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European Journal of Pharmacology 386 (1999) 289–295

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Lipopolysaccharides and cytokines downregulate the angiotensin II type 2 receptor in rat cardiac fibroblasts

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Received 24 June 1999; received in revised form 21 October 1999; accepted 26 October 1999

Abstract

The present study examines the effect of lipopolysaccharides and proinflammatory cytokines on the expression of the second isoform of the angiotensin II receptor (AT_2), which may have a role in lowering collagen deposition in cardiac tissue. Cardiac fibroblasts express high levels of both angiotensin II type 1 (AT_1) and type 2 receptors. Incubation with lipopolysaccharides for 24 h dose- and time-dependently decreased angiotensin II AT_2 receptor expression with no apparent difference in the affinity. Actinomycin D, cycloheximide, N^{ω}-nitro-L-arginine methyl ester and the protein tyrosine kinase inhibitor herbimycin A, but not the protein kinase C inhibitors bisindolylmaleimide and calphostin C, abolished the inhibitory action of lipopolysaccharides. The cytokines interleukin-1β and tissue necrosis factor-α mimicked the effect of lipopolysaccharides. All three compounds induced inducible nitric oxide synthase (iNOS). The nitric oxide donor sodium nitroprusside and the cGMP analog 8-bromoguanosine cyclic monophosphate downregulated angiotensin II AT_2 receptor expression. The findings are consistent with the pathway in which lipopolysaccharides or cytokines induce iNOS. The data suggest that lipopolysaccharide- or cytokine-dependent induction of iNOS and resultant production of nitric oxide leads to the production of cGMP, which in turn downregulates expression of the angiotensin II AT_2 receptor in cardiac fibroblasts.

Keywords: Lipopolysaccharide; Angiotensin II type 2 receptor; Cytokine; Nitric oxide (NO); Cyclic GMP; Cardiac fibroblast

1. Introduction

The renin–angiotensin system plays an important role in cardiac fibrosis (Weber, 1997; Sun and Weber, 1998). One of the most potent angiotensin peptides, angiotensin II, seems to be a key component in the etiology of cardiac fibrosis, since an inhibition of the angiotensin converting enzyme and a blockade of the angiotensin II type 1 (AT_1) receptor are effective in the prevention of the fibrosis (Takemoto et al., 1997; Holtz, 1998). Angiotensin II action is predominantly transmitted through the angiotensin II AT_1 receptor (Timmermans et al., 1993; Stroth and Unger, 1999). The angiotensin II AT_1 receptor plays a critical role in the production of the extracellular matrix in cardiac fibrosis (Brilla et al., 1997; Sun and Weber, 1998). However, recently a second isoform of the angiotensin II receptor (AT_2) has been cloned (Kambayashi et al., 1993; Mukoyama et al., 1993). The angiotensin II AT_2 receptor has been shown to counteract angiotensin II AT_1 receptor-mediated actions, such as cell proliferation in vascular endothelial cells (Nakajima et al., 1995; Stoll et al., 1995) and collagen deposition (Weber, 1997). Recent studies have also demonstrated that expression of the angiotensin II AT_2 receptor is upregulated in failing hearts from patients with myocardial infarction (Brink et al., 1996; Tsutsumi et al., 1998; Wharton et al., 1998) and idiopathic dilated cardiomyopathy (Tsutsumi et al., 1998). These results prompted us to speculate that the angiotensin II AT_2 receptor may have pathophysiological significance in other chronic heart diseases such as myocarditis. Lipopolysaccharides and/or proinflammatory cytokines, such as interleukin-1 and tissue necrosis factor (TNF)-α, often play an important role in the etiology of myocarditis

