



Original Article

Effect of aldosterone on epithelial-to-mesenchymal transition of human peritoneal mesothelial cells

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Background: Peritoneal fibrosis is one of the major causes of technical failure in patients on peritoneal dialysis. Epithelial-to-mesenchymal transition (EMT) of the peritoneum is an early and reversible mechanism of peritoneal fibrosis. Human peritoneal mesothelial cells (HPMCs) have their own renin-angiotensin-aldosterone system (RAAS), however, it has not been investigated whether aldosterone, an end-product of the RAAS, induces EMT in HPMCs, and which mechanisms are responsible for aldosterone-induced EMT.

Methods: EMT of HPMCs was evaluated by comparing the expression of epithelial cell marker, E-cadherin, and mesenchymal cell marker, α -smooth muscle actin after stimulation with aldosterone (1–100nM) or spironolactone. Activation of extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) and generation of reactive oxygen species (ROS) were assessed by western blotting and 2',7'-dichlorofluorescein diacetate staining, respectively. The effects of MAPK inhibitors or antioxidants (*N*-acetyl cysteine, apocynin, and rotenone) on aldosterone-induced EMT were evaluated.

Results: Aldosterone induced EMT in cultured HPMCs, and spironolactone blocked aldosterone-induced EMT. Aldosterone induced activation of both ERK1/2 and p38 MAPK from 1 hour. Either PD98059, an inhibitor of ERK1/2, or SB20358, an inhibitor of p38 MAPK, attenuated aldosterone-induced EMT. Aldosterone induced ROS in HPMCs from 5 minutes, and antioxidant treatment ameliorated aldosterone-induced EMT. *N*-acetyl cysteine and apocynin alleviated activation of ERK and p38 MAPK.

Conclusion: Aldosterone induced EMT in HPMCs by acting through the mineralocorticoid receptor. Aldosterone-induced generation of ROS followed by activation of ERK, and p38 MAPK served as one of the mechanisms of aldosterone-induced EMT of HPMCs.

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Introduction

Long-term peritoneal dialysis (PD) results in peritoneal damage that is characterized by a decreased ultrafiltration

capacity associated with submesothelial fibrosis, accumulation of extracellular matrix, and neoangiogenesis [1–3].

Recent data have revealed that peritoneal mesothelial cells play an important role in peritoneal fibrosis via phenotype transition and production of extracellular matrix. Yáñez-Mó et al [4] found that mesothelial cells isolated from dialysate effluents showed phenotypic transition. This phenomenon of epithelial-to-mesenchymal transition (EMT) denotes loss of epithelial characteristics and acquisition of a fibroblast-like phenotype, and is suggested as a key process in the onset and progression of peritoneal fibrosis.

There are several lines of evidence that the renin–angiotensin–aldosterone system (RAAS) is involved in organ fibrosis [5–8]. Activation of the RAAS is related to the development of tubulointerstitial fibrosis in the kidney, and hepatic and lung fibrosis [6,9,10]. Aldosterone, a final end-product of RAAS, is reported to be an important mediator of cardiac fibrosis [11], and spironolactone ameliorates peritoneal fibrosis in animal models of peritoneal fibrosis and inflammation [12].

To determine the effect of aldosterone on peritoneal EMT, we investigated whether aldosterone induced EMT of peritoneal mesothelium and examined the mechanism of aldosterone-induced phenotypic transition of the peritoneum.

Methods

Reagents

All chemicals and tissue culture plates were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Nunc Labware (Waltham, MA, USA), unless otherwise stated.

Isolation and culture of human peritoneal mesothelial cells

Human peritoneal mesothelial cells (HPMCs) were isolated from a piece of omentum, obtained from consenting patients receiving elective abdominal surgery according to the method described by Stylianou et al [13]. HPMCs were identified by phase-contrast microscopy according to morphological criteria and by immunofluorescence staining for the markers of mesothelial cells [14]. All experiments were performed using cells of the second to fifth cell passages. Tissue collection was approved by the Institutional Review Boards and informed consent was obtained from each patient.

Reverse transcriptase polymerase chain reaction of mineralocorticoid receptor

Total RNA was extracted from HPMCs that were prepared by using TRIzol reagent (Gibco Invitrogen, Carlsbad, CA, USA). The RNA pellet was suspended in DNase/RNase-Free distilled water and stored at -70°C until subsequent analysis. Mineralocorticoid receptor oligonucleotide primers used for the polymerase chain reaction (PCR) were as follows: forward primer: 5'-ACCAAGCATTCATGTTTCAGGCACC-3' and reverse primer: 5'-AGCTCCCCTCATCTGGTCTTGT-3'. PCR was performed in 10mM Tris–HCl (pH 9.0), 40mM KCl, 1.5mM MgCl_2 , and 0.1 U Taq polymerase (Promega), in a final volume of 20 μL . Reactions were carried out in a DNA thermal cycler (Perkin–Elmer, Boston, MA, USA). Following initial denaturation at 94°C for 5 minutes, mixtures were subjected 35 cycles at 94°C for 30 seconds,

annealing at 55°C for 30 seconds, primer extension at 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. PCR products were fractionated on 1% agarose gel, followed by staining with 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide (Gibco BRL, Grand Island, NY, USA). The amount of PCR products was normalized with a housekeeping gene, GAPDH (forward primer: 5'-ACCA-CAGTCCATGCCATCAC-3' and reverse primer: 5'-TCCAC-CACCCTGTTGCTGTA-3').

MTS assay for assessing cell proliferation

To measure cell proliferation, HPMCs (10^4 cells/well) were incubated with aldosterone (1–100nM) in 96-well plates. After incubation for 2–7 days at 37°C , MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; Promega] was added, and the amount of metabolized formazine was measured at 490 nm with an automated plate enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's instructions.

