



# 4-*O*-Methylascochlorin inhibits the prolyl hydroxylation of hypoxia-inducible factor-1 $\alpha$ , which is attenuated by ascorbate

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## Abstract

4-*O*-Methylascochlorin (MAC), a methylated derivative of ascochlorin, was previously shown to promote the accumulation of hypoxia-inducible factor (HIF)-1 $\alpha$  in human breast adenocarcinoma MCF-7 cells. In the present study, we further investigated the effects of MAC on the expression and function of HIF-1 $\alpha$  in human fibrosarcoma HT-1080 cells. MAC promoted the accumulation of the HIF-1 $\alpha$  protein without affecting its constitutive mRNA expression and augmented the transcriptional activation of HIF target genes. Ascorbate, but not *N*-acetylcysteine, attenuated MAC-mediated HIF-1 $\alpha$  accumulation. MAC-induced increases in HIF-1 $\alpha$  transcriptional activity were also attenuated by ascorbate. MAC inhibited the hydroxylation of HIF-1 $\alpha$  at the proline 564 residue, while it was reversed by ascorbate. MAC slightly decreased the intracellular concentration of ascorbate. The present results demonstrated that MAC promoted the accumulation of HIF-1 $\alpha$  by preventing prolyl hydroxylation, and ascorbate attenuated the MAC-mediated inhibition of HIF-1 $\alpha$  prolyl hydroxylation.

## Introduction

Oxygen is essential for aerobic respiration and ATP production by mitochondria [1]. Under low oxygen (hypoxic) conditions, hypoxia-inducible factors (HIF) play a critical role in the regulation of various cellular responses,

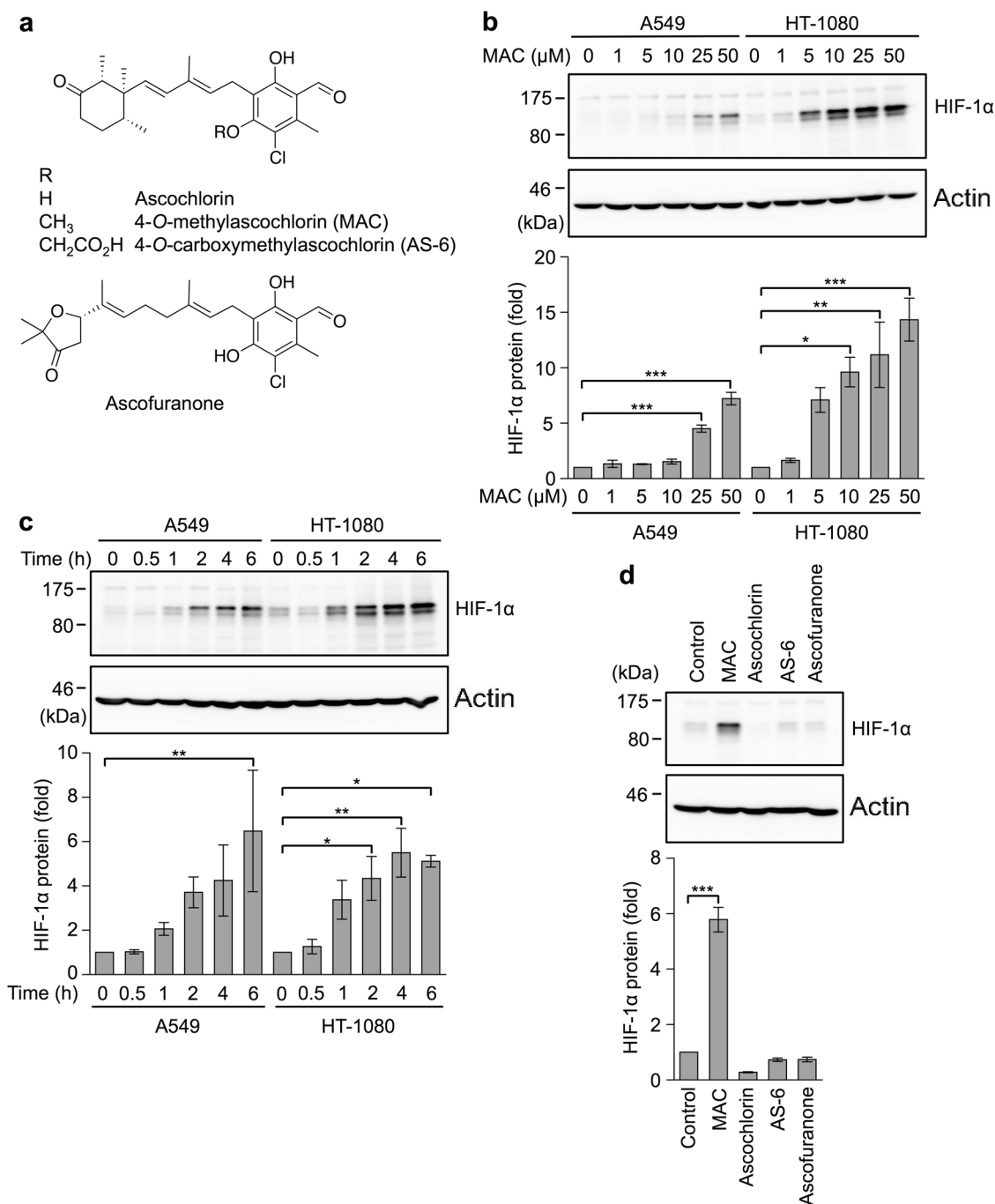
including cell metabolism, angiogenesis, tumorigenesis, and inflammation [2, 3]. HIF transcription factors are heterodimers composed of the stable HIF-1 $\beta$  subunit (also known as ARNT) and one of three hypoxia-inducible  $\alpha$  subunits [2]. HIF-1 $\alpha$  is expressed ubiquitously, whereas HIF-2 $\alpha$  and HIF-3 $\alpha$  are only present in some tissues [4]. Under normal oxygen conditions, HIF-1 $\alpha$  is hydroxylated at two proline residues by prolyl hydroxylase domain (PHD) family proteins [5]. PHD proteins require 2-oxoglutarate and oxygen as substrates and Fe<sup>2+</sup> and ascorbate as cofactors for their catalytic activity [6]. Of the three PHD proteins, PHD2 is most abundantly expressed in tissues, except for the testis and heart, in which PHD1 and PHD3, respectively, are strongly expressed [6, 7]. When HIF-1 $\alpha$  is hydroxylated, it is subsequently recognized by the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex, and undergoes polyubiquitination and proteasomal degradation, which maintains HIF-1 $\alpha$  at basal levels [8]. Under hypoxic conditions, PHD proteins become inactive and, thus, HIF-1 $\alpha$  accumulates in cells [6, 9]. HIF-1 $\alpha$  dimerizes with HIF-1 $\beta$ , and HIF heterodimers translocate to the nucleus and bind to hypoxia response element (HRE) in the target genes [8, 9].

