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Pericryptal Myofibroblast Growth in Rat Descending Colon Induced by Low-Sodium Diets Is Mediated by Aldosterone and not by Angiotensin II

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Abstract. Pericryptal myofibroblast growth in descending colonic crypts correlates with the activation of the renin-angiotensin-aldosterone system. Earlier work showed that during the transition from a high-Na^+ (HS) to low-Na^+ (LS) diet there are changes in the colonic crypt wall and pericryptal sheath. As LS diet increases both aldosterone and angiotensin II, the aim here was to determine their individual contributions to the trophic changes in colonic crypts. Experiments were conducted on control and adrenalectomized Sprague-Dawley rats fed an HS diet and then switched to LS diet for 3 days and supplemented with aldosterone or angiotensin II. The actions of the angiotensin-converting enzyme inhibitor captopril, the angiotensin receptor antagonist losartan and the aldosterone antagonist spironolactone on extracellular matrix proteins, claudin 4 and E-cadherin myofibroblast proteins, α-smooth muscle actin (α-SMA) and OB-cadherin (cadherin 11), angiotensin type 1 and TGFβr1 membrane receptors were determined by immunolocalization in fixed distal colonic mucosa. The LS diet or aldosterone supplementation following ADX in HS or LS increased extracellular matrix, membrane receptors and myofibroblast proteins, but angiotensin alone had no trophic effect on α-SMA. These results show that aldosterone stimulates myofibroblast growth in the distal colon independently of dietary Na^+ intake and of angiotensin levels. This stimulus could be a genomic response or secondary to stretch of the pericryptal sheath myofibroblasts accompanying enhanced rates of crypt fluid absorption.

Key words: Aldosterone — Angiotensin II — Colon — Myofibroblast — Fibrosis

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Introduction

A layer of myofibroblasts containing smooth muscle actin surrounds colonic crypts (Kaye, Lane & Pascal, 1968). These cells generate collagen IV and are held together predominantly by intercellular adhesion molecules, e.g., OB-cadherin (cadherin 11) (Danjo & Gipson, 1998; Hinz et al., 2004), which forms a link with the cytoskeletal smooth muscle actin (α-SMA) (Gabbiani & Badonnel, 1976) via adherens junctions in the cell membrane (Taliana et al., 2005). This cell layer has been shown to form a permeability barrier both to NaCl and dextran and to contribute to the accumulation of NaCl within the space lying between the crypt cell basal membranes and the sheath (Naftalin, Zammit & Pedley, 1995). In conditions leading to breakdown of this layer in acute post-irradiation (β or γ) colitis, when myofibroblasts apoptose within 6–12 h, or following high-sodium (HS) diet, when there is also loss of myofibroblasts, the crypts become leaky and are unable to generate a hypertonic absorbate or dehydrate feces (Thiagarajah et al., 2000; Naftalin, 2004; Moretó et al., 2005).

Colonic epithelial cells are held together mainly by the homophilic intercellular adhesion molecule, E-cadherin, via catenins at the adherens junctions (Takeichi, 1991). Tissue growth factor β (TGFβ) promotes synthesis of E-cadherins and integrins, which attach cells to the extracellular matrix at the basement membrane. Expression of both E-cadherin and integrins depends on the cells making contact with extracellular fibronectin and laminin, which activates protein kinase C, thereby maintaining the positive feedback cycle between the extracellular signal from cell contacts and the cell metabolism which maintains these contacts (Wang & Chakrabarty, 2001). Thus, the integrity of the mucosal barrier depends on the interactions between cellular and extracellular elements termed collectively cell

adhesions and cytokine stimulation cell membrane receptors (Wang et al., 2004).

Low NaCl concentrations at the macula densa segment of the renal distal tubule activate the renin-angiotensin-aldosterone system (RAAS) by increasing renin secretion, thereby inducing angiotensin I and angiotensin II (ANG II) synthesis and stimulating aldosterone (ALDO) synthesis and release in the adrenal zona glomerulosa cells (Peart, 1969). This increases both ANG II and ALDO plasma levels. Activation of RAAS by a low-sodium (LS) diet causes increased colonic fluid and Na^+ absorption (Thiagarajah et al., 2001). These effects are partly due to upregulation of the epithelial Na^+ conductance channel (ENaC) and also to changes in the barrier function of the layered structure of myofibroblasts in the pericryptal sheath, as shown in both rat (Naftalin & Pedley, 1999) and murine (Thiagarajah, Pedley & Naftalin, 2001) descending colonic crypts. Within three days of transition from HS to LS diet, the rat colonic crypt wall becomes less permeable to dextran and a much higher Na^+ concentration accumulates in the pericryptal space (Moretó et al., 2005). Retention of a high Na^+ concentration within the pericryptal space also requires that it does not drain too quickly either into the capillary circulation in the submucosa or by reflux via the paracellular route to the crypt lumen.

