Addition of a Nitric Oxide Donor to an Angiotensin II Type 1 Receptor Blocker May Cancel Its Blood Pressure-Lowering Effects

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Summary

While physiological levels of nitric oxide (NO) protect the endothelium and have vasodilatory effects, excessive NO has adverse effects on the cardiovascular system. Recently, new NO-releasing pharmacodynamic hybrids of angiotensin II (Ang II) type 1 (AT1) receptor blockers (ARBs) have been developed.

We analyzed whether olmesartan with NO-donor side chains (Olm-NO) was superior to olmesartan (Olm) for the control of blood pressure (BP). Although there was no significant difference in binding affinity to AT1 wild-type (WT) receptor between Olm and Olm-NO in a cell-based binding assay, the suppressive effect of Olm-NO on Ang II-induced inositol phosphate (IP) production was significantly weaker than that of Olm in AT1 WT receptor-expressing cells. While Olm had a strong inverse agonistic effect on IP production, Olm-NO did not. Next, we divided 18 C57BL mice into 3 groups: Ang II (infusion using an osmotic mini-pump) as a control group, Ang II (n = 6) + Olm, and Ang II (n = 6) + Olm-NO groups (n = 6). Olm-NO did not block Ang II-induced high BP after 10 days, whereas Olm significantly decreased BP. In addition, Olm, but not Olm-NO, significantly reduced the ratio of heart weight to body weight (HW/BW) with downregulation of the mRNA levels of atrial natriuretic peptide.

An ARB with a NO-donor may cancel BP-lowering effects probably due to excessive NO and a weak blocking effect by Olm-NO toward AT1, receptor activation. (Int Heart J 2015; 56: 656-660)

Key words: Cardiovascular disease, Inositol phosphate production, Inverse agonism

The renin angiotensin system plays an essential role in the pathophysiology of hypertension and cardiovascular disease.1) The clinical use of non-peptide angiotensin II (Ang II) type 1 (AT1) receptor blockers (ARBs) has been quite successful for the prevention of cardiovascular disease (CVD).2)

While most ARBs have common molecular structures (biphenyl-tetrazol and imidazole groups), their structures are also slightly different. Some of the benefits conferred by ARBs may not be class-specific (common) effects, and instead may be molecule-specific (differential) effects (eg, uricosuric effect and peroxisome proliferator-activated receptor activation).3-5) It is very difficult to demonstrate the molecular effects of ARBs via the clinical outcome because, at the outset of their development, ARBs were not intended to be used for their molecular effects. The development of next-generation ARBs that do more than just block AT1 receptors will be needed to further promote the prevention of hypertension (HTN) and CVD. Recently, multifunctional ARBs that not only block AT1 receptors but also stimulate beneficial molecular targets have been shown to be useful for preventing CVD [eg, dual AT1/endothelin A receptor blockers, nitric oxide (NO)-releasing pharmacodynamic hybrids of ARBs, and AT1 receptor-neprilysin inhibitors].6-9)

Editorial p.585

NO-releasing hybrids of losartan have been shown to have greater anti-ischemic cardio-protective effects than losartan itself.10) While physiological levels of NO protect the endothelium and have vasodilatory effects, excessive NO has adverse effects on the cardiovascular (CV) system.10) It is controversial whether NO has beneficial effects on the CV system. Therefore, we recently developed new NO-donor side chains for the ARB olmesartan (Olm-NO) and analyzed whether Olm-NO was superior to Olm with regard to cardio-protective effects including the control of blood pressure (BP).

Materials: The following antibodies and reagents were purchased or provided: Olm and Olm-NO (Figure 1, kindly provided by Daiichi-Sankyo Co. Ltd., Tokyo); [Sar1]Ang II and [Sar1, Ile8]Ang II (Sigma-Aldrich, St. Louis, MO, USA); and [125I-Sar1, Ile8]Ang II (Amersham Biosciences, Buckinghamshire, UK).

