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Short Communication

Increased MFG-E8 expression and its implications in the vascular pathophysiology of cocaine abuse

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Abstract: The aim of this study was to examine the possible involvement of smooth muscle cell remodeling and the induction of MFG-E8 (milk fat globule protein epidermal growth factor-VIII) in vascular pathophysiology during cocaine administration in cultured cells and rats. Cocaine exerts bifurcate effects on vascular cells; it stimulates vasoconstriction through enhancement of catecholamine release at low doses, while it suppresses cardiovascular functions through inhibition of ion channels at high doses. Short-term exposure to a high concentration of cocaine (3 mM, 24 hr) resulted in cell death of A7r5 rat aorta-derived smooth muscle cells. On the other hand, long-term exposure of the same cells to a low concentration (0.3 mM, ~7 days) resulted in a transient increase in MFG-E8 expression followed by an increased tendency toward cyclin D1, PCNA (proliferating cell nuclear antigen), and CDK4 (cyclin-dependent protein kinase-4) expression. Interestingly, autophagy was not induced, but rather was impaired, in cocaine-treated cells. Increased expressions of MFG-E8, PCNA, and CDK4 were also observed in the aortic vascular cells of rats administered cocaine (50 mg/kg, 2 days, i.v.), confirming that cocaine induced MFG-E8 expression *in vivo*. Taken together, the results show that MFG-E8 is induced in vascular cells exposed to cocaine, and that this induction is likely to be involved in the vascular toxicity elicited by cocaine abuse. (DOI: 10.1293/tox.2015-0072; J Toxicol Pathol 2016; 29: 131–138)

Key words: cocaine, vascular toxicity, autophagy, smooth muscle cells, MFG-E8

Cocaine is used as a local anesthetic, as it inhibits the pulse emitted by sensory nerves. Cocaine also stimulates the central nerves, and for that reason, it produces a pleasant feeling and psychic dependence. Cocaine is a common illegal drug traded throughout the world. It can be snorted, inhaled, smoked, or injected and is harmful to the human body; there are abundant reports describing its toxicity. For example, cocaine leads to such toxic effects on the cardiovascular system as heart stroke, chest pain, and atherosclerosis[1, 2]. Su *et al*. have shown that cocaine induces apoptosis in rat aortic vascular smooth muscle cells (VSMCs)[3], suggesting the possible involvement of cell death in vascular injuries caused by cocaine.

VSMCs comprise one component of blood vessels. They are located in the medial vascular layer, and are involved in the contractile function of the vessels. When exposed to extracellular stimuli, they sometimes change their phenotype from contractile to synthetic[4]. This remodeling is associated with an increased proliferative ability and migration of the cells to the intima[5]. These changes might be temporarily effective in stabilizing vascular tissues under stressful conditions, but a prolongation of this phenotypic change can result in vascular disorders such as intimal thickening and atherogenesis[6]. It has been demonstrated that in human coronary artery sections, foam cells derived from smooth muscle cells are abundantly observed in atherosclerotic plaques, suggesting the involvement of phenotype switching in human atherogenesis[7].

Autophagy, an intracellular pathway that degrades dysfunctional components including proteins and organelles[8], has recently been implicated in the regulation of VSMC remodeling. Several reports indicate a promoting role of autophagy in the remodeling of smooth muscle cells during exposure to advanced glycation end products[9] or platelet-derived growth factor (PDGF)[5], while another report describes an antagonistic role of autophagy against remodeling caused by drugs[10].

Milk fat globule epidermal growth factor-VIII (MFG-E8), also known as lactadherin and SED1, was originally discovered as an abundant protein in milk fat globules[11]. Later, MFG-E8 was identified as an "eat-me-signal" between apoptotic cells and macrophages[12]. Growing evidence has also shown that MFG-E8 is a key inflammatory mediator involved in the pathogenesis of various diseases. MFG-E8 accumulates in the arterial walls with aging and is correlated with cardiovascular remodeling and an accelera-

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tion of cardiovascular diseases[13–15]. It has been shown that the increase in MFG-E8 in vascular cells promotes vascular remodeling and resultant pathological changes in arteries[16]. Indeed, a proteomic analysis identified MFG-E8 as a component of human coronary atherosclerotic plaque, suggesting a potential role of MFG-E8 in the pathogenesis of coronary atherosclerosis[17]. In this report, we examined the possible involvement of MFG-E8, vascular remodeling, and autophagy in the vascular toxicity of cocaine using cultured VSMCs *in vitro* as well as experimental rats *in vivo*.

