Effects of Bay 10–6734 (Embusartan), a New Angiotensin II Type I Receptor Antagonist, on Vascular Smooth Muscle Cell Growth

L. IOUZALEN, O. STEPIEN, and P. MARCHE

Department of Pharmacology, CNRS URA 1482, University René Descartes, Necker Medical School, Paris, France

Accepted for publication November 4, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

Angiotensin II (AII), an important hypertrophic factor in the cardiovascular system, exerts most of its known effects in vivo through the AII receptor type 1 (AT1) subclass of AII receptors. These receptors are also responsible for the growth-related effects of AII in cultured vascular smooth muscle cells (VSMCs). We presently investigated the effects of BAY 10–6734 (Embusartan), a new orally active AT1 antagonist, on VSMC growth and proliferation of cultured VSMCs isolated from the aortae of Wistar Kyoto rats and spontaneously hypertensive rats. BAY 10–6734 and losartan (considered as AT1 receptor antagonist of reference), as well as their respective active metabolites, were studied for their inhibition of: 1) [125I]AII binding to its receptors, 2) AII-induced DNA and protein synthesis (by measuring the incorporation of 5-bromo-2′-deoxyuridine and [3H]leucine, respectively), and 3) AII-induced variations in intracellular Ca2+ concentration, using cells labeled with Fura-2. All of the tested compounds inhibited the aforementioned parameters in a concentration-dependent manner. Half-maximal inhibitory concentration values indicated that BAY 10–6734 was significantly more potent than losartan and that spontaneously hypertensive rat-derived VSMCs were more sensitive than Wistar Kyoto rat-derived ones. Neither BAY 10–6734 nor losartan affected the intracellular Ca2+ concentration of unstimulated VSMCs but both compounds inhibited both AII-induced Ca2+ mobilization from internal stores and Ca2+ influx. Neither compound affected arginine-vasopressin-, basic fibroblast growth factor-, or serum-induced DNA and protein synthesis. BAY 10–6734 appears therefore as a potent and specific new inhibitor of AII-induced growth-related events in VSMCs.

Abnormal accumulation of vascular smooth muscle cells (VSMCs) likely participates in smooth muscle hypertrophy, which is often associated with many vascular diseases including atherosclerosis, restenosis after balloon angioplasty, and primary hypertension (see Ross, 1993 for review). This may result from an uncontrolled proliferation of VSMCs in response to growth factors. Within the vasculature the renin-angiotensin system plays an important physiological role, not only through the regulation of blood pressure (Vallotton, 1987), but also through the modulation by its components, of the so-called vascular remodeling (Griffin et al., 1991). In this respect, the modulation in vitro by angiotensin II (AII) of VSMC growth, i.e., the modulation of mitogenic and trophic actions of AII, has been documented (Timmermans et al., 1993). BAY 10–6734 (Embusartan; 6-butyl-1-[(3-fluoro-2′-1H-tetrazol-5-yl-biphenyl-4-yl)-methyl]-2-oxo-1,2-dihydropropyridine-4-carboxylic) acid methylester is another prototype of AT1 receptor antagonists, has been demonstrated to be a potent antihypertensive agent that inhibits most of AII-induced intracellular responses including the growth-related cellular events (Ko et al., 1992; Lyall et al., 1992; Catalioto et al., 1995; Duff et al., 1995; Leduc et al., 1995). BAY 10–6734 (Embusartan; 6-butyl-1-[(3-fluoro-2′-1H-tetrazol-5-yl-biphenyl-4-yl)-methyl]-2-oxo-1,2-dihydropyridine-4-carboxylic) acid methylester is another newly developed orally active antihypertensive compound.

In vivo, the local AII production has been recently shown to directly affect vascular hypertrophy (Moriyama et al., 1994). Although the mechanisms whereby AII stimulates VSMC growth are not fully understood and remain controversial (Geisterfer et al., 1988; Paquet et al., 1990), it is widely accepted that these growth-related events are mediated through AT1 receptors (Timmermans et al., 1993; Sung et al., 1994).

