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ORIGINAL ARTICLE

Vasculoprotective effect of cilostazol in aldosterone-induced hypertensive rats

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Cilostazol (CILO), a selective inhibitor of phosphodiesterase 3 with potent antithrombotic property, has been shown to have a vasculoprotective effect in atherosclerosis animal models due to its potential anti-inflammatory and antioxidant actions. This study was undertaken to investigate whether CILO has in fact any vasculoprotective effects in aldosterone-induced hypertensive rats (Aldo-rats), and whether CILO affects Aldo-induced oxidative stress, nitric oxide (NO) production and pro-inflammatory gene expression. Treatment with CILO markedly ameliorated perivascular inflammatory changes in the coronary arterioles of Aldo-rats without affecting the systolic blood pressure and left ventricular weight. Treatment with CILO also prevented the increase in plasma levels of thiobarbituric acid-reactive substances, an oxidative stress marker, as well as decreased urinary NOx excretion in Aldo-rats. Furthermore, CILO almost completely inhibited a set of upregulated proinflammatory genes (*ICAM-1*, *MCP-1*, *PDGF-A*, osteopontin, *MMP-2* and *ACE*), as well as NAD(P)H oxidase components (p22phox, gp91phox, p47phox) and Aldo-inducible genes (*SGK-1* and *NHE-1*) in the aortic tissues from Aldo-rats. Taken together, this study showed for the first time that CILO prevented Aldo-induced vascular inflammation and injury without affecting the blood pressure, suggesting its vasculoprotective effect on Aldo-induced vascular injury independent of blood pressure.

INTRODUCTION

There is evidence suggesting that aldosterone (Aldo) is involved in the development and/or progression of cardiovascular injury through its direct effects on cardiovascular tissue through a mineralocorticoid receptor (MR).[1,2] Several studies have documented that vascular inflammation associated with increased expression of a variety of proinflammatory genes and oxidative stress is a hallmark of Aldo-induced cardiovascular injury, and such vascular changes are ameliorated by treatment with not only MR antagonists, but also antioxidants and/or NAD(P)H oxidase inhibitors.[3–7] Furthermore, it has been shown that Aldo directly increased superoxide generation and decreased nitric oxide (NO) synthesis in endothelial cells (ECs) *in vitro*.[8,9] In humans, it has been reported that patients with hyperaldosteronism have more severe endothelial dysfunction than the age- and blood pressure-matched patients with essential hypertension.[10] It is therefore suggested that the impairment of ECs caused by Aldo is the initial event in Aldo-induced vascular injury.

Cilostazol (CILO: 6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxyl]-3,4-dihydro-2 (1*H*)-quinolinone), a selective inhibitor of phosphodiesterase (PDE) 3 with a potent antithrombotic property, is clinically used for the treatment of peripheral artery disease and prevention of cerebral and myocardial infarction.[11] CILO exerts its antiplatelet aggregation and vasodilatory effects by increasing intracellular cyclic

AMP (cAMP) concentrations and subsequently activating protein kinase A.[11] Several experimental studies have shown that CILO exerts its vasculoprotective effect by inhibiting monocyte chemoattractant protein-1 (MCP-1) expression and superoxide generation, stimulating NO synthesis in ECs and inhibiting vascular smooth muscle cell proliferation.[12–15] It is noteworthy that an anti-atherosclerotic effect by CILO beyond its antiplatelet action has been recently reported in atherosclerotic animal models.[16,17] As the etiology of vascular injury process is common among various types of cardiovascular disease, it is possible to speculate that a diversity of effects by CILO also have a vasculoprotective role in Aldo-induced vascular injury. However, to the best of our knowledge, no study has been conducted to examine the effect of CILO on Aldo-induced vascular injury thus far.

These observations led us to investigate the following: (1) whether CILO has in fact any vasculoprotective effect in Aldo-induced hypertensive rats (Aldo-rats), and, if so, (2) whether CILO affects Aldo-induced oxidative stress, NO production and proinflammatory gene expression.

METHODS

Materials

Aldo was purchased from Acros Organics (Geel, Belgium). CILO was a generous gift from Otsuka Pharmaceutical (Tokyo, Japan). Thiobarbituric acid

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(TBA) was purchased from Sigma (St Louis, MO, USA); butylhydroxytoluene, trichloroacetic acid, n-butanol and methanol from Wako (Osaka, Japan).