In cardiac tissue, RAAS activation increases periarteriolar fibrotic tissue (Weber, Sun & Katwa, 1997). This leads to myocardial hypertrophy and subsequent heart failure. The primary cause of the increased myocardial fibrosis is invasion of the damaged tissue by macrophages and transformation of fibroblasts to myofibroblasts; these cells in turn produce cytokines, TGFβ, endothelin 1, and connective tissue growth factor that further stimulate myofibroblasts to generate collagen (Campbell & Katwa, 1997). The temporo-spatial coincidence of angiotensin-converting enzyme (ACE) activity and ANG II type 1 receptors (ATr1) has been observed at sites of fibrosis (Sun, Ramires & Weber, 1997). High densities of ACE antibody-binding have been observed at repair sites one week after myocardial infarction (Falkenhahn et al., 1995; Mezzano et al., 2003). The presence and density of ATr1 and TGFβr1 receptors has been shown to be a key factor in development of fibrosis. In cardiac myofibroblasts, Campbell, Janicki & Weber (1995) and Campbell and Katwa (1997) observed that ANG II has trophic effects, increasing α-SMA expression after 2 days, and increasing collagen synthesis after 14 days of infusion. TGFβ released by myofibroblasts leads to further release of cytokines by the myofibroblasts, resulting in a positive feedback loop, which leads to overproduction of collagen by the stimulated myofibroblasts and prevention of apoptosis (Zhang & Phan, 1999)

Angiotensin-converting enzyme inhibitors do not suppress ALDO synthesis completely. Mineralocorticoid receptors (MR) have been detected in cardiac myocytes and endothelial cells (Lombèd et al., 1995) and ALDO infusion produced cardiac fibrosis that can be prevented by spironolactone (Brilla et al., 1994). This leads to cardiac remodeling and eventual failure (Soberman, Chafin & Weber, 2002). The RALES trial (Zannad et al., 2000), which evaluated the benefits of spironolactone therapy on congestive heart failure, showed that ALDO-receptor blockade decreased morbidity and mortality associated with excessive myofibroblast stimulation.

As in heart and kidney, RAAS-related events in the distal colon stimulate fibrosis by stimulating myofibroblast growth (Thiagarajah et al., 2002).

Currently the specific effect(s) of ANG II and ALDO on myofibroblast growth are unclear. Transformation of fibroblasts to myofibroblasts has been attributed both to ALDO and ANG II and more recently to synergism between low concentrations of ALDO and ANG II (Min et al., 2005).

The main purpose of this present study is to discriminate between the two possible modes of stimulation of the trophic changes in colon by the RAAS. This has been done in two steps, first by observing the effects of ANG II inhibition by ACE inhibition with captopril, or ATr1 antagonism with losartan, or with the ALDO antagonist, spironolactone. As the results from these early studies left some uncertainty about whether ALDO alone, or ALDO in combination with ANG II was responsible for the trophic effects on colon, a second series of experiments was undertaken in which adrenalectomy (ADX) with infusion of either ALDO, or ANG II post-ADX, to replace one or other missing hormones, was tested to determine the extent to which these hormones activated the trophic effects on their own. Specifically, to test whether the adaptive effects in colon following LS are due to raised ALDO or to raised ANG II and whether the effects of the two hormones are synergistic. Although the current results unequivocally show that aldosterone is the trophic agent and ANG II alone does not cause a positive trophic effect on the myofibroblast layer—it may induce a negative trophic effect—doubts remain concerning synergy between ALDO and ANG II or synergism between ALDO and other factors influencing myofibroblast development.

Materials and Methods

Experimental Animals

Studies were performed on adult male Sprague-Dawley rats (Harlan Ibérica, Barcelona, Spain) weighing 200–250 g the day of experiment. Animals were housed one per cage under 12:12-h light-dark cycle,

and food and water were available *ad libitum*. Experimental procedures were approved by the ethical committee of the Universitat de Barcelona. The following two protocols were undertaken.

