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Angiotensin II bi-directionally regulates cyclooxygenase-2 expression in intestinal epithelial cells

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Abstract

We previously demonstrated that angiotensin II (Ang II) receptor signaling is involved in azoxymethane-induced mouse colon tumorigenesis. To clarify the role of Ang II in COX-2 expression in the intestinal epithelium, the receptor subtype-specific effect on COX-2 expression in a rat intestinal epithelial cell line (RIE-1) has been investigated. Ang II dose- and time-dependently increased the expression of COX-2, but not COX-1 mRNA and protein. This stimulation was completely blocked by the AT_1 receptor antagonist but not the AT_2 receptor antagonist. Ang II and lipopolysaccharide (LPS) additively induced COX-2 protein in RIE-1 cells, whereas the LPS-induced COX-2 expression was significantly attenuated by low concentrations of Ang II or the AT_2 agonistic peptide CGP-42112A only in AT_2 over-expressed cells. These data indicate that Ang II bi-directionally regulates COX-2 expression via both AT_1 and AT_2 receptors. Control of COX-2 expression through the Ang II signaling may have significance in cytokine-induced COX-2 induction and colon tumorigenesis.

Keywords

AT_1 receptor; AT_2 receptor; lipopolysaccharide; cyclooxygenase-2; intestinal epithelium

Introduction

Prostaglandins (PGs) play an important role in a broad range of physiological processes. Alterations in its production are implicated in pathophysiological processes, including angiogenesis, cancer, acute and chronic inflammation, etc. (1,2). Cyclooxygenase (COX) is the rate limiting enzyme catalyzing the conversion of arachidonic acid into prostaglandins and others eicosanoids, critically implicated in a variety of physiological and pathophysiological processes, including inflammation, vascular and renal homeostasis, and immune responses (3). Since the early 1990s, the existence of two isoforms of cyclooxygenases (COX-1 and COX-2) has been appreciated. COX-1 and COX-2 are recognized as constitutive and inducible COX isoenzymes, respectively. Conventional findings show that the therapeutics effects of non-steroidal anti-inflammatory drugs (NSAIDs such as aspirin, ibuprofen) are mostly dependent on COX-2 inhibition; therefore, agents that selectively inhibit COX-2 over COX-1 are desirable for treatment of inflammation (4). Increased expression of COX-2 and

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prostaglandins has been widely observed in human (5) and rodent intestinal tumors (6,7)] when compared with normal adjacent mucosa, whereas COX-1 expression is not differentially expressed. Among the consequences of elevated COX-2 expression and increased prostaglandin production are stimulation of angiogenesis, inhibition of apoptosis, and stimulation of cellular proliferation, all of which favor tumorigenesis (8).

Colorectal cancer is a major cause of death in developed countries, even though its prognosis has improved yearly due to advances in diagnostic and surgical techniques. Many reports indicate that the intake of NSAIDs decreases the risk of developing colorectal cancer in man (9) and in model animals (10,11). Inhibitors of COX-2 activity such as aspirin, NSAIDs, and specific COX-2 antagonists significantly reduce the occurrence of colorectal carcinoma in both animal and human studies (12). Studies on the mechanism by which NSAIDs decrease the risk of colorectal cancer suggests that inhibition of prostaglandin synthesis by cyclooxygenase-2 (COX-2) is the major mechanism in this chemoprevention (13,14). Therefore, the ideal procedure in the prevention of colorectal cancer is to keep prostaglandin levels low. However, few studies have reported on the endogenous negative regulation of prostaglandin production except with the use of nonspecific drugs such as dexamethasone (15)].

It has been suggested that angiotensin-converting enzyme inhibitors which are commonly employed in the treatment of human clinical hypertension also attenuate tumor growth in experimental animals (16–19)] and may reduce the risk of several human cancers (20). Angiotensin-converting enzyme inhibitors block the formation of angiotensin II (Ang II), an octapeptide which exerts the many diverse effects of the renin-angiotensin system through its receptors (21). This suggests that Ang II may have a modulating role in neoplasia. We recently demonstrated that Ang II type 2 receptor (AT_2)-mediated signaling is involved in azoxymethane-induced mouse colon tumorigenesis (22). Ang II, the effector of the renin-angiotensin system, has been shown to stimulate the release of prostaglandins in a variety of cells, including intestinal epithelial cells (23,24)]. Ang II-dependent increase of prostaglandin production has shown to be mediated through its type 1 receptor (AT_1) via the stimulation of COX-2 induction in intestinal cells (25). Although AT_1 receptor is believed to mediate most of the cardiovascular actions of Ang II (26), expression of AT_2 in the intestinal tissue is also noted (27–29). Since AT_2 often counteracts the AT_1-mediated Ang II signaling (30,31), if AT_2 signaling attenuates COX-2 induction, this might be an endogenous bidirectional regulation mechanism for COX-2 induction and resultant prostaglandin formation. This type of negative regulation is potentially an important target for the chemoprevention procedure of colorectal cancer.

In this study, we investigated whether Ang II regulates COX-2 expression in a rat intestinal cell line (RIE-1). We further examined the Ang II receptor subtype-specific role in this pathway.

Materials and Methods

Chemicals and reagents

Ang II, (Sar^1, Ile^8)-Ang II, AT_2 receptor antagonist PD123319, AT_2 receptor agonist CGP-42112A and lipopolysaccharides (LPS) from *Escherichia coli* were purchased from Sigma-Aldrich (St. Louis, MO). The AT_1 receptor antagonist losartan was obtained from the pharmacy at Vanderbilt University Medical Center. The anti-murine COX-2 polyclonal antibody and anti-mouse COX-1 monoclonal antibody were purchased from Cayman Chemical (Ann Arbor, MI). The actin monoclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture

Rat intestinal epithelial (RIE-1) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100,000 U/L penicillin G, and 100,000 μg/L streptomycin sulfate. For all experiments 90–95% confluent cultures were tendered quiescent by incubation in serum-free DMEM for 72 h before Ang II stimulation.