Animals and experimental protocol

These studies were conducted according to the guidelines of the Tokyo Medical and Dental University Guide for the Care and Use of Experimental Animals. Aldo-rats were used as previously described[4] with a minor modification. Briefly, 8-week-old Sprague–Dawley rats (weighing 200–250 g) (Charles River Japan, Tokyo Japan) were uninephrectomized and infused subcutaneously with $0.75\,\mu g\,h^{-1}$ Aldo through osmotic minipumps (Alzet 2004, Alza; Palo Alto, CA, USA). The rats were then administered with 0.9% NaCl and 0.3% KCl in tap water ad libitum for 4 weeks. The sham-operated rats that were provided with tap water ad libitum were used as controls. Aldo-rats were fed chow with or without 0.1% CILO. As daily intakes of chow by Aldo-rats were 25–30 g, $\sim 100\,mg\,kg^{-1}$ of CILO was ingested daily by Aldo-rats. The amount of CILO used in this in vivo study ($\sim 100\,mg\,kg^{-1}\,day^{-1}$), corresponding to the area under the curve of $10.41\,\mu g\,h^{-1}\,ml^{-1}$, is almost comparable with that in the clinical setting ($13.1\,\mu g\,h^{-1}\,ml^{-1}$ at 100 mg per individual).[17,18]

On 3 days before killing, the animals were placed in individual metabolic cages (KN-646B, Natume, Tokyo, Japan), and urine was collected every 24 h for 2 days. Urine samples were centrifuged, frozen and stored at $-80\,^{\circ}C$ until analysis. The systolic blood pressure (SBP) was recorded on the day before killing by the tail-cuff method (Manometer-Tachometer, model KN-21–1, Natsume Instruments, Tokyo, Japan) as previously described.[4] The rats were weighed and anesthetized with pentobarbital ($70\,mg\,kg^{-1}$, intraperitoneally) and blood samples were collected after decapitation.

For RNA and cAMP extraction, thoracic aorta was quickly excised, frozen immediately on dry ice and stored at $-80\,^{\circ}C$. The heart was excised and left ventricle was separated from the right ventricle, the atria and the greater vessels, and weighed. For histopathological analysis, the equatorial regions from the left ventricle were fixed in 10% formalin, routinely processed and then paraffin embedded. For histopathological study, sections (3-μm thick) from the left ventricle were stained with Masson's trichrome.[4] The stained sections were examined with a standard light microscopy (Olympus: AX-80, Tokyo, Japan) and the image was captured by a CCD color video camera (Olympus: DP70). The wall-to-lumen ratio and the perivascular fibrosis were defined and determined as previously described.[19] These structural changes were examined in coronary arterioles (lumen diameter $\leqslant 10^4\,\mu m^2$) using the Image J program (National Institute of Health).

Quantification of mRNA

The mRNA levels were quantified by real-time quantitative reverse transcriptase PCR using fluorescent SYBR green technology (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany) as previously described.[20,21] The sequence of PCR primer pairs were the following: serum and glucocorticoid-inducible kinase 1 (SGK-1) forward, 5′-GCCTGAGTATCTCGCTCCTGA-3′, and reverse, 5′-GCTGTGTTCCGGCTGTAGAAC-3′ (product size, 129 bp); Na/H exchanger-1 (NHE-1) forward, 5′-GGACCAGTTCATCATTGCCTA-3′, and reverse, 5′-CCAGGAACTGTGTGTGGATCT-3′ (product size, 242 bp); 18S-ribosormal RNA forward, 5′-GACACGGACAGGATTGACAG-3′, and reverse, R: 5′-AGAC AAATCGCTCCACCAAC-3′ (product size 90 bp). The sequence of PCR primer pairs for osteopontin, matrix metalloproteinase-2 (MMP-2), platelet-derived growth factor-A (PDGF-A), intercellular adhesion molecule-1 (ICAM-1), p22phox, gp91phox, p47phox and angiotensin-converting enzyme (ACE) were previously described.[4,21] Rat MCP-1 mRNA levels were quantified by TaqMan fluorescence methods as previously described.[22] Total RNA was extracted, first-strand cDNA synthesized and the amplification reaction was performed as previously described.[20,21] The mRNA levels of the target sequence were normalized with those of the 18S ribosome and the quantitative levels of each mRNA were calculated as previously described.[4,20,21]

Analytical procedures

Urinary excretion of nitrites and nitrates (NOx) was determined by the Griess method using a colorimetric NO assay kit (Dojindo, Kumamoto, Japan). The 24-h urinary excretion of NOx was corrected for the body weight (BW) of the animals. Oxidative stress was assessed by the measurement of plasma

thiobarbituric acid-reactive substances (TBARS) as previously described.[23] Contents of cAMP from the frozen aortic strips were measured as previously described,[24] and expressed as $nmol\,g^{-1}$ wet tissue.

