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Original Paper

Suppression of abdominal aortic aneurysm formation by inhibition of prolyl hydroxylase domain protein through attenuation of inflammation and extracellular matrix disruption

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Abstract

In the present study we sought to determine the effect of $CoCl_2$, an inhibitor of PHD (prolyl hydroxylase domain protein), on the development of AAA (abdominal aortic aneurysm). AAA was induced in C57BL/6 mice by periaortic application of $CaCl_2$ (AAA group). NaCl (0.9%)-treated mice were used as a sham control (SHAM group). Mice were treated with 0.05% $CoCl_2$ in the drinking water (AAA/$CoCl_2$ group). At 1 and 6 weeks after the operation, aortic tissue was excised for further examination. After 6 weeks of $CaCl_2$ treatment, aortic diameter and macrophage infiltration into the aortic adventitia were increased in the AAA group compared with the SHAM group. Treatment with $CoCl_2$ reduced the aneurysmal size and macrophage infiltration compared with the AAA group. Aortic expression of inflammatory cytokines and MCP-1 (monocyte chemoattractant protein-1) and the activities of MMP-9 (matrix metalloproteinase-9) and MMP-2 were enhanced in the AAA group and attenuated in the AAA/$CoCl_2$ group. Expression of cytokines and the activities of MMPs were already increased after 1 week of $CaCl_2$ treatment, but were suppressed by $CoCl_2$ treatment in association with reduced NF-κB (nuclear factor κB) phosphorylation. Treatment with $CoCl_2$ in mice prevented the development of $CaCl_2$-induced AAA in association with reduced inflammation and ECM (extracellular matrix) disruption. The results of the present study suggest that PHD plays a critical role in the development of AAA and that there is a therapeutic potential for PHD inhibitors in the prevention of AAA development.

Key words: abdominal aortic aneurysm, cobalt chloride ($CoCl_2$), cytokine, matrix metalloproteinase (MMP), prolyl hydroxylase domain protein (PHD)

INTRODUCTION

AAA (abdominal aortic aneurysm) is present in approximately 5–10% of men older than 65 years of age [1,2]. Most of the patients with AAA are asymptomatic and rupture may be the first manifestation of the disease, which is lethal in most cases. It is therefore important to diagnose AAAs in their early stages and to prevent progression. However, effective treatments for the prevention of AAA enlargement have not been established, suggesting that further clarification on the molecular mechanisms for the development and progression of AAAs is needed to identify effective therapeutic target molecules.

Previous studies have suggested that formation of AAAs is closely associated with chronic inflammation of the aortic wall, the local activation of proteinases and the degradation of matrix proteins [3]. In contrast with the atherosclerotic plaque, of

Abbreviations: AAA, abdominal aortic aneurysm; CAT, catalase; DAB, diaminobenzidine; DMOG, dimethyloxaloylglycine; ECM, extracellular matrix; EPO, erythropoietin; EVG, elastica Van Gieson; H/E, haematoxylin and eosin; HIF, hypoxia-inducible factor; HPRT, hypoxanthine phosphoribosyl transferase; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; PHD, prolyl hydroxylase domain protein; qRT-PCR, quantitative real-time reverse transcription– PCR; ROS, reactive oxygen species; SOD1, superoxide dismutase 1; TNFα, tumour necrosis factor α; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell.

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which inflammation is mainly observed in the intima, AAAs are characterized by transmural inflammation. Degradation of elastin in the media is also an important pathological feature of AAAs [4]. Degradation of matrix proteins is due to MMPs (matrix metalloproteinases) produced from infiltrated macrophages and medial VSMCs (vascular smooth muscle cells). Transcription factors, such as NF-κB (nuclear factor κB), are activated and expression of pro-inflammatory cytokines, such as IL-6 (interleukin-6) and TNFα (tumour necrosis factor α), are increased in AAAs [5,6].

PHD (prolyl hydroxylase domain protein) is responsible for the degradation of HIF-1α (hypoxia-inducible factor α) protein, a transcription factor activated in hypoxic conditions [7]. Hydroxylation of Pro^{402} and Pro^{564} of HIF-1α by PHD under normoxic conditions results in the ubiquitination and subsequent proteasomal degradation of HIF-1α. The activity of PHD is inhibited under hypoxic conditions and therefore HIF-1α is stabilized. HIF-1α then heterodimerizes with constitutively expressed HIF-1β and the resultant heterodimeric transcription factor HIF activates transcription of many genes involved in the attenuation of hypoxic damage, such as *VEGF* (vascular endothelial growth factor) and *EPO* (erythropoietin). The activity of PHD can be also inhibited by chemical inhibitors such as $CoCl_2$ and DMOG (dimethyloxaloylglycine) even under normoxic conditions [8]. In addition to degradation of HIF-1α, recent studies have revealed HIF-independent function of PHD [9]. We have reported previously that inhibition of PHD by chemical inhibitors or siRNA-mediated down-regulation suppressed LPS (lipopolysaccharide)-induced TNFα expression in a macrophage cell line independently of HIF [10]. These results prompted us to investigate whether inhibition of PHD attenuates the development of AAAs, in which inflammation plays an important role.