Protocol 1: The effects of HS and LS Diets and Pharmacological Treatment Modifying These

Animals received an HS diet (wheat and barley and drinking water containing 150 mM NaCI). After 4 days, animals were changed to a LS diet (wheat and barley and drinking water containing 150 μM NaCl) for 3 days. When appropriate, rats received pharmacological treatment: captopril (CAP, ACE inhibitor; 65 mg/kg·day in drinking water), losartan (LOS, ATr1 receptor inhibitor; 30 mg/kg.day in drinking water) or spironolactone (SPI, ALDO antagonist; 10 mg/kg·day by oral gavage).

Protocol 2: The Effects of HS and LS Diets in Adrenalectomized Rats Followed by Selective Hormonal Replacements

Rats were adrenalectomized (ADX) via bilateral flank under isofluorane (Inibsa®, Spain) anaesthesia. The protocol performed was as described before (Moretó et al., 2005). Briefly, osmotic minipumps (model 2002, Alzet, Palo Alto, CA) were implanted subcutaneously in the upper back region following ADX procedures. Pumps were filled with D-aldosterone (Sigma) dissolved in propylene glycol or with ANG II (Sigma) dissolved in saline, to deliver 450 μg/kg/day or 288 μg/kg/day, respectively. This protocol led to chronic increases in the plasma concentrations of both hormones. Groups without hormonal replacement were implanted with pumps delivering vehicle.

After surgery the animals were kept on the HS diet for 4 days. Thereafter, half of the animals were changed to LS diet for 3 days and the other half continued with HS diet. With this protocol 6 groups with different hormone profiles are obtained: ADX HS + ALDO, ADX HS + ANG, ADX HS, ADX LS + ALDO, ADX LS + ANG and ADX LS.

Tissue Preparation

Rats from both protocols were maintained in metabolic cages for the last three days prior to sacrifice, and the daily 24-h urinary output, water intake, food consumption and body weight were measured as described (Moretó et al., 2005). The descending colon was rapidly excised and the contents removed by washing with buffer. Mucosal sheets scraped from the underlaying serosa were fixed (4% paraformaldehyde in PBS) at 4°C for 24 h. The tissue was then washed and stored in PBS at 4°C for immunocytochemistry.

Immunocytochemistry

Colonic mucosal tissues (0.5 cm^2 pieces) were placed in 1.5 mL Eppendorf tubes. Tissues were then stained according to the following protocol. The procedure was the same for all antibodies used. Tissues were permeabilized in 0.2% Triton X-100 in blocking buffer (1% BSA in PBS and glycine) for 30 min. Samples were washed three times in PBS and incubated for 90 min with primary antibody (1:100), washed 3× in PBS and then incubated for 60 min in each secondary antibody (1:100) and washed again 3× in PBS.

Antibodies

The antibodies were obtained from the following agencies: Chemicon: RDI rabbit anti-collagen Type IV, mouse anti-angiotensin converting enzyme (ACE), monoclonal and rabbit anti-ANG II Type 1 receptor; Sigma: monoclonal anti-α smooth muscle actin α-SMA clone IA4; Santa Cruz: rabbit anti-TGFβ receptor 1 (R-20), goat anti-OB cadherin (Cadherin-11). For ANG II type 1 receptor-, TGFβr1-, and collagen IV-stained tissue, biotin anti-rabbit IgG was used as the secondary antibody followed by Alexa-488 NeutrAvidin. For ACE- and anti-α SMA-staining, Texas Red goat anti-mouse IgG was used. All secondary antibodies and Alexa-488 were obtained from Molecular Probes (Eugene, OR); goat anti-cadherin-E (human/mouse/rat), from Research Diagnostics (RDI, UK).

Confocal Images

Each piece of tissue was viewed in a Leica SPII confocal microscope using a 20 × oil immersion lens (Barcelona University), or using a Nikon Diaphot inverted microscope with Nikon Fluor 20× and 60× lenses attached to a BioRad MRC 600 confocal scanhead, equipped with two detection channels and an Ar/Kr mixed-gas laser allowing excitation at 488 nm and 568 nm (King's College London). *Z*-Axis movement with 0.1 μm resolution was provided by a software-controlled stepper motor attached to the fine focus control. The tissue was viewed from the mucosal side. The focus plane was initially taken to the surface of the tissue and images were captured at 5 μm steps from 0 μm down to 40–100 μm below the surface. The chosen images represented as much as possible the general level of staining throughout the whole tissue and were captured with the same optical conditions, gain and section size. Because the intensity of the image fluorescence varies with depth and because of slight tissue folding, the exact depth of the *Z*-plane cannot be accurately quantified to better than 5–10 μm; for this reason average intensity projection images in the *Z*-plane over the planes 5–40 μm were used to give comparability between samples from different pieces of tissue. This is easily done with colonic mucosa viewed *en face* since the crypts and their surrounding spaces lie perpendicular to the surface plane over a depth of at least 100 μm.