Cell morphology and immunocytochemistry

The morphology of HPMC cells was observed by an inverted phase contrast microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany) and the images were obtained using a digital camera (AxioCam HRC; Carl Zeiss). For staining, cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS (25 minutes at 20°C) and permeabilized with 1% Triton X-100 in PBS (15 minutes at 4°C). After washing with PBS, the cells were treated with 5% bovine serum albumin in PBS for 1 hour before incubation with primary antibodies specific for cytokeratin (1:50; DAKO Cytomation, Carpinteria, CA, USA) or α -smooth muscle actin (SMA,

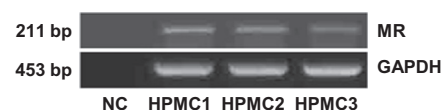


Figure 1. Expression of mineralocorticoid receptor mRNA in HPMCs. Mineralocorticoid receptor mRNA detected in cultured HPMCs. Representative reverse transcriptase polymerase chain reaction bands from three different patients are shown. NC (negative control) denotes the sample containing all reactants except cDNA. HPMC, human peritoneal mesothelial cell; MR, mineralocorticoid receptor.

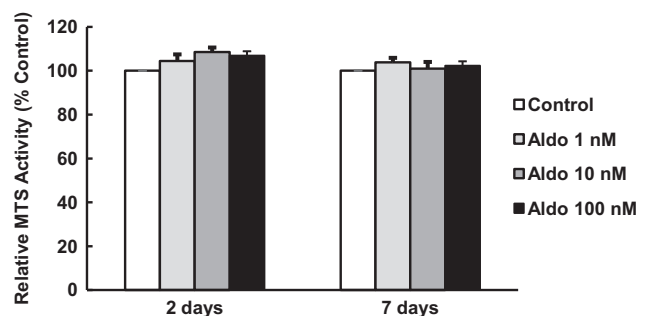


Figure 2. Effect of aldosterone on the proliferation of human peritoneal mesothelial cells. There was no effect of aldosterone (1–100nM) on MTS activity on Day 2 and 7. Data are presented as means \pm standard deviation. Aldo, aldosterone; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

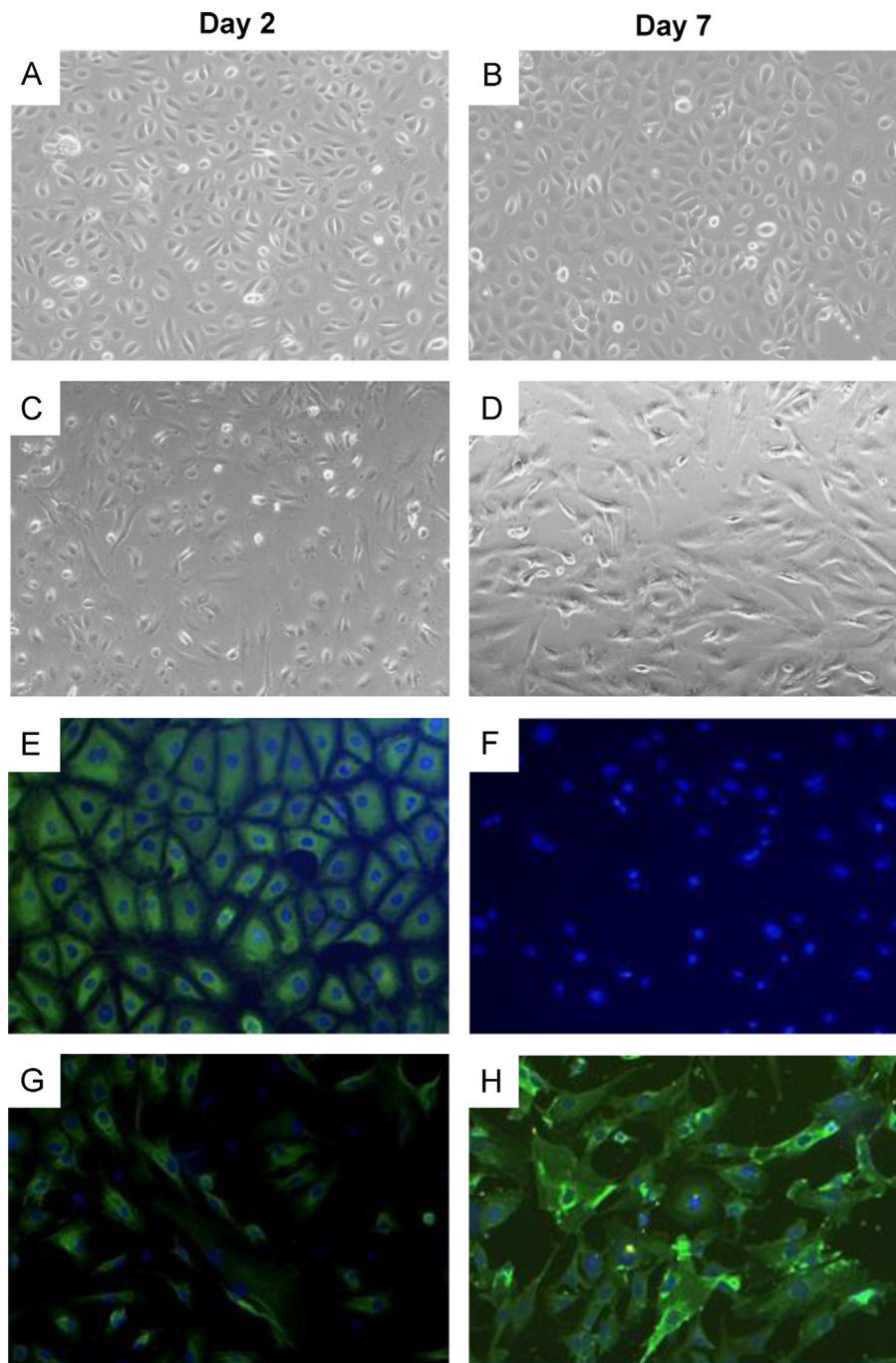


Figure 3. Effects of aldosterone on morphology and expression of cytokeratin and α -SMA in HPMCs. Compared with the cobblestone appearance of unstimulated HPMCs on Day 2 (A) and Day 7 (B), aldosterone (10nM) induced phenotypic transformation of cells. Cells began to elongate and acquire a fibroblast-like morphology after 2 days of aldosterone stimulation (C), which was further evident after 7 days (D). Immunofluorescence staining for cytokeratin revealed abundant cytoplasmic expression in unstimulated cells (E), which was markedly decreased upon aldosterone stimulation (G). Aldosterone upregulated α -smooth muscle actin organization (H) in contrast with almost negative staining in unstimulated cells (F). Magnification, 100 \times . HPMC, human peritoneal mesothelial cell; SMA, smooth muscle actin.