Statistical analysis

Data were expressed as the mean ± s.e.m. of five different rats. Differences between groups were examined for statistical significance using the unpaired t-test or analysis of variance with Dunn's post hoc test, if appropriate. P-values <0.05 were considered statistically significant.

RESULTS

SBP, BW and left ventricular weight (LVW) to BW ratio in the three groups are presented in Table 1. Aldo-rats showed markedly higher ($P<0.05$) SBP than the control, whereas treatment with CILO did not affect the SBP in Aldo-rats. BW was similar among the three groups. Aldo-rats had a significantly ($P<0.05$) higher LVW and LVW/BW than did the control, whereas treatment with CILO did not affect the LVW and LVW/BW in Aldo-rats. Marked perivascular inflammatory changes, such as myointimal thickening and adventitial fibrosis, were observed in the coronary arterioles of Aldo-rats, and these changes were ameliorated by treatment with CILO (Figures 1a–f). Morphometric analysis also revealed that both wall-to-lumen ratio and perivascular fibrosis in Aldo-rats were significantly ($P<0.05$) greater than those in control rats, whose effects were significantly ($P<0.05$) ameliorated by treatment with CILO (Figures 1g and h).

To determine whether CILO affects Aldo-induced oxidative stress and NO production, we measured the plasma concentrations of TBARS, an oxidative stress marker for lipid peroxidation, and 24-h urinary NOx excretion. Continuous Aldo infusion caused a significant ($P<0.05$) increase in plasma TBARS levels compared with the control, in which the effect of Aldo was abolished by CILO treatment (Figure 2a). In contrast, Aldo significantly ($P<0.05$) decreased urinary NOx excretion compared with control, in which this effect was significantly ($P<0.05$) reversed by CILO treatment (Figure 2b). Our results are consistent with the notion that treatment with CILO decreased oxidative stress and/or increased NO production in Aldo-induced hypertension.

To further clarify the molecular mechanism underlying the vasculoprotective effect by CILO on Aldo-induced vascular injury, the effect of CILO on gene expression of a set of proinflammatory molecules, NAD(P)H oxidase components and Aldo-inducible genes (SGK-1 and NHE-1)[25,26] in the aortic tissue from Aldo-rats was examined. Aldo significantly ($P<0.05$) increased the steady-state mRNA levels of a variety of proinflammatory genes (ICAM-1, MCP-1, PDGF-A, osteopontin, MMP-2 and ACE) and NAD(P)H oxidase components (p22phox, gp91phox, p47phox), and Aldo-inducible genes (SGK-1 and NHE-1); all these effects were significantly ($P<0.05$) inhibited by

Table 1 SBP, BW and LVW in control, Aldo-rats and Aldo-rats treated with cilostazol (CILO)

Parameter	Control	Aldo-rats	Aldo-rats with CILO
Number	5	5	5
SBP (mm Hg)	123 ± 6	187 ± 5*	179 ± 7*
BW (g)	396 ± 10	386 ± 12	390 ± 11
LVW (g)	0.82 ± 0.04	0.96 ± 0.05*	0.94 ± 0.06*
LVW/BW (g kg⁻¹)	2.03 ± 0.05	2.46 ± 0.06*	2.42 ± 0.07*