RNA extraction and RT-PCR

Total cellular RNA was extracted from confluent RIE-1 cells cultured in serum-free medium by TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and was quantified by measurement of absorbance at 260 nm in a spectrophotometer. Two μg of total RNA was subjected to first-strand cDNA synthesis using the oligo-dT primer and Ominiscript RT kit (Qiagen, Valencia, CA). The primer sets for the AT_1 receptor were 5'-GGA AAC AGCTTG GTG GTG-3' (sense) and 5'-BCA CAA TCG CCA TAA TTA TCC-3' (antisense), for the AT_2 receptor were 5'-CAC CAG CAG AAA CAC CAC-3' (sense) and 5'CCA AAC AAG GGG AAC TAC3' (antisense), for COX-2 were 5'-TTC ACC AGA CAG ATT GCT GGC-3' (sense) and 5'-AGT CTG GAG TGG GAG CCA CTT G-3' (antisense), and for GAPDH were 5'-CGC CTG GTC ACC AGG GCT GC-3' (sense) and 5'-CTT ACT CCT TGG AGG CCA TGT-3' (antisense). GAPDH was used as a control to demonstrate equal total RNA. The PCR cycle consisted of 94°C for 1 min, 60°C for 1 min (GAPDH 58°C, COX-2 62°C), 72°C for 1 min; these were repeated for 19 cycles for GAPDH, 25 cycles for AT1, 36 cycles for AT_2 and for COX-2.

Western blot analysis

Total cellular protein was extracted by lysing the cells in 250 μl of lysis buffer (0.25 M sucrose, 30 mM Tris, 1% Triton X-100, 0.4% SDS, 1 mM EDTA, one tablet Complete (Roche Applied Science, Indianapolis, IN) in 10 ml extraction solution. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL). Sixty microgram of protein underwent electrophoresis (SDS-PAGE) and were transferred to a nitrocellulose membrane, Protran (Schleicher & Schuell, Keene, NH). Proteins were detected using anti-COX-2 and -COX-1 antibodies, respectively. An actin monoclonal antibody was used as a control to demonstrate equal loading and transfer. ECL (Amersham Biosciences, Pittsburgh, PA) was used to detect the signals. Intensity of each protein band was quantified using Fluor-S multi-imager (Bio-Rad, Hercules, CA) and Quantity One software (Bio-Rad).

Preparation of adenoviral vectors

Overexpression of AT_2 in RIE-1 cells was achieved with an adenovirus encoding mouse AT_2 cDNA (Ad-AT_2). To generate recombinant replication-deficient adenovirus expressing AT_2 receptor, EcoRI-XbaI fragment of mouse AT_2 receptor cDNA construct (generous gift of Dr. Inagami, Vanderbilt University, Nashville, TN) was subcloned into a recombinant adenovirus shuttle vector pACCMVpLpA which has deletions in the *E1A*, *E1B* and *E3* genes. For preparation of virus stocks, 293 cells were infected at multiplicity of infection (MOI) 1–5 and harvested after cytopathic effect became visible (72–96 h). Cells were harvested and lysed in 10% FBS containing DMEM, and virus aliquots were stored at −80°C. Titers were determined by either plaque assay on 293 cells for plaque forming units/ml or spectrometer reading at 260 nm for particles/ml. Recombinant replication-deficient adenovirus encoding β-galactosidase (Ad-LacZ), which contains E. Coli LacZ gene, human cytomegalovirus promoter and SV40 polyadenylation signal (pHCMVsp1LacZ, generous gift of Dr. Myers, Vanderbilt University, Nashville, TN) (32)was used as control vector.

Virus Infection

For viral infections, 2×10^6 cells were plated in 60-mm tissue culture dishes and incubate for 48–72 h (90~95% confluent). Cells were infected by incubating with the adenoviral vectors at MOI of 50 in 200 μl of DMEM for 6 h at 37°C. After the infection, cells were washed and incubated with serum-free DMEM for 92 h, and then stimulated with either Ang II or both receptor antagonist and Ang II.

Radioligand-receptor-binding assay

The radioligand-receptor-binding assay was performed by using transfected cultured cells and [^{125}I](Sar^1,Ile^8)-Ang II in the presence of PD123319 for AT_1 or [^{125}I]CGP-42112A for AT_2. The [^{125}I]-labeled peptides were separately prepared from (Sar^1,Ile^8)-Angi II or CGP-42112A and [^{125}I]Na by the lactoperoxidase method. Infected cells were incubated with 0.5 nM radiolabeled peptide with or without 1 μM unlabeled peptide for 2 h at 24°C in the presence of 0.5 mg/ml bovine serum albumin. Unbound labeled ligand was thoroughly washed out with HBSS. Cells were solubilized with 0.5 N NaOH and the remaining radioactivity was counted. Specific binding was estimated by subtracting the non-specific binding obtained in the presence of 1 μM unlabeled ligand from the total binding. An aliquot of the solubilized cells was subjected to the bicinchoninic acid protein assay. Specific binding was normalized by the protein quantity per dish.

Expression of COX-2 in AT_2 transfected RIE-1 cell

In this study cells were transfected with AT_2 cDNA using the AT_2 adenoviral vector as described elsewhere. Briefly, the RIE-1 cells (3×10^5/well) were plated in a 6-well tissue culture plate and incubated for 48 h (90~95% confluent). Cells were infected with an adenovirus vector encoding mouse AT_2 (Ad-AT_2) or an adenovirus encoding LacZ (Ad-LacZ) with the adenoviral vectors MOI of 50 in 200 μl of DMEM for 6 h at 37°C. After washing with PBS the cells were incubated with 10% FBS-containing DMEM for 18 h and then serum free DMEM for 48–96 h. Cells were stimulated with various stimuli such as Ang II, CGP-42112A or LPS (5 μg/ml) for 6–8 h.. The AT_1-specific antagonist losartan (10^{-6} M) or AT_2-specific antagonist PD123319 (10^{-6} M) were added 30 min prior to the addition of stimuli. After stimulation cells were harvested and subjected to Western blot analysis for COX-2, GAPDH or actin as described above.

