent protein translation (34, 36). Both inhibition of Akt by a PI3K inhibitor, LY294002, and overexpression of dominant-negative mutant of Akt significantly turned down HIF-1α induction under hypoxia (26). Similarly, HIF-1–dependent gene transcription was blocked by overexpression of wild-type PTEN (phosphotase and tensin homolog), a negative modulator of PI3K/Akt (27, 37). Consistent with this, our results demonstrate that evodiamine diminishes HIF-1α accumulation through inhibition of PI3K/Akt signaling pathway.

In conclusion, evodiamine inhibits hypoxia-induced COX-2 expression and HIF-1α accumulation by blocking the phosphorylation of Akt and p70S6K in RAW264.7 macrophages, thus establishing a novel mechanism for anti-inflammatory action.

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