Anti-cleaved caspase-3, LC3, PCNA, and cyclin D1 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), anti-p62 was purchased from Medical and Biological Laboratories (Nagoya, Japan), anti-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA), anti-MFG-E8 was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and anti-Ki-67 was purchased from Abcam (Cambridge, MA, USA). Anti-CDK4 was from EMD Millipore (Temecula, CA, USA). A7r5 rat aortic smooth muscle cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. To evaluate A7r5 cell death morphologically, cells treated with cocaine were incubated for 10 min with propidium iodide (PI) (1 μg/mL) and Hoechst 33342 (1 μg/mL) to visualize plasma membrane integrity and nuclear morphology, respectively. The cells were observed under a fluorescence microscope (BZ-8100, Keyence, Osaka, Japan). For a lactate dehydrogenase (LDH) activity assay, A7r5 cells were inoculated on a 96-well plate two days before exposure to 0.5, 1, or 3 mM cocaine. After 24 hr of cocaine exposure, the percentages of LDH released into the medium were assessed using an LDH-Cytotoxic assay Test Wako (for cytotoxicity assay) according to the supplier's instructions (Wako, Osaka, Japan).

For western blot analysis, equal amounts (5–12 μg) of cellular protein were resolved under appropriate acrylamide concentrations in SDS gels according to Laemmli[18] and electroblotted onto PVDF membranes. After blocking with TBS-Tween [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.05% Tween 20] containing 3% skim milk, the membranes were incubated overnight at 4°C with diluted primary antibodies. The PVDF membranes were then washed with TBS-Tween and incubated with HRP-conjugated secondary antibodies at room temperature. Antigen signals were detected using a Western Lightning Chemiluminescence Reagent Plus Kit (PerkinElmer Life Sciences, Boston, MA, USA); signal intensities were quantified using an image analyzer (CS Analyzer, Atto, Tokyo, Japan).

All procedures for animal treatment were approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University. Cocaine hydrochloride (Shionogi & Co., Ltd., Osaka, Japan) was dissolved in isotonic saline, and administered at 50 mg/kg body weight intravenously (i.v.) to 8- to 12-week-old male Sprague-Dawley (SD) rats. Two days after administration, the rats were sacrificed by an overdose of sodium pentobarbital [40 mg/kg, intraperitoneal (i.p.)], and the hearts and thoracic aortas were immediately removed and stored at −80°C for later western blot analysis. Tissue samples were also fixed with formalin for immunohistochemical analysis. Immunohistochemical analysis was performed as described previously[19]. In brief, formalin-fixed tissues were embedded in paraffin and sectioned. The sections were deparaffinized, rehydrated, and subjected to microwave antigen retrieval in 10 mM sodium buffer for 8 min at 95°C. The sections were incubated with 3% hydrogen peroxide and 5% normal goat serum to block nonspecific reactions and then incubated overnight at 4°C with anti-MFG-E8 (1:50) and anti-Ki-67 (1:100). Antigens were visualized with a Histofine Simple Stain MAX PO kit (Nichirei Biosciences, Tokyo, Japan) using diaminobenzidine (DAB) as a substrate and examined under a light microscope (Olympus AX80). The data are expressed as the mean ± S.D. of at least 3 independent samples. Dunnett's test was used for comparisons of multiple experimental groups, while the Student's t test was used for comparisons of two pairs of experimental groups. P values less than 0.05 were considered statistically significant.

We first examined whether exposure to high concentrations (0.5–3 mM) of cocaine induces death of A7r5 VSMCs. Cells were exposed to 0, 0.5, 1, or 3 mM cocaine for 24 hr and observed under fluorescence microscopy. Cells exposed to 3 mM cocaine showed nuclear condensation, one of the main features of apoptosis (Fig. 1A, solid white arrows). On the other hand, an increased number of PI-positive cells (necrotic cells) was also observed in a small percent of cocaine-treated cells (3 mM, 24 hr; Fig. 1A). We next evaluated caspase activation by examining the levels of cleaved caspase-3 by western blotting. In cocaine-treated cells, caspase-3 was cleaved to its active form during cocaine exposure in both concentration- and time-dependent manners (Fig. 1B). We also detected a significant increase in LDH activity in the medium of cocaine-treated cells (3 mM, 24 hr; Fig. 1C), confirming the cytotoxicity of a high concentration of cocaine. Taken together, high concentrations of cocaine cause apoptotic as well as necrotic cell death in A7r5 cells.