Abbreviations: BW, body weight; LVW, left ventricular weight; SBP, systolic blood pressure.
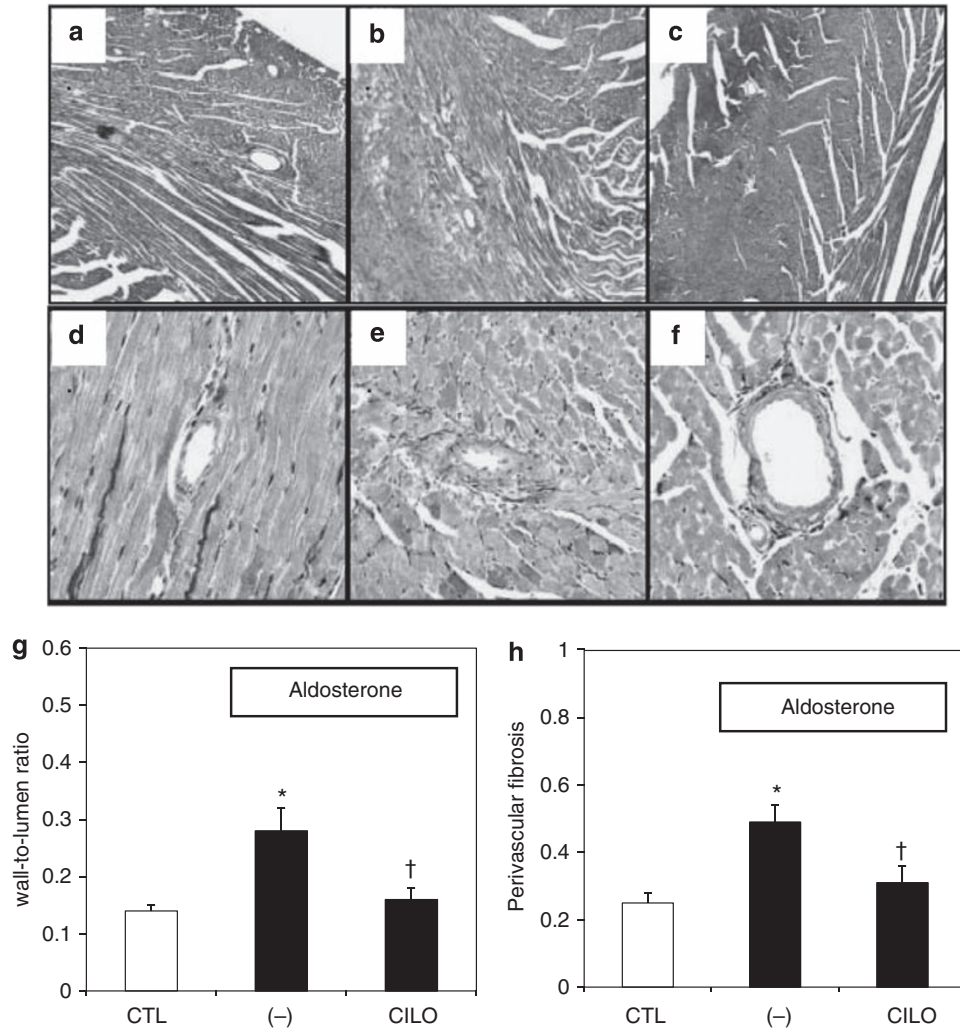Data are expressed as means ± s.e.m.
*$P<0.05$ vs. control.

Figure 1 Vascular inflammatory changes by aldosterone are prevented by treatment with CILO. Representative photomicrographs of the left ventricular tissue from control rats (**a**, **d**), Aldo-rats (**b**, **e**) and Aldo-rats with CILO (**c**, **f**) after 4 weeks of study. A prominent vascular inflammatory response (myointimal thickening and adventitial fibrosis) was observed in Aldo-rat (**b**, **e**), whose effect was prevented by treatment with CILO (**c**, **f**) (Masson's trichrome staining). Magnifications: $\times 40$ (**a**–**c**) and $\times 200$ (**d**–**f**). Wall-to-lumen ratio (**g**) and perivascular fibrosis (**h**) in three groups were measured. Values are means $\pm$ s.e.m. from five rats per group; $*P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. Aldo-rats.
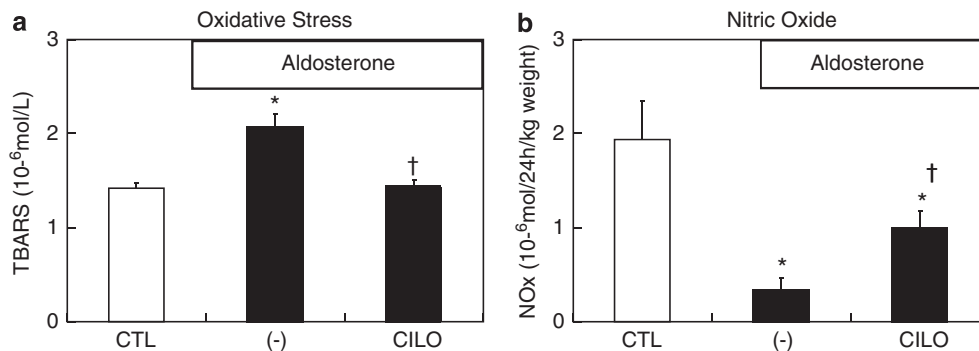

