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European Journal of Pharmacology 485 (2004) 243–249
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Angiotensin II stimulates superoxide production via both angiotensin AT_{1A} and AT_{1B} receptors in mouse aorta and heart

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Received 12 June 2003; received in revised form 20 November 2003; accepted 28 November 2003

Abstract

The present study was conducted to determine the roles of angiotensin AT_{1A} and AT_{1B} receptors in angiotensin II-induced superoxide anion production in mouse aorta and heart. Superoxide anion production in aorta was determined by the lucigenin chemiluminescence method, and thiobarbituric acid reactive substances in heart tissues were measured by biochemical assay. The basal production rate of superoxide anion in aorta of wild type (WT) mice was significantly higher than in angiotensin AT_{1A} receptor knockout (AT_{1A} KO) mice. Angiotensin II (2.8 mg/kg/day, s.c. for 13 days) significantly increased superoxide anion production in aorta of both AT_{1A} KO and WT mice. However, the superoxide anion production rate in aorta of angiotensin II-infused AT_{1A} KO mice was significantly lower than in angiotensin II-infused WT mice. Valsartan (40 mg/kg/day in drinking water) prevented angiotensin II-induced superoxide anion production in aorta of WT and AT_{1A} KO mice. Similarly, thiobarbituric acid reactive substances levels in heart tissues of angiotensin II-treated WT and AT_{1A} KO mice were significantly higher than those in vehicle-infused WT and AT_{1A} KO mice, respectively. Valsartan prevented angiotensin II-induced increases of thiobarbituric acid reactive substances levels in heart tissue of both WT and AT_{1A} KO mice. These results indicate that angiotensin II stimulates superoxide anion production via both angiotensin AT_{1A} and AT_{1B} receptors, and that angiotensin AT_{1A} receptors appear to play a predominant role in angiotensin II-induced superoxide anion production in mouse aorta and heart.

Keywords: Angiotensin II; Superoxide anion; Angiotensin AT_{1A} receptor; Angiotensin AT_{1B} receptor; Aorta; Heart

1. Introduction

A growing body of evidence indicates that angiotensin II stimulates the production of free oxygen radicals (Berry et al., 2000; Fukui et al., 1997; Griendling et al., 1994; Haas et al., 1999; Laursen et al., 1997; Rajagopalan et al., 1996; Romero, 1999) and potentiates the vasoconstrictor effects of angiotensin II (Laursen et al., 1997; Nishiyama et al., 2001b; Romero, 1999; Schnackenberg and Wilcox, 1999). Recent studies also suggest that angiotensin II activates NAD(P)H-dependent oxidases, an important enzymatic origin of superoxide anions in non-phagocytic cells, such as vascular smooth muscle cells, endothelial cells and heart tissue (Bendall et al., 2002; Berry et al., 2000; Griendling et al., 1994; Harrison, 1997; Ushio-Fukai et al., 1996), via angiotensin AT_1 receptor stimulation (Cifuentes et al., 2000; Fukui et al., 1997; Rajagopalan et al., 1996). Among the angiotensin AT_1 receptors, two subtypes, angiotensin AT_{1A} and AT_{1B}, have been identified in human, rat and mouse. Although it is known that these receptors are products of separate genes and share substantial sequence homology, currently available angiotensin AT_1 receptor antagonists cannot distinguish between angiotensin AT_{1A} and AT_{1B} receptor subtypes (Elton et al., 1992; Iwai and Inagami, 1992; Konishi et al., 1994; Murphy et al., 1992). Gene targeting is an alternative approach to clarify the functional significance of distinct angiotensin AT_1 receptor subtypes in vivo. Angiotensin AT_{1A} receptor knockout (AT_{1A} KO) mice have recently been developed. Resting blood pressure in AT_{1A} KO mice is substantially lower and acute pressure responses to administered angiotensin II are attenuated (Cervenka et al., 1999; Ito et al., 1995; Oliverio et al., 1997; Ruan et al., 1999; Sugaya et al., 1995). In contrast, angiotensin AT_{1B} receptor knockout mice (AT_{1B} KO mice) have normal blood pressure and angiotensin II causes similar dose proportional increases in