Recently, a novel class of AT1 receptor antagonists that are nonpeptidic and devoid of agonistic properties has been developed (Timmermans et al., 1993). In VSMC, losartan, the prototype of AT1 receptor antagonists, has been demonstrated to be a potent antihypertensive agent that inhibits most of AII-induced intracellular responses including the growth-related cellular events (Ko et al., 1992; Lyall et al., 1992; Catalioto et al., 1995; Duff et al., 1995; Leduc et al., 1995). BAY 10–6734 (Embusartan; 6-butyl-1-[(3-fluoro-2′-1H-tetrazol-5-yl-biphenyl-4-yl)-methyl]-2-oxo-1,2-dihydropyridine-4-carboxylic) acid methylester is another newly developed orally active antihypertensive compound.

ABBREVIATIONS: VSMC, vascular smooth muscle cell; AII, angiotensin II; AT1, angiotensin II type 1 receptor; [Ca2+]i, cystolic calcium concentration; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; BrdU, 5-bromo-2′-deoxyuridine; bFGF, basic fibroblast growth factor; TCA, trichloroacetic acid.
Embuvastaran is a nonpeptide-specific AT1 receptor antagonist that has proven to be efficacious in various animal models of hypertension (Stasch et al., 1997). The influence of BAY 10–6734 upon AII-induced vascular hypertrophy has not been investigated. This study was therefore undertaken to determine whether BAY 10–6734 could inhibit AII-stimulated growth of cultured VSMCs isolated from spontaneously hypertensive (SHR) and control normotensive Wistar Kyoto (WKY) rats. The effects obtained with BAY 10–6734 were compared to those obtained with losartan, used as a positive control. In addition, the effects of BAY 10–6735, the predominant therapeutically active moiety of BAY 10–6734, and those of EXP 3174, the active metabolite of losartan, were also investigated.

**Experimental Procedures**

**Materials.** Ten-week-old male WKYs and SHRs were purchased from Iffa-Credo (les Oncins, France). Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin, trypsin, and HEPES buffer were purchased from Life Technologies (Cergy Pontoise, France). Fetal calf serum (FCS) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). [125I]AII (81.4 TBq/mmol) and [3H]leucine (5.18 TBq/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled AII and all other chemicals were obtained from Sigma (St. Louis, MO). BAY 10–6734, BAY 10–6735 (the carboxylic acid derivative of BAY 10–6734), EXP 3174, and losartan were synthesized by Bayer AG (Leverkusen, Germany) for pharmacological research.

**Cell Culture.** VSMCs were cultured according to Ross (1971) as previously described (Stepien et al., 1998). Briefly, the media of thoracic aortae were isolated and cut into pieces of 1–2 mm². After removal of adventitia and endothelium using fine forceps, explants of the media were then cultured in DMEM containing 15% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 8 mM HEPES. Cells grown to confluence were detached with 0.125% trypsin and subcultured every week in a similar culture medium containing 10% FCS. For each experiment, the cells were allowed to grow for 4 to 5 days in 5% CO₂, 95% air at 37°C, until they were subconfluent. Then VSMCs were made quiescent, i.e., synchronized to the G0/G1 phase, by serum deprivation for 48 h before stimulation. Unless specified, subconfluent VSMCs between passages 4 and 13 were used for experiments.

**Measurement of Cytosolic Calcium.** Cytosolic calcium concentration ([Ca²⁺]i) was measured using the fluorescent Ca²⁺ indicator Fura-2 (Grynkiewicz et al., 1985). VSMCs were incubated with serum-free DMEM for 48 h and washed with buffered solution containing (in mM): 135 NaCl, 5.4 KCl, 44 NaHCO₃, 5 glucose, 0.8 MgSO₄, 0.9 NaH₂PO₄, and 10 HEPES, pH 7.4 at 37°C. VSMCs were then incubated in the same medium in the presence of 2 μM Fura-2/AM for 30 min at 37°C. The cells were washed twice to remove the external dye, and placed in the quartz cuvette for [Ca²⁺]i measurement at 37°C. Fluorescence was recorded with excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm on a spectrofluorometer SPEX CMIII (ISA-Jobin-Yvon, Longjumeau, France). [Ca²⁺]i was calculated as described (Grynkiewicz et al., 1985).