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00755-4

and the resultant development of fibrosis (Lane et al., 1991, 1993; Neumann et al., 1993; Kita et al., 1996; Bryant et al., 1998). Although lipopolysaccharide treatment has been shown to alter the angiotensin II AT_1 receptor levels in vascular smooth muscle cells (Burnier et al., 1995; Cahill et al., 1995), little is known about the angiotensin II AT_2 receptor in endotoxemia or in inflammation-mediated cardiac diseases. Since the angiotensin II AT_2 receptor often functions as a countermeasure against effects of the angiotensin II AT_1 receptor (Hein et al., 1995; Ichiki et al., 1995; Stoll et al., 1995; Chung et al., 1998; Ma et al., 1998), it is of interest to study the effect of lipopolysaccharides and proinflammatory cytokines on the angiotensin II AT_2 receptor. The purpose of the present study is to examine the effects of lipopolysaccharides and proinflammatory cytokines on the angiotensin II AT_2 receptor in vitro in cultured cardiac cells. Cardiac cells mainly consist of myocytes and fibroblasts, and fibroblasts from adult rats show strong immunohistochemical staining with specific anti-angiotensin II AT_2 receptor antibodies (Tamura et al., unpublished data). Expression of the angiotensin II AT_2 receptor has been shown to be associated with cardiac fibrosis in patients(Tsutsumi et al., 1998). Accordingly, the present study was carried out employing primary cultures of rat cardiac fibroblasts.

2. Materials and methods

2.1. Chemicals and reagents

Angiotensin II and [Sar^1, Ile^8] angiotensin II were purchased from Peninsula Laboratories. Collagenase A and DNase 1 were from Boehringer Mannheim. Trypsin was obtained from ICN Biomedical. Lipopolysaccharides from *Escherichia coli*, actinomycin D, cycloheximide, N^{ω}-nitro-L-arginine methyl ester (L-NAME), interleukin-1β, TNF-α, 8-bromoguanosine 3′,5′-cyclic monophosphate and sodium nitroprusside were from Sigma. Bisindolylmaleimide, calphostin C, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine, HCl (H-7) and herbimycin A were from Calbiochem-Novabiochem. Losartan was a gift from DuPont-Merck. The peptidic angiotensin II AT_2 receptor agonist CGP42112A and the non-peptidic angiotensin II AT_2 receptor antagonist PD123,319 were from RBI (Natick, MA). [^{125}I]Na was from DuPont New England Nuclear. Dulbecco's modified Eagle's medium (DMEM), Ham's F12, and fetal bovine serum were purchased from Gibco-BRL. Anti-mouse inducible nitric oxide synthase (iNOS) polyclonal antibodies were from Calbiochem. All other chemicals were of analytical grade.

2.2. Cell culture

Cardiac cells from newborn Sprague–Dawley rats were isolated according to a modified method described by Sadoshima et al. (1992). Briefly, a single litter of 1-day-old newborn rats was used for the primary culture. The hearts were quickly removed into chilled Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, and minced into 1–2 mm cubes. The cells were dissociated by 0.1% collagenase A, 0.1% DNase 1 and 0.1% trypsin in HBSS in the ice-bath for 90 min, then kept at 37°C for 10 min. The remaining tissue was treated to dissociate additional cells with the same enzyme solution at 37°C for 15 min. After washing, the cells were placed in 10 cm plastic culture dishes and incubated in a CO_2 incubator for 60 min. Attached cells were continuously cultured as fibroblasts. The floating cells were reseeded in plastic 6-well plates (Primaria, Falcon) in the presence of 0.1 mM 5-bromo-2′-deoxyuridine. The majority of the latter cells were characterized as cardiac myocytes by immunohistochemical staining of sarcomeric desmin and by observation of self-contraction. Cardiac cells were grown in DMEM/Ham's F12 (1:1) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 50 μg/ml gentamycin at 37°C under a humidified atmosphere of 95% air/5% CO_2. For determination of the receptor density, cells were plated in 12-well plates at a density of $2–3 \times 10^5$/well and cultured for 2–3 days. The medium was changed to serum-free DMEM 48 h prior to the binding assay unless otherwise stated.