We next evaluated the effects of low concentrations of cocaine on A7r5 cells. We first examined whether or not autophagy is induced during exposure to cocaine. Cells were treated with 0, 0.05, 0.1, or 0.3 mM cocaine for 7 days, and then LC3-II and p62, a marker for and a substrate of autophagy, respectively, were analyzed by western blot analysis. After exposure to 0.3 mM cocaine for 7 days, LC3-II levels decreased concentration-dependently and a significant decrease was observed at 0.3 mM (Fig. 2A). In contrast, p62 levels showed an increased tendency, with the maximal response at 0.05 mM cocaine exposure (Fig. 2B). These results suggest that autophagy is not induced but is rather suppressed by exposure to these concentrations of cocaine, though dose-related effects of cocaine on the levels of LC3-

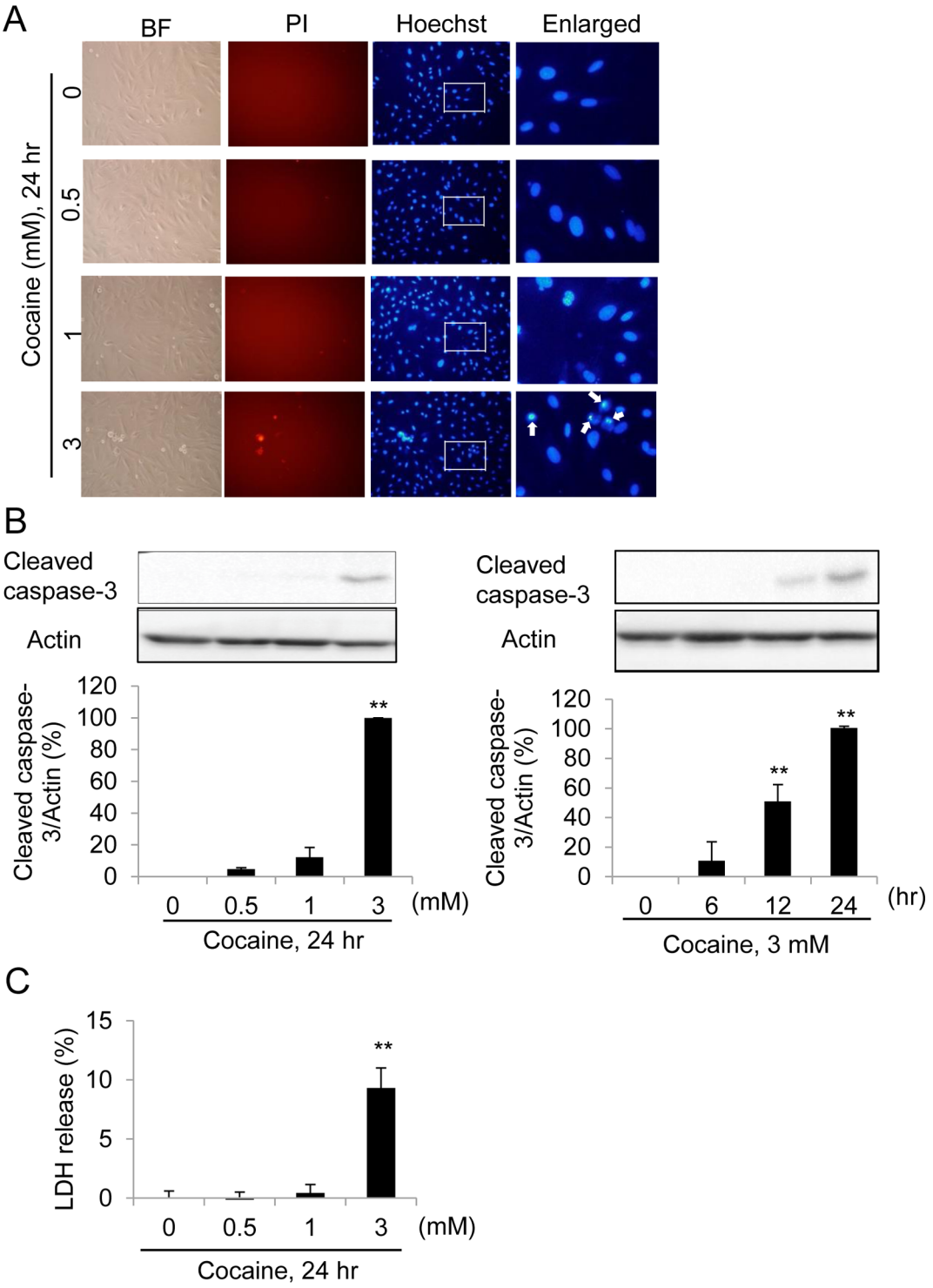

Fig. 1. High concentrations of cocaine cause death in A7r5 cells. (A) Morphological changes during cocaine exposure in A7r5 cells. Cells were exposed to 0, 0.5, 1, or 3 mM cocaine for 24 hr, stained with PI (red fluorescence) and Hoechst 33342 (blue fluorescence), and then observed under a fluorescence microscope. Enlarged images (enlarged) of Hoechst 33342 staining are also