Aldosterone induces myofibroblast EGF secretion to regulate epithelial colonic permeability

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Miró L, Pérez-Bosque A, Maijó M, Amat C, Naftalin RJ, Moretó M. Aldosterone induces myofibroblast EGF secretion to regulate epithelial colonic permeability. Am J Physiol Cell Physiol 304:C918–C926, 2013. First published March 6, 2013; doi:10.1152/ajpcell.00292.2012.—In vivo studies show that raised aldosterone (Aldo) during low-Na adaptation regulates the growth of pericryptal myofibroblasts and reduces the permeability of the colonic epithelium. The aim of this study was to reproduce in vitro the in vivo condition of increased Aldo using human CCD-18Co myofibroblasts and T84 colonic epithelial cells to measure myofibroblast and epithelial proliferation and the expression of intercellular junction proteins. Proliferation was quantified by measuring 5-bromo-2′-deoxyuridine incorporation. The myofibroblast expression of EGF, VEGFα, and transforming growth factor-β1 (TGF-β1) was measured by real-time PCR and the expression of junctional complex proteins by Western blot. Aldo stimulated the proliferation of myofibroblasts by 70% (P < 0.05) and increased EGF mRNA expression by 30% (P < 0.05) without affecting VEGFα and TGF-β1. EGF concentration in the incubation medium increased by 30% (P < 0.05) 24 h after Aldo addition, and these effects were prevented by the addition of spironolactone. Myofibroblast proliferation in response to Aldo was mediated by EGF receptor (EGFR) and involved both MAPKK and phosphatidylinositol 3-kinase pathways. When T84 cells were incubated with medium from myofibroblasts stimulated with Aldo (conditioned medium), the expression of β-catenin and claudin IV was increased by 30% (P < 0.05) and proliferation by 40% (P < 0.05). T84 proliferation decreased when α-EGF, or the EGFR antagonist AG1478, was present. Results in vivo indicate that rats fed a low-salt diet showed an increased expression of EGF and EGFR in the colonic mucosa. These results support the view that changes in colonic permeability during low-Na adaptation are mediated by the EGF secreted by myofibroblasts in response to raised Aldo.

aldosterone; EGF; proliferation

THE CRYPTS IN THE DESCENDING colon can absorb fluid despite a high hydraulic resistance in the lumen. The capacity to absorb a hypertonic absorbate requires Na pump activity and a physical barrier to Na movement from the pericryptal space into the crypt mucosa (19). This physical barrier is called the pericryptal sheath and is made up of a network of myofibroblast-like cells and the extracellular matrix surrounding the colonic crypts (13, 19, 22).

Low-Na intake activates the renin-angiotensin-aldosterone system (RAAS), which increases both angiotensin II (ANG II) and aldosterone (Aldo) plasma concentration. Activation of the RAAS increases colonic fluid and Na absorption (31). These effects are partly due to the upregulation of the epithelial sodium channel (ENaC) and changes in the barrier function of the pericryptal sheath, as has been shown in both rat and mouse descending colonic crypts (18, 32). Within 3 days of transition from high-Na to low-Na, the rat colonic crypt wall becomes less permeable to dextran and the pericryptal space can accumulate a much higher Na concentration (17). Vasopressin also has trophic effects on the rat distal colon, increasing pericryptal myofibroblast growth, and these effects are independent of Aldo (5).

Experiments conducted on adrenalectomized rats on low-Na and high-Na diets, implanted with osmotic pumps perfusing either Aldo or ANG II, showed that Aldo acts as a trophic agent on the myofibroblast layer and that ANG II has no direct role in this process (4, 17). Low-Na adaptation increased the expression of α-smooth muscle actin, specific adhesion molecules such as OB-cadherin, and collagen IV in the pericryptal sheath (4, 17, 33), and also resulted in functional changes in the crypt epithelium, including increased expression of junctional proteins such as claudin IV and E-cadherin. These effects were mediated by the mineralocorticoid receptor (MR) because spironolactone (SPI) prevented the effects of Aldo (4, 17).