2.3. Radioligand-receptor-binding assay

The radioligand-receptor-binding assay was performed by using intact cultured cells and [^{125}I][Sar^1, Ile^8] angiotensin II in the presence of PD123,319 for the angiotensin II AT_1 receptor or [^{125}I]CGP42112A for the angiotensin II AT_2 receptor (Tamura et al., 1999). The [^{125}I]-labeled peptides were separately prepared from [Sar^1, Ile^8] angiotensin II or CGP42112A and [^{125}I]Na by the lactoperoxidase method. Cells in 6- or 12-well plates were washed twice with HBSS and incubated with 0.5 nM radiolabeled peptide with or without 1 μM unlabeled peptide for 120 min at 24°C in the presence of 0.5 mg/ml bovine serum albumin. PD123,319 (1 μM) was added into the assay medium for the measurement of the angiotensin II AT_1 receptor since [^{125}I][Sar^1, Ile^8] angiotensin II binds to both receptor subtypes. 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 protein assay (BCA protein assay method, Pierce, Rockford, IL). Specific binding was normalized by the protein quantity per well.

2.4. Western blot analysis

After treatment with or without lipopolysaccharides or cytokines for 18 h, cells were washed with phosphate-

buffered saline (pH 7.4). Cells were lysed with 0.5 ml boiling lysis buffer (10 mM Tris–HCl, 1% sodium dodecyl sulfate (SDS) (pH 7.4)). After sonication, the cell lysate was boiled for 5 min and centrifuged. The resultant supernatant (15,000 rpm at 4°C for 10 min) was subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to a polyvinylidine fluoride (PVDF) membrane by electroblotting. The membrane was blocked for 16 h at 4°C with 5% non-fat milk in tris-buffered saline (10 mM Tris (pH 7.5), 100 mM NaCl) containing 0.1% Tween-20, and then incubated with anti-rabbit polyclonal iNOS antibodies for 1 h. After washing, the membrane was incubated for 1 h with goat anti-rabbit IgG conjugated to horseradish peroxidase; peroxidase activity was visualized with an enhanced chemiluminescence Western blotting detection system (Amersham).

2.5. Statistical analysis

Data obtained from the binding assay were averaged and are presented as mean ± S.E. Significant differences between groups were evaluated by one-way analysis of variance with the Student–Newman–Keuls test. A value of $P < 0.05$ was considered significant.

3. Results

Cardiac fibroblasts at passage #1 were cultured until confluent in 10% fetal bovine serum containing DMEM/Hams's F12 (1:1). The fibroblasts were kept in serum-free conditions for 72 h. The expression levels of angiotensin receptors were assessed by [^{125}I][Sar^1, Ile^8] angiotensin II-binding in the presence of 1 μM PD123,319 for the angiotensin II AT_1 receptor and [^{125}I]CGP42112A-binding for the angiotensin II AT_2 receptor. Cardiac fibroblasts (12 preparations) expressed both receptors. The angiotensin II AT_1 receptor-binding was 184 ± 44 fmol/mg protein, and the angiotensin II AT_2 receptor-binding was 35 ± 5 fmol/mg protein under standard assay conditions described in Section 2. In addition, it was confirmed that [^{125}I]CGP42112A-binding to the fibroblasts was displaceable by similar concentration ranges of angiotensin II and PD123,319, but not by losartan (Fig. 1). Angiotensin I showed very weak competition against [^{125}I]CGP42112A-binding to fibroblasts.

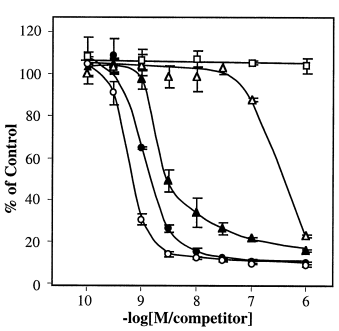

Fig. 1. Competitive binding curve of [^{125}I]CGP42112A with unlabeled CGP42112A (○), angiotensin II (●), angiotensin I (△), PD123,319 (▲) and losartan (□) in rat cardiac fibroblasts. Cells in 12-well plates were incubated with 0.5 nM [^{125}I]CGP42112A and various concentrations of unlabeled competitors for 120 min at 24°C. Assay was performed by the procedure described in Section 2. Each point is the mean ± S.E. of four determinations.

