Blockage of angiotensin II type 1 receptor regulates TNF-α-induced MAdCAM-1 expression via inhibition of NF-κB translocation to the nucleus and ameliorates colitis

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Mizushima T, Sasaki M, Ando T, Wada T, Tanaka M, Okamoto Y, Ebi M, Hirata Y, Murakami K, Mizoshita T, Shimura T, Kubota E, Ogawara N, Tanida S, Kataoka H, Kamiya T, Alexander JS, Joh T. Blockage of angiotensin II type 1 receptor regulates TNF-α-induced MAdCAM-1 expression via inhibition of NF-κB translocation to the nucleus and ameliorates colitis. Am J Physiol Gastrointest Liver Physiol 298: G255–G266, 2010. First published November 25, 2009; doi:10.1152/ajpgi.00264.2009.—Mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) plays an important role at the onset of IBD in experimental models of colitis (10, 35, 36, 47). These adhesion molecules are also upregulated in the inflamed human colon of CD and UC patients (6). MAdCAM-1 is believed to be the most important of the ECAMs for the development of colitis (10, 47). MAdCAM-1 is preferentially expressed on endothelial cells in intestinal mucosa, submucosa, and Peyer’s patches and plays a central role in leukocyte homing to the mucosal immune compartment (6). MAdCAM-1 orchestrates both rolling and firm adhesion of lymphocytes to gut endothelial cells through binding of L-selectin or α4β7-integrin, expressed on immunocytes (4). MAdCAM-1 expression is dramatically amplified during inflammation (10, 42, 44, 47). So either immunoneutralization of MAdCAM-1, or its ligand, α4β7-integrin, has been the focus of anti-MAdCAM-based therapy of IBD (19, 27, 55). Recently, humanized monoclonal antibodies to α4β7- and α4-integrin, vedolizumab and natalizumab, respectively, were approved for the therapy of IBD (18, 40, 54). Other adhesion molecules may also be involved in colitis, as an antibody against VCAM-1 has been reported to prevent colitis in animal models (3, 21, 41, 53).

In the last decade, many reports have shown that angiotensin II (ANG II) has significant proinflammatory actions in the vascular wall, including the production of reactive oxygen species (ROS), inflammatory cytokines, and adhesion molecules via nuclear factor-κB (NF-κB) and janus kinase/signal transducer and activator of transcription (5, 46). An ANG II type 1 receptor (AT1R) blocker (ARB) regulates artherosclerosis, cardiovascular death, stroke, and myocardial infarction via improvement of blood vessel endothelial cell function, plaque involution, and control of macrophage permeation via control of inflammation caused by ANG II (11, 39). Recently, ANG II receptor blockade has been shown to prevent endothelial inflammation by modulation of TNF-α-induced VCAM-1 expression via regulation of NF-κB activation (8, 9). These reports suggest pleiotropic effects of ARB, such as antioxidant effects, although the details of these effects are unclear.

ENDOTHELIAL CELL ADHESION molecules (ECAMs) play essential roles in the development of chronic inflammation by recruiting leukocytes, especially lymphocytes, to tissues and by supporting their adhesive behavior, including rolling, firm adhesion, and extravasation (20). Infiltration of tissues by leukocytes is a common hallmark of many chronic inflammatory states, including inflammatory bowel diseases (IBD), ulcerative colitis (UC), and Crohn’s disease (CD), as well as of coronary heart disease and renal disease. The expression of ECAMs, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1), plays an important role at the onset of IBD in experimental models of colitis (10, 35, 36, 47). These adhesion molecules are also upregulated in the inflamed human colon of CD and UC patients (6). MAdCAM-1 is believed to be the most important of the ECAMs for the development of colitis (10, 47). MAdCAM-1 is preferentially expressed on endothelial cells in intestinal mucosa, submucosa, and Peyer’s patches and plays a central role in leukocyte homing to the mucosal immune compartment (6). MAdCAM-1 orchestrates both rolling and firm adhesion of lymphocytes to gut endothelial cells through binding of L-selectin or α4β7-integrin, expressed on immunocytes (4). MAdCAM-1 expression is dramatically amplified during inflammation (10, 42, 44, 47). So either immunoneutralization of MAdCAM-1, or its ligand, α4β7-integrin, has been the focus of anti-MAdCAM-based therapy of IBD (19, 27, 55). Recently, humanized monoclonal antibodies to α4β7- and α4-integrin, vedolizumab and natalizumab, respectively, were approved for the therapy of IBD (18, 40, 54). Other adhesion molecules may also be involved in colitis, as an antibody against VCAM-1 has been reported to prevent colitis in animal models (3, 21, 41, 53).