Methods

Materials: The following antibodies and reagents were purchased or provided: Olm and Olm-NO (Figure 1, kindly provided by Daiichi-Sankyo Co. Ltd., Tokyo); [Sar1]Ang II and [Sar1, Ile8]Ang II (Sigma-Aldrich, St. Louis, MO, USA); and [125I-Sar1, Ile8]Ang II (Amersham Biosciences, Buckinghamshire, UK).
Intracellular Ca²⁺ as a second messenger of BP control because increases in transfected COS1 cells was measured to evaluate cell signaling. Label for 24 hours with WT and mutant AT₁ receptors were transiently transfection into COS1 cells using Lipoportamn 2000 liposomal reagent (Roche Applied Science) according to the manufacturer’s protocol.

Living cell-based competition binding study: The Kᵣ values of AT₁ receptor binding were determined by [³²P]-[Sar¹, Ile³] Ang II-binding experiments with ARBs (Olm and Olm-NO) under equilibrium conditions using COS1 cells expressing AT₁ WT receptors. Equilibrium binding kinetics were determined as previously described.¹¹,¹²

Inositol phosphate (IP) production assay: Agonist- or ARB-induced IP production by WT and mutant AT₁ receptors in transfected COS1 cells was measured to evaluate cell signaling as a second messenger of BP control because increases in the intracellular Ca²⁺ concentrations are a result of IP accumulation and induce vasoconstriction. The COS1 cells as surrogate model with the higher levels of AT₁ receptor expression are better suited for this study because it is easy to detect the differences in IP production between Olm and Olm-NO. Briefly, WT and mutant AT₁ receptor-transfected COS-1 cells were labeled for 24 hours with [³²P]-myoinositol at 37°C in DMEM containing 10% FBS. After labeling, the cells were washed with buffer three times and incubated with medium containing 10 mM LiCl for 20 minutes; 1 μM ARB or 0.1 μM [Sar¹]Ang II was added and incubation was continued for another 30 minutes at 37°C. After incubation, the medium was removed, and total soluble IP was extracted from the cells by the perchloric acid extraction method, as described previously.¹¹,¹²

Animal study: Male C57BL/6J mice at 8-9 weeks of age were purchased from Charles River Laboratories Japan, Inc. The experimental protocol was approved by the Animal Care and Use Committee of Fukuoka University, and all procedures conformed to the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources. We divided 18 mice into 3 groups: Ang II (600 ng/kg/minute infusion using an osmotic mini-pump) as a control group, Ang II (600 ng/kg/minute) + Olm-NO (4 mg/kg/day), and Ang II (600 ng/kg/minute) + Olm-NO (4 mg/kg/day). Ang II infusion was performed for 10 days, and Olm or Olm-NO infusion was started on the third day (from days 3 to 10). The rate of Ang II infusion was set to 600 ng/kg/minute (= 0.864 μg/kg/day) = 0.826 μmol/kg/day), and this rate of infusion induced a significant increase in BP according to a previous report.¹º The rate of Olm infusion was set to 4 mg/kg/day (= 8.96 μmol/kg/day) because this was about 10-fold higher than the rate of Ang II infusion and can completely block the rate of Ang II binding to AT₁ receptors. BP and pulse rate (PR) were measured at baseline (0), and on days 1, 3, 5, 7, and 10. We also analyzed the ratio of heart weight to body weight (HW/BW), plasma levels of NOx (NO/NO₃, Assay kit-C2 Colorimetric, DOJINDO), and mRNA levels of atrial natriuretic peptide (ANP), AT₁ receptors, and endothelial NO synthase (eNOS) in the left ventricle after 10 days. Ultrasound cardiography (UCG) was performed at baseline and after 10 days.

UCG measurement: UCG measurements were performed using a NEMIO SSA-550A (Toshiba, Tokyo), While mice were anesthetized by the inhalation of 1.5% isoflurane, short- and long-axis 2-dimensional views and M mode at the level of the papillary muscle were analyzed. Interventricular septum thickness (IVST), left ventricular internal dimension in diastole (LVDd), left ventricular posterior wall thickness diameter (LVPWd), left ventricular internal dimension in systole (LVDs), and heart rate were measured. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were calculated as: [100 × (volume in diastole - volume in systole)/volume in diastole] and [(LVDd - LVDs)/LVDd] × 100, respectively.