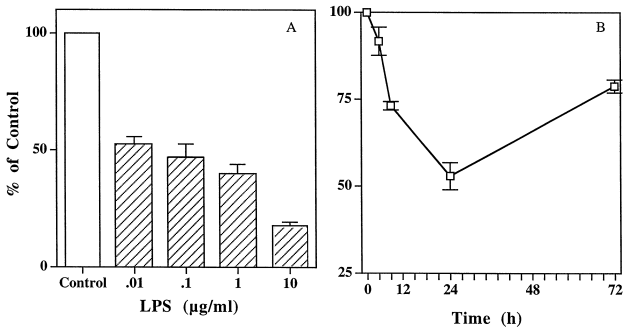

Fig. 2. Dose- and time-dependent effect of lipopolysaccharides (LPS) on expression levels of the angiotensin II AT_2 receptor in rat cardiac fibroblasts. Cells at confluency in 12-well culture plates were further cultured in serum-free DMEM for 48 h and then treated with various concentrations of lipopolysaccharides as indicated for 24 h (A) or with 1 μg/ml lipopolysaccharides for the indicated time (B) in a CO_2 incubator. The expression levels of the receptors were assessed by [^{125}I]CGP42112A-binding to the angiotensin II AT_2 receptor. Data are the means ± S.E. of two triplicate determinations.

The effect of lipopolysaccharides on the expression levels of the angiotensin II AT_2 receptor was determined in rat cardiac fibroblasts. The cells at confluency were cultured in serum-free DMEM for 48 h and then treated with various concentrations of lipopolysaccharides (10 ng/ml to 10 μg/ml) for 24 h. There was a progressive reduction in expression levels of the angiotensin II AT_2 receptor with increasing lipopolysaccharide concentration ranging from 50% reduction at the lowest lipopolysaccharide level to about 80% reduction at the highest lipopolysaccharide level (Fig. 2A). The reduction in receptor density measured at several time intervals was maximal at 24 h with partial recovery by 72 h (Fig. 2B). Scatchard analysis of binding data for specific [^{125}I]CGP42112A binding to angiotensin II AT_2 receptors yielded a linear plot demonstrating a single class of angiotensin II-binding sites, with a maximum number of binding sites of 365 fmol/mg cell protein and a dissociation constant (K_d) of 7.3 nM. Exposure of cells to 10 μg/ml lipopolysaccharides for 24 h reduced the angiotensin binding maximum to 210 fmol/mg protein and there was no significant change in the K_d value.

The addition of the transcription inhibitor actinomycin D or the translation inhibitor cycloheximide to the incubation medium 20 min prior to the addition of lipopolysaccharides abolished the inhibitory effect on angiotensin II

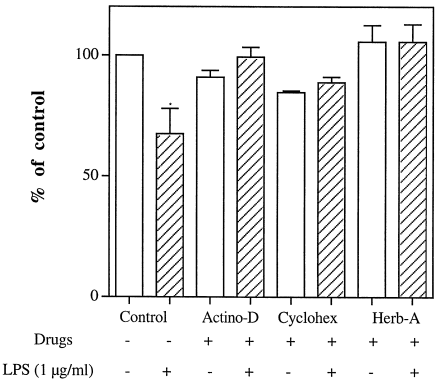

Fig. 3. Effects of actinomycin D, cycloheximide and herbimycin A on lipopolysaccharide (LPS)-induced downregulation of the angiotensin II AT_2 receptor in rat cardiac fibroblasts. Cells were incubated with actinomycin D (5 μg/ml), cycloheximide (5 μg/ml) or herbimycin A (1 μM) 20 min prior to the lipopolysaccharide (1 μg/ml) treatment. An [^{125}I]CGP42112A-binding assay was performed 6 h after the addition of lipopolysaccharides. Data are the means ± S.E. of two triplicate determinations. $^*P < 0.05$ compared to the control level.

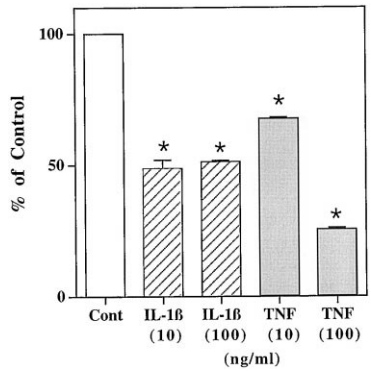

Fig. 5. Effect of interleukin (IL)-1β and tissue necrosis factor (TNF)-α on the expression levels of the angiotensin II AT_2 receptor in rat cardiac fibroblasts. Cells were incubated with various concentrations of interleukin-1β or TNF-α for 24 h as indicated. The receptor densities were determined by an [^{125}I]CGP42212A-binding assay. Data are the means ± S.E. of two triplicate determinations. $^*P < 0.05$ compared to the control level.