Statistical analysis

Data obtained from the RT-PCR and Western blot analysis were presented as means ±S.E (standard error). Statistical significance between groups were evaluated by one-way ANOVA with the Student-Newman-Keuls test. A value of $P < 0.05$ was considered significant.

Results

Both Ang II receptor subtypes were detected in RIE-1 cells

To evaluate the status of Ang II receptor expression in RIE-1-cells, the expression levels of both mRNA and functional proteins of the two receptor subtypes were determined by RT-PCR and radioligand receptor-binding assay, respectively (Fig. 1). Expression level of AT_1 mRNA was abundant whereas the level of AT_2 mRNA was approximately 10 % of the AT_1 expression levels. Expression levels of the functional AT_1 and AT_2 receptor proteins were revealed to be 226 and 12 fmol/mg protein, respectively. These data indicate that the rat intestinal epithelial cell line RIE-1 cells express a large amount of AT_1 but a small amount of AT_2. Since the expression level of AT_2 in adult tissues is known to be small but it is inducible (33), this cell line appears to be an appropriate cell line to study the effect of Ang II on cyclooxygenase expression.

Ang II stimulated COX-2 but not COX-1 expression through AT_1 receptor

Ang II increased COX-2 protein expression in RIE-1 cells in a concentration dependent manner as evaluated by Western blot analysis (Fig 2 A). Ang II (10^{-9} M) time-dependently increased COX-2 mRNA and protein level as shown in Fig. 2B and 2C, respectively, but not COX-1 protein level. The COX-2 mRNA response was observed as early as 30 min after Ang II treatment and the maximal increase was observed 1h after treatment. Ang II-induced COX-2 protein expression was first seen 2 h after treatment and the maximal response was observed at 4–6 h after the treatment and lasted until 24 h after treatment (Fig. 2 C and D).

The subtype–specific role of Ang II receptors in COX-2-induction was clarified by utilizing receptor-specific antagonists. Ang II-dependent induction of COX-2 was completely blocked by the AT_1-specific antagonist losartan (10^{-6} M) but not by the AT_2-specific antagonist PD123319 (Fig.3). This result clearly indicates that Ang II stimulates COX-2 induction via AT_1 because there is no expression of COX-2 in the presence of the AT_1-specific antagonist losartan or both AT_1 and AT_2-specific antagonists (Fig.3).

Ang II and LPS additively stimulated COX-2 expression

Although we obtained clear-cut results indicating that the Ang II stimulates COX-2 induction through the AT_1 in intestinal epithelial cells, the pathophysiological significance of Ang II in COX- 2 induction is still uncertain. In order to address this issue, the effect of Ang II on a typical COX-2 inducer, LPS-stimulated COX-2 induction was studied. Ang II (10^{-8} M) and LPS (5 μg/ml) additively increased COX-2 expression in RIE-1 cells and this additive effect was completely blocked by the AT_1-specific antagonist losartan (Fig.4). These results suggest that Ang II-dependent COX-2 induction in intestinal epithelial cells is likely to have pathophysiological significance.

Ang II attenuated LPS-induced COX-2 expression through over-expressed AT_2 receptor

Since the AT_2 protein expression level in this cultured cell is smaller than those reported (27, 34) the AT_2 protein level may not be sufficient to exhibit the AT_2-mediated Ang II effect in COX-2 expression. In this study, the AT_2 was over-expressed by transfecting cells with AT_2 cDNA using the AT_2 adenoviral vector. In this study the most efficient AT_2 receptor expression was observed at 4 days after transfection, and the expression of AT_2 was approximately 22% of the AT_1 expression level when 50 MOI vector was transfected (Fig.5). Accordingly, co-stimulation of the cells with LPS and Ang II was performed 4 days after transfection with 50 MOI AT_2 vector. Although Ang II (10^{-8} M) and LPS (5 μg/ml) additively increased COX-2 expression in both untransfected (Fig.4) and LacZ transfected RIE-1 cells (data not shown), low concentrations of Ang II and AT_2 agonist CGP-42112A significantly attenuated LPS-induced COX-2 expression when AT_2 receptor was transfected (Fig.6). The attenuation effect was more pronounced when lower concentration of LPS was used for the COX-2 induction. In addition, Ang II-dependent attenuation was pronounced in the presence of the AT_1 receptor antagonist losartan (1 μg/ml, data not shown). These results suggest that Ang II attenuates LPS-induced COX-2 expression through AT_2 mediated signaling. This implies that Ang II bi-directionally regulates COX-2 expression (AT_1 stimulates, but AT_2 attenuates COX-2 induction).

Discussion

It has been suggested that Ang II signaling may play important roles in tumorigenesis in several tissues (17,20,35–39). However, the specific role of the Ang II receptors in tumorigenesis has not been elucidated. We have previously demonstrated a pro-oncogenic role of the AT_2 receptor in chemical carcinogen azoxymethane–induced colon tumorigenesis (37)] and tobacco-specific nitrosamine–induced lung tumorigenesis (39). In the present study, we used cultured

normal intestinal epithelial cells to clarify the roles of Ang II and its receptors in cyclooxygenase-induction in intestine. Cyclooxygenases are the rate-limiting enzymes for the prostaglandin formation and have been reported to be associated with human colon cancer development (14,40,41) and other chronic bowel diseases (42). Elucidation of the role of Ang II and its receptors in colon tumorigenesis-associated cyclooxygenases are important in understanding the insight of upstream regulation of this enzyme.