This study focuses on the mechanisms of the effects of Aldo on colonic absorptive function during low-Na adaptation. We wanted to ascertain whether the effects on pericryptal sheath growth and epithelial permeability observed in vivo were directly attributable to Aldo or indirectly through mediators delivered by either the epithelial mucosa or myofibroblasts in response to Aldo regulation. Since the myofibroblast population of the pericryptal sheath and the crypt epithelium of the distal colon are targets of Aldo, we used an in vitro cell culture model with the human cell line CCD-18Co, which has the morphology of myofibroblasts, and the human T84 epithelial colonic cell line, which grows in monolayers, thus reproducing most of the characteristics of the colonic epithelium (7). The results indicate that RAAS activation has a dual effect on epithelial permeability: direct regulation by Aldo of pericryptal sheath growth and an indirect effect of Aldo on epithelial permeability, mediated by the EGF generated by the myofibroblasts surrounding the crypt.

MATERIALS AND METHODS

Chemicals and Growth Factors

Sodium bicarbonate, sodium pyruvate, nonessential amino acid solution, penicillin, streptomycin, FBS, trypsin, DMEM/F-12, and MEM were obtained from Gibco (Grand Island, NY). Aldo, SPI (an MR blocker), AG1478 [a blocker of the EGF receptor

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(EGFR), 5-bromo-2′-deoxyuridine (BrdU), protease inhibitor cocktail, PD98059 (a selective inhibitor of MAPKK), and LY249002 [a phosphatidylinositol 3-kinase (PI3K) inhibitor] were obtained from Sigma (St. Louis, MO). EGF and anti-human EGF (α-EGF) were obtained from Peprotech (London, UK).

**Cell Lines**

Human colonic fibroblasts CCD-18Co were obtained from American Type Culture Collection (ATCC, Manassas, VA) at passage 7, although they were used from passages 12 to 16. These cells were grown in MEM with 17.8 mM sodium bicarbonate, 1 mM sodium pyruvate, 0.1 mM nonessential amino acid solution, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS. Human colonic carcinoma T84 cells were also obtained from ATCC at passage 52, and cells were grown in 50% DMEM and 50% Ham’s F-12 supplemented with 15 mM HEPES, 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Both cell lines were serially passaged using 0.25% trypsin and 1 mM EDTA. Cell cultures were kept in continuous culture in a temperature- and humidity-controlled incubator at 37°C with 95% air-5% CO₂. The experiments for the study of cell proliferation were performed on coverslips in plates of 24 wells, and the expression of tight junctional proteins and RNA extraction was done in cells seeded in plastic plates.

**Animals**

Adult male Sprague-Dawley rats were supplied by Harlan Ibérica (Barcelona, Spain) and weighed 200–250 g on the day of the experiment. They were housed under a 12:12-h light-dark cycle, and food and water were available ad libitum. Experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona and the Catalan government and comply with the European Union regulation on animal experimentation. The protocol was similar to that described previously (17). All animals received a high-Na diet (wheat and barley and 150 mM NaCl in drinking water) for 4 days. Animals were divided into three groups: the HS group, which contained animals fed a high-Na diet; the LS group, which contained animals switched to a low-Na diet (0.15 mM NaCl in drinking water); and the LS + SPI group, which contained rats fed a low-Na diet and administered with spironolactone (10 mg·kg⁻¹·day⁻¹, by gavage).

**Treatment**

Myofibroblasts were seeded at 20,000 cells/cm². Seven days after seeding, cells were treated with Aldo 100 nM (Aldo group) dissolved in a medium with 0.2% ethanol. Control group (CTL) cells were incubated with medium containing 0.2% ethanol. In the Aldo + SPI group, myofibroblasts were incubated with Aldo and SPI and the SPI was dissolved in the medium with 0.2% ethanol to a final concentration of 10 μM. SPI was added 45 min before Aldo was added to the medium. Each treatment lasted for either 18 or 24 h, depending on the sample type (RNA isolation or immunohistochemistry, respectively).