Ascochlorin is a prenyl-phenol compound that was originally isolated as an anti-viral agent (Fig. 1a) [10, 11]. Ascochlorin and its derivatives, including 4-*O*-methylascochlorin (MAC), 4-*O*-carboxymethyl ascochlorin (AS-6), and ascofuranone (Fig. 1a), exhibit various

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**Fig. 1** MAC promoted the accumulation of the HIF-1 $\alpha$  protein in A549 cells and HT-1080 cells. **a** Structures of ascochlorin, MAC, AS-6, and ascofuranone. **b, c** A549 cells and HT-1080 cells were incubated with the indicated concentrations of MAC for 6 h (**b**). A549 cells and HT-1080 cells were incubated with MAC (50  $\mu$ M) for the indicated times (**c**). Whole-cell lysates were analyzed by western blotting. Representative blots are shown. The HIF-1 $\alpha$  protein relative to the actin protein (fold) is expressed as the mean  $\pm$  S.E. of three (**b**) and four (**c**) independent experiments. The values of MAC at 0  $\mu$ M

time 0 h are set to onefold. \* $P$  < 0.05, \*\* $P$  < 0.01, and \*\*\* $P$  < 0.001. **d** HT-1080 cells were incubated with MAC (50  $\mu$ M), ascochlorin (50  $\mu$ M), AS-6 (50  $\mu$ M), and ascofuranone (50  $\mu$ M) for 6 h. Whole-cell lysates were prepared independently by three experiments and then analyzed by western blotting. Representative blots are shown. The HIF-1 $\alpha$  protein relative to the actin protein (fold) is expressed as the mean  $\pm$  S.E. of three experiments. The value of the control is set to onefold. \*\*\* $P$  < 0.001

biological activities, such as hypolipidemic activity [12, 13], the prevention of hypertension [14], amelioration of diabetes [15–17], anti-tumor activity [18, 19],

immunosuppressive activity [20], and the induction of apoptosis [21]. We recently demonstrated that MAC suppressed the expression of c-Myc via the AMP-

activated protein kinase (AMPK)- and mechanistic target of rapamycin (mTOR)-dependent pathways in human leukemia cell lines [22]. MAC has also been shown to suppress the differentiation of murine 3T3-L1 pre-adipocytes by inhibiting peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) through the regulation of the AMPK/mTOR pathway [23]. These findings indicate that MAC promotes the activation of the AMPK pathway, thereby inhibiting the mTOR pathway, which regulates downstream cellular responses, such as apoptosis.

We previously reported that MAC promoted the accumulation of HIF-1 $\alpha$  via the activation of the AMPK pathway in human breast adenocarcinoma MCF-7 cells [24], while ascochlorin and ascofuranone suppressed HIF-1 $\alpha$  expression and transcriptional activity induced by EGF, but not by CoCl<sub>2</sub> in vitro, as well as EGF-induced angiogenesis in vivo [25, 26]. However, the mechanisms by which MAC promotes the accumulation of HIF-1 $\alpha$  have not yet been elucidated in detail. In the present study, we investigated the effects of MAC on HIF-1 $\alpha$  expression in human cancer cell lines. The results obtained showed that MAC inhibited the prolyl hydroxylation of HIF-1 $\alpha$  in human fibrosarcoma HT-1080 cells. Furthermore, ascorbate reversed the inhibitory effects of MAC on the prolyl hydroxylation of HIF-1 $\alpha$ .

## Materials and methods

### Cells

Human fibrosarcoma HT-1080 cells (JCRB9113) and human lung adenocarcinoma A549 cells (JCRB0076) were obtained from the National Institutes of Biomedical Innovation, Health and Nutrition JCRB Cell Bank (Osaka, Japan). HT-1080 and A549 cells were cultured with RPMI 1640 medium (Thermo Fisher Scientific, Grand Island, NY, USA), 500 ml of which was supplemented with 45 ml of heat-inactivated fetal calf serum (Nishirei Bioscience, Tokyo, Japan) and 5 ml of a penicillin-streptomycin antibiotic mixture (Nacalai Tesque, Kyoto, Japan). Since the amount of ascorbate in fetal calf serum was  $6.8 \pm 1.2 \mu\text{M}$  ( $n = 3$ ), the final concentration of ascorbate in the culture medium used in our experiments was estimated to be approximately  $0.56 \mu\text{M}$  unless otherwise specified.

### Reagents

Ascochlorin, ascofuranone, AS-6, and MAC were provided by Chugai Pharmaceutical (Tokyo, Japan). Ascorbate (Nacalai Tesque), *N*-acetylcysteine (Nacalai Tesque), MG-132 (Peptide Institute, Osaka, Japan), and oligomycin A (Cayman Chemical, Ann Arbor, MI, USA) were obtained from commercial sources.

### Antibodies

Primary antibodies reactive to HIF-1 $\alpha$  (54/HIF-1 $\alpha$ ; BD Biosciences, Franklin Lakes, NJ, USA), HIF-1 $\alpha$  (NB100-479; Novus Biologicals, Littleton, CO, USA), hydroxy-HIF-1 $\alpha$  (Pro564) (D43B5; Cell Signaling Technology, Danvers, MA, USA), AMPK $\alpha$  (D63G4; Cell Signaling Technology), phospho-AMPK $\alpha$  (Thr172) (40H9; Cell Signaling Technology), and  $\beta$ -actin (AC-15; Sigma-Aldrich, St. Louis, MO, USA),  $\gamma$ 1-actin (2F3; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were used. A horseradish peroxidase (HRP)-conjugated anti-mouse IgG (H + L) antibody and HRP-conjugated anti-rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used as secondary antibodies.

### Plasmids

The human cytomegalovirus (CMV) minimal promoter ( $-53 \sim +7$ ) [27, 28] was inserted into the *Xho*I and *Bgl*II sites of the pGL4.22[luc2P] luciferase reporter vector (Promega, Madison, WI, USA). The human enolase 1 (ENO1) promoter containing HRE ( $-416 \sim -349$ ) [29] was inserted into the *Kpn*I and *Xho*I sites upstream of the CMV minimal promoter.

### Western blotting

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with Triton X-100 lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Triton X-100, and 2 mM dithiothreitol) containing the protease inhibitor cocktail cOmplete<sup>TM</sup> (Sigma-Aldrich, St. Louis, MO, USA). After sonication, whole-cell lysates were collected as supernatants by centrifugation ( $15,300 \times g$ , 5 min). Protein samples (30  $\mu\text{g}$ ) were separated by SDS-PAGE and transferred to a ClearTrans<sup>®</sup> nitrocellulose membrane filter (0.22  $\mu\text{m}$ ; FUJIFILM Wako Pure Chemical). Membrane filters were incubated with primary antibodies and secondary antibodies. Protein bands were visualized with ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ, USA) or ImmunoStar<sup>®</sup> Zeta (FUJIFILM Wako Pure Chemical) and analyzed by Image Quant LAS 4000mini (GE Healthcare Japan, Tokyo, Japan).