Image Analysis

The images were analyzed using ImageJ program (http://rsb.info.nih.gov/ij/index.html). Fluorescence was quantified by dividing the field into either "crypt" or "intercryptal" areas by selecting regions of interest (ROI) within the relevant part of the image by drawing around the crypts with the freehand drawing tool. Three areas of both crypt and intercrypt were taken per image. After background subtraction, the mean and SEM of measurements from each image were calculated.

Statistics

Data were expressed as means ± SEM. Comparisons between HS and LS diet were made by ANOVA using SPSS-10.0 software (SPSS). To analyze the effects of pharmacological treatment, groups were compared with the LS group by ANOVA, followed by Scheffé's *post-hoc* test. In ADX groups, the groups with ALDO or ANG II replacement were compared with the groups without supplementation by ANOVA followed by Scheffé's *post-hoc* test. Differences were considered statistically significant when the test yielded $P < 0.05$.

Results

Characteristics of the Model

The changes in body weight, food and water intake, physiological variables relating to the urinary func-

tion, plasma ion and hormone concentrations are described in the previous paper (Moretó et al., 2005). Briefly, the groups on HS and LS diets showed the expected marked differences in urinary Na^+ excretion and ALDO and ANG II plasma concentration. In ADX animals (Protocol 2), hormonal replacement raised circulating ALDO (13.6 ± 4.8 nM in ADX HS + ALDO group, $n = 6$; 12.4 ± 1.5 nM in ADX LS + ALDO group, $n = 6$) and ANG II (104 ± 23 pg/mL in ADX HS + ANG, $n = 5$; 116 ± 17 pg/mL in ADX LS + ANG, $n = 5$). The two adrenalectomized groups without hormone infusion had no detectable levels of plasma ALDO.

Effect of HS and LS Diet on the Pericryptal Sheath

The hypothesis that ANG II regulates colonic fibrosis predicts that the receptors mediating this effect would be increased by the LS diet. After 3 days LS diet, TGFβr1 and ATr1 expression in the pericryptal region is increased by 5-fold and 38-fold respectively. These increases were prevented by captopril. However, neither losartan, nor spironolactone blocked the increases in ATr1. Since neither of the latter treatments interferes with ANG II synthesis, whereas losartan blocks ALDO synthesis and spironolactone blocks ALDO binding to the MR receptor, it may be inferred that ANG II is solely responsible for the observed increase in ATr1 (Fig. 1).

Similar effects of LS diet following HS diet on ACE density were observed. Following HS diet, there is a significant increase in expression of ACE, which is significantly reduced in LS by captopril (Figure 2).

Hormonal Effects on Paracellular Route

Myofibroblasts in the pericryptal layers are held together by OB-cadherin (Hinz et al., 2004) and colonocytes by E-cadherin (Takeichi, 1991) and both adhesion molecules are required for anchorage to underlying cytoskeletal elements. Results show that the LS diet increases by 14-fold and 2.4-fold the expression of OB-cadherin and E-cadherin, respectively, compared with the HS diet ($P < 0.05$ in all cases, Table 1). Captopril blocked the expression of OB-cadherin induced by diet, but did not prevent the expression of E-cadherin.

Claudins are junctional proteins that participate in epithelial barrier function and regulate paracellular permeability (Van Itallie, Rahner & Anderson, 2001; Matter & Balda, 2003). The expression of claudin IV was increased by an LS diet compared with the HS diet. The three pharmacological treatments prevented this effect (Fig. 1). In ADX groups, infusion of ALDO increased the expression of crypt colonocyte claudin IV independently of the sodium content of the diet ($P < 0.01$ for ALDO perfusion vs zero ALDO for both HS and LS) showing that ALDO can regulate the colonocyte tight junction structure. ALDO has recently been shown to induce an early acute effect on phosphorylation of claudin IV in cultured renal collecting duct cells (Le Moellic et al., 2005). This change is accompanied by reduced mannitol permeability.