Figure 2 Increased oxidative stress and decreased NO production in Aldo-rats are reversed by treatment with CILO. (**a**) Oxidative stress in plasma measured by TBARS assay and (**b**) 24-h urinary Nox excretion measured by Griess method, in control rats (CTL) and Aldo-rats without (−) and with CILO treatment after 4 weeks of the study. Values are means $\pm$ s.e.m. from five rats per group; $*P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. Aldo-rats.

CILO treatment (Figures 3 and 4). These results suggest that CILO blocks a set of upregulated proinflammatory genes, NAD(P)H oxidase components and Aldo-inducible genes in Aldo-induced hypertension.

Aldo significantly ($P < 0.05$) decreased cAMP contents (0.97 ± 0.11 nmol g^{-1} wet weight tissue) in aortic tissue compared with control (1.43 ± 0.12), whose effect was significantly ($P < 0.05$) reversed by CILO treatment (1.52 ± 0.17).
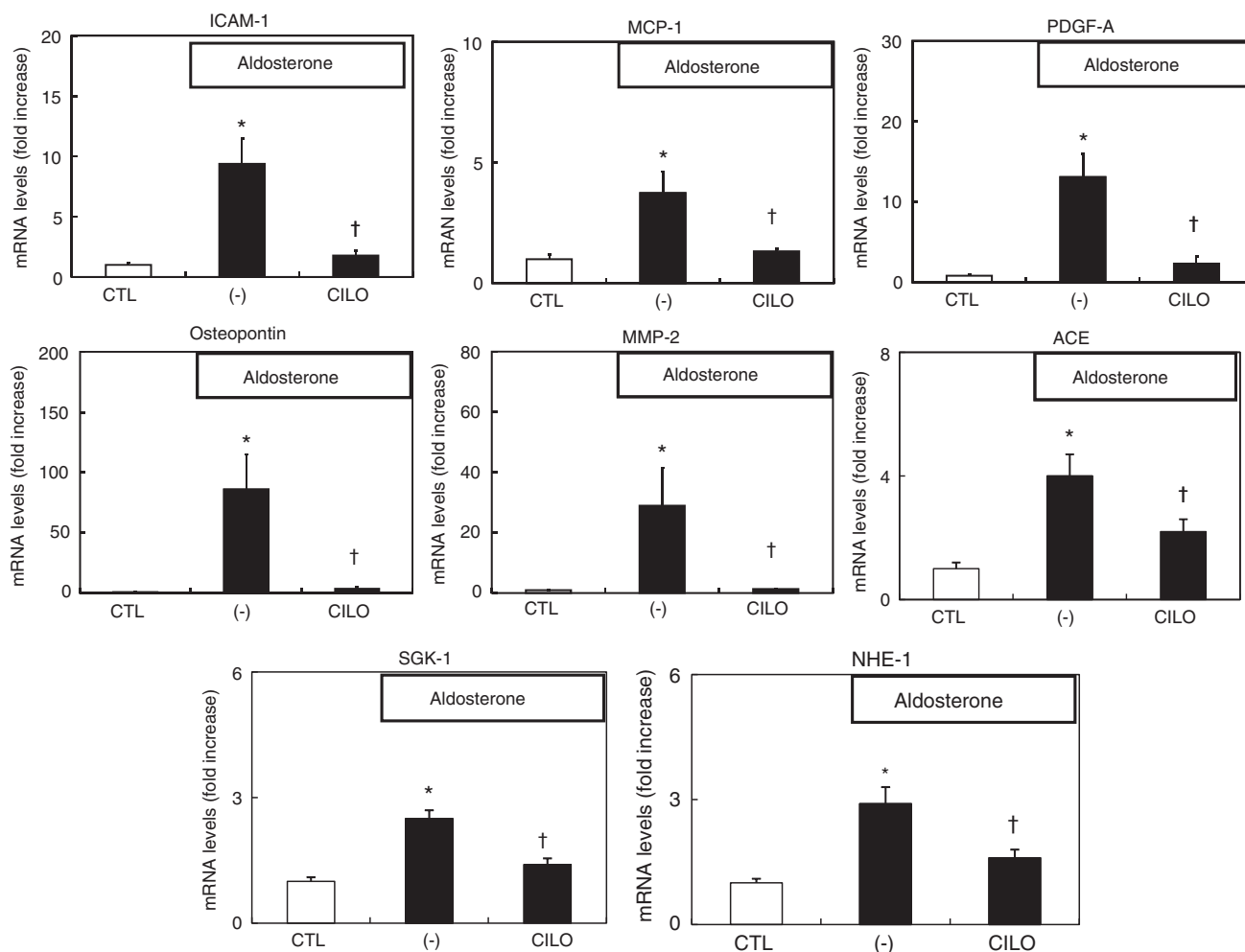
Figure 3 Increased gene expression of proinflammatory genes and Aldo-inducible genes in Aldo-rats are reversed by treatment with CILO. The steady-state mRNA levels for a set of proinflammatory genes (*ICAM-1*, *MCP-1*, *PDGF-A*, osteopontin, *MMP-2* and *ACE*) and Aldo-inducible genes (*SGK-1* and *NHE-1*) as measured by real-time reverse transcriptase PCR in aortic tissues from control rats (CTL), Aldo-rats without (−) and with CILO treatment are shown. Each column as shown by fold increase over control is the mean ± s.e.m. from five rats per group. *$P<0.05$ vs. control; †$P<0.05$ vs. Aldo-rats.