MATERIALS AND METHODS

Murine AAA model

All procedures were approved by the Animal Care and Use Committee of Kyushu University and were conducted in accordance with the institutional guidelines. AAA formation was induced in 6–7-week-old C57BL/6 mice by periaortic application of 0.5 mol/l $CaCl_2$ (Wako) as described previously [11], under anaesthesia by intraperitoneal injection of ketamine (100 mg/kg of body mass) and xylazine (10 mg/kg of body mass). The mice were purchased from Clea Japan and fed on a normal chow. Application of the appropriate level of anaesthesia was confirmed by the absence of a withdrawal response to a hard pinch of the foot. NaCl (0.9%) was substituted for $CaCl_2$ in the sham-operated mice (SHAM group; $n = 44$). Mice were treated with 0.05% $CoCl_2$ (Sigma–Aldrich) dissolved in water (AAA/$CoCl_2$ group; $n = 44$) or drinking water alone (AAA group; $n = 44$) for 1 week before the operations were performed. The estimated daily intake of $CoCl_2$ was 70 mg/kg of body mass.

At 1 and 6 weeks after the operations were performed, the blood pressures and heart rates of the mice were measured using the tail-cuff method while in a conscious state (BP-98A-L; Softron). The mice were then anaesthetized by intraperitoneal injection of ketamine (100 mg/kg of body mass) and xylazine (10 mg/kg of body mass) and perfusion-fixed with 10% formaldehyde in PBS at physiological perfusion pressure. The abdominal aorta was carefully excised so as not to disturb the adventitia and photographed to determine its external diameter. Aortic diameter was measured using computer-assisted image analysis software ImageJ (http://rsb.info.nih.gov/ij/). The aortic tissue was then subjected to histological analyses.

Histological analysis

Paraffin-embedded cross-sections of abdominal aorta (6-μm thick) were subjected to H/E (haematoxylin and eosin) and EVG (elastica Van Gieson) staining. For Mac-2 [also known as Lgals3 (galectin-3)] immunohistochemical staining, paraffin-embedded tissue sections were deparaffinized and rehydrated and then autoclaved in 10 mmol/l citrate buffer for antigen retrieval. Following quenching with endogenous peroxidase, sections were incubated with an anti-(mouse Mac-2) antibody (Cedarlane) at 4 °C overnight. After incubation with Simple Stain MAX-PO (Rat) (Nichirei Bioscience), sections were incubated in DAB (diaminobenzidine) solution and counterstained with haematoxylin. The number of Mac-2-positive cells was quantified by counting the total number of DAB-positively stained cells in 20 grid fields with a total area of 0.1 mm^2.

qRT-PCR (quantitative real-time reverse transcription–PCR)

The infrarenal portion of the aorta was removed, minced into small pieces, homogenized in ISOGEN solution (Nippon Gene) on ice and total RNA was extracted according to the manufacturer's protocol. A 1 μg sample of total RNA of the aorta was reverse-transcribed with the ReverTra Ace qPCR RT kit (Toyobo). Real-time qPCR analysis was performed using the Power SYBR Green PCR Master mix (Life Technologies) and the Applied Biosystems 7500 Real-Time PCR system according to the manufacturer's protocol. Expression of IL-1β, IL-6, MCP-1 (monocyte chemoattractant protein-1), TNFα, MMP-2 and MMP-9 are shown as the relative mRNA level of HPRT (hypoxanthine phosphoribosyl transferase) using the comparative C_T ($\Delta\Delta C_T$) method. The following primers were used: IL-1β, 5′-TCCAGGATGAGGACATGAGCAC-3′ (forward) and 5′-GAACGTCACACACCAGCAGGTTA-3′ (reverse); IL-6, 5′-CCACTTCACAAGTCGGAGGCTTA-3′ (forward) and 5′-GCAAGTGCATCATCGTTGTTCATAC-3′; MCP-1, 5′-GCATCCACGTGTTGGCTCA-3′ (forward) and 5′-CTCCAGCCTACTCATTGGGATCA-3′ (reverse); TNFα, 5′-AAGCCTGTAGCCCACGTCGTA-3′ (forward) and 5′- GGCACCACTAGTTGGTTGTCTTTG-3′ (reverse); HPRT, 5′-TTGTTGTTGGATATGCCCTTGACTA-3′ (forward) and 5′- AGGCAGATGGCCACAGGACTA-3′ MMP-2, 5′-GATAACCTGGATGCCGTCGTG-3′ (forward) and 5′-CTTCACGCTCTTGAGACTTTGGTTC-3′

Table 1 Body mass and haemodynamics
Results are means ± S.D. b.p.m., beats/min.