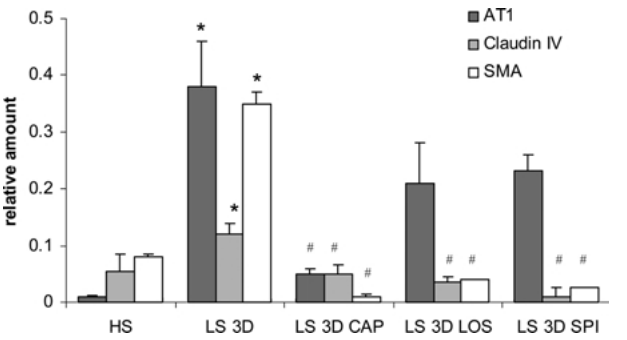

Fig. 1. Expression of ATr1, Claudin IV and α-smooth muscle actin (α-SMA) in control and pharmacologically treated groups. Values are means ± SEM ($n = 4$ animals). $^*P < 0.01$ HS *vs.* LS; $^\#P < 0.01$ LS *vs.* pharmacological treatment.

Effects on the Pericryptal Sheath Myofibroblasts Induced by Hormones

Once it was demonstrated in colon that expression of two receptors, TGFβr1 and ATr1, which activate fibrosis, ascribed to ANG II, was increased by the LS diet (Fig. 1), we decided to test whether ANG II had direct trophic effects on the pericryptal sheath similar to those reported in cardiac tissue (Campbell et al., 1995). Thus, expression of smooth muscle actin (α-SMA), a protein present in myofibroblasts (Gabbiani & Badonnel, 1976), and collagen IV, an exclusive myofibroblast product, was studied in the pericryptal sheaths of descending colonic crypts.

LS diet increases the amount of α-SMA in the pericryptal sheath by 4-fold (Fig. 1), while the fluorescence signal for collagen IV is increased 3-fold (Table 1). Captopril or losartan or spironolactone prevents the stimulatory effect of LS on α-SMA expression. In ADX animals, infusion of exogenous ALDO increased the amount of pericryptal α-SMA in both LS and HS conditions but to a greater extent in LS ($P < 0.01$ for both ALDO perfusion vs. control in HS and LS conditions) (Fig. 4). ANG II administration did not stimulate the expression of α-SMA in ADX rats. These results suggest that only ALDO has trophic effects on myofibroblast proliferation.

Discussion

Prolonged low Na^+ intake results in functional adaptations affecting the capacity of the distal

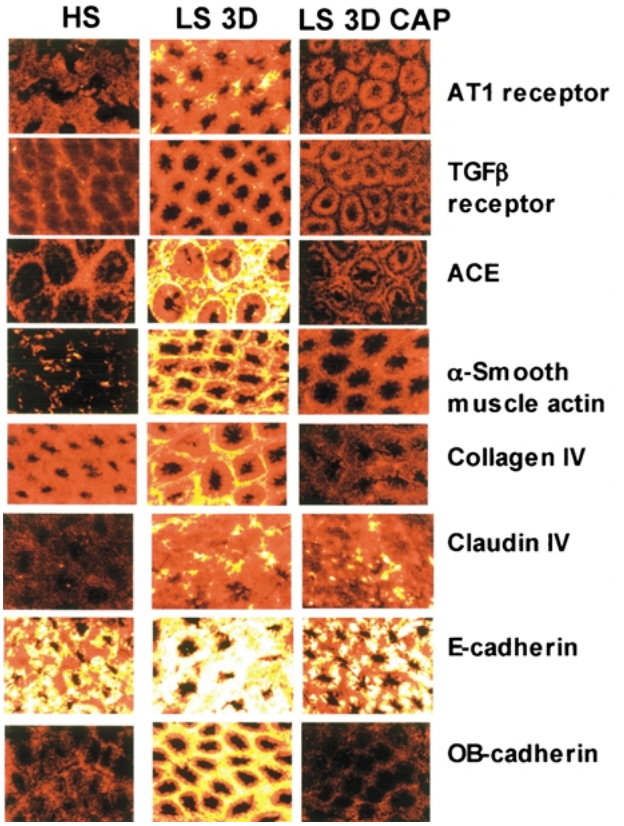

Fig. 2. Confocal images showing the LS diet ± captopril (*CAP*) effect on crypt antigen expression. All images were taken at a depth of 20 μm from the tissue surface.