### Luciferase reporter assay

Cells were transfected with the HRE-responsive firefly luciferase reporter plasmid and CMV promoter-driven *Renilla* luciferase reporter plasmid by the HilyMax Transfection reagent (Dojindo Laboratories, Kumamoto, Japan) for 4 h. Transfected cells were further cultured in the

luciferase activity were described previously [30]. Relative light units were measured with Lumitester C-110 (Kikkoman Biochemifa, Tokyo, Japan).

### Quantitative PCR

Total RNA was prepared with Sepasol<sup>®</sup>-RNA I Super G (Nacalai Tesque) and reverse-transcribed to cDNA with ReverTra Ace<sup>®</sup> (TOYOBO, Osaka, Japan) as described previously [31]. Real-time PCR was performed with Thermal Cycler Dice<sup>®</sup> Real Time System Lite (Takara Bio, Kusatsu, Japan) under the following conditions: 94 °C for 3 min and 45 cycles of 95 °C for 5 s, 58 °C for 30 s, and 72 °C for 30 s. Reactions were conducted by SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Tli RNase Plus) (Takara Bio) and primer pairs: 5'-CCTGAGCCTAATAGTCCC-3' (forward) and 5'-GTCTAAATCTGTGTCCTGAGTA-3' (reverse) for HIF-1 $\alpha$  [32], 5'-ACCTCCACCATGCCAAGTG-3' (forward) and 5'-TC TCGATTGGATGGCAGTAG-3' (reverse) for vascular endothelial growth factor (VEGF) [33], 5'-CGGGCCAAGAGTGTGCTAAA-3' (forward) and 5'-TGACGATACCGGAGCCAATG-3' (reverse) for glucose transporter 1 (GLUT-1) [34], 5'-AAAATATTCCCCCAAGGAGTT C-3' (forward) and 5'-ACGCTCGTGTTCCTCATGCT-3' (reverse) for Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) [35], and 5'-GGACATCCGCAAAGACCTGTA-3' (forward) and 5'-GCTCAGGAGGAGCAATGATCT-3' (reverse) for  $\beta$ -actin [36].

### Quantitation of ascorbate

The OxiSelect<sup>™</sup> Ascorbic Acid Assay Kit (FRASC) (Cell Biolabs, San Diego, CA, USA) was used to measure the amount of ascorbate according to the manufacturer's protocol. Absorbance at 570 nm was measured using the iMark<sup>™</sup> microplate reader (Bio-Rad Laboratories).

### Statistical analysis

Statistical analyses were assessed by one-way ANOVA followed by Tukey's test for multiple comparisons.

## Results

### MAC promoted the accumulation of the HIF-1 $\alpha$ protein in A549 cells and HT-1080 cells

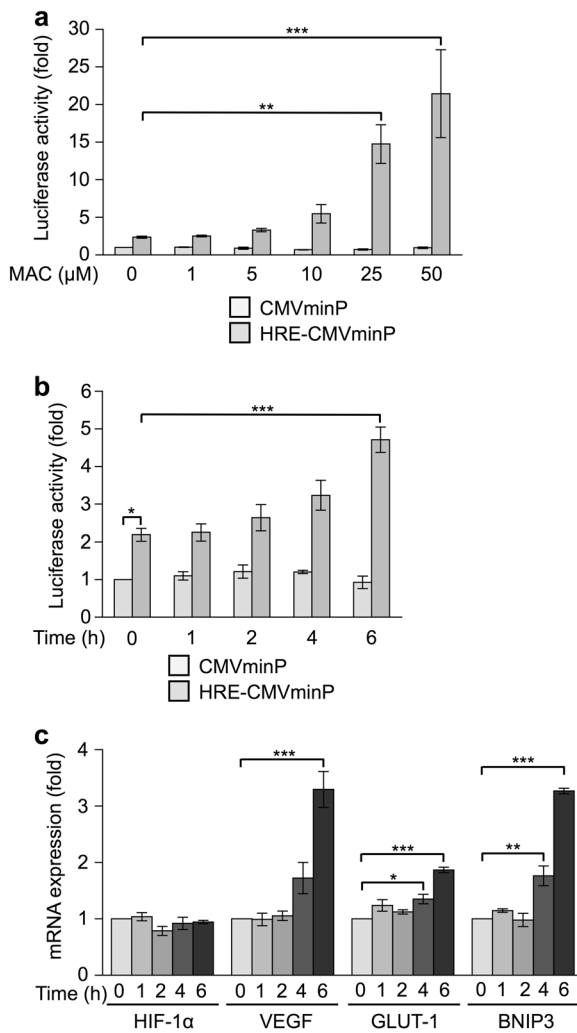
MAC was previously shown to promote the accumulation of the HIF-1 $\alpha$  protein in MCF-7 cells [24]. In order to generalize the effects of MAC on human cancer cell lines, A549 cells and HT-1080 cells were incubated with

different concentrations of MAC for 6 h. MAC up-regulated the expression of the HIF-1 $\alpha$  protein in a dose-dependent manner and at concentrations greater than 25  $\mu$ M and 5  $\mu$ M in A549 cells and HT-1080 cells, respectively (Fig. 1b). Under the same concentrations of MAC, HT-1080 cells expressed higher levels of the HIF-1 $\alpha$  protein than A549 cells (Fig. 1b). Time-course experiments showed that MAC at 50  $\mu$ M increased the expression of the HIF-1 $\alpha$  protein in a time-dependent manner in A549 cells and HT-1080 cells (Fig. 1c). In MAC-treated HT-1080 cells, the amount of the HIF-1 $\alpha$  protein started to increase from 0.5–1 h and reached a plateau at 4–6 h (Fig. 1c).

MAC and its three structural derivatives (ascochlorin, AS-6, and ascofuranone) were used to investigate structure-activity relationships (Fig. 1d). HT-1080 cells were incubated with ascochlorin derivatives at the same concentration (50  $\mu$ M) for 6 h. Unlike MAC, ascochlorin, AS-6, and ascofuranone did not increase HIF-1 $\alpha$  protein levels (Fig. 1d). Of the three ascochlorin derivatives examined, only ascochlorin appeared to decrease the basal level of the HIF-1 $\alpha$  protein (Fig. 1d). Consistent with our previous findings obtained using MCF-7 cells [24], these results indicate that HT-1080 cells respond more strongly to MAC than A549 cells, and, thus, we used HT-1080 cells in subsequent experiments.