**Data Analysis.** All experiments were performed in triplicate and values are expressed as means ± S.E.M. of n distinct experiments. When AT1 antagonists were tested, the experiments included the four antagonists, irrespective of the passage used. The IC₅₀ values were calculated by linear regression. Multiple comparisons and dose-response and time-dependent effects were examined by one-way ANOVA and posthoc Fisher’s test. Comparisons of dose-response effects between two different groups were assessed by two-way ANOVA.

**Results**

**Effect on Specific AII Binding.** The capacity of cultured VSMCs to bind AII was assessed in a first set of experiments. Both WKY- and SHR-derived VSMCs bound [125I]AII with high affinity, and Scatchard analysis of results (data not shown) indicated the presence of one single class of binding sites with K_D values of 0.51 ± 0.14 nM and 0.50 ± 0.08 nM, and a binding capacity of 45 ± 7 fmol/mg protein and 27 ± 2 fmol/mg protein, for WKY- and SHR-derived VSMCs, respectively. These characteristics are in agreement with what has already been reported (Sachinidis et al., 1993; Cahill et al., 1995).

In both WKY- and SHR-derived VSMC cultures, the specific binding of [125I]AII was inhibited in a concentration-dependent manner by the various AT1 antagonists tested (Fig. 1, A and B). Table 1 summarizes the IC₅₀ obtained with these compounds for VSMCs isolated from both rat strains. BAY 10–6734 appeared to exhibit more affinity than losar-
tan because its IC$_{50}$ value was significantly lower ($p < .001$) and, hence, appeared to have a better affinity for the AT$_{1}$ receptor. Table 1 also shows that IC$_{50}$ values were significantly lower in SHR-derived VSMCs compared with WKY-derived cells, indicating that the cells isolated from SHR were more sensitive than those from WKY.

**Effect of Bay 10–6734 on DNA Synthesis.** Exposure of VSMC cultures to AII significantly increased DNA synthesis in a concentration-dependent manner as indicated by the increase in BrdU incorporation ($p < .001$ for both, $n = 7–21$; Fig. 2A). In both SHR and WKY cultures, the maximum BrdU incorporation—3 to 5 times the incorporation in unstimulated cells—was reached with 1 $\mu$m AII (Fig. 2A). For AII concentrations above 10 nM, DNA synthesis was significantly increased in SHR compared to WKY, when analyzed by two-way ANOVA ($p < .01$).

In both WKY- and SHR-derived VSMCs, BAY 10–6734, losartan, and their respective metabolites did not promote DNA synthesis when added alone but inhibited AII-induced DNA synthesis in a concentration-dependent manner (Fig. 2, B and C). Statistical analysis (ANOVA) indicated that BAY 10–6734 was significantly more potent than losartan ($p < .001$) and EXP 3174 ($p < .05$), and BAY 10–6735 ($p < .05$) in WKY-derived VSMCs; in SHR-derived VSMCs, BAY 10–6734 was significantly more potent than losartan ($p < .001$) and EXP 3174 ($p < .05$). Table 2, which shows IC$_{50}$ values for each of the antagonists tested, clearly indicates that SHR-derived VSMCs were significantly more sensitive than WKY-derived VSMCs.

In another series of experiments, DNA synthesis was also stimulated in a concentration-dependent manner by basic fibroblast growth factor (bFGF), AVP, and FCS, as reported

---

**Table 1**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>WKY IC$_{50}$</th>
<th>SHR IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAY 10–6734</td>
<td>3.8 ± 0.5</td>
<td>0.07 ± 0.01$^{*}$</td>
</tr>
<tr>
<td>BAY 10–6735</td>
<td>4.9 ± 0.8</td>
<td>0.6 ± 0.1$^{*}$</td>
</tr>
<tr>
<td>Losartan</td>
<td>8.6 ± 1.3$^{*}$</td>
<td>1.2 ± 0.2$^{*}$</td>
</tr>
<tr>
<td>EXP 3174</td>
<td>0.95 ± 0.08</td>
<td>0.11 ± 0.02$^{*}$</td>
</tr>
</tbody>
</table>

Experiments were carried out as described in the legend of Fig. 1.