1:100; Abcam, Cambridge, UK) in 5% bovine serum albumin overnight at 4°C. After washing with 0.2% Tween 20 in PBS, the cells were incubated with goat anti-mouse IgG-fluorescein-isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at room temperature in the dark. The cell nuclei were counterstained with 4',6-diamidino-2-phenylindole. Immunofluorescence was visualized under an Axiovert 200 fluorescence microscope (Carl Zeiss) with 10 \times 0.3 numerical aperture and 20 \times 0.4 numerical aperture objectives equipped with an AxioCam

HRC digital camera (Carl Zeiss). Digital photographs were obtained with Axiovision release 4.3 (Carl Zeiss) and merged images were produced using Photoshop version 10 (Adobe Systems, Toronto, ON, Canada).

Western blot analysis

Protein samples were isolated from cell lysate (30 μ g), and boiled (95°C, 5 minutes) after mixing in reducing buffer. Proteins were resolved on 10% sodium dodecyl sulfate polyacrylamide

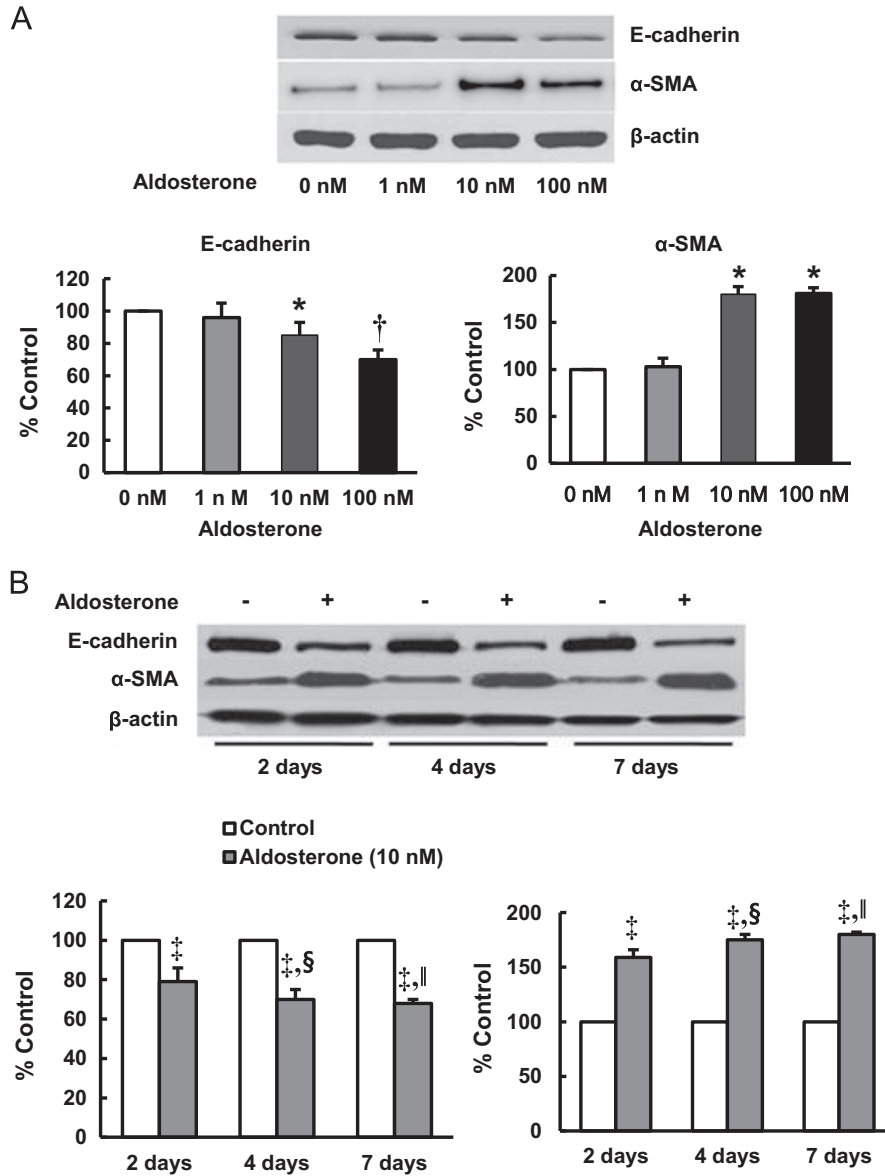


Figure 4. Effects of aldosterone on the expression of E-cadherin and α -SMA in HPMCs. Aldosterone induced a dose-dependent decrease in expression of E-cadherin and increase in α -SMA expression after 2 days stimulation (A). Aldosterone (10nM) also induced time-dependent changes in expression of E-cadherin and α -SMA between 2 days and 7 days (B). Representative western blots with a quantitation bar graph are shown. * $P < 0.05$ versus control (0nM) or 1nM aldosterone, † $P < 0.05$ versus 10nM aldosterone, ‡ $P < 0.05$ versus control at each time point, § $P < 0.05$ versus aldosterone at 2 days, || $P < 0.05$ versus aldosterone at Day 2 and Day 4 ($n = 5$). HPMC, human peritoneal mesothelial cell; SMA, smooth muscle actin.

gels, and transferred to polyvinylidene difluoride membranes. Membranes were blocked by incubation in 5% w/v nonfat milk powder in Tris-buffered saline for 30 minutes at room temperature, followed by incubation with primary antibodies directed against the following antigens overnight at 4°C: E-cadherin (BD Pharmingen, San Diego, CA, USA), α -SMA (Abcam), S1004A (DAKO), extracellular-signal-regulated kinase (ERK1/2, Santa Cruz Biotechnology), phospho-ERK (Santa Cruz Biotechnology), p38 mitogen-activated protein kinase (MAPK) (p38, Santa Cruz Biotechnology) and phospho-p38 MAPK (Santa Cruz Biotechnology). After washing the blot with PBS-Tween 20, blots were incubated with horseradish-peroxidase-conjugated secondary antibodies corresponding to each primary antibody, followed by enhanced chemiluminescence detection (Santa Cruz Biotechnology). Positive immunoreactive bands were quantified by densitometry and compared with the expression of human β -actin (Santa Cruz Biotechnology).