### MAC promoted the transcription of HIF-1 $\alpha$ target genes in HT-1080 cells

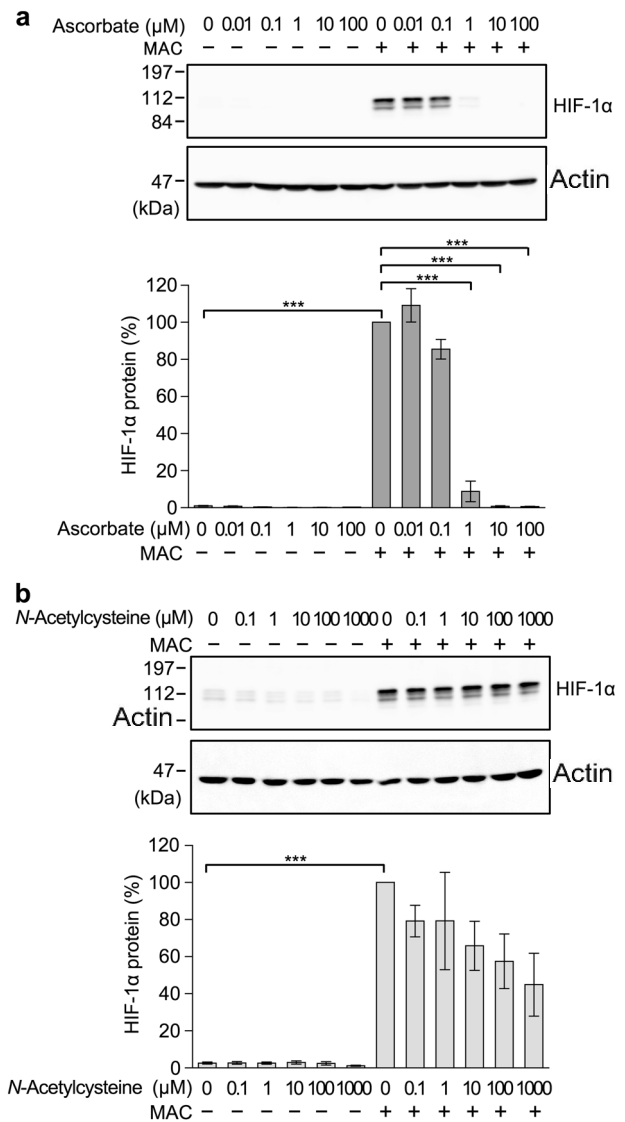
In order to evaluate HIF-1 $\alpha$ -dependent gene expression, the CMV minimal promoter (–53 to +7; CMVminP) fused to the human ENO1 promoter (–416 to –349) containing HRE was inserted into the luciferase reporter vector. HT-1080 cells were transiently transfected with luciferase reporter vectors and incubated with different concentrations of MAC for 6 h. Luciferase activity driven by HRE-CMVminP was augmented by MAC in a dose-dependent manner, while control CMVminP luciferase activity was unaffected (Fig. 2a). When HT-1080 cells were incubated with MAC at 10  $\mu$ M, luciferase activity driven by HRE-CMVminP increased at 6 h (Fig. 2b). Moreover, the mRNA expression of three HIF-1 $\alpha$  target genes (i.e., VEGF, GLUT-1, and BNIP3) was evaluated by real-time PCR. MAC increased VEGF mRNA, and BNIP3 mRNA levels by more than threefold and GLUT-1 mRNA levels by less than 2-fold at 6 h (Fig. 2c). In contrast, HIF-1 $\alpha$  mRNA levels were not affected by MAC during the 6-h incubation (Fig. 2c). Consistent with our previous findings obtained using MCF-7 cells [24], these results confirmed that MAC promoted the stabilization of the HIF-1 $\alpha$  protein in HT-1080 cells.



**Fig. 2** MAC increased HIF-1 $\alpha$ -dependent luciferase activity and mRNA expression in HT-1080 cells. **a, b** HT-1080 cells were transfected with the CMVminP firefly luciferase reporter plasmid (light gray bars) or HRE-CMVminP firefly luciferase reporter plasmid (dark gray bars) together with the CMV promoter-driven *Renilla* luciferase reporter plasmid for 24 h. Transfected cells were incubated with the indicated concentrations of MAC for 6 h (**a**). Transfected cells were incubated with MAC (10  $\mu$ M) for the indicated times (**b**). Luciferase activity (fold) is expressed as the mean  $\pm$  S.E. of three independent experiments. The values of CMVminP with MAC at 0  $\mu$ M or at time 0 h are set to onefold. \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001. **c** HT-1080 cells were incubated with MAC (10  $\mu$ M) for the indicated times. Total RNA was prepared and converted to cDNA, followed by quantitative PCR. The expression of HIF-1 $\alpha$ , VEGF, GLUT-1, and BNIP3 mRNA relative to  $\beta$ -actin mRNA (fold) is shown as the mean  $\pm$  S.E. of three independent experiments. The values of time at 0 h are set to onefold. \* $P$  < 0.05, \*\* $P$  < 0.01, and \*\*\* $P$  < 0.001

### Ascorbate attenuated the accumulation of the HIF-1 $\alpha$ protein and its transcriptional activity induced in MAC-treated HT-1080 cells

PHD proteins require Fe<sup>2+</sup> and ascorbate as cofactors for their activity [6, 7]. In order to investigate whether ascorbate affects the MAC-mediated accumulation of the HIF-1 $\alpha$



**Fig. 3** Ascorbate, but not *N*-acetylcysteine, attenuated the accumulation of the HIF-1 $\alpha$  protein by MAC in HT-1080 cells. **a, b** HT-1080 cells were preincubated with or without ascorbate (**a**) or *N*-acetylcysteine (**b**) for 1 h, and then incubated with (+) or without (-) MAC (10  $\mu$ M) for 4 h in the presence of ascorbate or *N*-acetylcysteine at the indicated final concentrations. Whole-cell lysates were prepared and then analyzed by western blotting. Representative blots are shown. The HIF-1 $\alpha$  protein relative to the actin protein (%) is expressed as the mean  $\pm$  S.E. of three independent experiments. The values of MAC at 10  $\mu$ M together with ascorbate at 0  $\mu$ M or *N*-acetylcysteine at 0  $\mu$ M are set to 100%. \*\*\* $P$  < 0.001

protein, HT-1080 cells were preincubated with serial dilutions of ascorbate and then incubated with 10  $\mu$ M MAC. Ascorbate markedly suppressed the accumulation of the HIF-1 $\alpha$  protein by MAC at concentrations greater than 1  $\mu$ M (Fig. 3a). Ascorbate is known to exhibit antioxidant activity [37]. In order to clarify whether the antioxidant activity of ascorbate is responsible for the MAC-mediated accumulation of HIF-1 $\alpha$ , a structurally different antioxidant