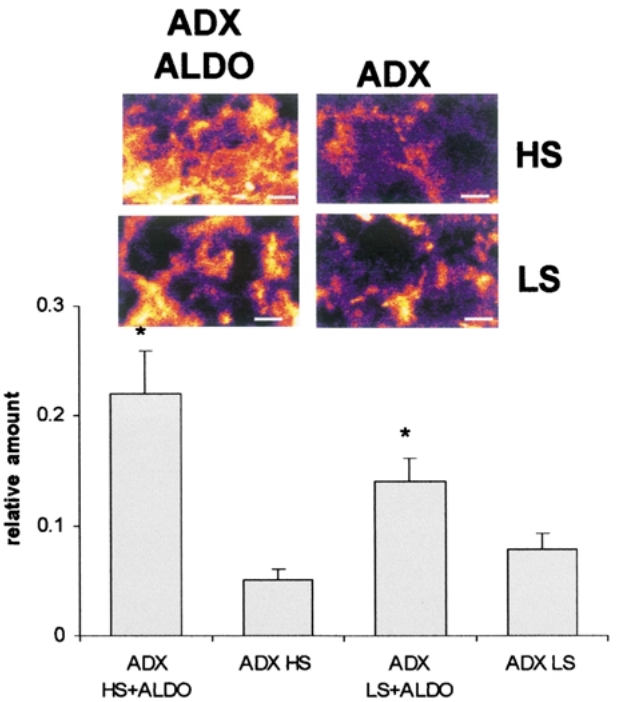

Fig. 3. Expression of Claudin IV in ADX groups supplemented with ALDO, ANG II or vehicle. Values are means ± SEM (n = 3–5 animals). Confocal images show Claudin IV-images taken at a depth of 20 μm from the tissue surface. *$P < 0.05$.

nephron and the distal colon to reabsorb Na^+; trophic effects accompany these functional changes in distal colon. Thiagarajah et al. (2002) showed that after 10 days of LS diet there was a growth stimulus of the pericryptal sheath cells surrounding the crypts of the descending colon, with increases in collagen IV deposition and OB-cadherin, smooth muscle actin, ACE and both ATr1 and TGFβr1 receptor expression. As an LS diet increases both ANG II and ALDO levels (Moretó et al., 2005), the main goal of the present study was to show whether ALDO or ANG II alone or in combination is required to generate the full trophic response of colonic crypts. The present study shows that these changes are detectable within 3 days of transition from HS to LS diet. Additionally, claudin IV, a protein that regulates tight junction permeability (Figs. 2 and 3) and E-cadherin (Fig. 2), an intercellular adhesion molecule, were increased by the LS diet. These results show that the growth of the pericryptal sheath during LS adaptation is concurrent with increased transepithelial Na^+ transport (Moretó et al., 2005), and confirm that colonic absorptive function depends both on crypt luminal cells and on myofibroblasts cells (Naftalin & Pedley, 1999).

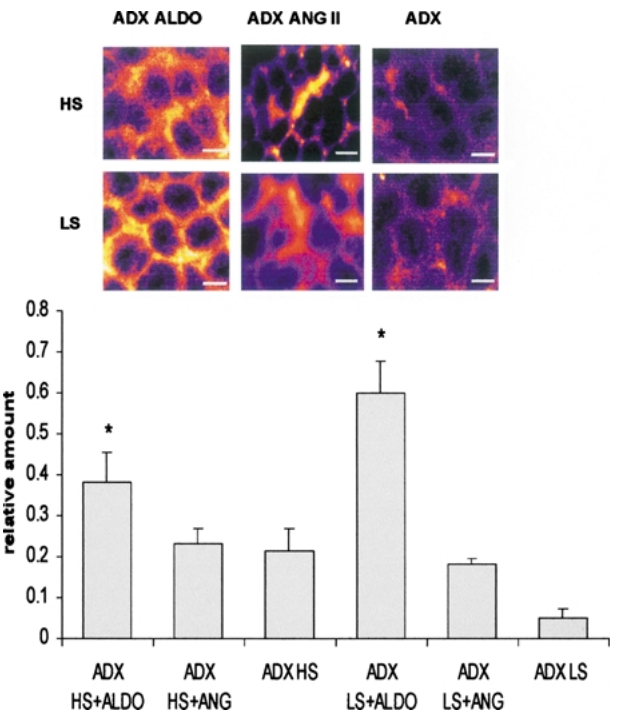

Fig. 4. Expression of α-smooth muscle actin (α-SMA) in ADX groups supplemented with ALDO, ANG II or vehicle. Values are means ± SEM (n = 5 animals). Confocal images show SMA images taken at a depth of 20 μm from the tissue surface. *$P < 0.05$.