shown. Solid white arrows indicate condensed nuclei. Bright field images (BF) of the cells were observed under a light microscope. (B) Concentration- and time-dependent activation of caspase-3 caused by cocaine exposure. Cells were treated with 0, 0.5, 1, or 3 mM cocaine for 24 hr (left) or 3 mM cocaine for 0, 6, 12, or 24 hr (right). Caspase-3 activation was evaluated by examining the levels of cleaved caspase-3 by western blotting (n = 3 per group). (C) Cytotoxicity of cocaine. LDH release assay of cells treated with 0, 0.5, 1, or 3 mM cocaine for 24 hr. Values represent percentages of LDH released from the cells into the medium. **P<0.01 versus the control.

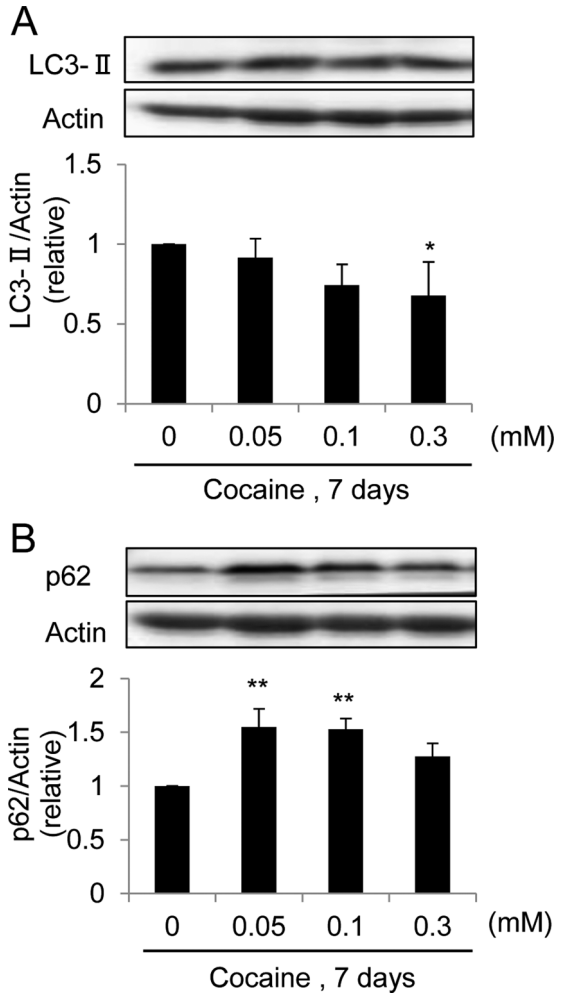

Fig. 2. Effect of low concentrations of cocaine on autophagy in A7r5 cells. (A and B) Low concentrations of cocaine suppress basal autophagy in A7r5 cells. Cells were exposed to 0, 0.05, 0.1, or 0.3 mM cocaine for 7 days, and subjected to western blot analysis. Representative western blot images and quantification of LC3-II (A) and p62 (B) levels relative to actin are shown (n = 3–4 per group). *P<0.05 versus 0 mM; **P<0.01 versus 0 mM.

II and p62 were somewhat different. We chose 0.3 mM cocaine for further analysis.