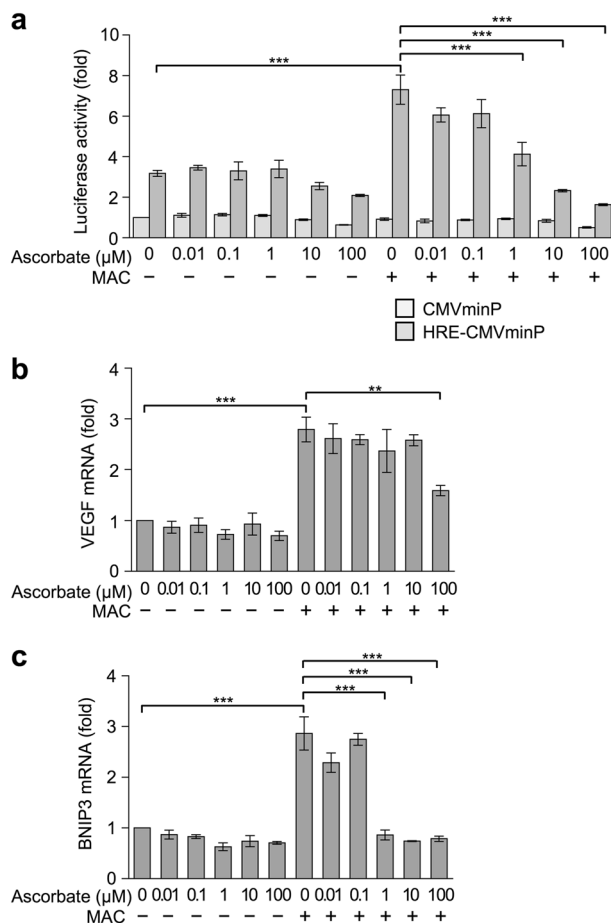
was used. *N*-acetylcysteine exhibits antioxidant activity [38]. In contrast to ascorbate, *N*-acetylcysteine at concentrations of 10–1000  $\mu$ M did not efficiently decrease the up-regulated expression of the HIF-1 $\alpha$  protein by MAC (Fig. 3b). These results suggest that ascorbate suppresses the effects of MAC on the accumulation of the HIF-1 $\alpha$  protein, not simply through its antioxidant activity, but through a specific mechanism.

Moreover, ascorbate suppressed increases in HRE-responsive luciferase activity in a dose-dependent manner in MAC-treated HT-1080 cells (Fig. 4a). The increase induced in BNIP3 mRNA levels by MAC was markedly suppressed by ascorbate at concentrations greater than 1  $\mu$ M, whereas that in VEGF mRNA levels was decreased by ascorbate at 100  $\mu$ M only (Fig. 4b, c). The VEGF promoter contains multiple consensus sites for transcription factors, other than HIF-1, and is regulated by various stimuli [39]. In addition to the HIF-1 $\alpha$  pathway, MAC may activate other transcription factors or increase the production of soluble factors that stimulate VEGF transcription.

### MAC inhibited HIF-1 $\alpha$ prolyl hydroxylation in HT-1080 cells

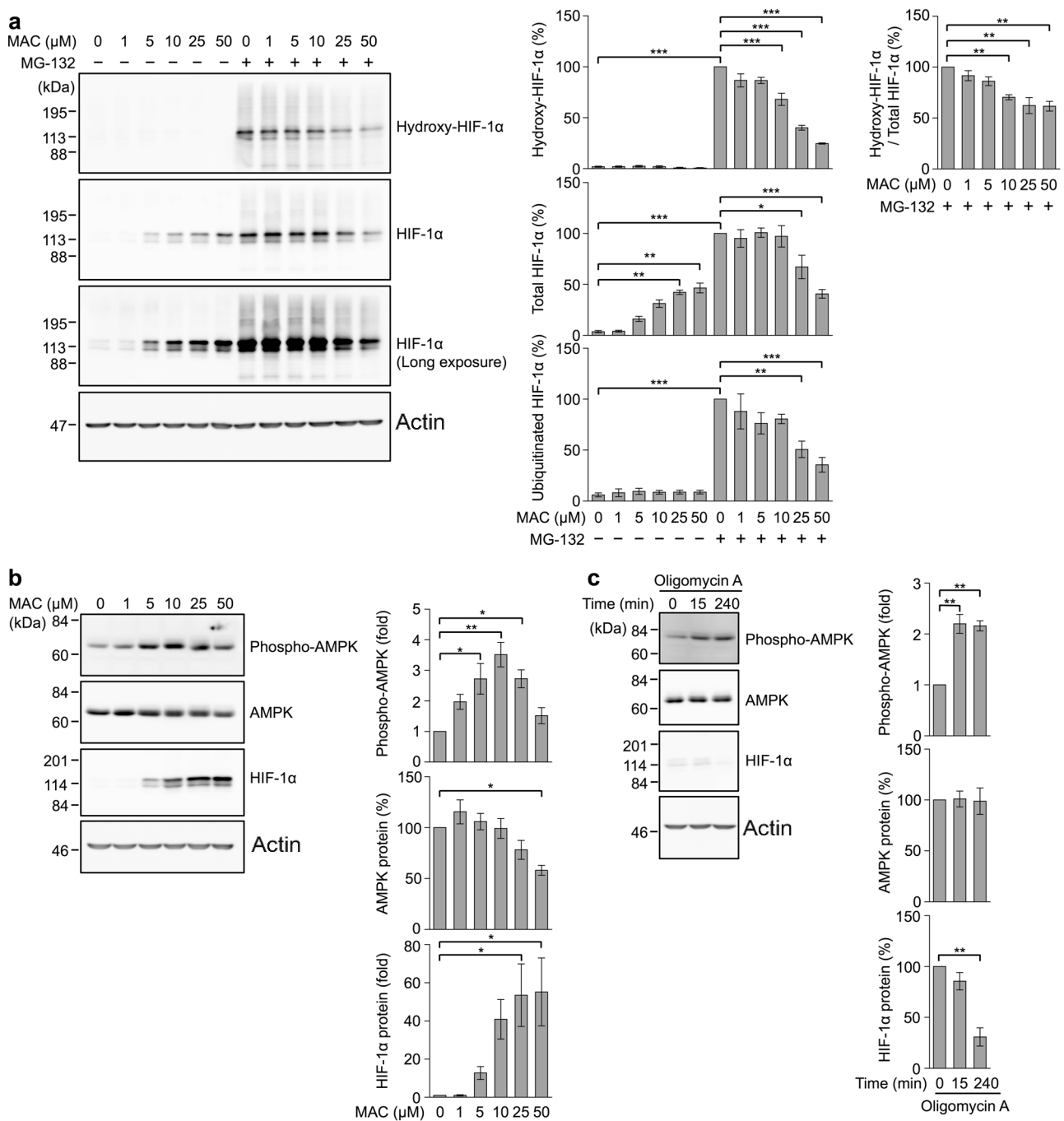
PHD proteins catalyze the hydroxylation of HIF-1 $\alpha$  at the proline 402 and proline 564 residues, and hydroxy-HIF-1 $\alpha$  undergoes ubiquitination and proteasomal degradation [8]. When HT-1080 cells were incubated with the proteasome inhibitor MG-132, hydroxy-HIF-1 $\alpha$  bands were clearly detectable using an antibody specific to hydroxyl proline 564 of HIF-1 $\alpha$  (Fig. 5a). MAC dose-dependently decreased the amount of hydroxy-HIF-1 $\alpha$  in the presence of MG-132 (Fig. 5a). Ubiquitinated HIF-1 $\alpha$ , migrating as higher molecular weight bands, was also reduced by MAC in the presence of MG-132, and these higher molecular weight bands were absent in MAC-treated cells despite the strong induction of HIF-1 $\alpha$  (Fig. 5a). It is important to note that MAC at 25 or 50  $\mu$ M reduced the total amount of HIF-1 $\alpha$  in the presence of MG-132 (Fig. 5a). The reason for this currently remains unclear. Therefore, further experiments are needed to clarify the cooperative effects of MAC and MG-132. Nevertheless, the ratio of hydroxy-HIF-1 $\alpha$  relative to total HIF-1 $\alpha$  was significantly reduced by MAC at concentrations of 10–50  $\mu$ M (Fig. 5a). These results indicate that MAC inhibits the prolyl hydroxylation of HIF-1 $\alpha$ .