First, we examined the Ang II receptor expression status of the rat intestinal epithelial cell line, which was utilized for the present study, using RT-PCR and ligand-receptor-binding assay. The present study demonstrated that the RIE-1 cell line expresses both AT_1 and AT_2 receptor mRNA's (Fig 1A). However, the proportion of the AT_1 protein was significantly larger than that of the AT_2 protein (Fig. 1B). Proportion of the AT_2 receptor expression levels seems smaller than the previous measurement of the receptor expression in intestinal mucosa *in vivo* (27). However this result seems reasonable, since AT_2 expression is suppressed during cell culture but is inducible by removing serum from the culture medium (34). Indeed, AT_2 protein expression in RIE-1 cells was noticeably increased by serum removal (data not shown). Therefore, the present study with cultured cells of RIE-1 should be a good model system to evaluate the role of Ang II in COX expression in intestinal epithelial cells.

In view of that, we evaluated the effect of Ang II on the expression of the COX-2 expression in the rat intestinal epithelial cell line using RT-PCR and Western blot analysis. The results clearly demonstrated that Ang II, dose- and time-dependently stimulated COX-2 but not COX-1 expression in the RIE-1 cell line (Fig. 2). The present study also indicates that the Ang II AT_1-mediated signal plays the primary role in COX-2 induction. These results are in good agreement with a recent report in which Ang II stimulates COX-2 expression in non-transformed rat intestinal cell line (25). Slice et al have shown that Ang II potently stimulated expression of COX-2 mRNA and protein as an immediate-early gene response through the Ang II type 1 receptor.

Although the present study also strongly suggests that AT_1 plays a critical role in Ang II-dependent COX-2-induction in intestinal epithelium, involvement of another Ang II receptor subtype in this reaction is unclear since the RIE-1 cell line expresses only a limited amount of AT_2 (Fig. 1). Therefore, the effect of Ang II on COX-2 expression was re-evaluated in the RIE-1 cell line expressing higher levels of AT_2. As demonstrated in the Fig. 5, the AT_2 transfected cells express much higher levels of the AT_2 protein in the cells. Although Ang II alone did not alter COX-2 expression in these cells, Ang II significantly attenuated the effect of a most powerful stimulator LPS-induced COX-2 expression (Fig. 6) and this attenuation was also mimicked by AT_2 agonist CGP-42112A. These results clearly demonstrated that the presence of the AT_2 receptor is potentially important in the regulation of COX-2 expression. This notion is potentially important, since tumor tissue consists of a variety of cell types such as infiltrated lymphocytes, macrophages, fibroblasts and endothelial cells. These cells seem to play an important role in tumor cell growth, invasion, metastasis, angiogenesis and evasion of the host immune system (43). Prostaglandins and various cytokines produced either in tumor cells or stromal cells are involved in many of these biological reactions (14,43–45).

Although a number of reports have described the role of Ang II in intestinal sodium and water absorption function (46,47), only a few reports describe subtype-specific Ang II receptor expression in the intestine (27,29,47). AT_2 receptor has been shown to be involved in absorption/secretion of water and electrolytes (47,48),and to induce mucosal release of nitric oxide (29). However, this receptor does not influence smooth muscular contractions (49,50). Since Ang II has similar affinity to both of its receptor subtypes it is suggested in most cases the AT_1 receptor counteracts AT_2 receptor-mediated effects. Johansson *et al.* have shown such an antagonistic relationship, in which activation of AT_1 receptor contributes to inhibition and

AT_2 receptor stimulates duodenal mucosal alkaline secretion (51). In terms of the potential mechanism by which AT_2 receptor signaling antagonizes AT_1 receptor signaling, it has been reported that AT_2 receptor signaling stimulates tyrosine phosphatase, thus antagonizes AT_1 receptor-mediated actions (52–54). It is also reported that the AT_2 receptor directly interacts with the AT_1 receptor, thus counteracting its specific function (22,55). Accordingly, the overall response to Ang II in COX-2 induction is the AT_1 receptor-mediated stimulation as shown in figures 2 and 3; this response may be modulated in part by AT_2 receptor-mediated signaling via regulation of de-phosphorylation of AT_1 receptor-dependent phosphoproteins or direct interaction of both receptor proteins.

On the other hand, it is difficult to speculate a precise mechanism by which AT_2 receptor signaling attenuated LPS-induced COX-2 expression. However, since LPS is shown to stimulate multiple kinase activities and regulate multiple gene expression (56–58), AT_2 receptor over-expression may have modulated LPS-dependent phosphoproteins via protein tyrosine phosphatase activation and hence attenuated LPS-dependent COX-2 protein expression. In addition, AT_2 receptor over-expression has shown to down regulate AT_1 receptor expression (22) and this may also be a part of a potential explanation for the AT_2 receptor-dependent attenuation of COX-2 protein expression. However, clarification of this mechanism needs to be evaluated by another study.

In summary, the present study demonstrated a bidirectional regulatory function of the Ang II receptors in cyclooxygenase-2 expression in non-transformed rat intestinal epithelial cell line. Since pharmacological control of the Ang II receptor function *in vivo* is a viable therapeutic technique (59), it is feasible to determine whether AT_1 receptor blockers attenuate colorectal tumorigenesis. Increasing AT_2 expression in intestinal epithelium, which attenuates COX-2 expression, may also be a potential route for a chemoprevention of colorectal cancer. This may lead to the development of a potential chemoprevention procedure for human colorectal cancer. However, the importance of the Ang II receptor function in human colorectal cancer awaits further study. To the best of our knowledge, the present study is the first to demonstrate that the Ang II AT_2 possesses a modulator function in COX-2 expression in intestinal cells.