Measurement of mRNA levels of ANP, AT₁, receptors, and endothelial NO synthase (eNOS): mRNA levels were quantified by real-time reverse transcriptase-polymerase chain reaction. After 10 days, the heart was removed and washed twice with PBS, frozen immediately in liquid nitrogen, and then stored at -80°C. Total RNA was extracted using a RiboPure RNA Purification Kit (Life Technologies, Carlsbad, CA). cDNA was produced using a Quantitect Reverse Transcription Kit (QiAGEN, Netherlands). Real-time PCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems) using a Quantitect SYBR Green PCR Kit (QiAGEN, Netherlands). ANP, AT₁, receptors, and eNOS were investigated. The primers used were 5'-AGGAGACGGGACTGCTTACAGG-3' and 5'-CAGCTGCGATCTGTGAGCT-3' for mouse ANP, 5'-AA GGCCAGAGGAACCTTGTTT-3' and 5'-CAAAGGGCTCCTGGA AACTTG-3' for mouse AT₁ receptor, 5'-GCACCCAGAGCTTTTCTTCTT-3' and 5'-GAACTTGGGGAGGAGGAAG-3' for mouse eNOS, and 5'-CCACACCCGGCCACCAGTTCC-3' and 5'-TACAGCCCGGGGAGCAGCCTG-3' for mouse β actin.

Statistical analysis: The results are expressed as the mean ± standard deviation (SD) of three or more independent determinations. Significant differences in measured values were evaluated with paired and unpaired Student’s t-test, as appropriate. Statistical significance was set at ≤ 0.05.

RESULTS

Kᵣ values of AT₁ receptor binding and IP production: The binding affinities of ARBs to the AT₁ receptor were analyzed. The Kᵣ value of Olm-NO (5.8 ± 1.6 nM) was comparable to that of Olm (3.3 ± 0.5 nM).

Next, we measured the production of IP as a second messenger of BP control using AT, WT and N111G mutant receptors (Figure 2A,B). Olm (1 μM and 0.1 μM), but not Olm-NO,
completely blocked 0.1 μM [Sar\(^1\)]Ang II-induced IP production (Figure 2A). Olm-NO (0.01 - 1 μM) did not block [Sar\(^1\)]Ang II-induced IP production completely, and the blocking effects of Olm-NO were significantly weaker than those of Olm. To analyze the inverse agonism of Olm and Olm-NO, we measured IP production in cells that expressed AT\(_1\) N111G mutant receptors (Figure 2B). Olm, but not Olm-NO, showed a strong inverse agonism because Olm significantly suppressed the basal level of IP production. Interestingly, Olm-NO partially stimulated IP production in cells that expressed AT\(_1\) N111G mutant receptors.

**Measurement of BP and PR in mice:** BP and PR in mice are
shown in Figure 3A-C. Systolic BP (SBP) at days 7 and 10 and diastolic BP (DBP) in the Olm group were significantly lower than those in the control group, whereas the Olm-NO group did not show significant reductions in SBP or DBP. Thus, Olm-NO did not block Ang II-induced high BP after 10 days, whereas Olm significantly decreased BP.

**HW/BW and UCG measurement:** We also measured HW/BW and UCG (Figure 3D and Table). Olm, but not Olm-NO, significantly lowered HW/BW (Figure 3D). On the other hand, there were no differences in any of the UCG parameters between baseline and after 10 days or among the control, Olm and Olm-NO groups (Table).

**mRNA levels of ANP, AT1 receptors, and eNOS and plasma levels of NOx:** The mRNA levels of ANP, AT1 receptors, and eNOS are shown in Figure 4A-C. The mRNA level of ANP in the Olm group was significantly lower than those in the control and Olm-NO groups (Figure 4A), while the mRNA level of AT1 receptors in the Olm group was significantly higher than that in the control group (Figure 4B). The eNOS levels in the Olm and Olm-NO groups were significantly higher than those in the control group (Figure 4C). Plasma levels of NOx in the Olm-NO group (43.4 ± 4.7 μM/L) were higher than those in the Olm group (33.3 ± 3.4 μM/L).

**DISCUSSION**

The main finding in the present study was that Olm-NO did not block Ang II-induced BP elevation, whereas Olm significantly decreased BP. In addition, Olm, but not Olm-NO, significantly suppressed HW/BW with the downregulation of mRNA levels of ANP, and upregulated the mRNA level of AT1 receptors.