Given the indication for decreased basal autophagy in A7r5 cells treated with 0.3 mM cocaine and the fact that decreased cellular autophagy is often accompanied by increased cellular proliferation[20], we next examined whether exposure to a low concentration of cocaine induces MFG-E8, an activator of smooth muscle cell proliferation[16]. Cocaine (0.3 mM) exposure for one day resulted in a significant induction (about 1.5- to 2-fold) of MFG-E8 expression (Fig. 3A), suggesting the possibility that cocaine may increase smooth muscle cell proliferation by inducing MFG-E8. Restoration of increased MFG-E8 expression to basal levels after exposure to cocaine for 3 days (Fig. 3A) might indicate that the effect of cocaine is transient due to the rapid degradation of this drug in plasma[21]. We thus examined whether the increase in MFG-E8 expression is linked to an increased proliferative status of A7r5 cells. Cyclin D1 and PCNA levels in cocaine groups were slightly higher than that in control groups (Fig. 3B, C). CDK4 levels showed a significant increase after exposure to 0.3 mM cocaine (Fig. 3D, Day 1). Taking the results shown in Fig. 3 together, a low concentration (0.3 mM) of cocaine induces MFE-G8 expression and increases the proliferative ability of A7r5 cells.

We further examined the expression of MFG-E8 and the proliferative markers (PCNA and CDK4) in the thoracic aorta and heart of rats administered cocaine. Since the peak day of VSMC remodeling is expected to be 1–2 days after cocaine administration (Fig. 3D), we administered cocaine at a sublethal dose (50 mg/kg, i.v.) to rats and sacrificed them 2 days after administration. The levels of MFG-E8 were significantly increased in the aorta (Fig. 4A), but not in the heart (Fig. 4B), following cocaine administration. A trend of increased PCNA level as well as a significant increase of CDK4 level was observed in the aorta exposed to cocaine compared with the control (Fig. 4C, D). In contrast, significant differences were not observed in the heart (data not shown). These results support the results obtained in A7r5 cells (Fig. 3) and indicate that cocaine increases vascular MFG-E8 expression and proliferative ability of vascular cells *in vivo*.

Immunohistochemical staining with anti-MFG-E8 as well as anti-Ki-67 antibodies revealed that immunoreactivity to MFG-E8 was increased in the intima of the arterial wall of cocaine-administered rats (Fig. 5A). The percentages of Ki-67-positive nuclei, which indicate proliferative cells, also seemed to be increased in the arterial wall of cocaine-administered rats (Fig. 5A). As a negative control, the immunoreactivity of sections stained with non-immunized normal serum from rats administered cocaine and control rats is also showen in Fig. 5A. The immunoreactivity of sections stained with normal serum was minimum both in control and cocaine-treated rat specimens (Fig. 5A), and HE staining yielded no obvious findings (Fig. 5B). These results imply that the expression of MFG-E8 and the activation of cellular proliferative ability might precede vascular toxicity during cocaine administration.

In this study, we present evidence that cocaine increases MFG-E8 expression in both A7r5 vascular smooth muscle cells *in vitro* and the rat thoracic aorta *in vivo*. Although plasma cocaine concentrations after administration of the drug in rats (50 mg/kg, i.v.) were not determined in this study, another study demonstrated that the peak plasma cocaine concentration reaches around 2,500 ng/ml (approx. 8 μM) after administration in rats (3 mg/kg i.v.)[22]. The peak plasma cocaine concentration after i.v. administration at 50 mg/kg might reach around or above 0.1 mM, since the dose-related peak plasma concentrations were already