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doi:10.1016/j.ejphar.2003.11.074

blood pressure in AT_{1B} KO mice and wild type (WT) mice (Oliverio et al., 1998). These findings suggest an important role for angiotensin AT_{1A} receptors in the regulation of angiotensin II-dependent blood pressure and vascular tone. However, it has also been reported that, when endogenous angiotensin II production is suppressed by angiotensin converting enzyme inhibitor, modest pressor and renal vasoconstrictor effects of angiotensin II can be detected in AT_{1A} KO mice. This pressure response was completely inhibited by an angiotensin AT_1 receptor antagonist (Oliverio et al., 1997; Ruan et al., 1999). Although these findings suggest some roles for angiotensin AT_{1B} receptors in the regulation of blood pressure, it is not clear whether angiotensin AT_{1B} receptors mediate any of the effects of angiotensin II on cardiovascular function.

It has not yet been determined which subtypes of angiotensin AT_1 receptors are involved in the angiotensin II-induced superoxide production. In this regard, AT_{1A} KO mice may provide a good opportunity to distinguish a role of angiotensin AT_{1B} receptors from that of angiotensin AT_{1A} receptors. Thus, the present experiment was designed to determine the relative contributions of angiotensin AT_{1A} and AT_{1B} receptors to angiotensin II-induced superoxide production using WT and AT_{1A} KO mice. Moreover, we used valsartan, an angiotensin AT_1 receptor antagonist, which is known to block both angiotensin AT_{1A} and AT_{1B} receptors, to determine the role of the angiotensin AT_{1B} receptor in AT_{1A} KO mice.

2. Materials and methods

2.1. Materials

Lucigenin (bis-*N*-methylacridinium nitrate), diethyldithiocarbamate (DETC), Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid) and angiotensin II (Asp-Arg-Val-Tyr-IIe-His-Pro-Phe) were purchased from Sigma (St. Louis, MO). Valsartan was obtained from Novartis Pharma (Basel, Switzerland). Lucigenin and Tiron were dissolved in Krebs-Hepes buffer (pH 7.4). DETC and angiotensin II were dissolved in bicarbonate buffer (pH 7.4) and 5% acetic acid, respectively.

2.2. Animal preparation

We used AT_{1A} KO mice generated from C57BL/6 mice by Sugaya et al. (1995). Male C57BL/6 AT_{1A} KO and WT mice were housed in separate cages, and maintained at room temperature on a 12-h light/dark cycle. Throughout the experiments, the animals had free access to water and standard mouse chow. All surgical and experimental procedures were performed according to the guidelines for the care and use of animals as established by Kagawa Medical University. AT_{1A} KO and WT mice weighing 25–30 g at the beginning of the experiments were randomly divided into four groups: sham (WT, n=8; AT_{1A} KO, n=8), angiotensin II (WT, n=9; AT_{1A} KO, n=9), valsartan (WT, n=9; AT_{1A} KO, n=9) and valsartan plus angiotensin II (WT, n=9; AT_{1A} KO, n=9) groups. The mice were anesthetized with inhaled diethyl ether. An incision was made in the midscapular region under sterile conditions and osmotic minipumps (Alzet model 2002; Alza Corporation) containing angiotensin II or vehicle (5% acetic acid) were implanted. The delivery rate of angiotensin II was 2.8 mg/kg/day for 13 days. Some of the animals in the sham and angiotensin II groups were given valsartan (40 mg/kg/day) in the drinking water, starting on the same day as the pump implantation, throughout vehicle or angiotensin II infusion. Systolic blood pressure was determined before starting and on the 13th day of treatment by tail-cuff plethysmography. Four to eight repeated values were averaged at each determination. On the 13th day of treatment, the mice were killed and the thoracic and abdominal aortas were removed. The heart was harvested in liquid nitrogen and stored at −70 °C.