$^{*}p < .001$ compared with WKY

$^{*}p < .001$ losartan versus BAY 10-6734

---
by others (Bobik and Campbell, 1993; Stepien et al., 1998). In such cases, preincubation of VSMCs with 100 nM BAY 10–6734 or losartan did not affect the stimulus-induced DNA synthesis (data not shown).

**Effect on Protein Synthesis.** In both WKY- and SHR-derived VSMCs, AII induced protein synthesis in a concentration-dependent manner, as assessed by [3H]-leucine incorporation (Fig. 3A; p < .001 for both, n = 5–7). Maximum [3H]-leucine incorporations were 2- and 3-fold the basal level in WKY- and SHR-derived VSMCs, respectively. For AII concentrations above 0.1 μM, [3H]-leucine incorporations were significantly higher in SHR VSMCs than in WKY ones (p < .01, n = 5–7).

In both WKY- and SHR-derived VSMCs, BAY 10–6734, losartan, and their respective metabolites did not promote [3H]-leucine incorporation when added alone. By contrast, preincubation of WKY- and SHR-derived VSMCs with each of these drugs inhibited AII-induced [3H]-leucine incorporation in a concentration-dependent manner (Fig. 3, B and C; p < .001, n = 4 for all tested drugs and with both WKY- and SHR-derived VSMCs). IC₅₀ values for each antagonist are given in Table 3. SHR-derived VSMCs were significantly more sensitive to BAY 10-6734 than WKY-derived VSMCs (Table 3). Moreover, in SHR-derived VSMCs, BAY 10–6734 appeared to be significantly more efficient than losartan (p < .001).

**Effect on All-Inhibited [Ca²⁺]ᵢ Variations.** In both WKY- and SHR-derived VSMCs, and in the presence of transmembrane calcium gradient, i.e., in the presence of 1 mM [Ca²⁺]₀x, AII (100 nM) induced a biphasic [Ca²⁺]ᵢ rise comprising a transient peak and a sustained phase represented by a plateau (Fig. 4). The observed transient and sustained phases likely reflect the release from internal stores and the calcium influx activation, respectively, because the absence of the transmembrane calcium gradient ([Ca²⁺]₀x = 50 nM) suppressed the latest phase without affecting the former (data not shown).

The effects of BAY 10–6734, losartan, or their respective metabolites on All-induced [Ca²⁺]ᵢ variations were measured by preincubating VSMCs with the antagonist (100 nM) before the addition of AII (100 nM), in the presence of 1 mM [Ca²⁺]₀x. Neither BAY 10–6734, losartan, nor their respective metabolites affected the basal [Ca²⁺]ᵢ level, which was 143 ± 24 nM and 116 ± 20 nM, respectively (NS/data not shown). By contrast, all AT₁ receptor antagonists tested drastically blunted both the All-induced transient calcium increase (expressed as the [Ca²⁺]ᵢ value at the peak diminished by the basal [Ca²⁺]ᵢ value) and the sustained phase (expressed as the [Ca²⁺]ᵢ value at the plateau level diminished by the basal [Ca²⁺]ᵢ value) (Fig. 4 for representative tracings and Fig. 5, A and B). The [Ca²⁺]ᵢ rise was totally suppressed when VSMCs were preincubated with 1 μM BAY 10–6734 or losartan (data not shown), indicating that these drugs acted in a concentration-dependent manner. Likewise, in a Ca²⁺-free external medium ([Ca²⁺]₀x=50 nM), All-induced [Ca²⁺]ᵢ increase was inhibited by BAY 10–6734 and