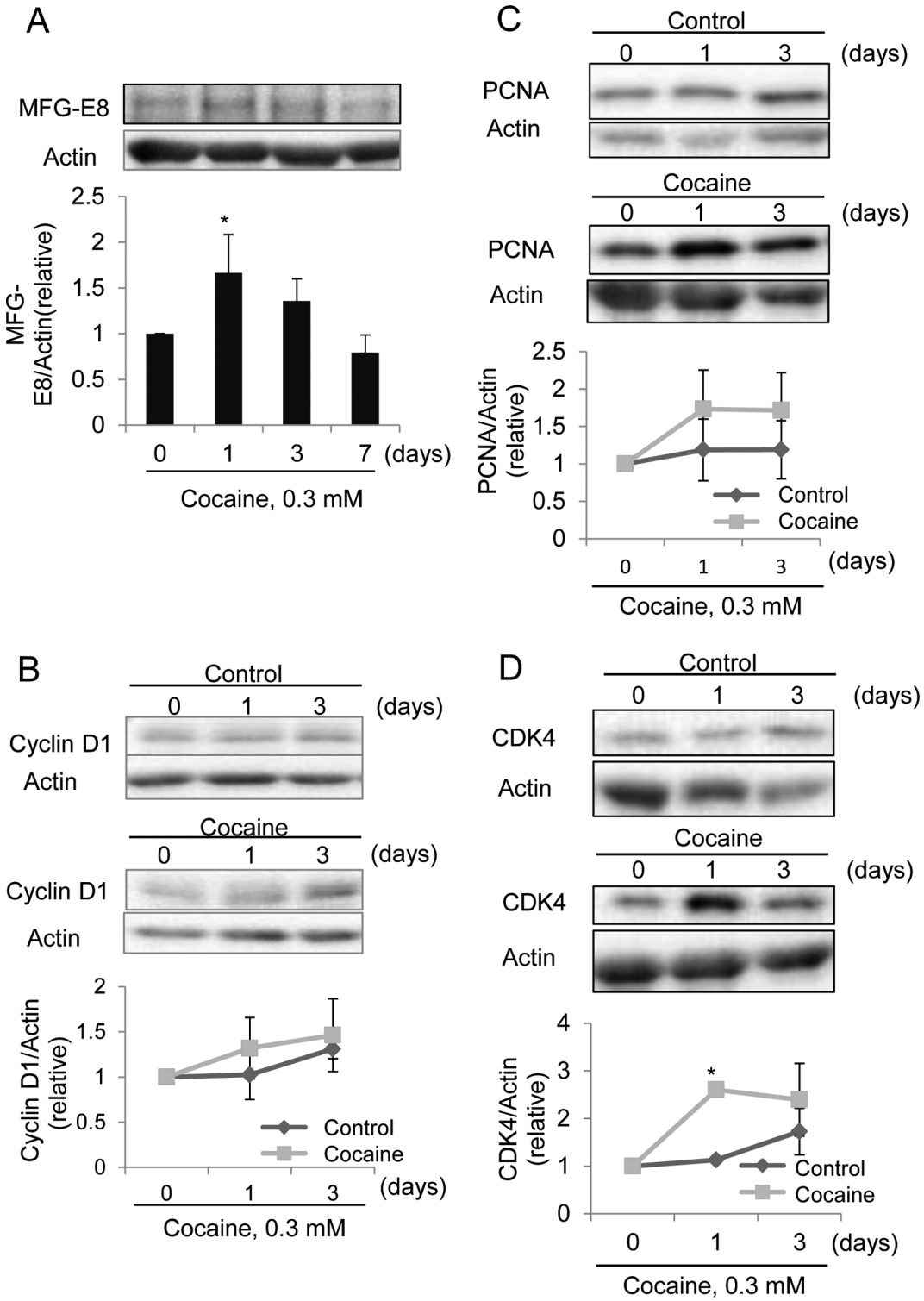

Fig. 3. Cocaine increases MFG-E8, cyclin D1, PCNA, and CDK4 expressions in A7r5 cells. Cells were exposed to 0.3 mM cocaine for 3 days and subjected to western blot analysis. Representative images and quantification of the levels of MFG-E8 (A), cyclin D1 (B), PCNA (C), and CDK4 (D) relative to actin are shown (n = 4 per group). *P<0.05 versus control.