2.3. Measurement of vascular superoxide anion production

Superoxide anion production in aortic segments from WT and AT_{1A} KO mice was determined using lucigenin chemiluminescence. The details of this assay have been published previously (Nishiyama et al., 2001b). Briefly, the animals were killed with an excess dose of pentobarbital sodium, and the aorta was quickly removed and placed on a petri dish containing pre-warmed bicarbonate buffer composed of (in mmol/l) NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 5.5 and EDTA 0.026. Perivascular tissue was carefully removed and the vessels were repeatedly washed to remove adherent blood cells, and then cut into 0.5-cm long rings. The aorta was placed in a test tube containing bicarbonate buffer and DETC (10 mM), and incubated for 30 min at 37 °C and pH 7.4 by gassing with 95% O_2–5% CO_2. DETC was used to inhibit the Cu^{2+}/Zn^{2+} form of superoxide dismutase (Heikkila and Cohen, 1977; Omar et al., 1991). After equilibration, the rings were rinsed with pre-warmed (37 °C) modified Krebs-HEPES buffer composed of (in mmol/l) NaCl 119, HEPES 20, KCl 4.6, $CaCl_2$ 1.2, KH_2PO_4 0.4, $MgSO_4$ 1.0, Na_2HPO_4 0.15, $NaHCO_3$ 25.0, glucose 5.5 (pH 7.4). The aorta was then transferred into test tubes containing 1 ml of lucigenin (250 μmol/l of Krebs-HEPES buffer) and equilibrated in the dark for 10 min at 37 °C. Chemiluminescence was then recorded every 30 s for 5 min with a luminescence reader (BLR-301; Aloka). After measurements of lucigenin chemiluminescence in DETC-treated aortic rings, Tiron (20 mmol/l), a cell-permeant, non-enzymatic scavenger of superoxide anion (Gyellenhammar, 1987) was then added to quench all superoxide anion-dependent chemiluminescence (Omar et al., 1991) and 18 more cycles were read; the final 10 values, which appeared to be maximally reduced, were averaged. Lucigenin chemiluminescence was expressed as counts per minute per milligram of dry tissue weight (cpm/mg of dry tissue weight). The differences between the values obtained

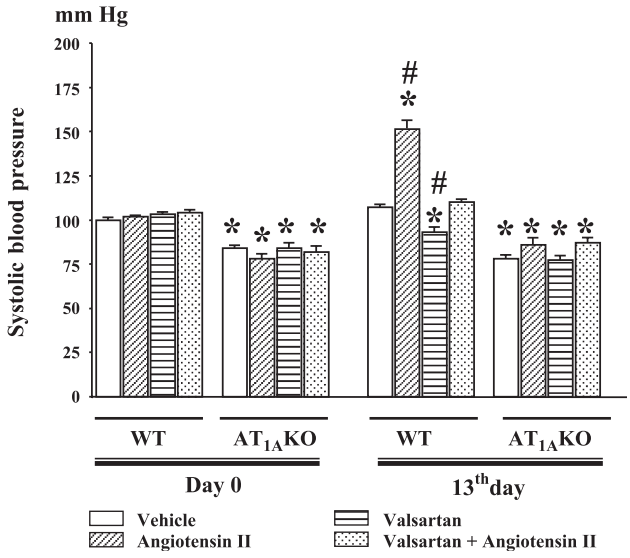

Fig. 1. Bar graph shows systolic blood pressure in WT and angiotensin AT_{1A} KO mice. 0 and 13th day indicate before starting and on the 13th day of treatment, respectively. Data represent means±S.E.M. *P<0.05 versus WT sham and #P<0.05 versus day 0 in the same group.

before and after adding Tiron were calculated and defined as the Tiron-quenchable lucigenin chemiluminescence. The superoxide anion production rate in the aorta was assessed by Tiron-quenchable lucigenin chemiluminescence.

2.4. Measurement of thiobarbituric acid reactive substances in heart tissues

Thiobarbituric acid reactive substances levels in heart tissue were measured according to the method of Kikugawa et al. (1992). Briefly, heart tissue was homogenized (5% wt/vol) in a solution containing 0.15 M KCl and 0.02 M Tris–HCl (pH 7.4). The homogenate was mixed with 15% trichloroacetic acid and 0.375% thiobarbituric acid. Butylated hydroxytoluene (0.01%) was added to the assay mixture to prevent autoxidation of samples. The mixture was heated at 100 °C for 15 min. After cooling, the mixture was centrifuged at 3500 rpm for 20 min. Absorbance of the organic phase was measured at 535 nm. The amount of thiobarbituric acid reactive substances in the sample was determined with a known standard and expressed as nmol/g of wet tissue weight.