Generation of reactive oxygen species

HPMCs were incubated with 10 μ M 2',7'-dichlorofluorescein diacetate (DCF-DA) (Invitrogen, Carlsbad, CA, USA) prior to exposure to aldosterone (10nM) and/or spironolactone (1 μ M). Serial fluorescence was measured using a fluorescent plate reader at excitation 485 nm and emission 535 nm (Molecular Devices).

Effect of MAPK inhibitors or antioxidants on aldosterone-induced EMT and activation of MAPK

To investigate the signal transduction pathway responsible for aldosterone-induced EMT, HPMCs were pretreated with inhibitors of ERK (PD98059, 10 μ M) or p38 MAPK (SB203580, 10 μ M), and stimulated with aldosterone for 48 hours. We also examined the role of reactive oxygen species (ROS) in

phenotypic transition of HPMCs by examining the effect of various antioxidants, *N*-acetyl cysteine (NAC, 5mM, ROS scavenger), apocynin [10 μ M, an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase], and rotenone (1 μ M, an inhibitor of mitochondrial electron transfer chain subunit I) on aldosterone-induced EMT and activation of MAPK.

Extraction of total RNA and real-time PCR

The ABI PRISM 7000 sequence detection system was used for determining the level of transcripts with SYBR Green I as a double-stranded DNA-specific dye (Applied Biosystems, Foster City, CA, USA). The PCR was performed in 5 μ M cDNA, 10 μ M SYBR Green PCR Master Mix, and 5pM sense and antisense primers of E-cadherin: (forward primer: 5'-ACCCCTGTGGTGTCTTT-3', reverse primer: 5'-TTCGGGCTGTGTCATTCT-3'), α -SMA: (forward primer: 5'-GGGAATGGGACAAAAGACA-3', reverse primer: 5'-CTCAGGGGCAACACGAA-3') for a final volume of 20 μ M per reaction. Optimal primer concentrations were determined by preliminary experiments. The comparative cycle threshold method was used to assess the relative mRNA expression levels of the target genes. For statistical analysis, all PCR procedures were replicated at least three times. The amount of PCR products was normalized with the housekeeping gene, β -actin, to determine the relative expression ratios for each mRNA in relation to the control group.

Statistical analysis

Data are shown as mean \pm standard deviation. All statistical analyses were carried out using SPSS for Windows version 11.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using a Kruskal–Wallis nonparametric test, Student's *t* test or one-way analysis of variance for multiple comparisons. Statistical significance was determined when *P* was < 0.05.

Results

Expression of mineralocorticoid receptor in HPMCs

There was a constitutive expression of mineralocorticoid receptor (MR) in HPMCs. Fig. 1 shows the mRNA expression of MR in HPMCs isolated from three different patients.

Effect of aldosterone on proliferation of HPMCs

Stimulation of HPMCs with aldosterone (1–100nM) for 2–7 days did not induce an alteration of cell proliferation assessed by MTS assay (Fig. 2).

Aldosterone-induced EMT of HPMCs

Aldosterone induced morphological changes in cultured HPMCs from Day 2 (Fig. 3). The typical cobblestone-shape of the HPMC monolayer disappeared on Day 2 of aldosterone stimulation with a change into an elongated morphology. After 7 days of aldosterone exposure, there was a loss of cell contact with a fibroblast-like phenotype. Immunofluorescent staining demonstrated a gradual decrease and redistribution in cyto-keratin and epithelial cell marker, and acquisition of α -SMA as early as 2 days after aldosterone stimulation (Fig. 3).

Exposure of HPMCs to aldosterone (1, 10 and 100nM) for 2–7 days resulted in a dose- and time-dependent decrease in mRNA and protein expression of epithelial cell marker and E-cadherin, associated with an increase in expression of mesenchymal marker α -SMA (Figs. 4 and 5). The expression of S100A4, another specific marker of myofibroblasts, was increased by stimulation with aldosterone (Fig. 6). Aldosterone-induced changes in the expression of E-cadherin and α -SMA were almost completely inhibited by treatment with an MR antagonist, spironolactone (1 μ M) (Fig. 7).

Effect of aldosterone on ERK and p38 MAPK activation

Aldosterone at a concentration of 10nM significantly increased phosphorylation of ERK1/2 from 1 hour of stimulation, which was blocked by spironolactone (Fig. 8). p38 MAPK was also activated by aldosterone at 1 hour, and showed a

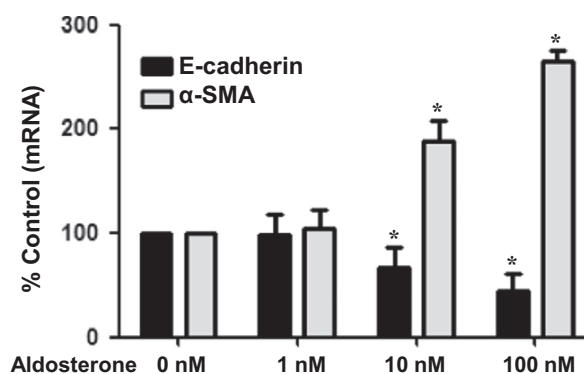


Figure 5. Effects of aldosterone on mRNA expression of E-cadherin and α -SMA in HPMCs. Aldosterone induced a significant decrease in the mRNA expression of E-cadherin and an increase in α -SMA at concentrations > 10nM after 2 days. **P* < 0.05 versus 0 and 1nM aldosterone (*n* = 4). HPMC, human peritoneal mesothelial cell; SMA, smooth muscle actin.