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The renin-angiotensin system has become a target of digestive disease research, and it has been shown that the angiotensin-converting enzyme inhibitor attenuates pancreatic inflammation (28). Moreover, it has been reported that ANG II is increased in colonic mucosa of CD patients (24), and, in animal experiments of colitis, the degree of colitis is attenuated either by administration of angiotensin-converting enzyme inhibitors (7, 50, 57) or by using angiotensin gene-deficient mice (23). These reports suggest the involvement of the renin-angiotensin system in colonic inflammation. The importance of AT1R for colitis was recently demonstrated in a colitis model using AT1R-knockout mice (26). However, the precise mechanism by which AT1R influences colitis is unclear.

In this paper, we focused on elucidation of the regulation of AT1R and MadCAM-1, which are key molecules in the pathogeneses of colitis.

MATERIALS AND METHODS

Reagents

Antibodies to ICAM-1 and VCAM-1 were purchased from R&D systems (Minneapolis, MN), an antibody to MadCAM-1 (clone MECA 367) was purchased from Pharmingen (San Diego, CA), an antibody to AT1R was purchased from Santa Cruz Biotechnology (Delaware, CA), and antibodies to phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182), phospho-IκBα, and phospho-IκBα (Ser32) were purchased from Cell Signaling (Danvers, MA). Recombinant mouse TNF-α was purchased from Sigma (St. Louis, MO). ANG II was purchased from the Peptide Institute (Minoh-shi, Osaka, Japan). Candesartan (ARB) was purchased from Takeda (Osaka, Japan).

Cell Culture

The SVEC4-10 cell line is an endothelial cell line derived by SV40 (strain 4A) transformation of murine small vessel endothelial cells originally isolated from the axillary lymph node vessels of an adult male C3H/Hej mouse (31). This cell line was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% antibiotics/antimycotics solution (Life Technologies), 1% nonessential amino acids (Life Technologies), 1% MEM (Immortomouse) (2). This cell line was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine (Life Technologies, Rockville, MD), 1% nonessential amino acids (Life Technologies), 1% MEM vitamins solution (Life Technologies), 1% antibiotics/antimycotics (Life Technologies), and 10 U/ml of IFN-γ (Endogen, Woburn, MA). The cells were plated onto 25-cm² flasks (precoated with 1% gelatin). The addition of IFN-γ was used to activate the myosin heavy chain H-2Kb class I promoter, which regulates the level of the large T antigen protein in the Immortomouse-derived cells (25). This cell line was maintained at the permissive temperature (33°C) in an atmosphere of 95% air and 5% CO₂.

Mice

Female wild-type C57BL/6J (AT1R+/+) mice were purchased from SLC (Hamamatsu, Japan). AT1R knockout male mice (Agtr1a<sup>tm1Unc</sup>) were purchased from Jackson Laboratory (Bar Harbor, ME). After being backcrossed at least seven times to the C57BL/6 strain, we bred AT1R+/+ (littermate control) and AT1R−−/− (AT1R knock out) by crossing AT1R+/+ and AT1R−−/− mice. The mice were bred and maintained in the Animal Care Committee of Nagoya City University, under temperature- and humidity-controlled conditions, with light-dark cycles of 12:12 h. The study protocol was approved by the Animal Care Committee of Nagoya City University (approval number: 05106).

Experimental Design

Female mice of 9 wk of age were divided into three groups: an AT1R knockout (AT1R−−/−) colitis group [AT1R−−/− dextran sulfate sodium (DSS) group], a wild-type (AT1R+/+) colitis group (wild DSS group), and a wild-type (AT1R+/+) noncolitis group (control group) (n = 8 per group). The mice in the colitis group were treated with 2% DSS (MP Biomedicals, Morgan, CA), dissolved in their drinking water, which was provided ad libitum, and were killed at the end of 5 days of DSS treatment. The mice in the noncolitis group were given plain drinking water, provided ad libitum, for 5 days.