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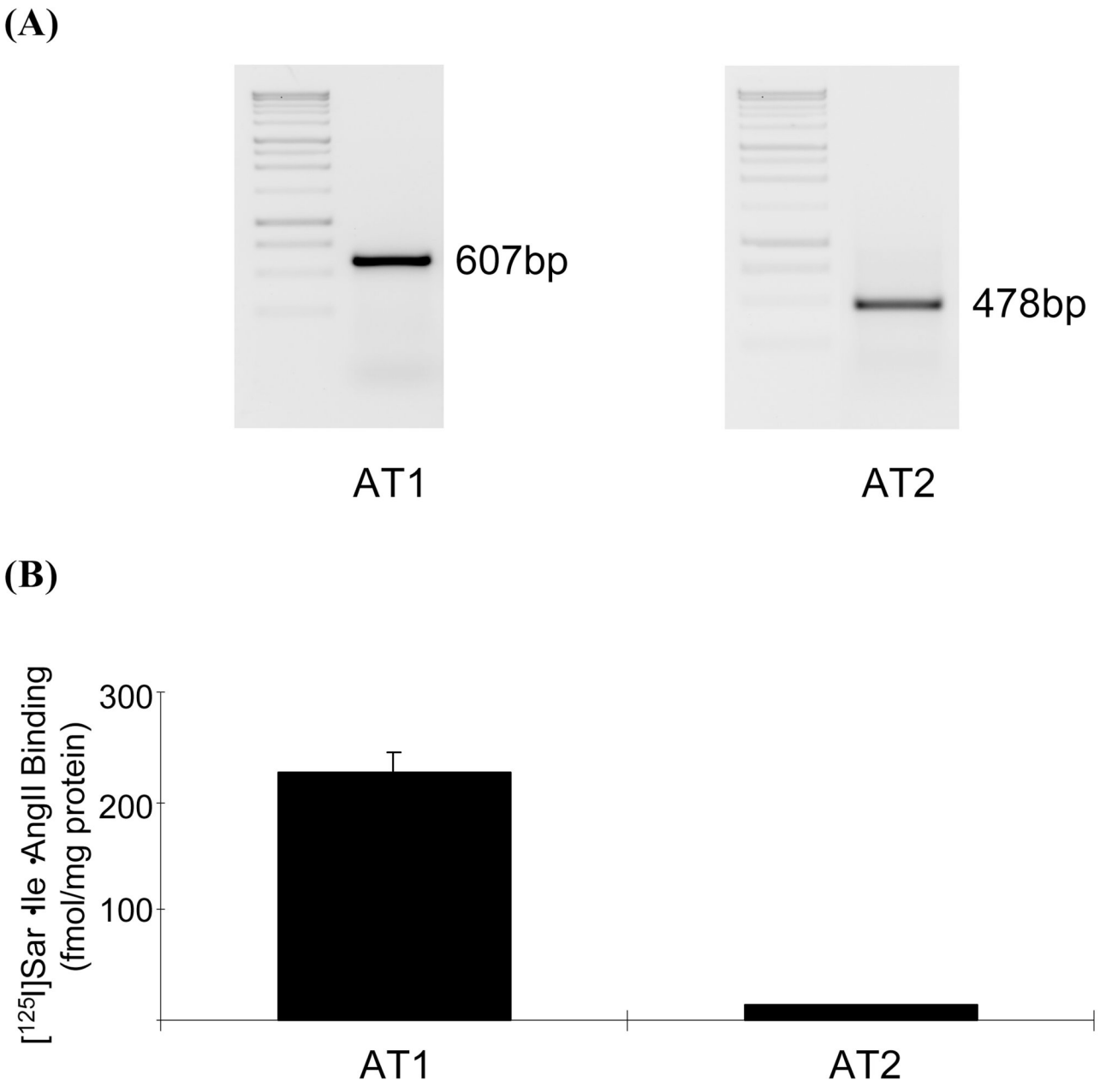

Fig. 1.

Detection of Ang II type 1 (AT_1) and type 2 (AT_2) receptors in RIE-1 cell line. A, Total RNA was isolated from confluent RIE-1 cells cultured in serum-free medium as described in the Materials and Methods section. The expression of specific mRNA was detected by RT-PCR. B, Receptor protein expression was determined by ligand receptor–binding assay as described in the Materials and Method section using cultured whole intact cells and [^{125}I](Sar^1,Ile^8)-Ang II. Data are representative of triplicate determinations.