DISCUSSION

This study clearly shows that treatment with CILO prevented the vascular inflammatory changes associated with increased oxidative stress and decreased NO bioavailability in Aldo-rats without affecting blood pressure; the upregulated gene expression of the set of proinflammatory factors, as well as the NAD(P)H oxidase components in the aortic tissue were completely reversed by treatment with CILO.

We previously reported that treatment with the angiotensin II type1 receptor antagonist candesartan and a superoxide dismutase mimetic tempol, as well as the selective MR antagonist eplerenone resulted in vasculoprotective effects on Aldo-induced cardiovascular injury even with a modest fall in blood pressure.[4] In contrast, this study showed that CILO prevented vascular inflammatory changes in Aldo-rats without affecting blood pressure, suggesting that its vasculoprotective effect was independent of blood pressure. In addition, treatment with CILO did not affect LVW in Aldo-rats, suggesting that high blood pressure *per se* is a key determinant of LV hypertrophy in this hypertensive animal model. To the best of our knowledge, this is the first study showing a vasculoprotective effect of CILO on Aldo-rat without affecting blood pressure. Our data are consistent with those of a recent study showing that CILO exerts its vasculoprotective and anti-inflammatory effects on atherosclerotic lesions in apolipoprotein E- and low-density lipoprotein receptor-deficient mice.[16,17] Taken together, it is suggested that CILO, besides having an antiplatelet action, has a profound vasculoprotective effect on cardiovascular injury associated not only with atherosclerosis but also with Aldo-induced vascular injury.

The antioxidant effect of CILO as shown by the decrease in plasma TBARS, a marker for oxidative stress, in this study is in accordance with the previous studies showing that CILO decreased superoxide production *in vivo*[16] and *in vitro*.[15] As oxidative stress has a pivotal role in the development of Aldo-induced vascular injury,[2–7] it is strongly suggested that the antioxidant effect of CILO is responsible for its vasculoprotective effects in the Aldo-induced hypertension model.