For the ARB treatment study, female mice at 9 wk of age were randomly designated to receive an intraperitoneal injection of either placebo (normal saline, 0.5 ml) or the ARB, candesartan (Takeda; 0.4 mg/kg, i.p., once daily for 5 days).

Fig. 1. Effect of candesartan [a selective angiotensin II type 1 receptor (AT1R) blocker] on TNF-α-induced mucosal addressin cell adhesion molecule-1 (MadCAM-1) protein expression in SVEC and MJC-1 cells. A: SVEC cells were pretreated with or without candesartan at a concentration of 0, 10, 30, or 50 μM for 1 h and stimulated with or without TNF-α (20 ng/ml) for 24 h before all experiments to inactive the SV40 large T antigen.

B: MJC-1 cells were pretreated with or without candesartan (50 μM) for 1 h and stimulated with TNF-α (20 ng/ml) for 24 h, and then inhibition of MadCAM-1 expression by candesartan was analyzed by Western blotting.
mg/kg, injection volume 0.5 ml) once a day (n = 8 per group). The treatment (placebo or candesartan) was administered daily for as many days as the mice received DSS. Mice were given 2% DSS dissolved in their drinking water, which was provided ad libitum, and were killed at the end of 5 days of DSS treatment.

Data and Specimen Collection

Body weight, stool blood, and the stool form of each mouse were recorded daily. The presence of occult blood was measured by using a guaiac-card test (Shionogi, Osaka, Japan). At the end of 5 days, mice were euthanized with a high dose of ketamine/xyalazine, and laparotomy with total colectomy was immediately performed. The colon was washed with phosphate-buffered saline (PBS) to remove fecal materials.

Western Blotting

Cells and distal colon tissues of mice were washed with PBS (−) and subsequently dissolved in 1× cell lysis buffer (Cell Signaling Technology) containing 20 mmol/l Tris · HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l Na2EDTA, 1 mmol/l EGTA, 1% Triton, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na3VO4, and 1 μg/ml leupeptin. After disruption using a Bio-ruptor sonicator (Cosmo Bio) or a homogenizer for 15 s (cells) or for 30 s (colon tissues), respectively, lysates were centrifuged at 20,000 g for 10 min (cells) or for 30 min (colon tissues) at 4°C. On the other hand, nuclear extracts of SVEC cells were prepared by using Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL), according to the manufacturer’s instructions. Each sample was normalized on an equal protein concentration by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Protein samples from cells (75 μg each) or from colon tissues (60 μg each) were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). For all blots, the membrane was blocked with 5% skimmed milk in PBS (−) for 1 h at room temperature. The membrane was then incubated with the primary antibodies indicated in the text, either anti-MAdCAM-1, ICAM-1, or VCAM-1 for 2 h at room temperature, or with AT1R, phospho-p38 MAPK, p38 MAPK,
phospho-IκB-α, IκB-α, or NF-κB p65 overnight at 4°C and then washed with 0.05% Tween 20 in PBS (–) three times at 5-min intervals. The membrane was incubated with secondary antibody for 1 h at room temperature, followed by three washes with 0.05% Tween 20 in PBS (–) three times at 5-min intervals. The membrane was then treated with enhanced chemiluminescence detection reagents (Amer sham Pharmacia Biotechnology) for 1 min at room temperature and exposed to scientific imaging films (Eastman Kodak), and protein bands were visualized. Filters were stripped and reprobed with a monoclonal β-actin antibody (Abcam Plc., Cambridge, MA), or an anti-proliferating cell nuclear antigen antibody (Cell Signaling) as internal controls. The expression of MAdCAM-1, ICAM-1, VCAM-1, and β-actin was quantified by analyzing the density of the scanned gel band with imaging analyzer. The densitometric result of MAdCAM-1, ICAM-1, and VCAM-1 was normalized against that of β-actin in each lane, and the data were presented as fold of control.