Exogenous EGF dissolved in the medium was added to a final concentration of 50 ng/ml. When myofibroblasts were treated with inhibitors, AG1478 (5 μM, dissolved in DMSO:MEOH, 1:1), PD98059 (10 μM, dissolved in DMSO), LY249002 (10 μM, dissolved in DMSO), or α-EGF (0.5 μg/ml dissolved in medium) was added 45 min before the incubation was started with Aldo. Twenty-four hours before hormone treatments, FBS was removed from the incubation medium.

**Immunohistochemistry**

BrdU was used for the quantification of proliferating cells. Proliferation studies were carried out when the cells were near 70% confluence. BrdU was added to the culture media at the same time as the hormones, and its incorporation was quantified using immunohistochemistry, as previously described (20) but with some modifications. Slides were fixed in 4% paraformaldehyde at room temperature and maintained at 4°C with PBS. Afterwards, they were washed with PBS, denatured with 4 M HCl, and then neutralized with sodium tetraborate 0.1 M. The primary antibody used was anti-BrdU (dilution 1/1,000, monoclonal antibody; Sigma). The slides were incubated with a secondary anti-mouse Alexa 488 antibody (Molecular Probes, Eugene, OR), counterstained with nuclear marker Hoechst 33258 (Calbiochem, Darmstadt, Germany), and mounted in Mowiol (Calbiochem). Negative controls were performed without the primary antibody. The samples were stored at 4°C until observed with an Olympus microscope. Proliferation was quantified using ImageJ software (http://imagej.nih.gov/ij/), and the results were expressed by the percentage increase of total cells numbers.

**Determination of the Concentration of EGF Protein in the Cell Culture Medium**

The concentration of EGF present in the medium after CCD-18Co incubation with Aldo for 24 h was determined using a commercial EGF FlowCytomix Simplex kit (Bender MedSystems, Vienna, Austria). The assay was performed in accordance with the manufacturer’s instructions. The detection limit for this kit was 22.5 pg EGF/ml.

**Fig. 1.** CCD-18Co cell proliferation after aldosterone (Aldo) treatment. A: representative images of the proliferation of myofibroblasts CCD-18Co after addition of Aldo (100 nM) or Aldo + spironolactone (SPI; 10 μM) for 24 h [SPI (10 μM) was added 45 min before the addition of Aldo (100 nM)]. Top row shows the cells that have proliferated [5-bromo-2′-deoxyuridine (BrdU⁺)]; middle row shows the total cells (stained by Hoechst); in bottom row the yellow spots are the results of merging both stains. B: quantification of cell proliferation of CCD-18Co after Aldo (100 nM) incubation. Results are expressed by the percentage of cell proliferation. Results are expressed as means ± SE of 6–9 replicates for each experimental condition, and each experiment was repeated at least 3 times. Means without a common letter differ: a,bP < 0.05.
stimulation with Aldo (100 nM) for 24 h. Results are expressed as means
the addition of Aldo (100 nM).
incubated with Aldo (100 nM) for 18 h. SPI (10

Triton X-100, and 2% vol:vol of protease inhibitor cocktail (Sigma).
buffer containing 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1% vol:vol
homogenizing the cells. The cell lysing buffer composition was as
a 24- or 48-h incubation with the CM was obtained by scraping and
analysed by Western blot. The total cellular fraction of T84 cells after
the treatments were applied. Cell proliferation and tight junction
expression were studied using a conditioned medium (CM) from
the CM had been incubated with Aldo or Aldo + SPI. The
CM was collected and added directly to T84 cells for 24 or 48 h.

Western Blot Analysis

In vitro experiments. The experiments to determine the expression
of junctional proteins were carried out 15 days after seeding the cells,
onece they had formed a consistent and stable monolayer (100% confluence). EGFR, phosphorylated EGFR (phosphorylation of Tyr
1197), claudin IV, E-cadherin, and β-catenin protein expression were
analysed by Western blot. The total cellular fraction of T84 cells after
a 24- or 48-h incubation with the CM was obtained by scraping and
homogenizing the cells. The cell lysis buffer composition was as follows: 50 mM HEPES, 1% (vol/vol) Triton X-100, 0.2% (vol/vol)
sodium deoxycholate, 0.1% (vol/vol) SDS, 150 mM NaCl, 1.5 mM
MgCl2, 1 mM EGTA, and a cocktail of protease inhibitors (2% vol/vol).