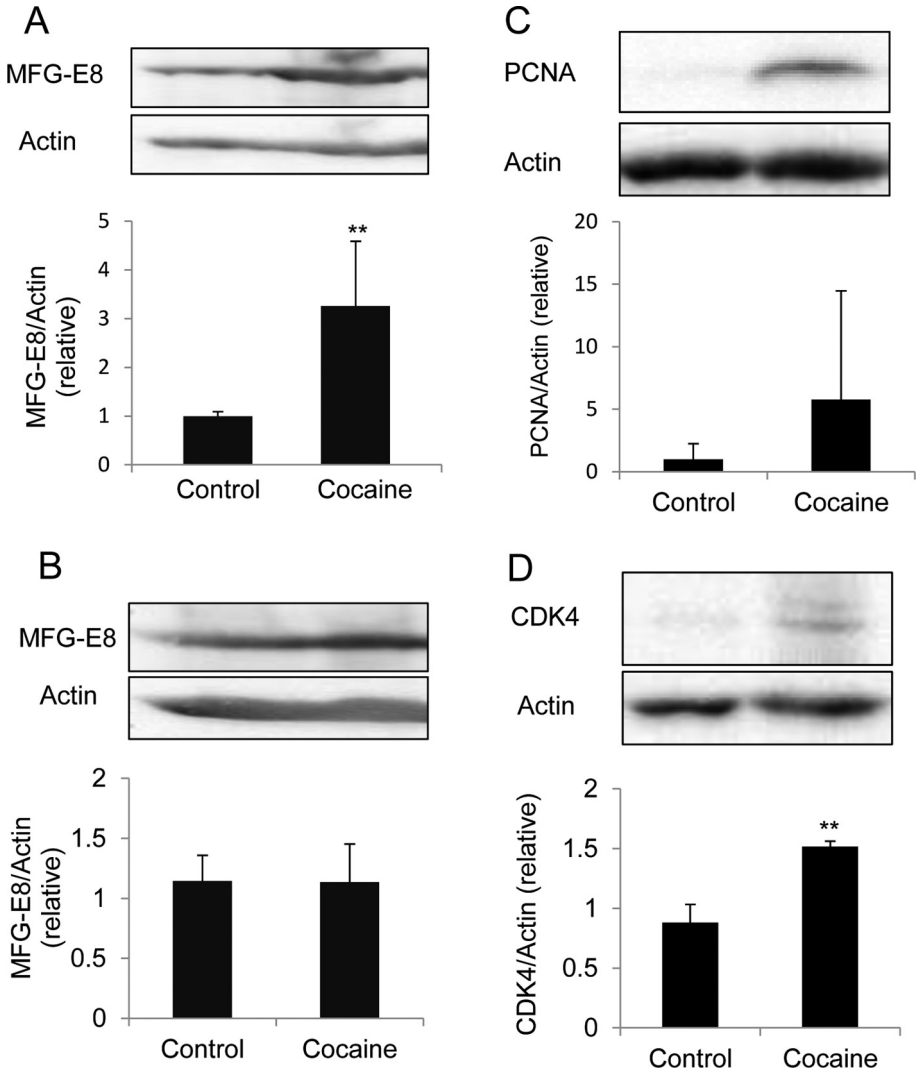

Fig. 4. Cocaine increases MFG-E8, PCNA, cyclin D1, and CDK4 expressions in the thoracic aorta and heart of rats. Rats were administered cocaine intravenously (50 mg/kg). Two days after cocaine administration, the thoracic aorta (A) and hearts (B) were extracted and subjected to western blot analysis. Representative western blot images and quantification of MFG-E8 levels relative to actin are shown. Levels of PCNA (C) and CDK4 (D) were also examined in the thoracic aorta (n = 3–5 per group). **P<0.01 versus control.

observed around the doses of 0.5–3.0 mg/kg[22]. Our current data also show that increased MFE-G8 expression is associated with a tendency for increased expression of cellular proliferation marker proteins (cyclin D1, PCNA, CDK4, and Ki-67) in cocaine-treated vascular cells. Thus, it might be possible to speculate that VSMC remodeling is followed by intimal thickening and subsequent stenosis or plaque expansion. These events might cause cardiovascular disorders like atherosclerosis in the long term. Interestingly, cocaine does not induce autophagy, but rather suppresses the basal levels of autophagy in A7r5 cells (Fig. 2). Consistent with our results, recent reports have shown that suppression of autophagy exacerbates vascular changes like atherosclerosis[23]. In A7r5 cells, CDK4 was decreased by 0.3 mM cocaine transiently within 3 days (Fig. 3D), whilst a long duration of LC3-II decreases (~7 days) was observed (Fig. 2A). These results might indicate that autophagy is a downstream event of VSMC remodeling, though further analysis is required to elucidate the role of autophagy in VSMC remodeling by cocaine. To our knowledge, this is the first report showing that cocaine increases MFE-G8 expression and decreases autophagy activity. These changes appear to be involved in the vascular toxicity of cocaine.