(a) After 1 week

Parameter	SHAM ($n=5$)	AAA ($n=5$)	AAA/$CoCl_2$ ($n=5$)
Body mass (g)	19.1 ± 0.44	19.2 ± 0.50	18.7 ± 0.64
Systolic blood pressure (mmHg)	103 ± 7	105 ± 6	102 ± 6
Heart rate (b.p.m.)	560 ± 10	570 ± 9	561 ± 11

(b) After 6 weeks

Parameter	SHAM ($n=8$)	AAA ($n=10$)	AAA/$CoCl_2$ ($n=8$)
Body mass (g)	24.5 ± 2.3	25.1 ± 1.3	24.6 ± 1.6
Systolic blood pressure (mmHg)	105 ± 8	107 ± 9	105 ± 8
Heart rate (b.p.m.)	562 ± 32	578 ± 26	588 ± 31

(reverse); MMP-9, 5′-CATTCGCGTGGATAAGGAGT-3′ (forward) and 5′-ACCTGGTTCACCTCATGGTC-3′ (reverse); SOD1 (superoxide dismutase 1), 5′-AGCATTCCATCATTGGCCGTA-3′ (forward) and 5′-TACTGCGCAATCCCAATCACTC-3′ (reverse); CAT (catalase), 5′-TCTACACAAAGGTGTTGAACGAGGA-3′ (forward) and 5′-AGTCAGGGTGGACGTCAGTGAA-3′ (reverse); and VEGF, 5′-GCACATAGGAGAGATGAGCTTCC-3′ (forward) and 5′-CTCCGCTCTGAACAAGGCT-3′ (reverse).

Gelatin zymography

To estimate the activities of MMP-2 and MMP-9, gelatin zymography was performed using extracts from AAA tissue. Frozen descending aorta was homogenized in lysis buffer composed of RIPA buffer, 1% aprotinin, 10 μmol/l pepstatin A, 1 mmol/l PMSF and 2.5 μg/ml leupeptin. After centrifugation at 12 000 ***g*** for 20 min at 4 °C the supernatant was collected. An equal amount of protein (15 μg) was subjected to SDS/PAGE (10% gel) copolymerized with 0.1% gelatin as a substrate. Gels were washed with 2.5% Triton X-100 and incubated at 37 °C for 48 h with reaction buffer (50 mM Tris/HCl, pH 6.8, containing 10 mM $CaCl_2$) and then stained with staining buffer (0.8% Brilliant Blue R) so that protein bands with gelatinolytic activity were easily identified as clear lytic bands. Band intensities were quantified using ImageJ software. The total of MMP-2 and pro-MMP-2 or MMP-9 was measured as the total MMP-2 or MMP-9 activity. Human MMP marker (Primary Cell) was used to determine the molecular mass.

Western blot analysis

For Western blotting, preparation of aortic tissue lysate was performed as described above. Equal amounts (10 μg) of protein samples were subjected to SDS/PAGE and transferred on to PVDF membranes (Immobilon-P; Millipore). After blocking with 5% dried non-fat skimmed milk powder, the membrane was incubated with the primary antibodies against phospho-p65 and p65 (Cell Signaling Technology), followed by exposure to the secondary antibody. Actin (Santa Cruz Biotechnology) immunoblotting was used as a protein loading control. Blots were detected using an ECL Prime Western Blotting Detection reagent (GE Healthcare). The signals were quantified by ImageJ software.

Statistical analysis

The experimental data were analysed by one-way ANOVA and Tukey's HSD (honestly significant difference) test. Steel–Dwass tests were used if the data were not normally distributed. Results are means ± S.E.M. or S.D. and $P < 0.05$ was considered statistically significant.

RESULTS

PHD inhibition by $CoCl_2$ prevents the development of AAA

The body mass, blood pressure and heart rate were comparable among the three groups after 1 and 6 weeks (Table 1). No obvious toxicity of $CoCl_2$ treatment was observed. No significant changes in aortic diameter were observed after 1 week of $CaCl_2$ treatment (results not shown). The maximal external diameter of the abdominal aorta at 6 weeks was larger in the AAA group than in the SHAM group ($P < 0.01$). Dilatation of the aorta was attenuated in the AAA/$CoCl_2$ compared with the AAA group ($P < 0.01$) (Figures 1A and 1B).

PHD inhibition by $CoCl_2$ attenuated inflammation and preserved ECM (extracellular matrix) structure

H/E staining showed enlargement of the luminal area, modest thinning of the media and marked thickening of the adventitia in the AAA group after 6 weeks (Figures 1C–1H). However, these changes were attenuated by $CoCl_2$ treatment. EVG staining showed the wavy structure of the elastic lamellae in the SHAM group, which was lost in the AAA group (Figures 1I–1N). Treatment with $CoCl_2$ preserved the elastin structure of the aorta.

Immunohistochemical staining for macrophages with an anti-Mac-2 antibody showed a marked infiltration of macrophages into the adventitia of the aorta in the AAA group (Figures 1O–1R). $CoCl_2$ treatment decreased the number of infiltrated Mac-2-positive macrophages (Figures 1O and 1P).