In vivo experiments. The descending colon was rapidly excised, and
the contents were removed by washing with buffer. Mucosal
sheets scraped from the underlying serosa and samples were stored at
−80°C. The samples of colonic mucosa were homogenized in lysis
buffer containing 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1% vol:vol
Triton X-100, and 2% vol:vol of protease inhibitor cocktail (Sigma).

The homogenate was centrifuged at 4°C at 14,000 g for 10 min, and
EGFR and phospho-EGFR were determined in the supernatant. The
protein concentration of the supernatant was determined using the
Bradford method (Bio-Rad). Blots were incubated with a primary
antibody of rabbit polyclonal anti-β-catenin at a dilution of 1:2,000
(NeoMarkers, Fremont, CA), mouse monoclonal anti-claudin-IV at a
dilution of 1:3,000 (Zymed Laboratories, South San Francisco, CA),
rabbit polyclonal anti-E-cadherin at a dilution of 1:2,000 (Santa Cruz
Biototechnology), rabbit polyclonal anti-EGFR and anti-phosphorylat-
ed-EGFR at a dilution of 1:500 (Signalway Antibody, Pearlnd, TX),
and mouse monoclonal anti-β-actin at a dilution of 1:10,000 (Sigma)
onight at 4°C. The blots were measured using WesternDot 625
Western blot kits (Life Technologies, Paisley, UK). The assay was
carried out in accordance with the manufacturer’s instructions. After
their detection, hybridization bands were quantified using ImageJ gel
analyzer software.

RNA Extraction and Real-Time PCR

The total RNA was extracted with TRIzol reagent (Life Technol-
ologies) following the manufacturer’s instructions. The extraction and
retrotranscription of RNA were carried out as previously described
(21). Real-time PCR was determined using a template of cDNA in a
20-μl reaction containing 0.2 μmol/l of each primer and 2 SyBr
GreenER SuperMix (Life Technologies). The human primers used
were as follows: EGF forward, 5′-CTT GTC ATG CTG CTC CTG-3′
and EGF reverse, 5′-TGC GAC TCC TCA CAT CTC TGC-3′ (fragment size 118 bp); transforming growth factor-β (TGF-
β1), forward 5′-TGC GGC AGC TGT ACA TTG A-3′ and TGF-β1

**Fig. 2. Expression of growth factors in CCD-18Co after Aldo treatment.** A: mRNA expression of VEGFa. B: mRNA expression of transforming growth factor-β (TGF-β1). C: mRNA expression of EGF. Myofibroblasts were incubated with Aldo (100 nM) for 18 h. SPI (10 μM) was added 45 min before the addition of Aldo (100 nM). D: concentration of EGF in the medium after stimulation with Aldo (100 nM) for 24 h. Results are expressed as means ± SE (n = 6; representative of 3 different experiments). Means without a common letter differ: a,b, P < 0.05.

**Effect of Conditioned Medium on Epithelial Cell Line**

Colonocytes were seeded at concentrations of 10⁵ cells/cm² in 12-
or 24-well plates. Cells were incubated without FBS for 24 h before
the treatments were applied. Cell proliferation and tight junction
expression were studied using a conditioned medium (CM) from
CCD-18Co that had been incubated with Aldo or Aldo + SPI. The
CM was collected and added directly to T84 cells for 24 or 48 h.

**Western Blot Analysis**

In vitro experiments. The experiments to determine the expression
of junctional proteins were carried out 15 days after seeding the cells,
onece they had formed a consistent and stable monolayer (100% confluence). EGFR, phosphorylated EGFR (phosphorylation of Tyr
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homogenizing the cells. The cell lysis buffer composition was as follows: 50 mM HEPES, 1% (vol/vol) Triton X-100, 0.2% (vol/vol)
sodium deoxycholate, 0.1% (vol/vol) SDS, 150 mM NaCl, 1.5 mM
MgCl₂, 1 mM EGTA, and a cocktail of protease inhibitors (2% vol/vol).