2.5. Statistical analysis

All values are expressed as means±S.E.M. Analyses of blood pressure data were performed with two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. Statistical evaluation of the lucigenin chemiluminescence levels in the aorta and thiobarbituric acid reactive substances levels in heart was performed using an unpaired t-test. A value of P<0.05 was considered statistically significant.

3. Results

3.1. Systolic blood pressure in vehicle, angiotensin II, valsartan and valsartan plus angiotensin II-treated WT and AT_{1A} KO mice

Fig. 1 shows systolic blood pressure in vehicle, angiotensin II, valsartan and valsartan plus angiotensin II-treated WT and AT_{1A} KO mice. Before starting treatment (0 day), systolic blood pressure was not different among the animals receiving vehicle, angiotensin II, valsartan or valsartan plus angiotensin II in WT mice or AT_{1A} KO mice. Baseline systolic blood pressure was significantly lower in AT_{1A} KO mice than in WT mice. Vehicle infusion for 13 days did not affect systolic blood pressure in either WT mice (100±2 mm Hg on 0 day versus 107±2 mm Hg on day 13) or AT_{1A} KO mice (84±2 mm Hg on 0 day versus 78±2 mm Hg on day 13). Angiotensin II (2.8 mg/kg/day, s.c.) infusion for 13 days significantly increased systolic blood pressure in WT mice but did not change it in AT_{1A} KO mice. Valsartan (40 mg/kg/day, in drinking water) significantly decreased systolic blood pressure in WT mice but did not alter it in AT_{1A} KO mice. Valsartan completely prevented the angiotensin II-induced increases in systolic blood pressure in WT mice.

3.2. Superoxide anion production in aorta of vehicle, angiotensin II, valsartan and valsartan plus angiotensin II-treated WT and AT_{1A} KO mice

Fig. 2 shows the Tiron-quenchable lucigenin chemiluminescence in aorta of WT and AT_{1A} KO mice on the 13th day of vehicle, angiotensin II, valsartan or valsartan plus angiotensin II infusion. The basal levels of lucigenin chemiluminescence in aorta of vehicle-infused WT mice were 1.6-fold

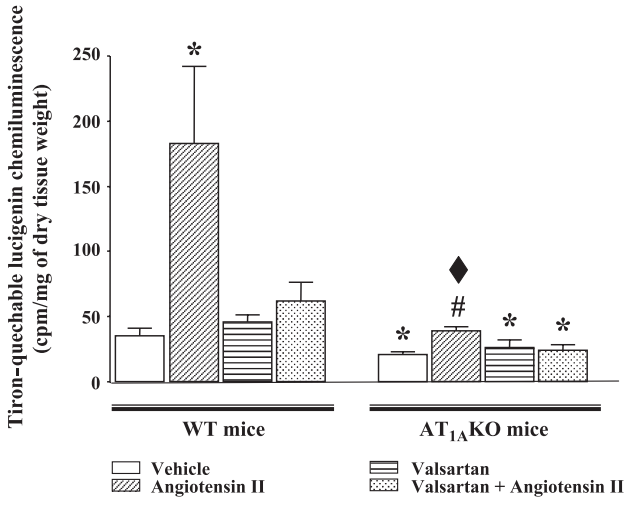

Fig. 2. Bar graph shows Tiron-quenchable lucigenin chemiluminescence in DETC-treated aortic rings of WT and angiotensin AT_{1A} KO mice. cpm indicates counts per minute. Data represent means±S.E.M. *P<0.05 versus WT sham, ♦P<0.05 versus AT_{1A} KO sham and #P<0.05 between angiotensin II-treated WT and AT_{1A} KO mice.