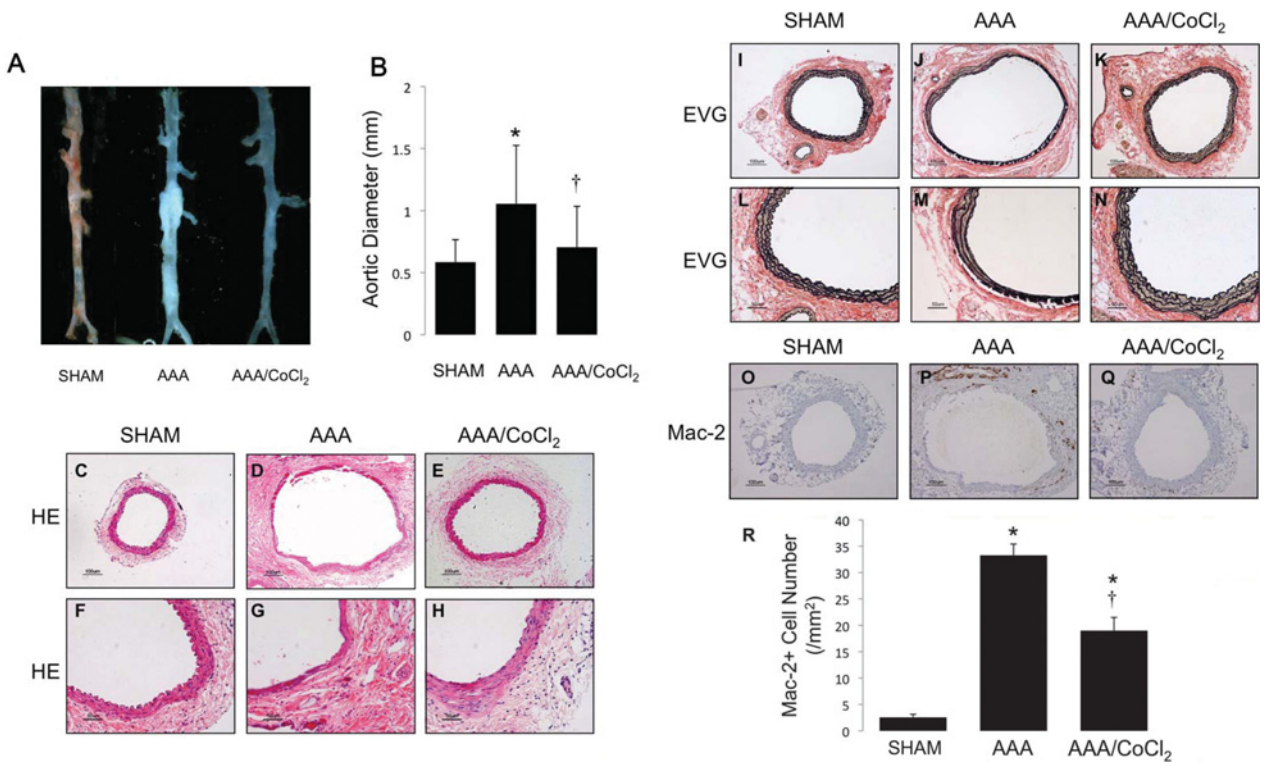

Figure 1 **Inhibition of PHD by $CoCl_2$ attenuated $CaCl_2$-induced AAA in mice**

(**A**) Representative macroscopic appearances of the aorta in sham-operated control mice (SHAM), AAA mice (AAA) and $CoCl_2$-treated AAA mice (AAA/$CoCl_2$) after 6 weeks are shown. (**B**) The maximum external diameter of the abdominal aorta after 6 weeks of $CaCl_2$ treatment is larger in the AAA group than in the SHAM group. The diameter is smaller in the AAA/$CoCl_2$ group than in the AAA group. Results are means $\pm$ S.E.M. ($n = 10$ for each group). $^*P < 0.05$ compared with the SHAM group and $\dagger P < 0.05$ compared with the AAA group. (**C**–**N**) Representative microphotographs of the H/E (**C** and **F**, SHAM; **D** and **G**, AAA; and **E** and **H**, AAA/$CoCl_2$) and EVG (**I** and **L**, SHAM; **J** and **M**, AAA; and **K** and **N**, AAA/$CoCl_2$) staining of the aortic wall. Scale bars, 100 μm in (**C**–**E** and **I**–**K**) and 50 μm in (**F**–**H** and **L**–**N**). (**O**–**Q**) Immunohistochemical staining for Mac-2 in the mouse aortic wall. Scale bars, 100 μm. (**R**) Mac-2-positive cells were counted in each slice and are indicated as the number of cells per mm^2. Results are means $\pm$ S.E.M. (SHAM, $n = 7$; AAA, $n = 10$; and AAA/$CoCl_2$, $n = 8$). $^*P < 0.05$ compared with the SHAM group and $\dagger P < 0.05$ compared with the AAA group.