We previously showed that MAC promoted the activation of AMPK in MCF-7 cells [24]. In HT-1080 cells, MAC increased the phosphorylation of AMPK at concentrations higher than 5  $\mu$ M, while it decreased the total amount of AMPK, particularly at 50  $\mu$ M (Fig. 5b). These concentrations were similar to those required for the accumulation of HIF-1 $\alpha$  and the inhibition of HIF-1 $\alpha$



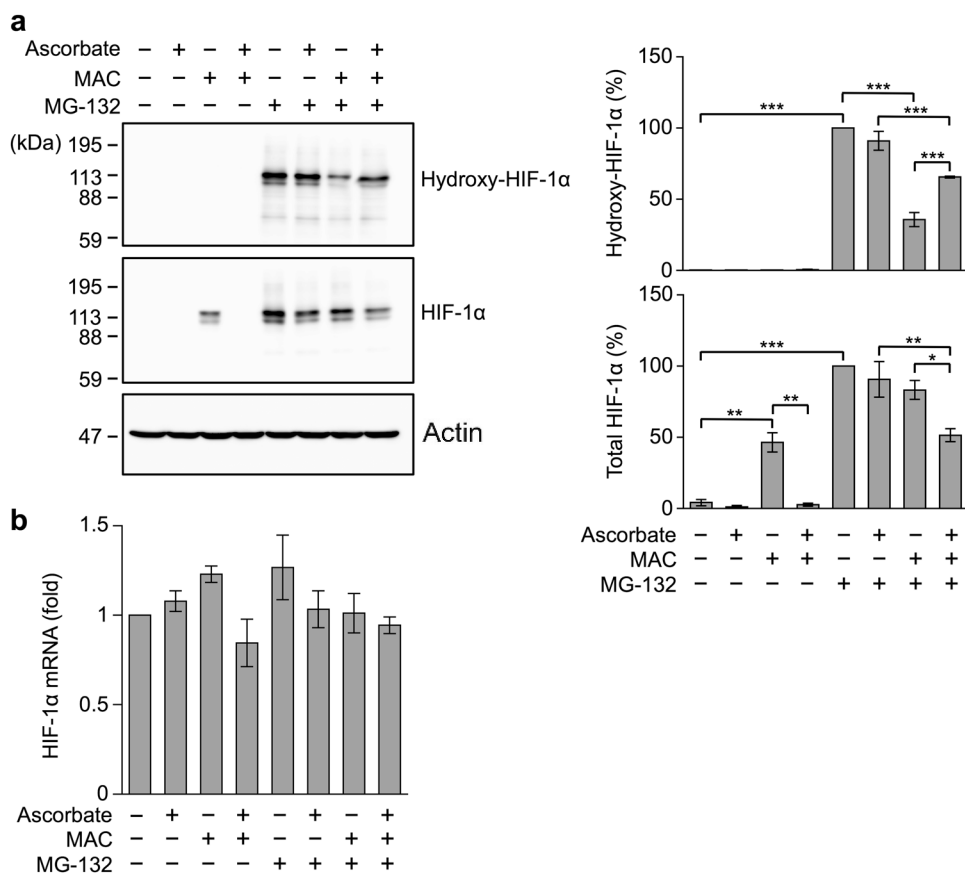
**Fig. 4** Ascorbate attenuated increases in HIF-1 $\alpha$ -dependent luciferase activity and mRNA expression by MAC in HT-1080 cells. **a** HT-1080 cells were transfected with the CMVminP firefly luciferase reporter plasmid (light gray bars) or HRE-CMVminP firefly luciferase reporter plasmid (dark gray bars) together with the CMV promoter-driven *Renilla* luciferase reporter plasmid for 24 h. Transfected cells were preincubated with ascorbate for 1 h and then incubated with (+) or without (–) MAC (10  $\mu$ M) for 6 h in the presence or absence of ascorbate at the indicated final concentrations. Luciferase activity (fold) is expressed as the mean  $\pm$  S.E. of three independent experiments. The values of CMVminP with MAC at 0  $\mu$ M and ascorbate at 0  $\mu$ M are set to onefold. \*\*\* $P$  < 0.001. **b, c** HT-1080 cells were preincubated with or without ascorbate for 1 h and then incubated with (+) or without (–) MAC (10  $\mu$ M) for 6 h in the presence or absence of ascorbate at the indicated final concentrations. Total RNA was prepared and converted to cDNA, followed by quantitative PCR. The expression of VEGF mRNA (**b**) and BNIP3 mRNA (**c**) relative to  $\beta$ -actin mRNA (fold) is shown as the mean  $\pm$  S.E. of three independent experiments. The values of MAC at 0  $\mu$ M and ascorbate at 0  $\mu$ M are set to onefold. \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001

hydroxylation (Figs. 1b and 5a). It is important to note that oligomycin A, an inhibitor of mitochondrial ATP synthase, did not augment the amount of HIF-1 $\alpha$  but rather decreased its basal level at 240 min despite the phosphorylation of AMPK (Fig. 5c), suggesting that the activation of AMPK alone is insufficient for promoting the accumulation of HIF-1 $\alpha$ .