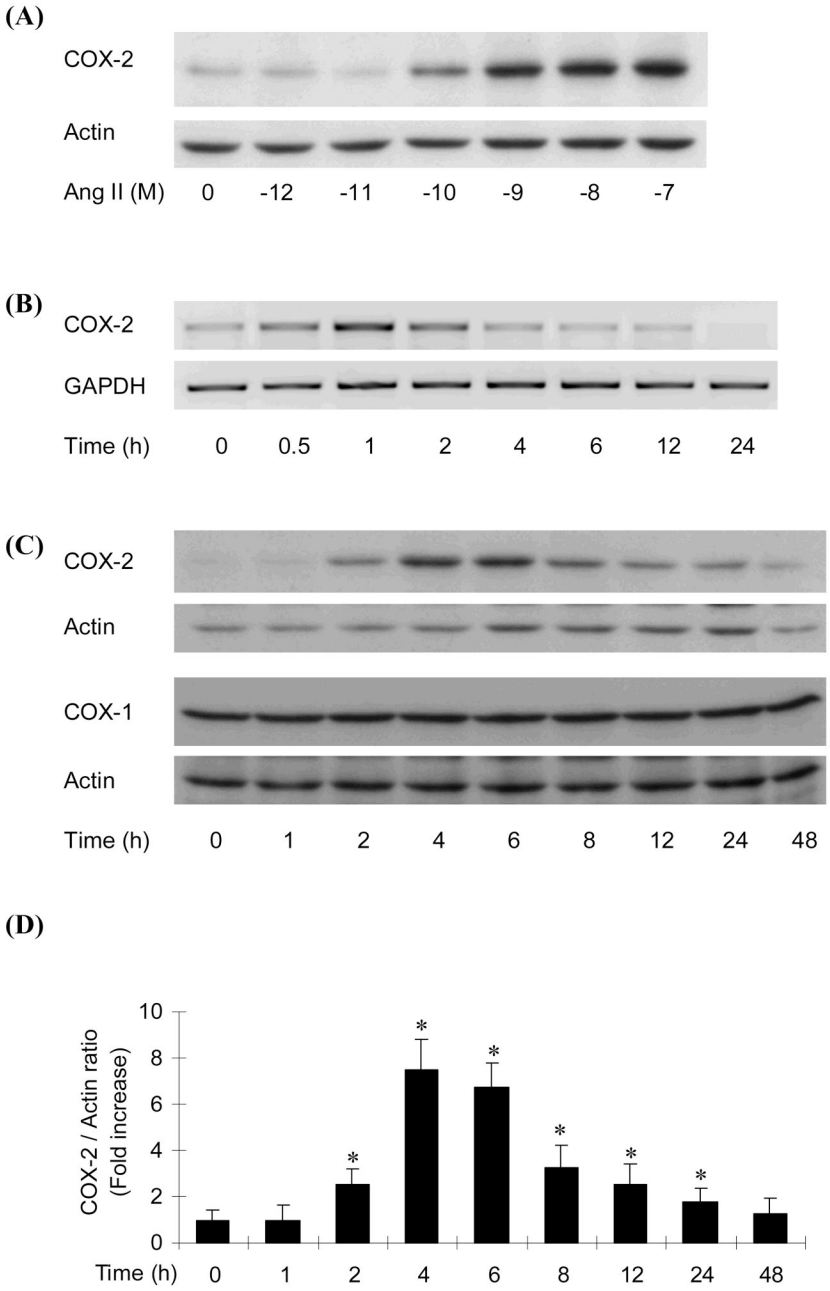

Fig. 2.

Effect of Ang II on COX expression in RIE-1 cells. A. Concentration dependent expression of COX-2 protein in RIE-1 cells by Ang II. B. Time dependent expression of COX-2 mRNA in RIE-1 cells by Ang II (10^{-9} M). C. Time dependent expression of COX-2 and COX-1 protein by Ang II (10^{-8} M). The whole homogenates were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with anti-murine COX-1 and COX-2 antibodies. D. Quantification of COX-2 protein in panel C was carried out by scanning densitometry and averages of the expression levels (n=5) normalized by actin levels are displayed in the histogram. *, $P \leq 0.05$ as compared to the level of time 0h.

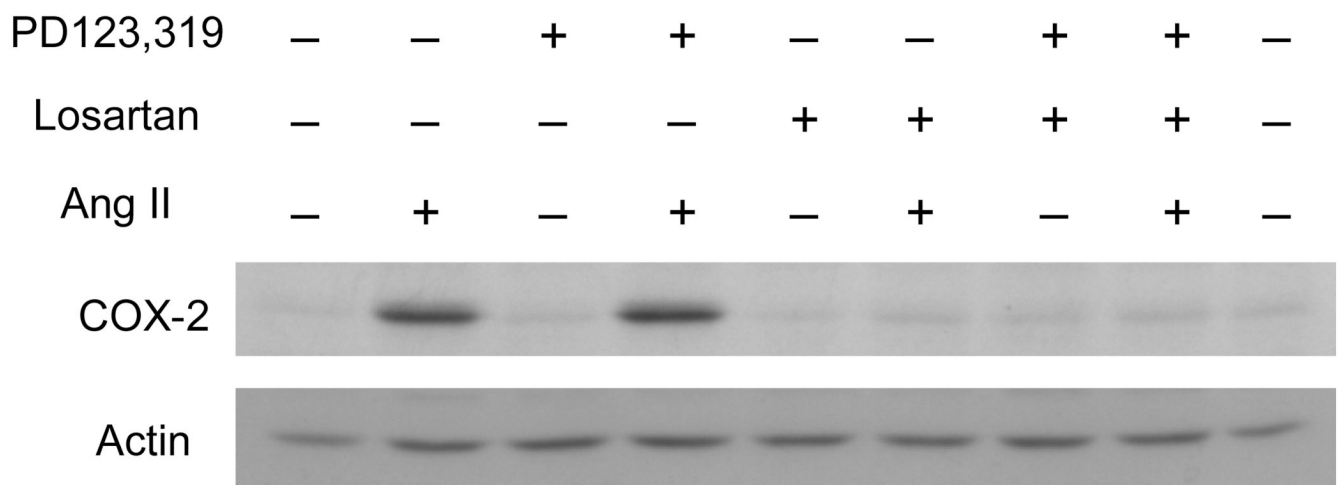

Fig. 3.

Effect of Ang II and its receptor antagonists on COX-2 protein expression in RIE-1 cells. Cells were pretreated with either AT_1-specific antagonist losartan (LOS; 10^{-6}M) or AT_2-specific antagonist PD123319 (10^{-6} M) for 30 min, and then stimulated with Ang II (10^{-8} M) for 6 h. Western blot analysis of the COX-2 protein was carried out as described in the Materials and Methods section.