**TABLE 2**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>WKY IC₅₀ (nM)</th>
<th>SHR IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAY 10-6734</td>
<td>8.8 ± 1.2</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>BAY 10-6735</td>
<td>12.7 ± 1.9</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Losartan</td>
<td>279 ± 46</td>
<td>104 ± 23</td>
</tr>
<tr>
<td>EXP 3174</td>
<td>35 ± 5</td>
<td>1.12 ± 0.17</td>
</tr>
</tbody>
</table>

Experiments were carried out as described in the legend of Fig. 2.

a p < .001 compared with WKY
b p < .001 losartan versus BAY 10-6734

cp < .001 compared with WKY

---

**TABE 3**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>WKY IC₅₀ (nM)</th>
<th>SHR IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAY 10-6734</td>
<td>102 ± 9</td>
<td>1.1 ± 0.12</td>
</tr>
<tr>
<td>BAY 10-6735</td>
<td>~1</td>
<td>0.9 ± 0.15</td>
</tr>
<tr>
<td>Losartan</td>
<td>90 ± 13</td>
<td>82 ± 9</td>
</tr>
<tr>
<td>EXP 3174</td>
<td>~1</td>
<td>9.1 ± 1.7</td>
</tr>
</tbody>
</table>

Experiments were carried out as described in the legend of Fig. 4.

a p < .001 compared with WKY
b p < .001 losartan versus BAY 10-6734
The present investigations were designed to determine the effects of BAY 10–6734, a newly developed orally active antihypertensive drug, which has been classified as an AT1 receptor antagonist (Stasch et al., 1997; Bohm et al., 1998), on AII-induced hypertrophy/hyperplasia of VSMCs. The in vitro effects of BAY 10–6734 on AII-induced growth-related events were, therefore, studied in VSMCs isolated from normotensive and hypertensive rats. Using WKY- and SHR-derived cultured VSMCs, we examined the effect of BAY 10–6734 and losartan, and their metabolites BAY 10–6735 and EXP 3174, respectively, on AII binding, AII-induced DNA and protein synthesis, and AII-induced variations in intracellular [Ca²⁺]. There is compelling evidence that such AII-induced hyperplasia and hypertrophy are mediated by the type I AII receptors (Geisterfer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Bunkenburg et al., 1992). Moreover, in culture, VSMCs have been demonstrated to be devoid of type II AII receptors (de Gasparo et al., 1990).

In the present study, BAY 10–6734 has been shown to antagonize the specific binding of [¹²⁵I]AII to both WKY- and SHR-derived VSMCs (Fig. 1). In VSMCs isolated from WKY, both BAY 10–6734 and losartan inhibited AII binding with the same affinity, as indicated by their IC⁵⁰ values (Table 1). However, BAY 10–6734 was significantly (>10 times) more potent than losartan for displacing [¹²⁵I]AII binding to VSMCs isolated from SHR (Table 1). IC⁵⁰ values similar to those obtained here have been reported for other AT1 receptor antagonists, including TCV-116, UP 269–6, HR 720, and SK&F 108566 (Sung et al., 1994; Flesch et al., 1995; Dunn et al., 1997; Virone-Oddos et al., 1997). Regarding the potency of each AT1 receptor antagonist used to inhibit [¹²⁵I]AII binding, Table 1 also indicates that SHR VSMCs were more sensitive than WKY ones, as already reported with UP 269–6 (Virone-Oddos et al., 1997).

The hyperplastic effect of AII in cultured VSMCs is still controversial (Geisterfer et al., 1988; Berk et al., 1989; Sachindis et al., 1993). Under the present experimental conditions, AII behaved as a weak mitogen for VSMCs (EC⁵⁰; 10⁻¹⁰ M). Nevertheless, AII did stimulate DNA synthesis in a concentration-dependent manner in both WKY- and SHR-derived VSMCs, the latter being significantly more sensitive (Fig. 2A), consistent with previous reports (Hamada et al., 1990; Paquet et al., 1990; Morton et al., 1995). In both cell types examined, BAY 10–6734 and losartan, as well as their respective metabolites, inhibited AII-induced DNA synthesis.
in a concentration-dependent manner (Fig. 2, B and C). IC50 values similar to those presented in Table 2 have been reported for other AT1 antagonists, including SK&F 105665, TCV-116, losartan, and their respective metabolites CV 11974 and EXP 3174 (Briand et al., 1994; Sung et al., 1994; Flesch et al., 1995; Itazaki et al., 1995). In both WKY- and SHR-derived VSMCs, BAY 10–6734 was significantly more potent that losartan (Table 2). BAY 10–6734 was as potent as its metabolite BAY 10–6735 (Table 2); however, EXP 3174 was more potent than losartan, its parent compound, in agreement with a previous report (Sachinidis et al., 1993). As might be expected with a specific AT1 antagonist, BAY 10–6734 did not influence serum-, AVP-, or bFGF-induced DNA synthesis (not shown).