AT_2 receptor expression (Fig. 3). Pretreatment with the specific protein kinase C inhibitors bisindolylmaleimide (~ 1 μM) and calphostin C (1 μM) had no effect on the lipopolysaccharide inhibition of angiotensin II AT_2 receptor expression (data not shown). While the nonspecific protein kinase inhibitor H-7 (100 μM) only slightly attenuated the effect of lipopolysaccharides (26%), herbimycin A, a specific protein tyrosine kinase inhibitor, completely abolished the lipopolysaccharide effect (Fig. 3). Treatment of the cells for 30 min with L-NAME, a nonspecific inhibitor of NOS, abolished the inhibitory effect of lipopolysaccharides on angiotensin II AT_2 receptor expression (Fig. 4).

Since the cytokines interleukin-1β and TNF-α, as well as lipopolysaccharides, induce iNOS in vascular smooth muscle cells (Koide et al., 1993; Beasley and Eldridge, 1994), we examined the effect in rat cardiac fibroblasts of interleukin-1β and TNF-α on the expression of the angiotensin II AT_2 receptors. When rat cardiac fibroblasts were incubated with interleukin-1β for 24 h the receptor density declined approximately 50% (Fig. 5). TNF-α also lowered the receptor density in a dose-dependent fashion (Fig. 5).

In order to confirm the iNOS induction, rat cardiac fibroblasts were incubated for 18 h with lipopolysaccharides or the cytokines interleukin-1β or TNF-α. The induction of iNOS protein was then visualized by Western blot analysis with polyclonal mouse iNOS antibodies. All three agents induced the expression of iNOS protein (Fig. 6).

In order to determine if the NO effect is mediated by cGMP, rat cardiac fibroblasts were incubated for 6 h with lipopolysaccharides, with the NO donor sodium nitroprusside or with a stable cGMP analog 8-bromo-cGMP. Both the NO donor and the cGMP analog, as well as the

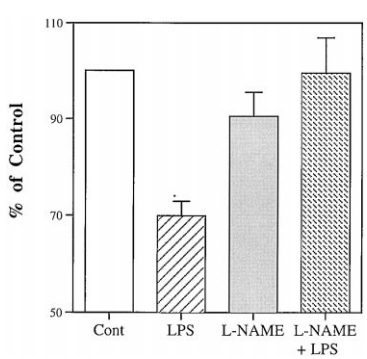

Fig. 4. Effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) on lipopolysaccharide (LPS)-induced downregulation of the angiotensin II AT_2 receptor in rat cardiac fibroblasts. Cells were incubated with L-NAME (200 μM) 30 min prior to lipopolysaccharide (1 μg/ml) treatment. The receptor levels were determined 24 h after the lipopolysaccharide treatment by an [^{125}I]CGP42212A-binding assay. Data are the means ± S.E. of two triplicate determinations. $^*P < 0.05$ compared to the control level.

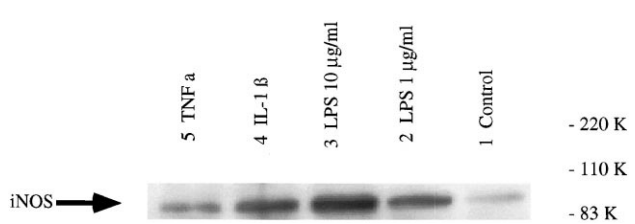

Fig. 6. Effects of lipopolysaccharides (LPS), interleukin (IL)-1β and tissue necrosis factor (TNF)-α on inducible nitric oxide synthase (iNOS) protein in rat cardiac fibroblasts. Rat cardiac fibroblasts were incubated for 18 h with lipopolysaccharides (lane 2: 1 μg/ml; lane 3: 10 μg/ml), interleukin-1β (lane 4: 10 ng/ml) or TNF-α (lane 5: 10 ng/ml). The cells were then lysed and subjected to SDS-polyacrylamide gel electrophoresis (approximately 30 μg protein per lane) followed by immunoblotting with polyclonal mouse iNOS antibodies. Data are representative of three experiments.