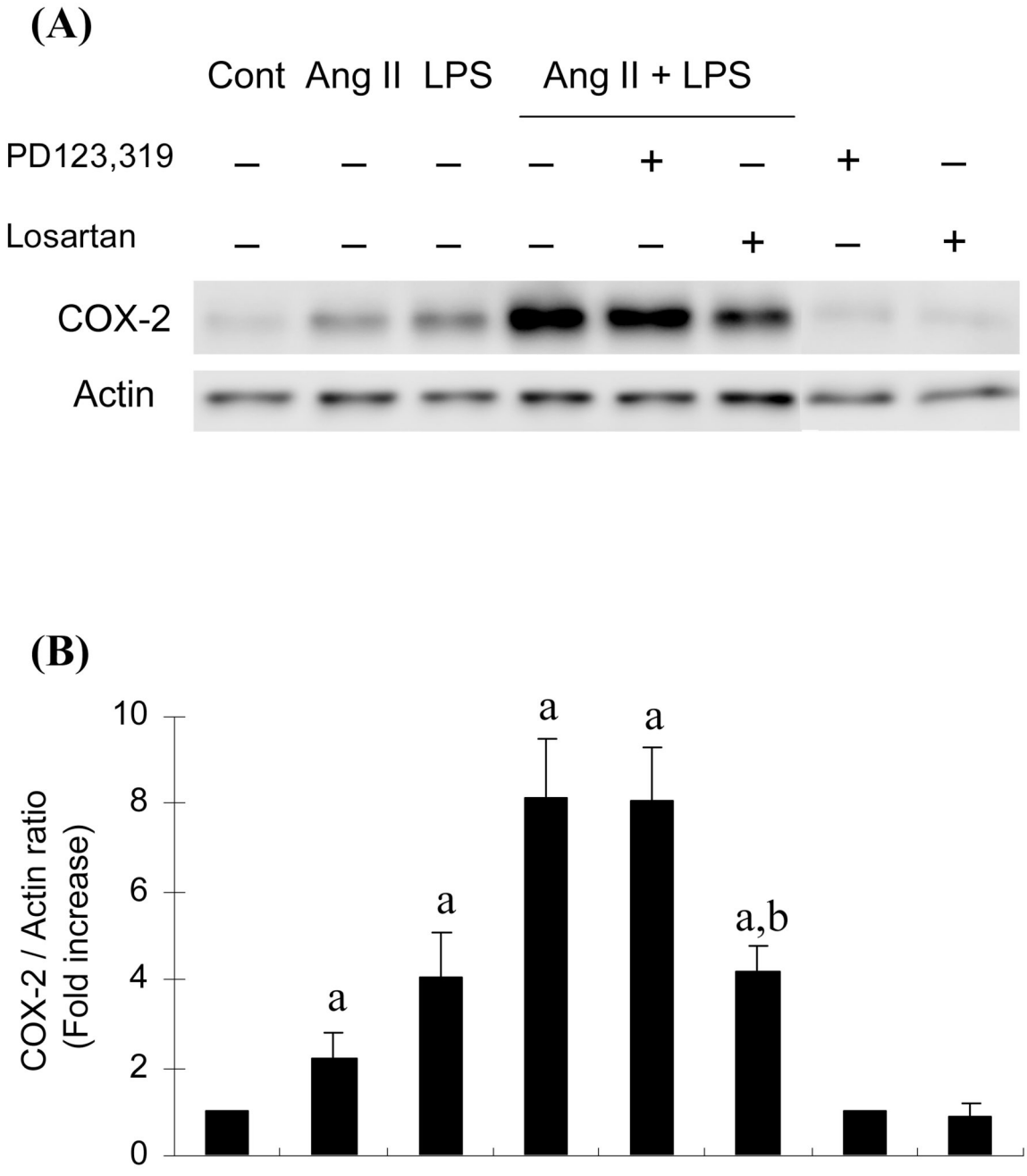

Fig. 4.

Effect of Ang II and LPS on COX-2 protein expression in RIE-1 cells. Cells were treated with either Ang II (10^{-8} M), LPS (5 μg/ml) or both for 6 h in the presence or absence of AT_1-specific antagonist losartan (10^{-6}M) or AT_2-specific antagonist PD123319 (10^{-6} M). Western blot analysis of the COX-2 protein was carried out as described in the Materials and Methods section. Quantification of COX-2 protein in panel B was carried out by scanning densitometry and averages of the three expression levels normalized by actin levels are displayed in the histogram. a, $P \leq 0.05$ as compared to the level in the untreated control. b, $P \leq 0.05$ as compared to the level in Ang II and LPS treated cells.

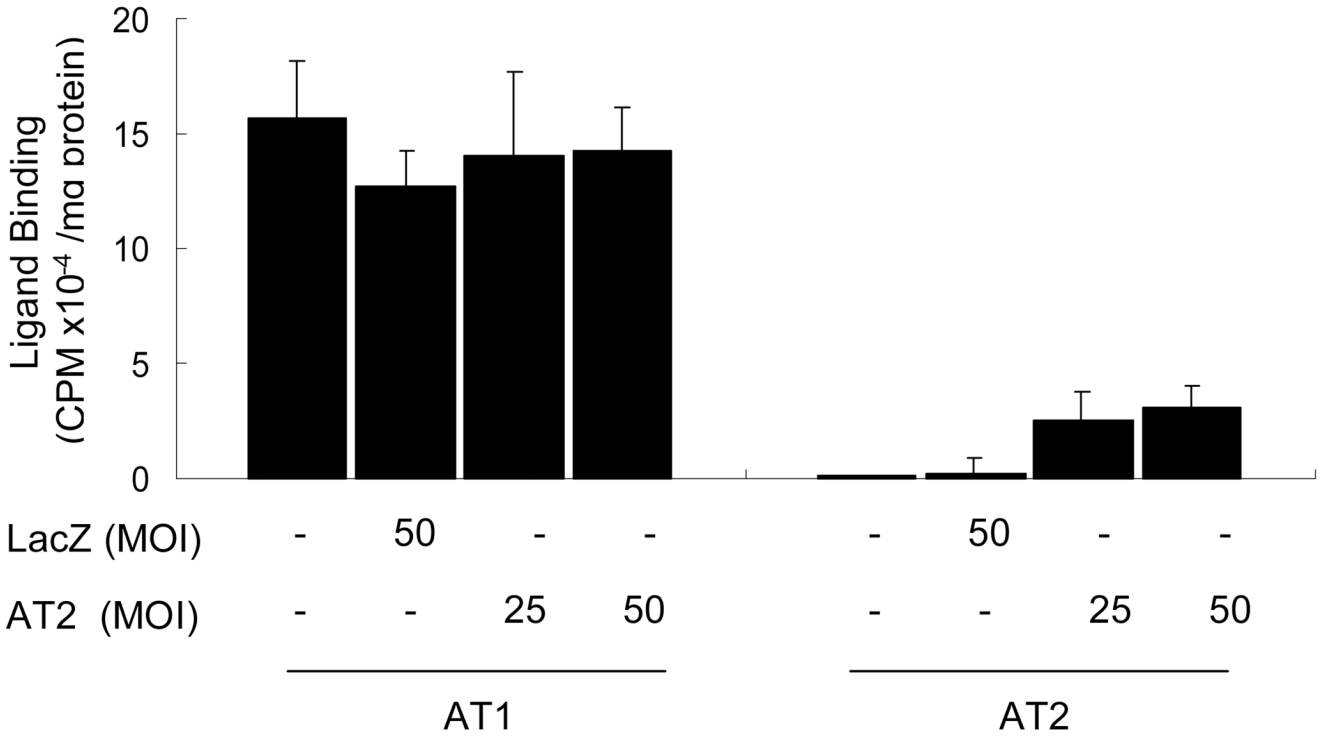

Fig. 5.