In vivo experiments. The descending colon was rapidly excised, and
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buffer containing 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1% vol:vol
Triton X-100, and 2% vol:vol of protease inhibitor cocktail (Sigma).

**Fig. 3. Proliferation of CCD-18Co exposed to Aldo.** A: cells were incubated
with Aldo (100 nM) or EGF (50 ng/ml) for 24 h. The antibody α-EGF (0.5
μg/ml) and the EGFR receptor (EGFR) inhibitor AG1478 (5 μM) were added 45
min before the addition of Aldo. B: CCD-18Co were preincubated with a
highly selective inhibitor of MAPKK (PD98059, 10 μM) and with the PI3K
inhibitor (LY294002, 10 μM) were added 45 min before the addition of Aldo. Results are
expressed as the percentage of cell proliferation; means ± SE (n = 6;
representative of 3 different experiments). Means without a common letter differ: a,b, P < 0.05. PD, PD98059; LY, LY294002.
RESULTS

Aldo Effects on Myofibroblasts

We first demonstrated by RT-PCR that CCD-18Co myofibroblasts express MR (not shown). Incubation with Aldo increased CCD-18Co proliferation by 70% (P = 0.05; Fig. 1B), and preincubation with SPI prevented Aldo-stimulated proliferation. Treatment with SPI alone did not have any effect on this variable (data not shown).

Aldo increased the mRNA expression of EGF by 30% (P < 0.05; Fig. 2C). Preincubation of cells with SPI prevented the effects of Aldo. The mRNA expression of VEGFa (Fig. 2A) and TGF-β1 (Fig. 2B) was unaffected by Aldo and the Aldo + SPI treatment.

The incubation of CCD-18Co myofibroblasts with Aldo increased the release of EGF by 30% (P < 0.05; Fig. 2D). When myofibroblasts were preincubated with SPI, the concentration of this protein in the medium was similar to that of the CTL group.

Pathways Involved in the Effects of Aldo on CCD-18Co

In preliminary experiments we checked that CCD-18Co cell express EGFR (not shown). When myofibroblasts were incubated with exogenous EGF they showed increased proliferation (P < 0.05; Fig. 3A) and the results were similar to those observed in Aldo-incubated cells (P < 0.05). Preincubation of CCD-18Co with anti-EGF or AG1478 before the addition of Aldo prevented the effects of Aldo (Fig. 3A). Myofibroblasts

Determination of Aldo in Serum

Blood was collected by cardiac puncture at the end of the experimental period to obtain serum that was stored at −80°C for subsequent analyses. The concentration of Aldo was determined by radioimmunoassay using a commercial kit (RIA aldosterone kit; Immuno-tech, France).

Statistical Analysis

Data are presented as means ± SE. Data shown are representative of three different experiments. Each experiment was carried out using six to nine different wells for each condition, and it was repeated three times. Statistical analysis of data was performed using SPSS-17 software, which assessed the effect of different treatments by studying the variance of one factor (ANOVA), following Scheffé’s method in the case of homogeneous variances or the Tamhane test in the case of nonhomogeneous variances. Data were considered significant at P < 0.05.
incubated with AG1478 or anti-EGF alone did not have any
effect on cell proliferation (data not shown).

The results also showed that incubation with inhibitors of the
Ras/Raf/MAPK or PI3K/Akt pathways prevented proliferation
in CCD-18Co in response to incubation with Aldo (Fig. 3B).
Treatment with both inhibitors at the same time in myofibro-
blasts had similar proliferation values to the CTL group.

**Incubation of T84 Cells with CM**

Direct addition of Aldo to T84 cells did not change the
proliferation or the expression of junctional complex proteins
(data not shown). However, incubation of T84 cells with CM
obtained from CCD-18Co treated with Aldo for 24 h showed a
significant increase in proliferation ($P < 0.05$; Fig. 4A). This
effect was not observed when SPI was present in the medium.