The addition of a NO-donor to an ARB may cancel its BP-lowering effect. There are at least two possible explanations for why Olm-NO did not block the Ang II-induced elevation of BP: Olm-NO may be associated with excessive NO production and a weak blocking effect toward AT1 receptor activation. NO is a well-known endothelium-derived relaxant of vascular smooth muscle cells, and Olm-NO may lower BP. On the other hand, excessive NO can have deleterious effects on the CV system. A large amount of NO interacts with molecular oxygen and anion superoxide to form reactive nitrogen species. Excessive production of superoxide can inactivate NO and shut down the cGMP pathway with coronary vasoconstriction. The abundant synthesis of NO generates a free radical cascade, and this may contribute to cardiomyopathy. In fact, Olm significantly suppressed HW/BW and decreased mRNA levels of ANP compared to Olm-NO in an Ang II-infused model in this study. The balance between the physiologic and pathologic production of NO may be critical in regulating cardiac function. In this study, plasma levels of NOx in the Olm-NO group were higher than those in the Olm group. NO may be released from Olm-NO because the binding affinity of Olm-NO was only slightly worse as compared with that of Olm in a living cell-based binding study. Thus, excessive NO production from Olm-NO may have a deleterious effect on BP-lowering. Another possibility in which Olm-NO did not block the Ang II-induced elevation of BP is the weak blocking effect on AT1 receptor activation including a lack of inverse agonism. Lower concentrations of Olm-NO did not block Ang II-induced IP production compared to those of Olm, although there was no significant difference in binding affinity to AT1 WT receptors between Olm and Olm-NO. In addition, Olm has inverse agonist activity with regard to IP production, which is Gq-dependent signal transduction. As shown in Figure 2B, Olm-NO did not have inverse agonist activity in AT1 N111G receptor-expressing COS1 cells. The inverse agonist activity of Olm requires two important interactions: that between the hydroxyl group of Olm and Tyr in the AT1 recept-

Table. Ultrasound Cardiography (UCG) Parameters in the Control, Olm, and Olm-NO Groups

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<th>IVS/PW (mm)</th>
<th>LVDd (mm)</th>
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<td>Pre</td>
<td>Post</td>
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</tr>
<tr>
<td>Control</td>
<td>0.5/0.6</td>
<td>0.6/0.6</td>
<td>3.5</td>
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<tr>
<td>Olm</td>
<td>0.5/0.6</td>
<td>0.6/0.6</td>
<td>3.7</td>
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<tr>
<td>Olm-NO</td>
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There were no significant changes in any parameter between before and after Ang II infusion in all groups.
tor and that between the carboxyl group of Olm and Lys and His in the receptor. When ARB did not have carboxyl and hydroxyl groups, the additive effects of Olm, which include the suppression of renal dysfunction in rats and a reduction in BP in hypertensive patients, may be lost. A carboxyl group of Olm was modified with a linker so that it could interact with NO to construct Olm-NO, and this modification may affect the inverse agonist activity of Olm. In addition, Olm-NO exhibited partial agonism, as shown in Figure 2B. Since the AT1 N111G receptor is constitutively active and stabilized in a partial active state, this state may be easily activated by Olm-NO. Therefore, the structure of Olm-NO after NO release would not be Olm itself. Generally, ARBs upregulate the expression of AT1 receptors in compensation for completely blocking the activation of AT1 receptors. Olm, but not Olm-NO, upregulated mRNA levels of AT1 receptors in rats, which indicates that Olm-NO cannot completely block activation of this receptor. In addition, the hybrid compound losartan with NO donor (Los-NO) had AT1-antagonist effects in esterase inhibitor-free conditions. On the other hand, the presence of esterase inhibitor dramatically reduced this antagonism. Although the AT1-blocking activity was due to hydrolytic cleavage of the side chain and release of the “native” losartan, we did not analyze the esterase activity in our experimental systems.

In conclusion, the addition of a NO donor to Olm may cancel its BP-lowering effect, probably due to excessive NO and a weak blocking effect toward AT1 receptor activation.

DISCLOSURE

Conflict of interest: K.S. is a Chief Director and S.M. is a Director of NPO Clinical and Applied Science, Fukuoka, Japan. K.S. has an Endowed “Department of Molecular Cardiovascular Therapeutics” supported by MSD, Co. LTD. S.M. and Y.U. belong to the Department of Molecular Cardiovascular Therapeutics, which is supported by MSD, Co. LTD.

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