higher than those in vehicle-infused AT_{1A} KO mice (35±6 cpm/mg of dry tissue weight versus 21±2 cpm/mg of dry tissue weight in WT and AT_{1A} KO mice, respectively), indicating that superoxide anion production rate in aorta of vehicle-infused WT mice was significantly higher than that in vehicle-infused AT_{1A} KO mice. In aorta of angiotensin II-treated WT and AT_{1A} KO mice, the levels of lucigenin chemiluminescence were significantly higher than those in vehicle-infused WT and AT_{1A} KO mice, respectively. However, the level of lucigenin chemiluminescence in aorta of angiotensin II-treated AT_{1A} KO mice (1.8-fold higher than that in vehicle-infused AT_{1A} KO mice) was significantly lower than that in angiotensin II-treated WT mice (5.2-fold higher than that in vehicle-infused WT). These results clearly show that angiotensin II stimulates superoxide anion production even in AT_{1A} KO mice. The levels of lucigenin chemiluminescence in aorta of valsartan plus angiotensin II-treated WT and AT_{1A} KO mice were not significantly different from those in vehicle-infused WT and AT_{1A} KO mice, respectively.

3.3. Thiobarbituric acid reactive substances levels in heart tissue of vehicle, angiotensin II, valsartan and valsartan plus angiotensin II-treated WT and AT_{1A} KO mice

Fig. 3 shows the thiobarbituric acid reactive substances levels in heart tissue of WT and AT_{1A} KO mice on the 13th day of vehicle, angiotensin II, valsartan or valsartan plus angiotensin II infusion. The basal levels of thiobarbituric acid reactive substances in heart tissue of vehicle-infused WT (n=5) and AT_{1A} KO (n=7) mice were 75.7±7.6 and 64.9±4.9 nmol/g of wet tissue weight, respectively. In angiotensin II-treated WT (n=5) and AT_{1A} KO mice (n=5), the levels of thiobarbituric acid reactive substances in heart tissue were significantly higher than those in vehicle-infused WT and AT_{1A} KO mice, respectively. However, the level of thiobarbituric acid reactive substances in heart tissue of angiotensin II-treated AT_{1A} KO mice was significantly lower than that in angiotensin II-treated WT mice. Valsartan prevented angiotensin II-induced increases of thiobarbituric acid reactive substances levels in heart tissue of WT (n=5) and AT_{1A} KO mice (n=5).

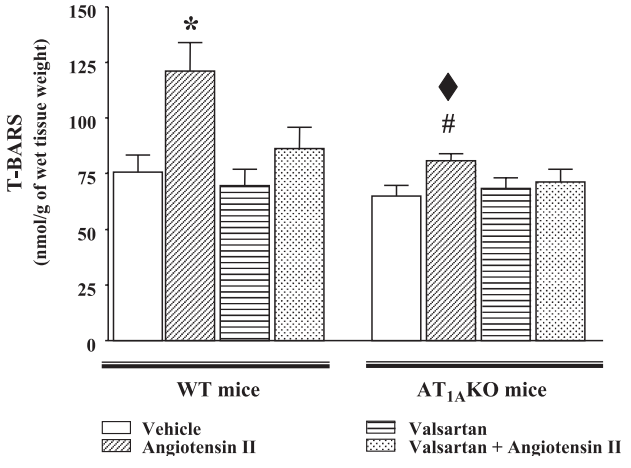

Fig. 3. Bar graph shows thiobarbituric acid-reactive substances (T-BARS) levels in heart tissue of WT and angiotensin AT_{1A} KO mice. Data represent means±S.E.M. *P<0.05 versus WT sham, ♦P<0.05 versus AT_{1A} KO sham and #P<0.05 between angiotensin II-treated WT and AT_{1A} KO mice.

4. Discussion

The aim of the present study was to determine the roles of angiotensin AT_{1A} and AT_{1B} receptors in angiotensin II-induced superoxide anion production in mouse aorta and heart. The results of the present experiments clearly showed that angiotensin II stimulates superoxide anion production in vascular and heart tissue via angiotensin AT_{1B} as well as AT_{1A} receptors.