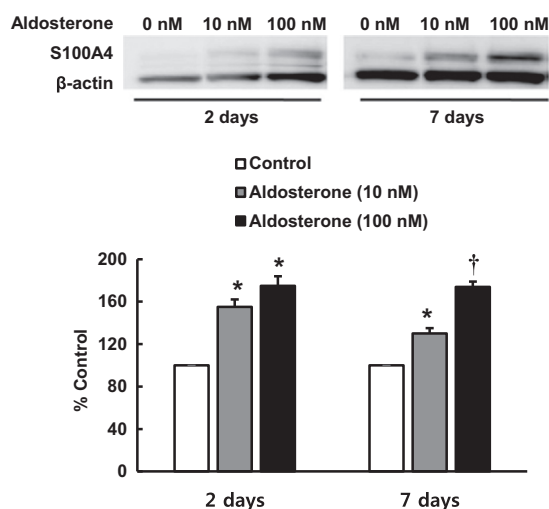


Figure 6. Effect of aldosterone on expression of S100A4 in HPMCs. Aldosterone (10–100nM) increased expression of S100A4 protein on Day 2 and Day 7 in a dose-dependent manner. Representative western blots with a quantitation bar graph are shown. **P* < 0.05 versus control (0nM) at each time point, †*P* < 0.05 versus control and 10nM aldosterone (*n* = 3). HPMC, human peritoneal mesothelial cell.

secondary peak of phosphorylation at 24 hours and 48 hours. Aldosterone-induced phosphorylation of p38 MAPK was inhibited by treatment with spironolactone.

Effect of MAPK inhibitors on aldosterone-induced EMT

Both PD98059, an inhibitor of ERK pathway, and SB20358, an inhibitor of p38 MAPK, alleviated aldosterone-induced EMT of HPMCs (Fig. 9).

Effect of antioxidants on aldosterone-induced EMT and MAPK activation

Aldosterone significantly increased the generation of intracellular ROS in HPMCs from 5 minutes (Fig. 10), which was blocked by spironolactone. NAC, apocynin and rotenone ameliorated E-cadherin downregulation and upregulation of α -SMA induced by aldosterone both at the protein and mRNA levels (Fig. 11). Aldosterone-induced ERK1/2 and p38 MAPK

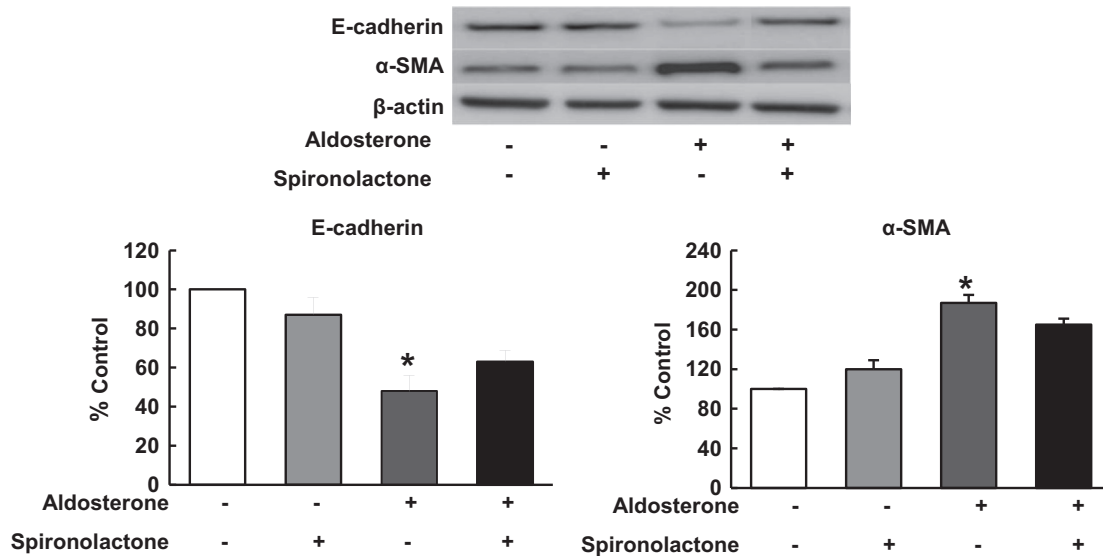


Figure 7. Effect of spironolactone on aldosterone-induced epithelial-to-mesenchymal transition in HPMCs. Aldosterone (10nM)-induced changes in expression of E-cadherin and α -SMA were blocked by pretreatment with spironolactone (1 μ M). Representative western blots with a quantitation bar graph are shown. * $P < 0.05$ versus others ($n=5$). HPMC, human peritoneal mesothelial cell; SMA, smooth muscle actin.