When colonocytes were incubated with CM from cells
incubated with Aldo for 48 h. The expression of E-cadherin
was not affected (Fig. 4E), but the expression of $\beta$-catenin
increased by 30% ($P < 0.05$; Fig. 4C) and that of claudin IV
by 24% ($P < 0.05$; Fig. 4D). These effects were prevented by
the addition of SPI. The group incubated with the CM con-
taining SPI gave similar results to those of the CM-CTL group
(data not shown).

Incubation of colonocytes with exogenous EGF at 10, 20,
50, and 100 ng/ml increased proliferation in a dose-response
profile ($P < 0.05$; Fig. 5A). The addition of exogenous EGF to
the CM increased the proliferation of T84 cells ($P < 0.05$; Fig.
5B). This effect was similar to that observed in response to
Aldo (proliferation increased by 28% in both the CM-EGF and
CM-Aldo groups). Incubation of T84 cells with $\alpha$-EGF or
AG1478 reduced proliferation to similar levels observed in the
CM-CTL group.

**Results In Vivo**

Once adapted to the HS diet, animals were either kept on the
HS diet or switched to the LS diet and killed in the following
24, 48, or 72 h. The expression of EGF and EGFR in the colon
mucosa and the concentration of Aldo in serum samples were
analyzed. The concentration of Aldo in the HS groups re-
mained low and always below 0.2 nM ($n = 9$), but it was
increased after 24 h (0.83 ± 0.22 nM; $n = 6$), 48 h (0.87 ±
0.20 nM; $n = 6$), and 72 h (1.16 ± 0.30 nM; $n = 8$) on the LS
diet (all $P < 0.05$ vs. the HS groups). The expression of EGF
was increased 17-fold in the LS 24 group ($P < 0.05$) and 5-fold
in the LS 48 group, and both effects were prevented by the
addition of SPI. However, this effect was no longer evident in
the LS 72 group (Fig. 6A). Expression of EGFR and the
phosphorylated form of EGFR was increased 88 and 54%,
respectively, in the LS 24 animals ($P < 0.05$) and was also
prevented by the addition of SPI (Fig. 6, C and D).

**DISCUSSION**

Low-Na adaptation stimulates Na resorption in the kidney
and in the distal intestine and activates the growth of peric-
ryptal myofibroblasts, and these effects are attributed to the
RAAS. There is evidence that ANG II and TGF-$\beta$ are involved
in myofibroblast activation and extracellular matrix deposition
in several tissues (22). However, studies conducted on rats
perfused with ANG II and Aldo, subjected to both low-Na and
high-Na conditions, support the view that increased Aldo alone
can stimulate myofibroblast growth and reduce paracellular
and pericytbral sheath permeability (4, 17).

This study analyzed the mechanism of action of Aldo in the
adaptive changes that take place in the distal colon during
low-Na adaptation, using cell lines of human colonic fibro-
blasts and epithelial cells. In some tissues, Aldo acts directly
through MR while, in others, Aldo stimulates the production
of cellular products that behave as mediators of Aldo action. For
example, the inflammatory effects of Aldo are mediated by
Aldo-induced cytokines and the profibrotic effects of Aldo in
the kidney are in part mediated by TGF-$\beta$ (2). Therefore, the
morphological and functional changes that take place in the
mucosa of the distal colon during low-Na adaptation may
involve direct and indirect effects of Aldo.

Our working hypothesis was that the myofibroblasts sur-
rounding colonic crypts are the main cell target for Aldo,
which stimulates their proliferation and therefore results in a
thicker and tighter pericytbral sheath. Moreover, the sheath,
which is anatomically close to the epithelium (22), may even-
tually regulate its permeability through a mechanism involving
both stimulation of epithelial growth and the expression of
intercellular adhesion molecules.