The relative abundance of angiotensin AT_{1A} receptors among AT_1 receptor subtypes is generally thought to mediate most of the angiotensin II effects in almost all tissues and organs. However, currently available pharmacological agents do not discriminate between angiotensin AT_{1A} and AT_{1B} receptors. Thus, it is not clear whether angiotensin AT_{1A}, angiotensin AT_{1B} or both receptors are involved in the regulation of cardiovascular function, and in the vascular superoxide anion production. We have considered gene targeting to be an alternative approach to clarify the functional significance of the distinct angiotensin AT_1 receptor subtypes in vivo. The present experiment using WT and AT_{1A} KO mice was conducted to clarify the relative contribution of angiotensin AT_{1A} and AT_{1B} receptors to the angiotensin II-induced superoxide production in vascular and heart tissues, since several researchers have already reported that chronic infusion of angiotensin II stimulates free oxygen radicals via angiotensin AT_1 receptors in vascular tissues (Cifuentes et al., 2000; Rajagopalan et al., 1996). In addition, Bendall et al. (2002) showed that chronic infusion of angiotensin II activated the NAD(P)H system in myocardium. Previous studies (Cervenka et al., 1999; Ito et al., 1995; Oliverio et al., 1997; Ruan et al., 1999; Sugaya et al., 1995) demonstrated that the resting blood pressure in AT_{1A} KO mice was significantly lower than that of WT mice, and that acute administration of angiotensin II increased blood pressure in WT mice but not in AT_{1A} KO mice. In agreement with results of previous studies, our results showed that resting blood pressure in AT_{1A} KO mice was significantly lower than that of WT mice. Furthermore, chronic infusion of angiotensin II significantly increased systolic blood pressure in WT mice but not in AT_{1A} KO mice. Thus, these findings indicate a predominant role of the angiotensin AT_{1A} receptors in mediating vascular responses to angiotensin II and in blood pressure regulation. On the other hand, we found that the basal superoxide level in aorta of AT_{1A} KO mice was lower than

that in aorta of WT mice. However, chronic infusion of angiotensin II significantly increased superoxide levels in aorta of both AT_{1A} KO mice and WT mice. To determine the role of angiotensin AT_{1B} receptors in angiotensin II-induced superoxide production, we used valsartan, which antagonizes both angiotensin AT_{1A} and AT_{1B} receptors. During the blockade of angiotensin AT_1 receptors by valsartan, angiotensin II did not increase systolic blood pressure in WT mice, indicating a complete blockade of angiotensin AT_1 receptors. We found that angiotensin II-induced increases of superoxide anion production in aorta of WT mice were completely inhibited by valsartan. These results support other findings (Cifuentes et al., 2000; Fukui et al., 1997; Ortiz et al., 2001; Rajagopalan et al., 1996) that angiotensin II stimulates superoxide production via angiotensin AT_1 receptor activation. We also found that valsartan completely inhibited angiotensin II-induced superoxide production in aorta of AT_{1A} KO mice. This result indicates that in aorta of AT_{1A} KO mice, angiotensin II stimulated superoxide production via angiotensin AT_{1B} receptors.

Lipid peroxidation is a major biochemical consequence of reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyradical attacks on biological tissue. We therefore determined the degree of lipid peroxidation in heart tissue. Thiobarbituric acid reactive substances levels in heart of vehicle-infused AT_{1A} KO mice were slightly lower than those in WT mice. However, these changes were not statistically significant. We have no good explanation of why superoxide levels in aortic tissues were lower in AT_{1A} KO mice than those in WT mice, whereas thiobarbituric acid reactive substances levels in the heart were similar in these animals. Further studies will be required to determine angiotensin AT_{1A} receptor-dependent and -independent superoxide production pathways in different organs and tissues. However, similar to superoxide production rate in aorta of WT and AT_{1A} KO mice, thiobarbituric acid reactive substances levels in heart tissue of angiotensin II-treated WT and AT_{1A} KO mice were significantly higher than those in vehicle-infused WT and AT_{1A} KO mice, respectively. Valsartan prevented angiotensin II-induced increases of thiobarbituric acid reactive substances levels in heart tissue of both WT and AT_{1A} KO mice. Taken together, these results indicate that angiotensin II may stimulate superoxide anion production via angiotensin AT_{1B} as well as AT_{1A} receptors in vascular and heart tissues.