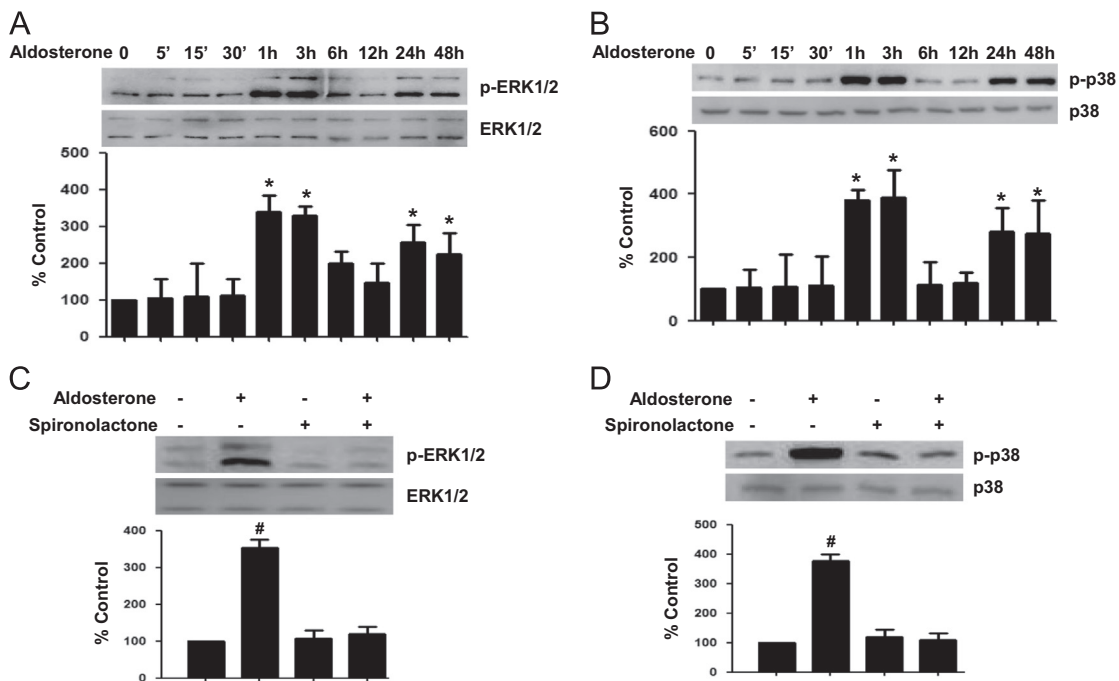


Figure 8. Effects of aldosterone on the activation of ERK1/2 and p38 MAPK in HPMCs with or without spironolactone pretreatment. Aldosterone (10nM) significantly increased phosphorylation of ERK1/2 (A) and p38 MAPK (B) in HPMCs. Aldosterone-induced activation of ERK1/2 and p38 MAPK was inhibited by spironolactone (1 μ M) (C, D). Representative western blotting and its quantitation graphs are shown ($n=4$). * $P < 0.05$ versus others. ERK, extracellular signal-regulated kinase; HPMC, human peritoneal mesothelial cell; MAPK, mitogen-activated protein kinase.

activation was ameliorated by NAC and apocynin, but not by rotenone (Fig. 12).

Discussion

This study shows that aldosterone *per se* induces the phenotypic transition of HPMCs. The effect of aldosterone is MR dependent and involves intracellular ROS generation and activation of ERK1/2 and p38 MAPK. Antioxidants or MAPK inhibitors ameliorate aldosterone-induced EMT, suggesting these two mechanisms are responsible for aldosterone-induced changes in cell phenotype.

Long-term PD is frequently associated with functional and structural alterations in the peritoneal membrane [15,16]. Complex interactions of host and local factors, hypertonic glucose-based dialysate, acidity of lactate-buffered solution, and the presence of glucose degradation products with activation of inflammatory cytokines and various growth factors are known to be responsible for the changes in the peritoneal membrane [17,18]. Recent data suggest that EMT is an early

and reversible step of peritoneal fibrosis [19]. EMT is a process by which epithelial cells lose their polarity and intercellular adhesion, and undergo remodeling of the intracellular cytoskeleton [20]. Concurrent with a loss of epithelial phenotype, cells undergoing EMT acquire the expression of mesenchymal components and manifest migratory and invasive properties [17,21,22]. Although EMT is a physiologically important process in embryogenesis and wound healing, it can impose unfavorable effect by promoting tissue fibrosis in nonphysiological conditions.

HPMCs possess their own renin-angiotensin system (RAS) with expression of angiotensinogen, angiotensin-converting enzyme, or angiotensin II type I receptor [23,24]. High glucose concentration results in RAS activation in HPMCs, and RAS blockers ameliorate the production of transforming growth factor- β and fibronectin, preserve ultrafiltration, and decrease peritoneal fibrosis [23]. As a continuum of previous studies, we found that HPMCs also constitutively express MR, indicating the presence of almost all components of the RAAS in peritoneal mesothelial cells. Profibrotic and proinflammatory effects of angiotensin II have been shown, along with demonstration of beneficial effects of angiotensin-converting enzyme inhibitor and angiotensin II type I receptor blocker on tubulointerstitial fibrosis or peritoneal fibrosis [25-27]. Another end-product of the RAAS is aldosterone, a mineralocorticoid hormone, which is produced in the adrenal cortex, endothelial and vascular smooth muscle cells in the heart, blood vessels, and brain. Besides the classical effect on sodium and potassium transport, aldosterone is known to play a role in the development of cardiac and renal fibrosis. Chronic aldosterone infusion causes myocardial fibrosis in rats with high salt intake [28]. Similarly, aldosterone causes glomerular injury and tubulointerstitial fibrosis [29]. Previous studies also demonstrated that spironolactone decreases peritoneal thickening and inflammation in animal models of bacterial peritonitis and peritoneal fibrosis induced by mechanical scraping [30,31]. One study has investigated the effect of spironolactone (25 mg) in a small number of PD patients (8 in placebo group vs. 8 in spironolactone group). Spironolactone administration for 6 months resulted in less inflammation and collagen deposition in peritoneal biopsy specimens [32]. However, these studies in animal models and PD patients neither address the mechanism of protective effect of spironolactone on peritoneal fibrosis nor focus on EMT as a potential

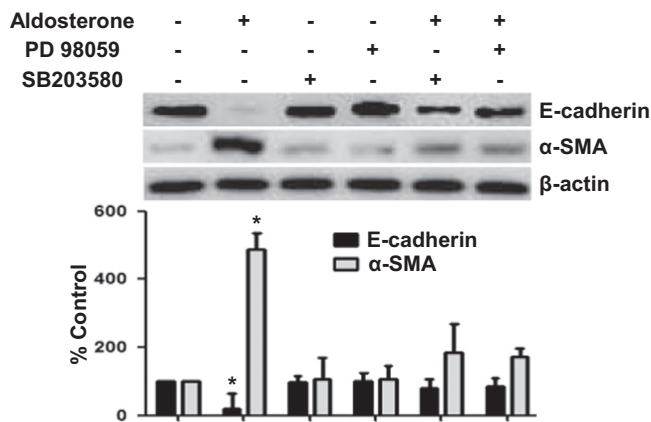


Figure 9. Effect of MAPK inhibitors on aldosterone-induced EMT. ERK 1/2 inhibitor (PD98059, 10 μ M) and p38 inhibitor (SB203589, 10 μ M) alleviated the decrease of E-cadherin and acquisition of α -SMA in aldosterone-exposed cells at 48 hours. Representative western blotting and its quantitation graphs are shown ($n=6$). * $P < 0.05$ versus others. EMT, epithelial-to-mesenchymal transition; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; SMA, smooth muscle actin.