The experiments were conducted using two human colonic
cell lines, the T84 line, with an epithelial phenotype, and the
CCD-18Co fibroblast/myofibroblast cell line. T84 cells were
characterized by the expression of typical markers involved in
the control of epithelial permeability, such as ENaC, claudin
IV, β-catenin, E-cadherin, and zonula occludens 1 (23), and they grew to confluence with stable and reproducible permeability properties. Some reports indicate that T84 cells do not express MR (34), while others demonstrate that MR is indeed present (9). When T84 cells were incubated with butyrate, the expression of ENaC subunits increased, thus confirming the previous findings of Fukushima et al. (8) and demonstrating that butyrate can induce the expression of both MR and α- and γ-ENaC in T84 cells. When mounted in monolayers in Ussing chambers, the T84 monolayer can generate a short-circuit current, albeit low (16). However, although the T84 cells expressed MR, they did not respond to Aldo since no changes in ENaC expression, cell proliferation, or epithelial junctional protein expression were observed. This may mean that Aldo has no direct effect on this cell line or that MR is not functional.

Alternatively, some of the effects may be indirect, i.e., mediated by local signals released by myofibroblasts in response to Aldo. This hypothesis was checked in CCD-18Co fibroblasts, a cell line that expresses structural proteins such as α-smooth muscle actin, vimentin, and cadherin 11, as previously shown by Simmons et al. (27), and also expresses MR (this study). Our results show that Aldo stimulates myofibroblast proliferation and that this effect is mediated by MR, as it can be blocked by SPI. These findings indicate that the in vitro model reproduces the effects of Aldo on pericryptal growth observed in vivo (4, 18). Since colonic myofibroblasts regulate important functions of epithelial cells via distinct secreted cytokines and autacoids (22), the next step was to study the secretory response of the CCD-18Co cell line to Aldo stimulation. From the many candidates that may mediate the myofibroblast-epithelial interaction, we decided to study TGF-β1 [since it is secreted by primary adult human colonic subepithelial myofibroblasts and enhances barrier function (1) and it is also secreted in response to radiation injury (30)]; VEGF [since it plays a role in epithelial repair and is secreted by CCD-18Co under hormonal regulation (3)]; and EGF [since it is indispensable in the activation of intestinal epithelial cell proliferation (29)].

Of the three candidates, Aldo stimulated only EGF expression. The lack of effect on TGF-β expression was unexpected because this cytokine is an important mediator of hormonal stimulation of fibroblast proliferation (2, 25, 26). Incubation of myofibroblasts with exogenous EGF resulted in proliferation similar in magnitude to that observed when cells were incubated with Aldo. When EGF-neutralizing antibodies were included in the medium, the effects of Aldo on proliferation were abrogated, and when cells were incubated with an EGFR blocker in the presence of Aldo, the proliferative effects of Aldo were again prevented. These results support the view that the mechanism of action of Aldo on myofibroblast proliferation is mediated by MR and requires both EGF secretion and EGFR activation. These findings constitute new evidence for Aldo interaction with peptide hormone signaling, as previously shown by Aldo-stimulated mesangial cell proliferation (12) and for cardiac Na/H exchanger expression (6). Our findings confirm that Aldo leads to enhanced EGFR expression via interaction with the EGFR promoter, as previously shown by Grossmann et al. (10) in the Madin-Darby canine kidney C7 cell line. The phosphorylated form of EGFR, although present, could not be quantified because the amount collected from the CCD-18Co cells was too low to enable accurate analysis. These in vitro results imply that increased ANG II and Aldo in vivo stimulate proliferation through independent pathways:

Fig. 6. EGF expression in rat colonic mucosa

A: mRNA expression of EGF. B: representative images of phosphorylated (P)-EGFR and EGFR by Western blot. C: quantification of total EGFR in rat colonic mucosa by Western blot. D: quantification of phosphorylated form of EGFR in rat colonic mucosa by Western blot. Rats were fed with a high-Na diet (HS), a low-Na diet (LS) or an LS diet + spironolactone (LS + SPI). Animals were killed at 24, 48, and 72 h after switching to the low-Na diet. Results are expressed as means ± SE (n = 4). Means without a common letter differ: P < 0.05.
ANG II-inducing TGF-β and its downstream mediators resulting in matrix accumulation, inflammation, and apoptosis (24), and Aldo-inducing EGF, promoting myofibroblast and fibroblast growth and proliferation. Recently, a similar conclusion has been reached that Aldo stimulates ENaC expression in rat distal colon via the MR but not the glucocorticoid receptor whereas it stimulates an increase in SGK-1 via the glucocorticoid receptor (11).