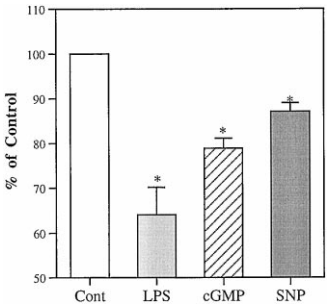

Fig. 7. Effects of the cyclic GMP analog 8-bromo-cGMP and nitric oxide donor sodium nitroprusside (SNP) on angiotensin II AT_2 receptor densities in rat cardiac fibroblasts. Cells were incubated with 8-bromo-cGMP, SNP, or lipopolysaccharides as indicated in the figure. An [^{125}I]CGP42212A-binding assay was performed 6 h after the addition of each chemical. Data are the means ± S.E. of two triplicate determinations. $^{*}P < 0.05$ compared to the control level.

lipopolysaccharides, significantly ($P < 0.05$) decreased the angiotensin II AT_2 receptor density (Fig. 7).

4. Discussion

The angiotensin II AT_2 receptor is postulated to play a role in cardiac fibrosis (Ohkubo et al., 1997; Matsubara, 1998; Tsutsumi et al., 1998). However, its pathophysiological significance in post-inflammatory myocarditis has not been clarified. The present study focused on determining the regulation mechanism of angiotensin II AT_2 receptor expression in a primary culture of neonatal cardiac fibroblasts in the presence or absence of lipopolysaccharides. Since cardiac fibroblasts are one of the cell types that express both the angiotensin II AT_2 and AT_1 receptors in adult tissues, the primary cultured fibroblasts are good models for the study of the regulation of angiotensin II AT_2 receptor expression.

In the present study we have confirmed the expression of both type 1 and type 2 angiotensin II receptors in rat cardiac fibroblasts. The angiotensin II AT_2 receptor in rat cardiac fibroblasts was dose- and time-dependently downregulated by lipopolysaccharides (Fig. 2). Similar downregulation was observed in the presence of the cytokines interleukin-1β and TNF-α (Fig. 5). Since lipopolysaccharide-dependent downregulation of the angiotensin II AT_2 receptor is blocked by actinomycin D and cycloheximide (Fig. 3), it is suggested that DNA transcription and new protein synthesis precedes the receptor downregulation. The nitric oxide synthase inhibitor L-NAME prevents the receptor downregulation by lipopolysaccharides (Fig. 4), implying that nitric oxide synthase must be transcribed and expressed prior to the initiation of the downregulation. Indeed, the present study demonstrated that lipopolysaccharides, interleukin-1β or TNF-α stimulated an induction of iNOS, the calcium-independent inducible form of nitric oxide synthase, in cardiac fibroblasts (Fig. 6). Furthermore, sodium nitroprusside, an NO donor, and 8-bromo-cGMP, a stable cGMP analog, mimicked the downregulation of the angiotensin II AT_2 receptor (Fig. 7). Accordingly, the summarized data suggest that lipopolysaccharide- or cytokine-dependent downregulation of the angiotensin II AT_2 receptor is mediated through an induction of iNOS and the resultant production of NO and cGMP. Further downstream signaling of NO and cGMP in this downregulation may involve either a decrease in the transcription of the angiotensin II AT_2 receptor gene or a destabilization of the angiotensin II AT_2 receptor mRNA. Clarification of these possibilities requires another series of study.