Table 1. TGFβr1, OB-cadherin, E-cadherin, ACE and collagen IV expression using Protocol 1

	HS	LS 3D	LS 3D CAP
TGFβ receptor	0.05 ± 0.01 (5)	0.23 ± 0.008 (5)*	0.08 ± 0.002 (5)$^{\#}$
OB-cadherin	0.30 ± 0.03 (5)	4.31 ± 1.01 (5)*	0.23 ± 0.01 (5)$^{\#}$
E-cadherin	0.05 ± 0.02 (5)	0.12 ± 0.01 (5)*	0.12 ± 0.04 (5)
ACE	1.45 ± 0.20 (5)	2.08 ± 0.17 (5)*	1.51 ± 0.12 (5)$^{\#}$
Collagen IV	0.01 ± 0.0005 (5)	0.03 ± 0.0010 (5)*	0.01 ± 0.0005 (5)$^{\#}$

All experiments were carried out in the distal colon segment from rats fed HS or LS diets and rats treated with captopril (CAP). Values are relative amounts, expressed as means ± SEM; (number of animals in parentheses). *$P < 0.001$ HS *vs.* LS; $^{\#}P < 0.001$ LS *vs.* pharmacological effect.

The ACE inhibitor, captopril, the ATr1 receptor antagonist, losartan, and the ALDO antagonist, spironolactone, were investigated to determine which specific regulatory agent is involved in the trophic changes in descending colon (Fig. 1). Unfortunately for present purposes, inhibition of ANG II synthesis also indirectly decreases ALDO synthesis (Moretó et al., 2005), so the inhibition of the effect of LS with captopril or losartan could be due to loss of stimulation from either ANG II or ALDO.

The large reductions of the expression of myofibroblast smooth muscle actin and the intercellular junctional proteins, claudin IV and E- and OB-cadherin, seen in LS modulated by spironolactone, are unequivocal evidence for the requirement of ALDO (Table 1, Fig. 1). The results (Fig. 4) also indicate that ANG II alone has no trophic effect on crypt myofibroblasts. However, the possibility that there is a synergistic effect of ANG II and ALDO cannot be excluded by this result.

Until recently, our hypothesis was that ALDO controlled ENaC and Na^+ pump levels, whilst ANG II controlled the myofibroblastic pericryptal sheath growth. This was based on our experiments with the ACE inhibitor captopril, which slowed colonic adaptation to LS diet. However, the more recent experiments with osmotic minipumps (Moretó et al., 2005) confound these simple findings. ADX animals maintained on HS diets, where ANG II levels are low, given ALDO by continuous injection with a minipump, have a smooth muscle actin expression as high as colon adapted to LS diet (Table 1). Apparently, raised ALDO alone can stimulate myofibroblast growth. Similar findings have been reported in cardiac muscle (Funder, 2001).

Although our results in both this and the companion paper (Moretó et al., 2005) indicate that ANG II perfusion in the ADX has no positive trophic effect on the extracellular matrix. Indeed, in view of the raised dextran permeability and transepithelial tissue resistance observed in this condition, it is likely that ANG II perfusion in the absence of aldosterone inhibits collagen synthesis-possibly by ANG II-dependent activation of adrenomedullin, which in turn activates collagenases in the extracellular matrix (Mishima et al., 2003; Tsuruda et al., 1999, 2004).

It is also uncertain that the positive trophic response of myofibroblasts to ALDO treatment is entirely a genomic response. Several recent studies indicate that as well as ALDO-induced growth of myofibroblasts, mechanical tension stress modulates myofibroblasts' smooth muscle actin synthesis and contractile strength (Hinz et al., 2004; Taliana et al., 2005). Additionally, the rapid phosphorylation of claudin 4 in response to ALDO (Le Moellic et al., 2005) suggests that other non-genomic factors, e.g., local mechanical stress induced by fluid transport itself, could be factors in the apparent trophic effects of ALDO in mesenchymal tissue surrounding the crypts.

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