PHD inhibition by $CoCl_2$ reduced the expression of inflammatory cytokines

We examined the expression of inflammatory cytokines in the aortic walls after 1 week of $CaCl_2$ application (Figure 2A), when enlargement of the aorta is minimal [12]. Expression of IL-1β, IL-6 and MCP-1 was up-regulated in the AAA group compared with the SHAM group. Expression of IL-1β, IL-6 and MCP-1 was significantly suppressed in the AAA/$CoCl_2$ group. However, we did not observe any effect of $CoCl_2$ on TNFα expression.

qRT-PCR analysis of the mRNA at the aneurysmal sites after 6 weeks of $CaCl_2$ treatment revealed up-regulation of IL-1β, IL-6, MCP-1 and TNFα in the AAA group compared with the SHAM group (Figure 2B). Although the mRNA level of IL-1β, IL-6, MCP-1 and TNFα showed a trend towards a reduction in the AAA/$CoCl_2$ group, the difference was not statistically significant.

The mRNA expression of *VEGF*, a target gene of *HIF*, was increased after both 1 and 6 weeks, suggesting that $CoCl_2$ treatment suppressed PHD activity and thereby activated *HIF*.

PHD inhibition by $CoCl_2$ restored the expression of antioxidant genes

In the AAA group, expression of *SOD1* and *CAT* were down-regulated compared with the SHAM group after 1 week of $CaCl_2$ treatment. At the same time point the expression of *SOD1* and *CAT* were reversed in the AAA/$CoCl_2$ group (Figure 2A). Although the mRNA levels of *SOD1* and *CAT* after 6 weeks of $CaCl_2$ treatment showed a trend towards an increase in the AAA/$CoCl_2$ group compared with the AAA group, the difference was not statistically significant (Figure 2B). The expression of the Nox2, Nox4 and p47 NADPH oxidase subunits was not changed significantly among the three groups after both 1 and 6 weeks (results not shown).

PHD inhibition by $CoCl_2$ suppressed the activity of MMPs

We examined the effect of $CoCl_2$ treatment on MMP activity by gelatin zymography. Gelatin zymography showed that the abundance of the active forms of MMP-9 and MMP-2 in the aortic walls was up-regulated strongly after 1 week (Figures 3A–3C)