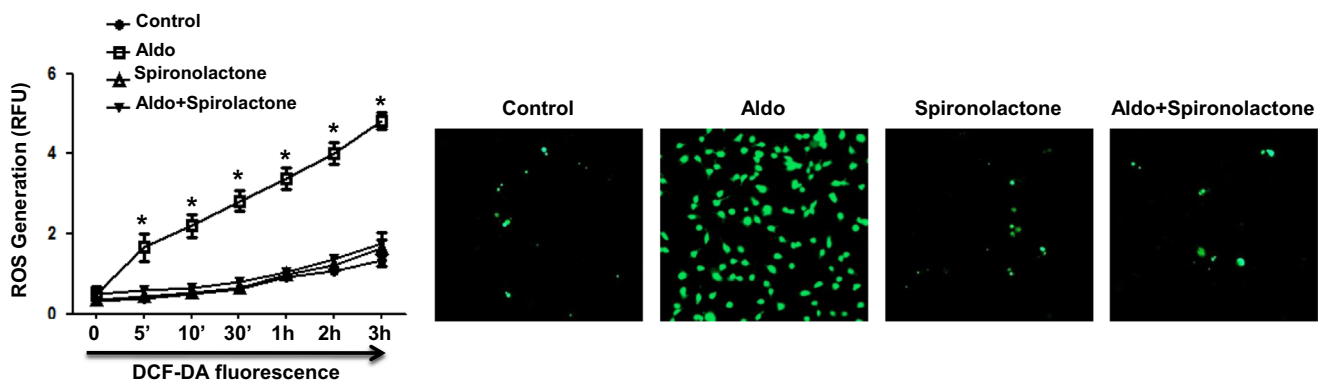


Figure 10. Effect of spironolactone on aldosterone-induced ROS production. Aldosterone (10nM) significantly increased generation of intracellular ROS in human peritoneal mesothelial cells after 5 minutes; the effect was blocked by co-treatment with spironolactone (1 μ M). Representative DCF-DA staining at 30 minutes of aldosterone treatment is shown ($n=5$). * $P < 0.05$ versus others. Aldo, aldosterone; DCF-DA, 2',7'-dichlorofluorescein diacetate; ROS, reactive oxygen species.

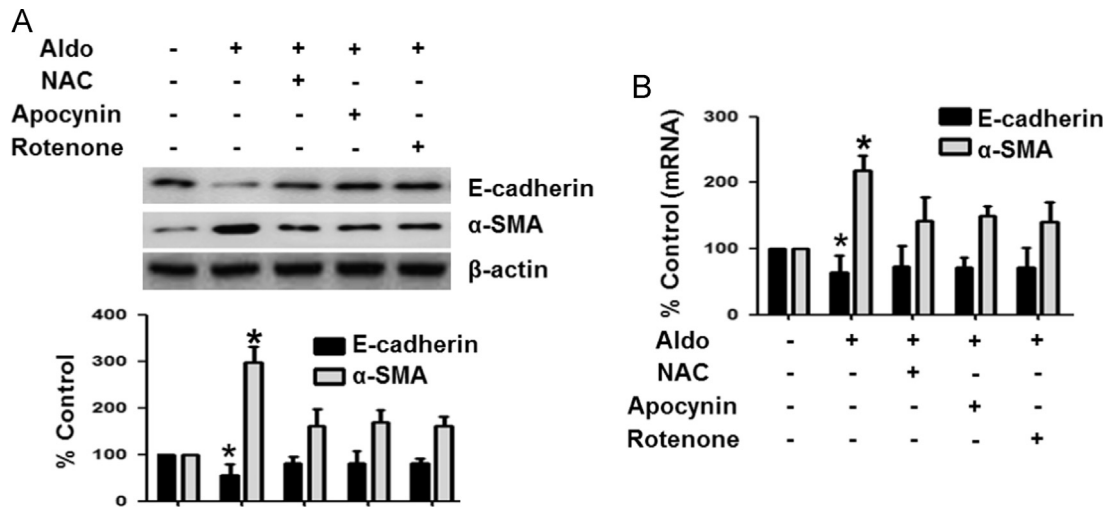


Figure 11. Effects of antioxidants on aldosterone-induced EMT. Aldosterone-induced changes in expression of E-cadherin and α -SMA were blocked by NAC (5mM), apocynin (10 μ M), and rotenone (5 μ M) ($n=5$). Representative western blotting with its quantitation graph (A) and relative mRNA expression (B) are shown. * $P < 0.05$ versus others. Aldo, aldosterone; EMT, epithelial-to-mesenchymal transition; NAC, *N*-acetyl cysteine; SMA, smooth muscle actin.