Several clinical studies have shown that endothelial function, which is assessed by NO-mediated endothelium-dependent vasodilation, is impaired to a greater extent in hyperaldosteronism patients than that in patients with essential hypertension and treatment of hyperaldosteronism results in the improvement of endothelial dysfunction,[10,27] suggesting that impaired endothelial NO production is involved in the Aldo-induced vascular injury. It has recently been reported that Aldo
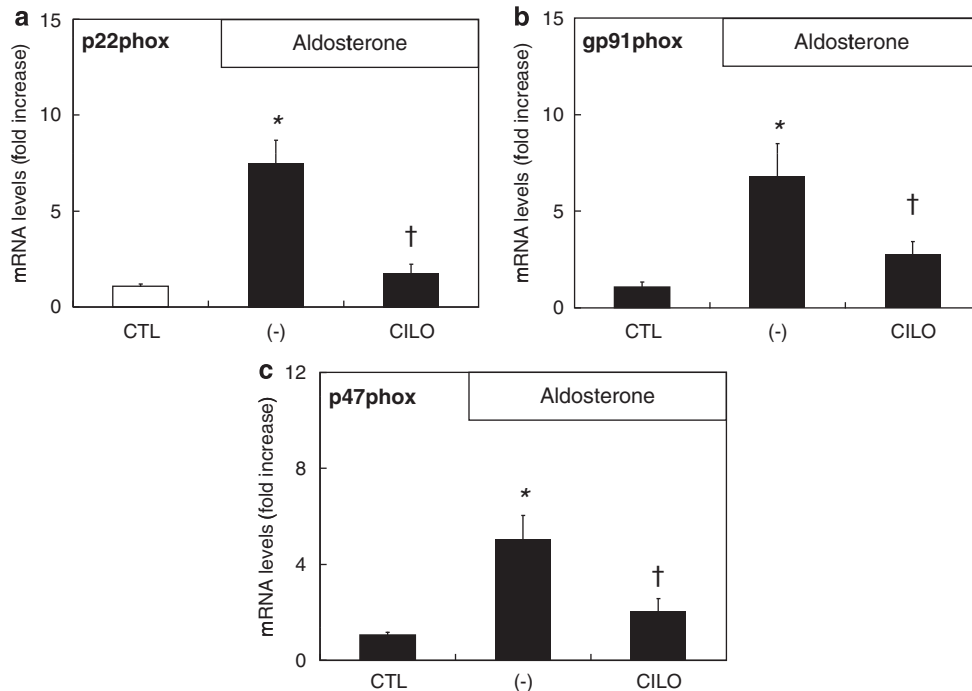
Figure 4 Increased gene expression of NAD(P)H oxidase components in aortic tissue of Aldo-rats are reversed by treatment with CILO. The steady-state mRNA levels of NAD(P)H oxidase components, (**a**) p22phox, (**b**) gp91phox and (**c**) p47phox, as measured by real-time reverse transcriptase PCR in aortic tissues from control rats (CTL), Aldo-rats without (−) and with CILO treatment are shown. The data are plotted as described in Figures 3. *$P<0.05$ vs. control; †$P<0.05$ vs. Aldo-rats.

decreased endothelial NO synthase (eNOS) activity because of reactive oxygen species-mediated eNOS uncoupling in human umbilical vein ECs.[9] In this study, the reversal of decreased urinary NOx excretion by CILO treatment suggests that NO is possibly involved in the improvement of Aldo-induced endothelial dysfunction. This notion is further supported by a recent *in vitro* study showing that CILO stimulates endothelial NO production by eNOS activation mediated by the cAMP/protein kinase A-mediated AKT pathway.[12] Alternatively, it is assumable that the antioxidant effect of CILO could prevent scavenging NO by free radicals derived from oxidative stress[28] in Aldo-induced hypertension.

We and other investigators have shown that increased proinflammatory gene expression in vascular tissue is a hallmark of Aldo-induced cardiovascular injury.[4,29,30] This study clearly showed that CILO almost completely blocked the upregulation of aortic proinflammatory gene expressions, including osteopontin, MCP-1, PDGF-A, ICAM-1, MMP-2 and ACE, in Aldo-induced hypertension. Our results are partly in agreement with those of the inhibition of aortic vascular cell adhesion molecule-1 and MCP-1 expression by CILO *in vivo*[16] and *in vitro*.[14,31] As the upregulation in vascular proinflammatory gene expression in Aldo-rats is mediated by a redox-sensitive mechanism,[2,4,7,32] such an inhibitory effect on the expression of a set of proinflammatory gene expression by CILO may be partly due to its antioxidant action. The pathogenesis of Aldo-induced vascular injury could be initiated by the upregulation of a variety of proinflammatory genes based on the following lines of evidence: (1) upregulated expression of adhesion molecules (ICAM-1) and chemokine (MCP-1) seem to be involved in monocyte/macrophage accumulation during Aldo-induced vascular injury,[4,7,33] (2) upregulated growth factor (PDGF-A) expression is likely to be responsible for vascular myointimal thickening because of its migratory and proliferative effects on

vascular smooth muscle cells,[4,34] and (3) upregulated matrix metalloprotease (MMP-2) and extracellular matrix component (osteopontin) expression are highly likely to be involved in perivascular fibrosis through extracellular matrix remodeling.[4,35–37] We previously reported that activation of vascular RAS caused by ACE upregulation is involved in Aldo-induced vascular injury.[4] Thus, the blockade of CILO on the upregulated vascular proinflammatory genes, including ACE, in Aldo-induced vascular injury could represent its vasculoprotective effect.