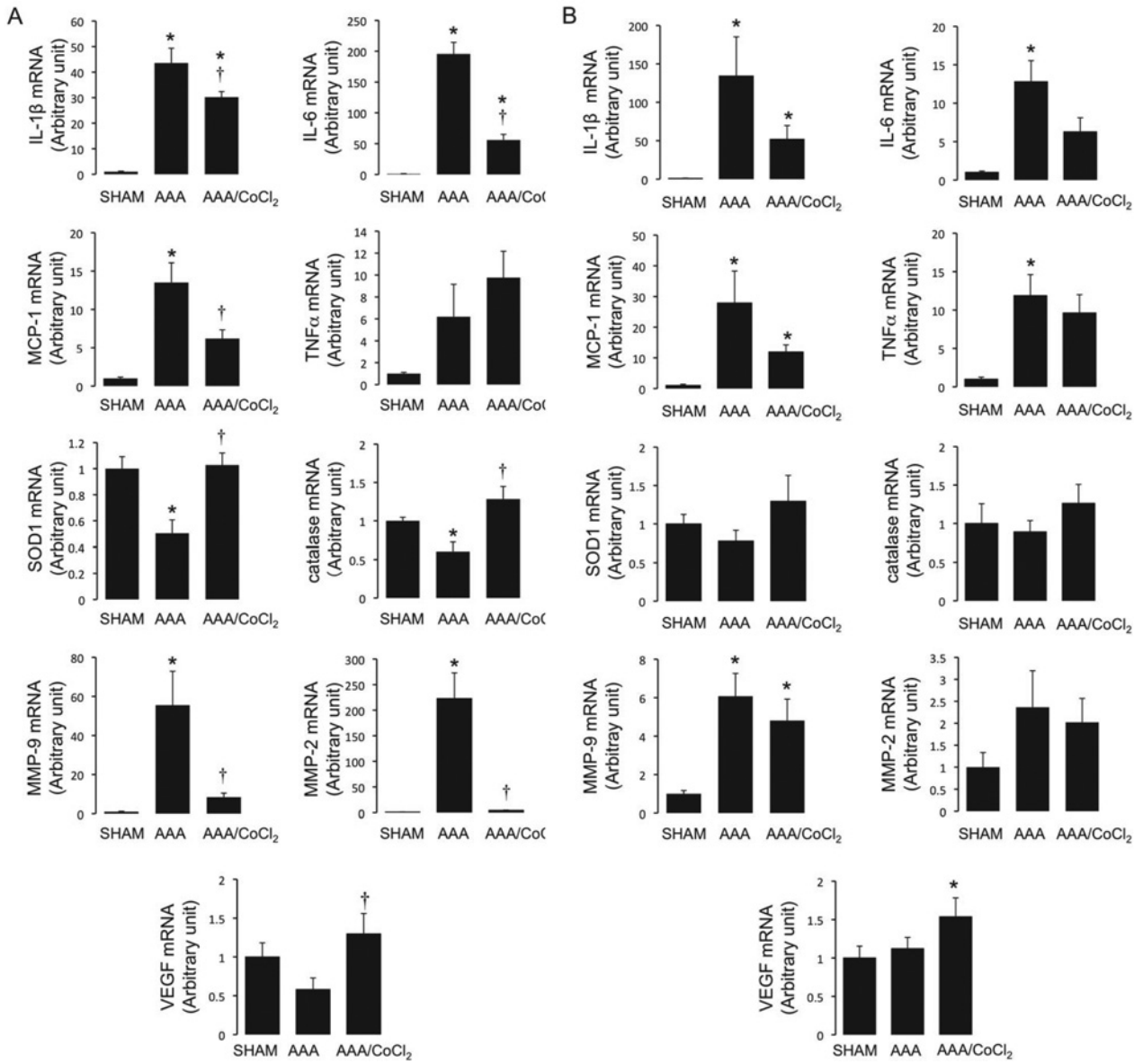

Figure 2 Inhibition of PHD by $CoCl_2$ attenuated the expression of pro-inflammatory cytokines in $CaCl_2$-treated aortas

(**A**) Expression levels of IL-1β, IL-6, MCP-1, TNFα, SOD1, CAT, MMP-2, MMP-9 and VEGF in the aorta of $CaCl_2$- or saline-treated sites after 1 week. Expression of each gene is shown as the relative mRNA level to that of HPRT. Expression levels in the saline-treated aortas (SHAM) were set to 1. Results are means $\pm$ S.E.M. ($n = 5$ for each group). *$P<0.05$ compared with the SHAM group and †$P<0.05$ compared with the AAA group. (**B**) Expression levels of IL-1β, IL-6, MCP-1, TNFα, SOD1, CAT, MMP-2, MMP-9 and VEGF at aneurysmal sites after 6 weeks are expressed as the ratio of those at sites treated with saline (SHAM). Expression of each gene was presented as the relative mRNA level to that of HPRT. Expression levels in the saline-treated aortas (SHAM) were set to 1. Results are means $\pm$ S.E.M. ($n = 10$ for each group). *$P<0.05$ compared with the SHAM group and †$P<0.05$ compared with the AAA group.

and increased modestly after 6 weeks (Figures 3D–3F) of $CaCl_2$ treatment in the AAA group compared with the SHAM group. Their abundances were decreased by $CoCl_2$ treatment.

The mRNA levels of *MMP-2* and *MMP-9* were increased greatly after 1 week of $CaCl_2$ treatment (Figure 2A), which was suppressed significantly by $CoCl_2$ treatment. *MMP-9* mRNA was still significantly increased after 6 weeks, but the increase in *MMP-2* expression was not significant (Figure 2B). The effect of treatment with $CoCl_2$ on *MMP-2* and *MMP-9* expression was not observed at this point.

PHD inhibition by $CoCl_2$ suppressed p65 phosphorylation of NF-κB

NF-κB plays an important role in the up-regulation of inflammatory cytokines and MMPs. Protein expression of the p65 subunit of NF-κB was increased slightly in the AAA group; however, the

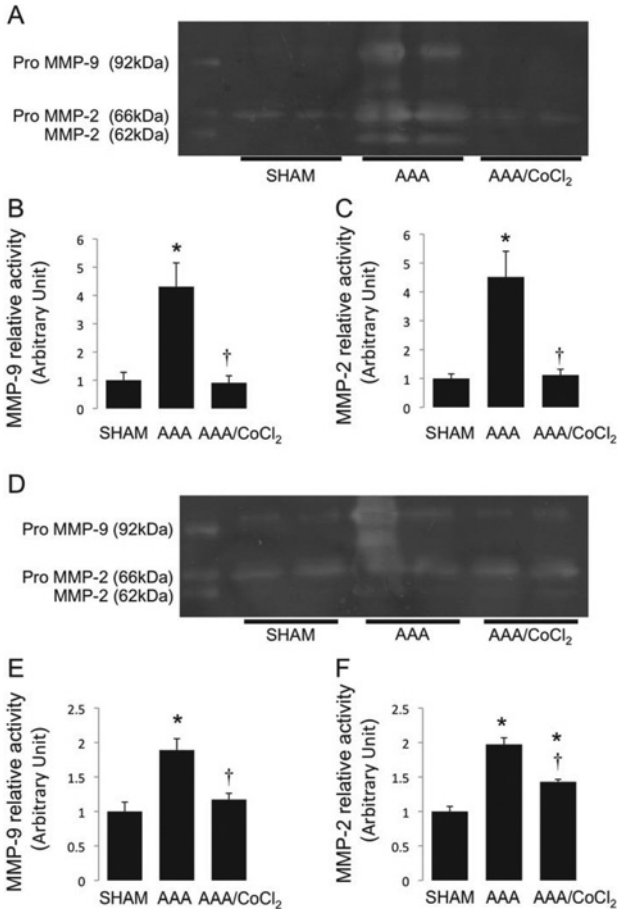

Figure 3 Inhibition of PHD by $CoCl_2$ suppressed MMP activity in the aortic wall after 1 and 6 weeks of $CaCl_2$ treatment