Angiotensin II-induced superoxide production in aorta and heart tissues of WT mice was significantly higher than that of angiotensin II-treated AT_{1A} KO mice. These findings may indicate that angiotensin AT_{1A} receptors play a predominant role in angiotensin II-induced superoxide production. A plausible explanation for this result is that the differences in responses between WT mice and AT_{1A} KO mice were likely to be related to the ratio of angiotensin AT_{1A}/AT_{1B} receptors in aorta and heart tissue, because angiotensin AT_{1A} receptors are much more highly expressed than angiotensin AT_{1B} receptors in vascular and cardiac tissues (Burson et al., 1994; Harada et al., 1998; Kitami et al., 1992; Llorens-cortes et al., 1994). In the present study, we did not determine the ratio of angiotensin AT_{1A} and AT_{1B} receptors in the aorta and heart tissue of WT mice. However, previous studies using the same mice demonstrated that the angiotensin AT_{1B} gene in the heart of AT_{1A} KO mice is <10% of the angiotensin AT_1 receptor in WT mice (Harada et al., 1998). Moreover, Zhu et al. (1998) showed that the ratio of mRNA for angiotensin AT_{1A}/AT_{1B} receptor is 1.7 in cultured aortic smooth muscle cells from WT mice. In addition, it has been shown that both angiotensin AT_{1A} and AT_{1B} receptors share substantial sequence homology and common pathways for signal transduction (Zhu et al., 1998). If it is assumed that angiotensin AT_{1B} receptor expression was not changed in aorta and heart of AT_{1A} KO mice, the differences in responses between WT mice and AT_{1A} KO mice may be explained based on the density of angiotensin AT_{1A} and AT_{1B} receptors.

Results of previous studies suggest that in the absence of angiotensin AT_{1A} receptors, angiotensin AT_{1B} receptors may partially take over the function of angiotensin AT_{1A} receptors in regulation of blood pressure and angiotensin II-mediated vasoconstriction (Oliverio et al., 1997; Ruan et al., 1999; Zhu et al., 1998). In the present study, we did not observe an increase in blood pressure during chronic infusion of angiotensin II in AT_{1A} KO mice. At present, we have no conclusive explanation, as to why angiotensin II failed to increase blood pressure in AT_{1A} KO mice. One possible explanation is that the presence of increased endogenous angiotensin II levels, as was reported for AT_{1A} KO mice (Cervenka et al., 1999; Sugaya et al., 1995) might be blunting the vasoconstrictor effect of angiotensin II. Another possibility is that in the absence of angiotensin AT_{1A} receptors, angiotensin AT_{1B} receptor-mediated small increases of blood pressure might be opposed by AT_2 receptor-mediated increased levels of NO (Rahman et al., 2002; Siragy et al., 1999; Nishiyama et al., 2001a) in angiotensin II-treated AT_{1A} KO mice.

A previous study of ours suggested that superoxide, at least in part, participates in the production of angiotensin II-dependent hypertension (Nishiyama et al., 2001b). In the present experiment, it was not clear whether angiotensin AT_{1B} receptor-mediated superoxide contributed to the angiotensin II-induced increases of blood pressure or not. However, it is reasonable to assume that the angiotensin AT_{1B} receptors play a primary role in angiotensin II-induced superoxide production when angiotensin AT_{1A} receptors are down-regulated or angiotensin AT_{1B} receptors are up-regulated.

In conclusion, the present study clearly showed that angiotensin II stimulates superoxide anion production via both angiotensin AT_{1A} and AT_{1B} receptors, and that angiotensin AT_{1A} receptors appear to play a predominant role in

mediating angiotensin II-induced superoxide anion production in mouse aorta and heart.

Acknowledgements

We are grateful to Tanabe Pharmaceutical Co. (Osaka, Japan) for supplying the wild type and AT_{1A} KO mice, and also to Novartis Pharma (Basel, Switzerland) for supplying valsartan.

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan. We thank Ms. Yomi Ihara for secretarial services.

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