**Fig. 5** MAC reduced the prolyl hydroxylation of HIF-1 $\alpha$  and promoted the phosphorylation of AMPK in HT-1080 cells at similar concentrations. **a** HT-1080 cells were preincubated with (+) or without (-) MG-132 for 1 h, and then treated with the indicated concentrations of MAC for 4 h in the presence or absence of MG-132 (10  $\mu$ M) as the final concentrations. Whole-cell lysates were analyzed by western blotting. Hydroxy-HIF-1 $\alpha$  relative to actin (%), total HIF-1 $\alpha$  relative to actin (%), ubiquitinated HIF-1 $\alpha$  (higher molecular weight bands) relative to actin (%), and hydroxy-HIF-1 $\alpha$  relative to total HIF-1 $\alpha$  (%) are shown as the means  $\pm$  S.E. of three independent experiments.

The values of MG-132 at 10  $\mu$ M and MAC at 0  $\mu$ M are set to 100%. \* $P$  < 0.05, \*\* $P$  < 0.01, and \*\*\* $P$  < 0.001. **b**, **c** HT-1080 cells were treated with the indicated concentrations of MAC for 4 h as the final concentrations (**b**) or with oligomycin A (10  $\mu$ M as the final concentration) at the indicated times (**c**). Whole-cell lysates were analyzed by western blotting. Phospho-AMPK $\alpha$  relative to actin (fold), total AMPK $\alpha$  relative to actin (%), and HIF-1 $\alpha$  relative to actin (fold for **b** and % for **c**) are shown as the means  $\pm$  S.E. of three independent experiments. The values of MAC at 0  $\mu$ M or time at 0 h for oligomycin A (**c**) are set to onefold or 100%. \* $P$  < 0.05 and \*\* $P$  < 0.01



**Fig. 6** Ascorbate reversed the reduced hydroxylation of HIF-1 $\alpha$  by MAC in HT-1080 cells. **a** HT-1080 cells were preincubated with (+) or without (-) MG-132 for 30 min, incubated with (+) or without (-) ascorbate for 30 min, and then treated with (+) or without (-) MAC for 4 h in the presence or absence of MG-132 (10  $\mu$ M), ascorbate (100  $\mu$ M), or MAC (25  $\mu$ M) as the final concentrations. Whole-cell lysates were analyzed by western blotting. Hydroxy-HIF-1 $\alpha$  relative to actin (%) and total HIF-1 $\alpha$  relative to actin (%) are shown as the means  $\pm$

S.E. of three independent experiments. The value of ascorbate (-) MAC (-) MG-132 (+) is set to 100%. \* $P$  < 0.05, \*\* $P$  < 0.01, and \*\*\* $P$  < 0.001. **b** HT-1080 cells were treated as described in Fig. 6a. Total RNA was prepared and converted to cDNA, and this was followed by quantitative PCR. The expression of HIF-1 $\alpha$  mRNA relative to  $\beta$ -actin mRNA (fold) is shown as the mean  $\pm$  S.E. of three independent experiments. The value of ascorbate (-) MAC (-) MG-132 (-) is set to onefold. No significant difference was observed

### Ascorbate reversed the inhibitory effect of MAC on HIF-1 $\alpha$ hydroxylation in HT-1080 cells

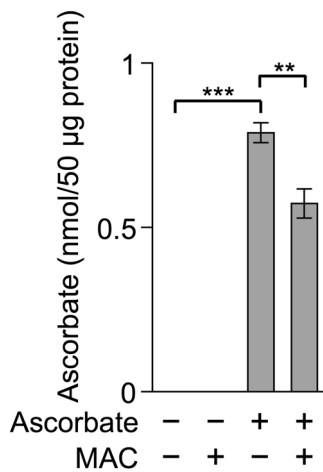
MAC decreased the amount of hydroxy-HIF-1 $\alpha$  in the presence of MG-132 (Fig. 5a). We further examined whether ascorbate affected the amount of hydroxy-HIF-1 $\alpha$  in the presence of MAC and MG-132. The amount of hydroxy-HIF-1 $\alpha$  decreased by MAC was recovered by ascorbate at concentrations of 100  $\mu$ M (Fig. 6a) and 10  $\mu$ M (Figure S1), and to a lesser extent at 1  $\mu$ M (Figure S2). These results clearly indicate that MAC inhibits the prolyl hydroxylation of HIF-1 $\alpha$ , which is reversed by ascorbate. We also assessed the amount of HIF-1 $\alpha$  mRNA under these conditions. MAC, together with MG-132 and/or ascorbate, did not markedly influence the expression of HIF-1 $\alpha$  mRNA (Fig. 6b), suggesting that ascorbate affects post-

transcriptional events in the expression of the HIF-1 $\alpha$  protein.

### MAC slightly reduced the amount of intracellular ascorbate in HT-1080 cells

In order to clarify whether MAC affects intracellular ascorbate levels, we measured the amount of ascorbate in culture media and cells. The concentration of ascorbate in culture media was approximately 0.56  $\mu$ M, as described in the Materials and Methods. The amount of intracellular ascorbate was undetectable when HT-1080 cells were incubated with these culture media (Fig. 7). In contrast, the amount of intracellular ascorbate markedly increased when HT-1080 cells were incubated in the presence of ascorbate (100  $\mu$ M) for 4 h (Fig. 7). Under these





**Fig. 7** MAC slightly reduced the amount of intracellular ascorbate in HT-1080 cells. HT-1080 cells were incubated with (+) or without (-) ascorbate for 30 min and then treated with (+) or without (-) MAC for 4 h in the presence or absence of ascorbate (100  $\mu$ M) or MAC (25  $\mu$ M) as the final concentrations. The amount of intracellular ascorbate was measured. The lysates (50  $\mu$ g protein) of control HT-1080 cells corresponded to  $9.7 \pm 0.5 \times 10^5$  cells ( $n = 3$ ). The amount of ascorbate (nmol per 50  $\mu$ g protein) is shown as the mean  $\pm$  S.E. of three independent experiments. \*\* $P < 0.01$  and \*\*\* $P < 0.001$

conditions, MAC reduced the amount of intracellular ascorbate by 27%.

## Discussion

We previously reported that MAC enhanced HIF-1 $\alpha$  protein accumulation in human breast adenocarcinoma MCF-7 cells [24]. In the present study, we also showed that MAC promoted the accumulation of the HIF-1 $\alpha$  protein in additional cancer cell lines, i.e., human fibrosarcoma HT-1080 cells and human lung adenocarcinoma A549 cells. Collectively, these results indicate that the accumulation of HIF-1 $\alpha$  is one of the common biological activities of MAC in human cancer cell lines. We also revealed that MAC prevented the prolyl hydroxylation of HIF-1 $\alpha$  without affecting its mRNA levels in HT-1080 cells. Moreover, ascorbate canceled the inhibition of HIF-1 $\alpha$  prolyl hydroxylation and suppressed the accumulation of the HIF-1 $\alpha$  protein in MAC-treated HT-1080 cells. The present results demonstrated that MAC promoted the accumulation of HIF-1 $\alpha$  by preventing prolyl hydroxylation and ascorbate reversed the processes induced by MAC.