MAPKs play an important role in cell growth and differentiation. Aldo stimulates proliferation in different cell types, such as renal M1-CCD (14) and mesangial cells (12). In all cases, preincubation with the MAPKK inhibitor PD98059 blocked proliferation. Our results also demonstrate that Aldo-induced myofibroblast proliferation depends in part on extracellular signal-regulated kinases (ERK) 1/2 phosphorylation, since blocking ERK1/2 activation with PD98059 inhibited Aldo-induced cell proliferation. Another signaling pathway involved in Aldo-induced mesangial cell proliferation (12) is the PI3K cascade. PI3K mediates various cellular processes such as proliferation and survival (15). The present study shows that the PI3K inhibitor LY294002 completely blocks the Aldo-induced CCD-18Co growth, which indicates the importance of this pathway in myofibroblast proliferation. Our results indicate that Aldo has a role in EGFR activation with the resulting CCD-18Co proliferation blocked by the EGFRI kinase inhibitor AG1478.

A further step was to determine whether the incubation medium from Aldo-stimulated CCD-18Co cells (the CM) affected the growth and function of T84 cells. As mentioned above, T84 cells did not respond directly to incubation with Aldo. However, when they were incubated with CM from CCD-18Co, there was an increase in T84 proliferation. This effect was not observed when SPI was present during CCD-18Co incubation with Aldo. Therefore, a product resulting from stimulation by Aldo of myofibroblasts and present in the CM induced the expression of β-catenin and claudin IV in T84 cells. This effect was not observed when the medium was obtained from CCD-18Co cells simultaneously incubated with Aldo and SPI. Furthermore, the CM from myofibroblasts incubated with Aldo resulted in a potent stimulation of T84 proliferation. This stimulation of T84 cell proliferation by Aldo was blocked by α-EGF and the EGFR kinase inhibitor AG1478.

These findings support the view that EGF is the key product in the CM secreted by myofibroblasts in response to Aldo. This

![Proposed model for the mechanism of action of Aldo on myofibroblast and colonocyte proliferation.](http://ajpcell.physiology.org/)
demonstrates the existence of a TGF-β-independent, EGFR-mediated pathway for Aldo-induced proliferation and the resultant functional increase in transepithelial electrical resistance.

The aim of the in vivo experiments was to confirm that the mechanisms observed in vitro were also present in vivo. The low sodium intake induced a secondary aldosteronism, within 24 h after switching to the low-Na diet, as previously described (5, 17). Aldo stimulated the expression of EGF and EGFR in the colon mucosa at 24 and 48 h but not after 72 h. This indicates that the change in EGF expression occurs transiently, shortly after switching to the low-Na diet. The phosphorylated form of EGFR was also increased only in the LS 24-h group. These results suggest that the Aldo-MR-EGFR pathway has a regulatory role in vivo as observed in vitro and are consistent with the observations of Sinphitukkul et al. (27), demonstrating that Aldo can induce EGFR phosphorylation in the rat kidney.

In conclusion, the results of the present study support the view that the effects of Aldo on distal colon crypts involve both direct and indirect mechanisms. Our findings are summarized in Fig. 7: Aldo acts directly on myofibroblasts through a pathway involving EGFR transactivation, resulting in cell proliferation (hence pericryptal growth). Aldo also stimulates EGF secretion. The effects of Aldo on crypt epithelium are indirect, mediated by EGF secreted by myofibroblasts in response to Aldo, resulting in stimulated cell proliferation and expression of junctional and adhesion molecules. Although these conclusions come from in vitro studies, in vivo results indicate that Aldo-induced EGF secretion is involved in the mucosal response to low-Na adaptation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
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