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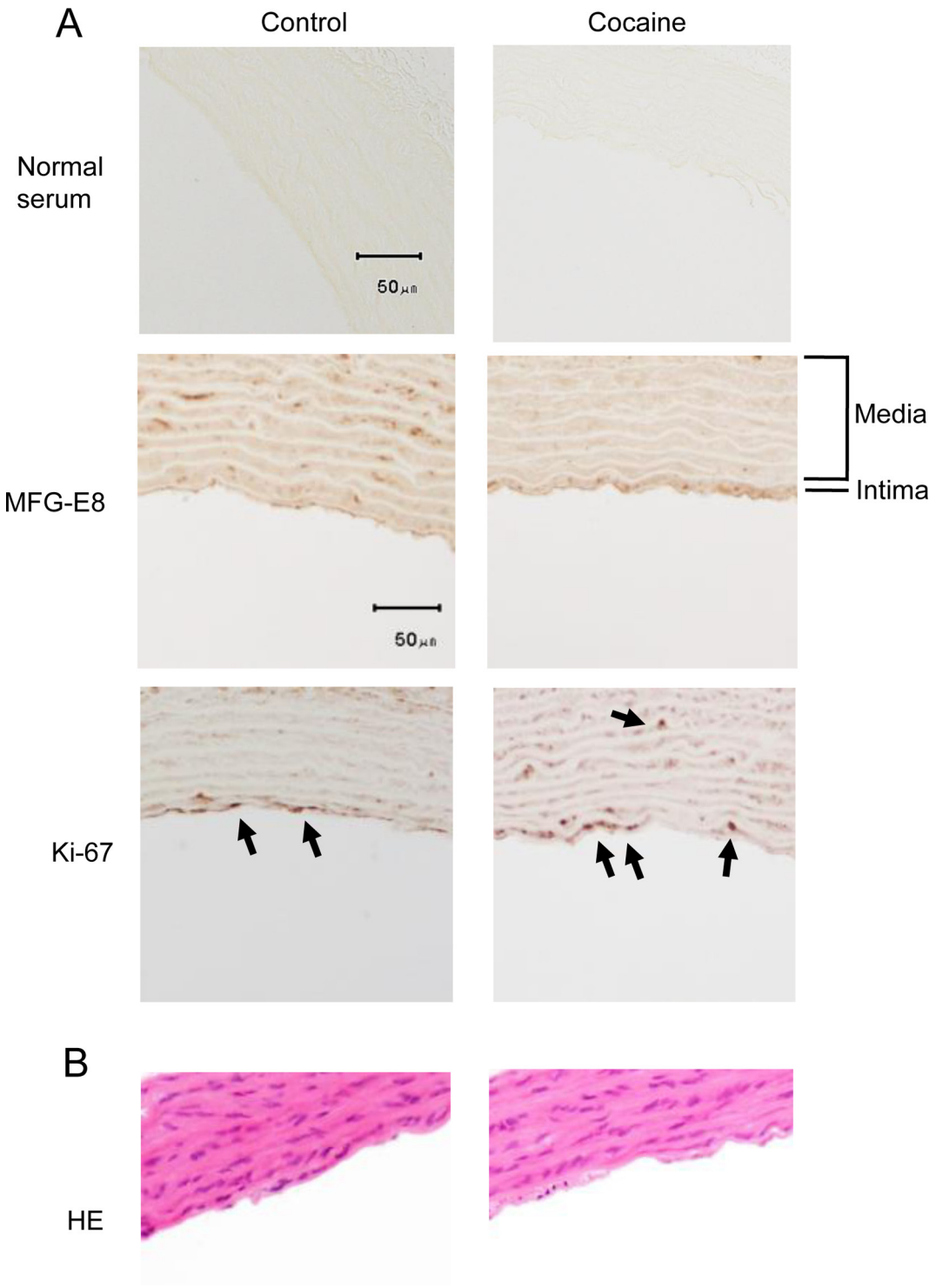

Fig. 5. Immunohistochemical analysis of MFG-E8 and Ki-67 in the thoracic aortic wall of rats administered cocaine. (A) Sections of the thoracic aortic wall from rats administered cocaine (50 mg/kg, 2 days) or untreated control rats were stained with anti-MFG-E8 and anti-Ki-67 antibodies. Representative images are shown (n = 4 per group). Sections were also stained with non-immunized normal serum (normal serum). Arrows indicate Ki-67-positive nuclei. (B) Hematoxylin and eosin (HE) staining of the tissues is also shown.

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