Expression levels of Ang II receptors in the RIE-1 cells transfected with AT_2 cDNA were assessed by ligand receptor-binding assay as described in the Materials and Methods section. AT_2 protein expression was markedly increased by AT_2 cDNA transfection, whereas AT_1 expression was not significantly altered as compared to LacZ transfected cells. The averages of three separate experiments are displayed in the histogram.*, $P \leq 0.05$ as compared to the level of LacZ transfected cells.

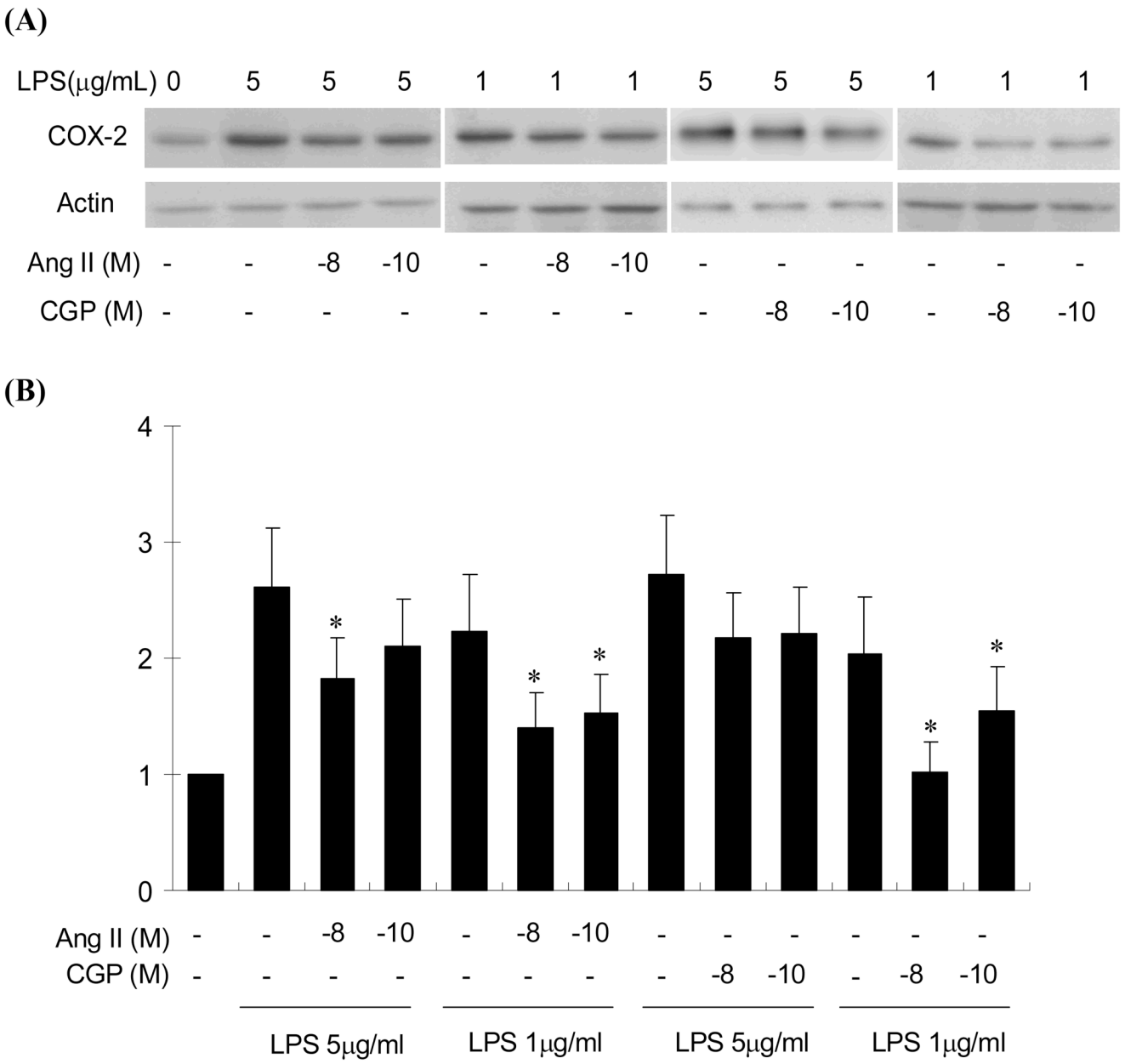

Fig. 6.

Effect of Ang II on LPS-induced COX-2 expression in AT_2 over-expressed RIE-1 cells. AT_2 transfected RIE-1 cells were pretreated with LPS (1–5 μg/ml) and then stimulated with either Ang II or CGP-42112A (CGP, 10^{-10} or 10^{-8} M) for 8 h as indicated in the figure. Top panel, COX-2 protein expression was assessed by Western blot analysis as described in Materials and Methods. Bottom panel, Semi-quantification of COX-2 protein expression was carried out according to the method described in the Materials and Methods using scanning densitometry. The average expression levels normalized by actin level (n=3) are displayed in the histogram. *, $P \leq 0.05$ as compared to the level of LPS alone treatments.