Several previous studies have shown that the expression of NAD(P)H oxidase components were significantly upregulated in the renal cortex and/or cardiac tissues in Aldo/salt-induced hypertensive rats, whose effects were ameliorated by treatment with MR antagonist or antioxidant.[4,7,32,38] In this study, the mRNA levels of NAD(P)H oxidase components in the aortic tissues were upregulated in Aldo-rats, which were ameliorated by the treatment with CILO. The expression of gp91phox subunit is restricted to intima and adventitia of the blood vessel and leukocytes, whereas both p22phox and p47phox are expressed in the whole vasculature.[39] Therefore, the increased gp91phox mRNA expression in the aortic tissue as shown in this study could represent the intimal and adventitial sites, and/or leukocytes infiltrated into the vasculature. It has been shown that CILO inhibits the activation of NADPH oxidase by blocking Rac-1 and/or PI3 kinase pathways.[15,40] Hence it is possible to assume that the inhibitory effect of CILO on NADPH oxidase has a crucial role in its vasculoprotective effect on Aldo-induced vascular injury.

In this study, we found increased expression of SGK-1 and NHE-1, both Aldo-inducible genes,[25,26] in the aortic tissue from Aldo-rats compared with control rats, whose effect was reversed by treatment with CILO. As both SGK-1 and NHE-1 have been shown to be

regulated by a redox-dependent pathway,[41,42] CILO may directly block such Aldo-inducible gene expression through its antioxidant effect.

The detailed molecular mechanism(s) of vasculoprotective effects of CILO in Aldo-induced vascular injuries has not been clarified from this study. As CILO, a selective inhibitor of PDE3, increases intracellular cAMP levels in platelets and cardiovascular tissue, including ECs and vascular smooth muscle cells,[13,43] cAMP is most likely to be a crucial intracellular mediator involved in its vasculoprotective effect. In fact, this study showed that Aldo decreases vascular cAMP levels, which is ameliorated by treatment with CILO, possibly through the inhibition of PDE3. Taken together with the vasculoprotective role of cAMP *in vitro* as well as in vascular injury models,[21,44–46] this findings suggest that reversal of decreased vascular cAMP levels by CILO is, in part, involved in its vasculoprotective effect on Aldo-induced vascular injury.

As the prevalence of cardiovascular complication in hyperaldosteronism is greater than that in essential hypertension,[47–49] clinical significance of Aldo as a cardiovascular risk hormone has been widely recognized. Thus, the vasculoprotective effect by CILO independent of blood pressure on Aldo-induced vascular injury as shown in this study lend strong support to the contention that Aldo-induced cardiovascular injury resulting from its proinflammatory and pro-oxidative actions on cardiovascular tissue could be a potential therapeutic target by drugs with both antioxidant and anti-inflammatory properties, such as CILO.

The limitation of this study is that we could not exclude the possibility that the vasculoprotective effect of CILO on the Aldo-induced vascular injury is mediated by its antiplatelet action, as we did not test and compare the effects of other antiplatelet agents to that of CILO in this study. To the best of our knowledge, however, no experimental study has documented that platelet aggregation has a role in the development of mineralocorticoid-induced vascular injury. On the contrary, several previous reports[50–52] have shown that platelet aggregation is indeed reduced in mineralocorticoid-induced hypertensive animal model, suggesting diminished platelet activation under such pathophysiological condition. Even if some antiplatelet aggregation inhibitors with antioxidant and anti-inflammatory actions, such as aspirin,[53,54] may potentially have a vasculoprotective role in Aldo-induced vascular injury, such experimental study seems beyond the scope of this study.

In conclusion, this study showed for the first time that CILO prevented vascular inflammation and injury in Aldo/salt-induced hypertension without affecting blood pressure, suggesting its direct blocking effect on Aldo-induced vascular changes independent of hypertension *per se*. The vasculoprotective effect of CILO could be attributed to multiple modes of action, including reduction in oxidative stress, increase in NO bioavailability, and blockade on the gene expression of several proinflammatory factors and NADPH oxidase components.

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