Ichiki et al. (1995) have shown that the angiotensin II AT_2 receptor is rapidly downregulated by the stimulation of protein kinase C activity and this may in some instances account for its low or absent expression in adult animals. High levels of lipopolysaccharides, which are amphiphilic compounds, allow lipopolysaccharides to cross the plasma membrane and bind to the phosphatidyl serine site of protein kinase C, thereby activating protein kinase C (Sweet and Hume, 1996). McKenna et al. (1995) reported that exposure of cardiac myocytes to lipopolysaccharides (100 ng/ml) for 6 h activated protein kinase C and that induction of iNOS by lipopolysaccharides was prevented by protein kinase C inhibitors. In the present study, however, the addition of specific protein kinase C inhibitors to the culture medium prior to the lipopolysaccharide treatment did not affect the angiotensin II AT_2 downregulation. In addition, a much lower concentration of lipopolysaccharides (10 ng/ml) significantly downregulated the angiotensin II AT_2 receptor (Fig. 2A). These data suggest that lipopolysaccharide -dependent downregulation of the angiotensin II AT_2 receptor in cardiac fibroblasts is not mediated through the protein kinase C pathway. Although this result is contradictory to the above-mentioned studies with cardiac myocytes (McKenna et al., 1995), it may be explained by the following. The protein kinase C-dependent downregulation of the angiotensin II AT_2 receptor is mediated through one particular isozyme of protein kinase C, which may be a novel protein kinase C isozyme missing in cardiac fibroblasts. This possibility seems reasonable, since cardiac fibroblasts are stromal cells which synthesize various proteins, such as fibronectin and collagens, to physically support myocytes, whereas cardiac myocytes are contractile and appear to be particularly sensitive to protein kinase C-dependent signaling in general. On the other hand, the protein tyrosine kinase inhibitor herbimycin A completely abolished the lipopolysaccharide effect, indicating that tyrosine phosphorylation is involved in the effect of lipopolysaccharides on the angiotensin II AT_2 receptor expression. This is consistent with reports that describe lipopolysaccharide- and cytokine-dependent induction of iNOS in macrophages (Wienstein et al., 1991) and in vascular endothelium (Marczin et al., 1993).

The levels of the angiotensin II AT_2 receptor have been shown to greatly increase in fibroblasts at sites of fibrosis in human hearts (Brink et al., 1996; Tsutsumi et al., 1998; Wharton et al., 1998) and in failing myopathic hamster hearts (Ohkubo et al., 1997). The angiotensin II AT_2 receptor in these cells has been shown to inhibit fibronectin and collagen synthesis (Ohkubo et al., 1997; Sabri et al., 1997) and directly antagonize the effects of the angiotensin II AT_1 receptor-mediated angiotensin II-dependent increase in collagen synthesis (Ohkubo et al., 1997). In myocarditis or chronic bacterial infection, the angiotensin II AT_2 receptor in cardiac fibroblasts is most likely downregulated by endotoxin and pro-inflammatory cytokines. Thus, downregulation of the angiotensin II AT_2 receptor may accelerate progression of the fibrosis. Our earlier study indicated that NO is ineffective against angiotensin II-dependent collagen deposition in coronary vasculature (Rizvi and Myers, 1997). This may also support the above hypothesis. It would therefore be important to clarify the pathophysiological significance of lipopolysaccharide- or proinflammatory cytokine-dependent downregulation of the angiotensin II AT_2 receptor. Nevertheless, the results provide potential evidence that the angiotensin II AT_2 receptor is negatively associated in the early developmental stage with the etiology of chronic inflammatory-dependent cardiac fibrosis. Whether the tissue-specific expression of the angiotensin II AT_2 receptor in cardiac fibroblasts prevents the development of cardiac fibrosis and protects cardiac function is of great interest. To the best of our knowledge, the present study is the first to demonstrate that the angiotensin II AT_2 receptor is regulated by lipopolysaccharides and proinflammatory cytokines.

Acknowledgements

We are deeply indebted to Dr. Tadashi Inagami (Department of Biochemistry, Vanderbilt University) for his enthusiastic support and encouragement. We are grateful to Ms. Pamela Tamura (Department of Chemistry, Vanderbilt University) for critical reading and constructive comments during the preparation of the manuscript. This work was supported by a Veterans Administration Merit Review (P.R.M.), a pilot project grant from the Center in Molecular Toxicology, Vanderbilt University (3P30-ES00267-32, M.T.) and a Grant in Aid from the American Heart Association (National Center, 9750624N, M.T.).

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