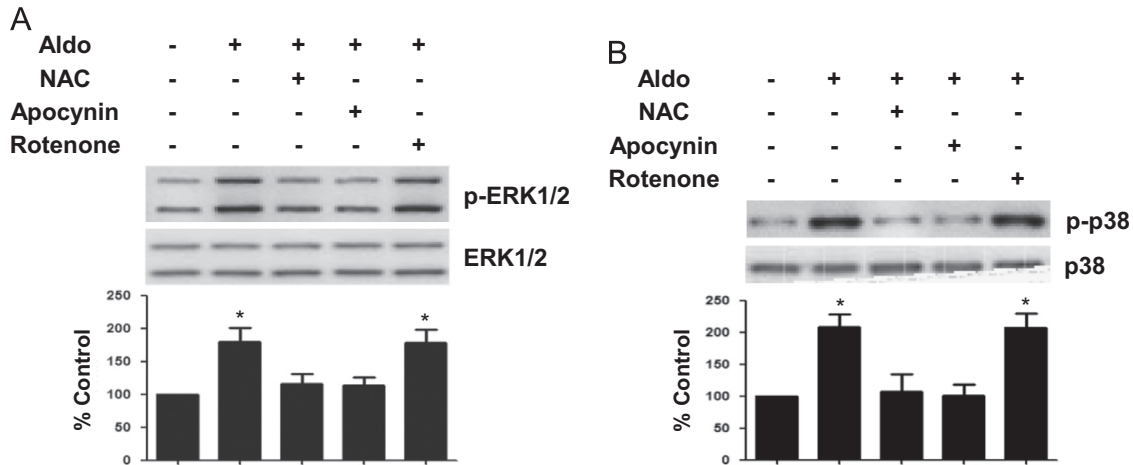


Figure 12. Effect of antioxidants on aldosterone-induced MAPK activation. Aldosterone (10nM) significantly increased phosphorylation of ERK1/2 (A) and p38 MAPK (B) in human peritoneal mesothelial cells. Aldosterone-induced activation of ERK1/2 and p38 MAPK was significantly ameliorated by antioxidants, NAC (5mM) or apocynin (10 μ M). However, rotenone (5 μ M) did not alter the aldosterone-induced activation of ERK1/2 and p38 MAPK. Representative western blotting and its quantitation graphs are shown ($n=4$). * $P < 0.05$ versus others. Aldo, aldosterone; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NAC, *N*-acetyl cysteine.

mechanism of aldosterone-induced EMT. Our data suggest that blocking ROS generation with amelioration of ERK1/2 and p38 MAPK activation is the mechanism for the beneficial effect of spironolactone on aldosterone-induced EMT and fibrosis.

Aldosterone activated both ERK1/2 and p38 MAPK in HPMCs. Interestingly, aldosterone induced an activation of both MAPKs at 1–3 hours, followed by secondary phosphorylation at 24–48 hours. The significance of each peak of ERK and p38 activation needs to be further investigated. The initial activation of MAPK seems not to be related to the genomic effect of aldosterone, whereas the secondary peak may be the consequence of activation of genomic pathways by aldosterone. ERK1/2 and p38 MAPK are known to be important mediators of the intracellular signal transduction pathway responsible for collagen synthesis and tissue fibrosis [33]. ERK is implicated in cell proliferation and differentiation, whereas p38 mediates cellular stresses such as inflammation and death. A previous study demonstrated that angiotensin II-

mediated EMT of renal tubular cells was ameliorated by MAPK inhibitors [34]. In our study, both ERK1/2 and p38 MAPK inhibitors alleviated aldosterone-induced EMT of HPMCs, indicating the role of MAPK in the development of EMT and peritoneal fibrosis.

In this study, intracellular ROS generation was one of the earliest findings of aldosterone-induced changes in HPMCs, which was observed after 5 minutes of aldosterone stimulation. There have been several studies demonstrating the role of ROS in the development of peritoneal fibrosis [35,36]. High glucose and glucose-based PD solutions induced generation of ROS in cultured HPMCs [37–39]. Furthermore, treatment with antioxidants, NAC or catalase, effectively reversed high-glucose-induced change in E-cadherin and α -SMA [35]. The role of ROS in aldosterone-induced EMT was also examined in renal tubular cells [40]. In human proximal tubular cells, aldosterone increases intracellular ROS production and induced EMT. Aldosterone-induced EMT in renal tubular cells

is completely blocked by mitochondrial respiratory chain complex I inhibitor [40]. In this study, we also found antioxidants including ROS scavenger, NADPH oxidase inhibitor, and mitochondria-targeted antioxidant ameliorated aldosterone-induced EMT of HPMCs, which was consistent with the previous study performed in renal tubular cells.

Importantly, ERK1/2 and p38 MAPK activation in aldosterone-stimulated HPMC was blocked by NAC and apocynin, but not by rotenone. This finding suggests ROS generation by NADPH oxidase precedes MAPK activation, which is followed by mitochondrial ROS generation. Mitochondrial ROS generation in HPMCs was observed after 6 hours of aldosterone stimulation (data not shown), in contrast to positive DCF-DA staining as early as after 5 minutes, which may have been due to activation of NADPH oxidase.

Aldosterone concentration used in this study was 0.1–100nM. The normal plasma level of aldosterone is 0.14–0.80nM in sitting and standing positions, and 0.08–0.30nM in the lying position, which is lower than the levels that induced EMT in this study. However, in patients with kidney disease, liver cirrhosis, heart failure and a condition of increased intra-abdominal pressure, aldosterone level is increased [41]. Plasma level of aldosterone is reported to be higher in continuous ambulatory PD (CAPD) patients compared with other patients with chronic kidney disease. According to the previous study, mean plasma and dialysate level of aldosterone in 27 stable CAPD patients were 1.5 ± 0.6 nM and 0.7 ± 0.3 nM, respectively [42]. Higher plasma level of aldosterone in CAPD patients was considered to be due either to increased secretion or decreased clearance. The instillation of 2 L dialysate into the peritoneal cavity during CAPD may reduce hepatic blood flow and reduce the endogenous metabolic clearance rates of aldosterone, which results in a high level of aldosterone in peritoneal dialysate [43,44]. Although aldosterone concentrations inducing EMT in our *in vitro* study are higher than the aldosterone level in peritoneal dialysate, continuous exposure of peritoneal mesothelium to certain concentrations of aldosterone in PD patients may trigger phenotypic transition of mesothelial cells.

In conclusion, our study demonstrated that aldosterone induced EMT in HPMCs for the first time. Aldosterone-induced ROS generation plays a key role in aldosterone-induced EMT via activation of intracellular ERK1/2 and p38 MAPK. Our data suggest a potential therapeutic use of MR antagonist as well as MAPK inhibitor or specific antioxidants in peritoneal fibrosis.

Conflict of interest

The authors have declared that no competing interest exists.

Acknowledgments

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