HT-1080 and A549 cells were maintained in RPMI 1640 medium supplemented with fetal calf serum. Ascorbate is not a basic component of typical culture media, including RPMI 1640 medium, and fetal calf serum is the only source of ascorbate. The concentration of ascorbate in the culture media used in the present study was estimated to be approximately 0.56  $\mu$ M. The amount of intracellular

ascorbate was undetectable in HT-1080 cells in these culture media, while it markedly increased when HT-1080 cells were incubated in the presence of ascorbate (100  $\mu$ M). Assuming that the intracellular water volume of HT-1080 cells is similar to that of two human adenocarcinoma cells (WiDr and Ishikawa) and HUVEC reported in a previous study (1.29, 2.45, and 2.81  $\mu$ l per  $10^6$  cells, respectively) [40], the intracellular concentration of ascorbate in HT-1080 cells incubated with ascorbate (100  $\mu$ M) is estimated to range between 290 and 630  $\mu$ M. Consistent with previous studies [40, 41], intracellular ascorbate levels were several-fold higher than its extracellular levels in HT-1080 cells. In the presence of ascorbate (100  $\mu$ M), MAC decreased the amount of intracellular ascorbate by 27%. A previous study reported that nickel and cobalt at 0.25 mM each inhibited the amount of intracellular ascorbate by more than 80%, and the addition of ascorbate rescued the inhibition of HIF-1 $\alpha$ -dependent gene expression by nickel and cobalt [41]. Since the inhibitory effects of MAC were weaker than those of nickel and cobalt, we speculate that the intracellular level of ascorbate is not a primary target of MAC to inhibit HIF-1 $\alpha$  hydroxylation.

MAC induced the accumulation of HIF-1 $\alpha$  without an increase in hydroxy-HIF-1 $\alpha$ , while the proteasomal inhibitor MG-132 promoted the accumulation of HIF-1 $\alpha$  with increases in hydroxy-HIF-1 $\alpha$ . Moreover, MAC decreased the accumulation of hydroxy-HIF-1 $\alpha$  induced by MG-132. These results clearly show that MAC inhibited the prolyl hydroxylation of HIF-1 $\alpha$ . Hydroxy-HIF-1 $\alpha$  is recognized by VHL E3 ubiquitin ligase, and this is followed by poly-ubiquitination and proteasomal degradation [8]. PHD proteins require 2-oxoglutarate and oxygen as substrates and  $Fe^{2+}$  and ascorbate as cofactors for the prolyl hydroxylation of HIF-1 proteins [6]. A recent study proposed that ascorbate acts as a co-substrate of PHD proteins by competing with 2-oxoglutarate in the active center [42]. Previous studies showed that ascorbate suppressed the accumulation of HIF-1 $\alpha$  induced by  $CoCl_2$  and the iron chelator desferrioxamine [40, 41, 43]. Ascorbate has been suggested to sustain an active form of PHD proteins by preventing the loss of  $Fe^{2+}$  or  $Co^{2+}$  substitution [37]. We found that ascorbate suppressed the accumulation of the HIF-1 $\alpha$  protein in MAC-treated cells. MAC did not affect the constitutive expression of HIF-1 $\alpha$  mRNA. These results suggest that MAC affects the process downstream of HIF-1 $\alpha$  translation and most likely interferes directly or indirectly with the catalytic activity of PHD proteins. Further experiments are needed in order to clarify this issue.

Previous studies showed that MAC promotes the phosphorylation of AMPK and suppresses the phosphorylation of mTOR and its target proteins p70S6K and 4E-BP-1 [22–24]. AMPK knockdown or an AMPK inhibitor canceled the MAC-mediated accumulation of HIF-1 $\alpha$  [24], indicating that

AMPK activation is indispensable for the accumulation of HIF-1 $\alpha$  by MAC. mTOR complexes have been classified into mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [44]. AMPK negatively controls mTORC1 signaling [45, 46]. mTORC1 mediates the phosphorylation of downstream effectors and promotes the synthesis of proteins and other components [47]. Rapamycin has been shown to inhibit HIF-1 $\alpha$  expression, indicating that HIF-1 $\alpha$  is positively regulated by mTORC1 [46, 47]. However, mTORC1 does not appear to be involved in the MAC-mediated accumulation of HIF-1 $\alpha$  because MAC decreased the phosphorylation of mTOR, p70S6K, and 4E-BP-1 and rapamycin did not exert any inhibitory effects on the MAC-mediated accumulation of HIF-1 $\alpha$  in MCF-7 cells [24]. Ascofuranone also suppressed mTOR, which is assumed to be responsible for the suppression of EGF-induced HIF-1 $\alpha$  activation in breast cancer cells [26]. In the present study, we confirmed that MAC promoted the phosphorylation of AMPK in HT-1080 cells. In contrast, the present results showing that oligomycin A activated AMPK without augmenting the expression of HIF-1 $\alpha$  suggest that the activation of AMPK alone is insufficient for promoting the accumulation of HIF-1 $\alpha$ . Although the mechanistic link between MAC-mediated AMPK activation and HIF-1 $\alpha$  accumulation currently remains unclear, it seems more likely that MAC, but not other ascochlorin derivatives, selectively inhibits the process of HIF-1 $\alpha$  prolyl hydroxylation. Unlike HIF-1 $\alpha$ , AMPK activation and mTORC1 inhibition by MAC are known to be responsible for the down-regulation of c-Myc and PPAR $\gamma$  in cancer cell lines [22, 23].

In conclusion, we herein revealed that MAC prevents the prolyl hydroxylation of HIF-1 $\alpha$ . PHD inhibitors are considered to activate the HIF pathway by preventing the prolyl hydroxylation and degradation of HIF proteins. PHD inhibitors have potential as therapeutics for ischemic diseases, chronic kidney diseases, intestinal bowel diseases, and anemia [48–52]. The HIF pathway is activated in many cancers and plays critical roles in multiple steps of cancer development [2–4]. Thus, while small molecule inhibitors targeting the HIF pathway are regarded as anti-cancer drugs, activators of the HIF pathway are important for understanding the molecular mechanisms of the HIF signaling pathway and its cellular responses in cancer. MAC may be a useful bioprobe for investigating the molecular mechanisms underlying the regulation of HIF-1 $\alpha$  prolyl hydroxylation.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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