Representative gelatin zymography images after 1 week (**A**–**C**) and 6 weeks (**D**–**F**) of saline (SHAM) or $CaCl_2$ treatment (AAA). Representative photographs of the gels are shown. The left-hand lane is the MMP marker derived from human fibrosarcoma cells and the bands showed pro-MMP-9 (92 kDa), pro-MMP-2 (66 kDa) and MMP-2 (62 kDa) according to the manufacturer's instructions. Densitometric analysis of the gels was performed for MMP-9 (**B** and **E**) and MMP-2 (**C** and **F**). The abundance of MMPs in the SHAM group was set to 1. Results are means ± S.E.M. (1 week, $n = 4$ and 6 weeks, $n = 8$). $^*P < 0.05$ compared with the SHAM group and $\dagger P < 0.05$ compared with the AAA group.

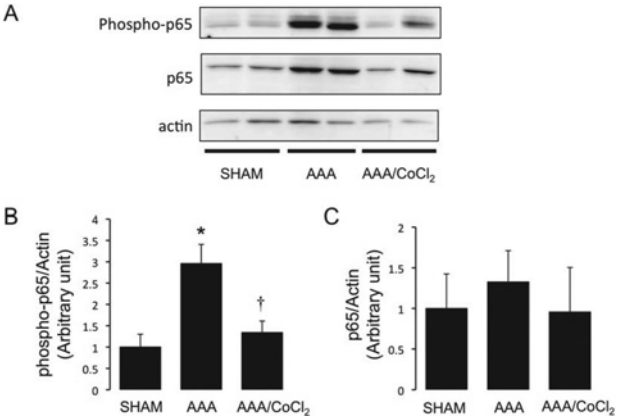

Figure 4 Inhibition of PHD by $CoCl_2$ suppressed p65 phosphorylation in the aortic wall after 1 week

(**A**–**C**) Representative immunoblotting analysis of phospho-p65 and p65 of NF-κB in the mouse aortic wall lysate. Results are means ± S.E.M. ($n = 6$ for each group). Because the expression of p65 was mildly increased in the AAA group, the p65 and phospho-p65 levels were normalized to the β-actin expression. Histograms indicate the relative ratio of phospho-p65 to β-actin (**B**) and p65 to β-actin (**C**). $^*P < 0.05$ compared with the SHAM group and $\dagger P < 0.05$ compared with the AAA group.

difference was not statistically significant. The phosphorylation level of p65 was significantly greater in the AAA group than the SHAM group. Treatment with $CoCl_2$ significantly attenuated the phosphorylation of p65 (Figure 4).

DISCUSSION

In the present study, we have shown that treatment with $CoCl_2$ attenuated the $CaCl_2$-induced dilatation of the abdominal aorta in association with a reduction in inflammatory cytokine expression, MMP activity and NF-κB phosphorylation. The causal relationship between the reduction in cytokines and MMPs and the suppression of AAA formation is not clear from the present study. However, $CoCl_2$ treatment suppressed cytokine expression and MMP activity after 1 week of $CaCl_2$ treatment, when dilatation of the aorta is minimal in this model [12]. Therefore the suppression of cytokines and MMPs by $CoCl_2$ treatment may be involved in the attenuation of AAA formation in our model.

The effect of cobalt on inflammation is controversial. Kalpana et al. [13] reported that hypoxia-induced NF-κB activation in the rat brain was inhibited by pre-treatment with $CoCl_2$. Cobalt suppressed the hypoxia-induced NF-κB activation and the production of ROS (reactive oxygen species), resulting in the suppression of the production of MCP-1 and IFNγ (interferon γ). These data are consistent with the present study and our previous study that DMOG, another PHD inhibitor, or knock down of PHD suppressed LPS-induced expression of TNFα, IL-6 and IL-1β in association with the suppression of NF-κB-dependent gene transcription in macrophages [10]. Another study reported renal protection by $CoCl_2$ [14]. Treatment of hypertensive rats with Type 2 diabetes and nephropathy with $CoCl_2$ attenuated proteinuria. The reduction of proteinuria was associated with attenuation of kidney injury and suppression of Nox2, a subunit of NADPH oxidase, and TGF-β (transforming growth factor-β) expression. Treatment with cobalt also attenuated cyclosporine A-induced tubulointerstitial injury in association with a reduction in macrophage infiltration into the renal tubulointerstitium [15]. Cobalt suppressed cyclosporine A-induced NF-κB phosphorylation, a marker of its activation, in human renal tubular epithelium cells. The results of these studies and the present study suggest that $CoCl_2$ suppresses inflammation. Activation of HIF by cobalt-mediated PHD inhibition is proposed to be involved in the anti-inflammatory effect. Although we confirmed

PHD inhibition/HIF activation in the AAA/$CoCl_2$ group by up-regulation of *VEGF* mRNA in the aorta, the role of HIF in the anti-inflammatory effects of $CoCl_2$ remains to be determined.