As already published by others (Berk et al., 1989; Catalioto et al., 1995; Dunn et al., 1997; Vironne-Oddos et al., 1997), AII also stimulated protein synthesis in VSMCs (Fig. 3A). The various compounds tested in this study inhibited AII-induced protein synthesis in a concentration-dependent manner in both WKY- and SHR-derived VSMCs (Fig. 3, B and C). Although BAY 10–6734 and losartan exhibited similar affinity for inhibiting protein synthesis in WKY-derived VSMCs, the affinity of BAY 10–6734 appeared ~10 times greater than that of losartan in SHR-derived VSMCs (Table 3). Table 3 also indicates that IC50 values for BAY 10–6734 and its metabolite were in the range of those reported for other AT1 antagonists (Dunn et al., 1997; Vironne-Oddos et al., 1997) and SHR-derived VSMCs were more sensitive to BAY 10–6734 than WKY-derived cells.

In VSMC, AII-induced elevation of [Ca2+], is a primary signaling event for stimulating mitogen-activated protein kinase pathways (see Schmitz and Berk, 1997 for review). AII-induced [Ca2+], variations presented here (Fig. 4) are consistent with what has been previously reported (Neusser et al., 1993; Sachinidis et al., 1993; Koh et al., 1994). BAY 10–6734, losartan, and the metabolites tested did not affect the basal level of [Ca2+], but they inhibited AII-induced Ca2+ movements (Figs. 4 and 5). Irrespective of the cells examined, BAY 10–6734 and its metabolite BAY 10–6735 tremendously reduced AII-induced transient Ca2+ elevation (i.e., AII-induced Ca2+ mobilization from internal stores) and also abolished the sustained phase (i.e., AII-induced Ca2+-influx). Losartan and its metabolite behave similarly, as expected from previous reports dealing with AT1 receptor antagonists (Ko et al., 1992; Sachinidis et al., 1993; Koh et al., 1994; Itazaki et al., 1995). Both BAY 10–6735 and EXP 3174 appeared more potent than their respective parent compounds; this observation has already been reported for the losartan metabolite (Sachinidis et al., 1993). As a specific AT1 receptor antagonist, BAY 10–6734 did not affect thrombin-, AVP-, or bFGF-induced variations of [Ca2+], (results not shown), indicating that the compound did not inhibit cellular Ca2+-ATPases.

Taken together, our results demonstrate that BAY 10–6734 and its active metabolite BAY 10–6735 behave as other AT1 antagonists and are potent and specific inhibitors of AII-induced growth-related events in VSMCs. One may envisage that such a potency participates in the antihypertensive properties of BAY 10–6734 in the various animal models of hypertension (Stasch et al., 1997), particularly in the SHR, where AII has been shown to exert more marked hyperplastic and hypertrophic effects (Bunkenburg et al., 1992 and this study) and where cellular hyperactivity has been well documented (Marche et al., 1995).

Acknowledgments

We thank Dr. Andreas Knorr for steady encouragement throughout the study.

References


Morton C, Baines R, Masood I, Ng L and Boarder MR (1995) Stimulation of two...


Send reprint requests to: Dr P. Marche, Pharmacologie, CNRS URA 1482, Université René Descartes, Faculté de Médecine Necker, 156 rue de Vaugirard, 75015 Paris, France. E-mail: marche@necker.fr