Real-time Reverse Transcription Polymerase Chain Reaction

MAdCAM-1 mRNA expression in response to TNF-α treatment in SVEC and MJC-1 cells, or mRNA levels of monocyte chemotactic protein-1 (MCP-1), TNF-α, and MAdCAM-1 in the mice colon were measured by real-time reverse transcription polymerase chain reaction (real-time RT-PCR). For this assay, SVEC and MJC-1 were first pretreated for 1 h with or without 50 μM candesartan and were then incubated for 4 h with or without 20 ng/ml TNF-α. Total RNA was extracted from SVEC and MJC-1 cells, or from distal colon tissue of mice by using Trizol reagent (Invitrogen, Carlsbad, CA), and 2 μg RNA were reverse transcribed into complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Tokyo, Japan), according to the manufacturer’s instructions. Primers for mouse MAdCAM-1, TNF-α, MCP-1, and control mouse glyceraldehyde-3-phosphate dehydrogenase were obtained from Applied Biosystems (MAdCAM-1, Mm00522088_m1; TNF-α Mm00443258_m1; MCP-1, Mm00441242_m1; glyceraldehyde-3-phosphate dehydrogenase, 4352339E). Real-time RT-PCR was carried out using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Each experiment was carried out in a 20-μl reaction volume containing 18 μl TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 1 μl cDNA, and 1 μl primers. Uniform amplification of the products was rechecked by analysis of the melting curve of the amplified products. All reactions were carried out in triplicate to assess reproducibility of the data. The data were presented as fold of internal control.

Transfection of Short Interfering RNA Oligonucleotides

Two short interfering RNAs (siRNAs) were used for AT1R knockdown. The first siRNA [AT1R no. 1 (AT1 siRNA sc-29751)] was purchased from Santa Cruz Biotechnology, and the second siRNA [AT1R no. 2 (Agtr1a siRNA ID: s62143)] was purchased from Applied Biosystems. Silencer Negative Control no. 1 (scrambled) siRNA (Applied Biosystems) was used as control siRNA. The day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics, and transferred to 24-well plates. Transient transfection of siRNAs was carried out using Oligofectamine (Invitrogen) according to the manufacturer’s instructions, resulting in a final siRNA concentration of 200 nM added to the cells. Cells were usually assayed 36–72 h after transfection. Specific silencing was confirmed by at least three independent experiments.

Immunofluorescence Microscopy

Subconfluent SVEC cells were preincubated with or without candesartan (50 μM) for 1 h and then stimulated with TNF-α (20 ng/ml) for 1 h, following which the subcellular localization of NF-κB p65 was analyzed by immunofluorescence. For this assay, the cells were fixed with ethanol and acetone. Incubation with primary antibodies against NF-κB p65 was generally carried out in PBS containing 0.1% milk at room temperature. Sections were then incubated with the appropriate secondary antibody, and all sections were counterstained with 4’,6-diamidino-2-phenylindole (Kirkegaard and Perry Laborato-

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**Fig. 3.** Lack of effect of candesartan on TNF-α-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase, and on the phosphorylation and degradation of IκB, in SVEC (A) and MJC-1 (B) cells. The endothelial cells were pretreated with or without candesartan (50 μM) for 1 h and then stimulated with TNF-α (20 ng/ml) for 0, 2, 5, 10, or 30 min. Transient phosphorylation of p38 MAPK and of IκB, as well as degradation of IκB, in SVEC (A) and MJC-1 (B) cells were assayed by Western blot and were unaffected by candesartan treatment.
ries, Gaithersburg, MD). Images were obtained using an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

**Histological Scoring**

The 5-mm tissue of the distal colon was used for histological assessment. Hematoxylin-eosin (H&E) staining was performed using common methods. Inflammation and crypt damage in the H&E-stained sections were assessed by using the criteria described by Dieleman et al. (14). The crypt was scored on a scale of 0 to 4 grade: grade 0, intact crypt; grade 1, loss of the basal one-third of the crypt; grade 2, loss of two-thirds of the crypt; grade 3, loss of entire crypt with surface epithelium remaining intact; grade 4, loss of the entire crypt and surface epithelium. These changes were quantified as to the percent involvement by the disease process: grade 1, 1–25%; grade 2, 26–50%; grade 3, 51–75%; grade 4, 76–100%. The crypt damage score was determined as the multiplication of the grade of the crypt and percent area score (0–16). Inflammation severity was subjectively evaluated on a scale of 0 to 3 grade: grade 0, none; grade 1, slight; grade 2, moderate; grade 3, severe. The extent of disease involvement was estimated as follows: grade 0, none; grade 1, mucosa; grade 2, mucosa and submucosa; grade 3, transmural. The inflammation score was determined as the multiplication of the grade of the inflammation severity and inflammation extent (0–9). Histological scoring was performed in a blind manner.