In contrast, it has also been reported that treatment with $CoCl_2$ (160 μmol/l) induced IL-6 and TNFα production in microglia, resident macrophages in the central nervous system [16]. Cobalt also induced expression of iNOS (inducible nitric oxide synthase) and NO production. Yang et al. [17] showed that cobalt increased COX-2 (cyclooxygenase-2) expression and the secretion of IL-6 and IL-8 in human skin keratinocytes in association with the phosphorylation of NF-κB. Exposure to $CoCl_2$ induced overproduction of ROS and a reduction in cell viability.

It is difficult at present to reconcile these discrepant results; however, *in vivo* experiments generally have shown protective effects of $CoCl_2$ against inflammatory changes. Although these results are not supported by other *in vitro* experiments, this may be due to a very high concentration of $CoCl_2$ used in these *in vitro* experiments. Alternatively, some secondary protective effects may play a role in the *in vivo* effect of $CoCl_2$.

We have also shown in the present study that treatment with $CoCl_2$ reduced the activities of MMP-2 and MMP-9 in the aorta; however, several *in vitro* studies have shown that cobalt activates MMPs. Wang et al. [18] have shown that $CoCl_2$ activates MMP-2 in ligament cells. It has also been shown that the response to cobalt is different between the anterior cruciate ligament and medial collateral ligament. Another study showed that $CoCl_2$ increases MMP-2 secretion in articular chondrocytes [19].

It is also difficult to explain the discrepancy in the effects of cobalt on the activity of MMPs between the present study and previous studies. One of the possible explanations is that the effect of cobalt is cell-type specific. Therefore further study is needed to examine the effect of $CoCl_2$ on MMP production in macrophages or VSMCs in the future.

Vascular oxidative stress is thought to play an important role in the pathogenesis of AAA and previous studies have shown that oxidative stress enhanced AAA development [20,21]. Dubick et al. [22] reported low levels of Cu,Zn-SOD (copper,zinc SOD) activity in human aneurysm. Another study reported that $CoCl_2$ inhibited hypoxia-induced oxidative stress in the brain via the HIF-1α signalling mechanism [23]. The present study showed that $CoCl_2$ restored the expression of SOD1 and CAT, which was suppressed in aneurysmal sites. These data suggest that $CoCl_2$ inhibited the formation of AAA via antioxidant effects and thereby suppression of NF-κB phosphorylation and cytokine expression.

Cobalt is a trace element in the body essential for the normal function of vitamin B_{12}. However, it is well known that excessive intake of cobalt causes cytotoxicity and neurotoxicity and therefore cobalt cannot be used to treat patients. Recently, several PHD inhibitors are under development for clinical use as a drug for the treatment of anaemia because PHD inhibition causes up-regulation of EPO through HIF stabilization [24]. PHD inhibitors are also expected to be beneficial for ischaemic disorders [8]. Although it is not clear whether these PHD inhibitors show the same anti-inflammatory effect as has been shown for $CoCl_2$ in the present study and therefore further study is needed, PHD inhibitors may be novel promising drugs for the treatment of AAA.

There are several limitations of the present study. The first is that, although previous studies [13–15] and the present study showed that $CoCl_2$ activates the HIF pathway, we could not exclude the possibility of non-specific effects of $CoCl_2$. The second is that $CoCl_2$ treatment was started before the topical application of $CaCl_2$ to the aorta and therefore it is not clear whether treatment with $CoCl_2$ prevents the progression of dilatation of established AAA. The third is that Mac-2 can also localize to fibroblasts and therefore we may have overestimated the number of adventitial macrophages in Mac-2 staining, which may explain the larger change in the number of Mac-2-positive cells between the groups than the changes in aortic dimension (Figures 1B and 1R). Further studies are needed to address these issues.

In conclusion, we have shown in the present study that PHD inhibition by $CoCl_2$ administration attenuated AAA formation induced by $CaCl_2$ in association with a reduction in inflammatory cytokines and the activity of MMP-2 and MMP-9. Although further study is required to elucidate the detailed mechanisms, PHD inhibition may be a novel strategy for the prevention of AAA progression.

CLINICAL PERSPECTIVES

- Inhibition of PHD has been shown to suppress LPS-induced TNFα expression in a macrophage cell line in a previous study, but the effect of a PHD inhibitor on AAA development is unknown.
- In the present study, $CoCl_2$, a PHD inhibitor, prevented the development of $CaCl_2$-induced AAA in association with reduced inflammation and ECM disruption.
- These findings suggest that PHD plays a critical role in the development of AAA and that there is a therapeutic potential of PHD inhibitors for the prevention of AAA development.

AUTHOR CONTRIBUTION

Aya Watanabe, Toshihiro Ichiki and Kenji Sunagawa conceived and designed the the study and wrote the paper. Aya Watanabe performed the experiments. Chikahiro Sankoda, Yusuke Takahara, Jiro Ikeda and Eriko Inoue helped with the experiments. Tomotake Tokunou and Shiro Kitamoto gave technical advice on the experiments.

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