**Statistical Analysis**

Results are expressed as the mean ± SE. Data were analyzed using Kruskal-Wallis test and Mann-Whitney’s U-test. P values < 0.05 were considered as statistically significant.

**RESULTS**

**Candesartan Prevented TNF-α-induced MAdCAM-1 Expression in SVEC and MJC-1 Cells**

To evaluate the effect of AT1R on the regulation of MAdCAM-1 expression, we first assayed the effect of the selective inhibitor of AT1R, candesartan, on TNF-α-induced MAdCAM-1 protein ex-
pression. SVEC cells were pretreated with or without candesartan for 1 h at concentrations varying from 10 to 50 μM and were then stimulated with TNF-α (20 ng/ml) for 24 h (n = 4). MadCAM-1 protein expression was detected by Western blot analysis and quantified by analyzing the density of the scanned MadCAM-1 gel band with an imaging analyzer. TNF-α-activated MadCAM-1 expression was significantly inhibited by candesartan in a dose-dependent manner (Fig. 1A). To confirm the effect of candesartan on MadCAM-1 expression, a similar experiment was carried out using another endothelial cell line, MJC-1, which was isolated from mouse colon. Candesartan also inhibited TNF-α-activated MadCAM-1 mRNA expression in MJC-1 cells (Fig. 1B). Second, we evaluated the effect of candesartan on TNF-α-activated MadCAM-1 mRNA expression in SVEC and MJC-1 cells by real-time RT-PCR analysis. The endothelial cells were pretreated with or without 50 μM candesartan for 1 h and then stimulated with TNF-α (20 ng/ml) for 4 h. Candesartan significantly inhibited TNF-α-activated MadCAM-1 mRNA expression in both SVEC (TNF-α vs. TNF-α + candesartan: 0.00029 ± 0.00004 vs. 0.00016 ± 0.00003-fold of control, P < 0.05) and MJC-1 cells (TNF-α vs. TNF-α + candesartan: 0.00012 ± 0.00002 vs. 0.00006 ± 0.00001-fold of control, P < 0.05).

Candesartan Prevented TNF-α-activated ICAM-1 and VCAM-1 Expression in SVEC Cells

We next assayed the effect of candesartan on TNF-α-activated ICAM-1 and VCAM-1 protein expression. SVEC cells were pretreated with or without candesartan (50 μM) and then stimulated with TNF-α (20 ng/ml) for 6 h (n = 4). ICAM-1 and VCAM-1 protein expression was detected by Western blot analysis and quantified by analyzing the density of the scanned ICAM-1 and VCAM-1 gel band with an imaging analyzer. TNF-α-activated ICAM-1 and VCAM-1 expression was significantly inhibited by candesartan (ICAM-1: TNF-α vs. TNF-α + candesartan, 0.40 ± 0.02 vs. 0.29 ± 0.02-fold of control, P < 0.05); VCAM-1: TNF-α vs. TNF-α + candesartan, 0.40 ± 0.03 vs. 0.30 ± 0.03-fold of control, P < 0.05).

AT1R Knockdown Inhibited TNF-α-activated MadCAM-1 Expression in SVEC and MJC-1 Cells

To clarify the effect of the specific blockage of AT1R on TNF-α-activated MadCAM-1 expression, the AT1R gene was knocked down using an siRNA technique. Figure 2A shows the knockdown of the AT1R gene by two different AT1R siRNAs (no. 1 or no. 2) in SVEC and MJC-1 cells, respectively. Thirty-six hours after siRNA transfection, SVEC and MJC-1 cells were stimulated with TNF-α (20 ng/ml) for 24 h; following this, MadCAM-1 expression was detected by Western blotting analysis and quantified by analyzing the density of the scanned MadCAM-1 gel band with an imaging analyzer (n = 4). TNF-α-activated MadCAM-1 expression was significantly prevented by AT1R knockdown in both cell types (Fig. 2B).

AT1R Knockdown Inhibited TNF-α-activated ICAM-1 and VCAM-1 Expression in SVEC Cells

To clarify the effect of the specific blockage of AT1R on TNF-α-activated ICAM-1 and VCAM-1 expression, the AT1R gene was knocked down using an siRNA technique. Thirty-six hours after siRNA transfection, SVEC cells were stimulated with TNF-α (20 ng/ml) for 6 h, following which ICAM-1 and VCAM-1 expression was detected by Western blotting analysis and quantified by analyzing the density of the scanned ICAM-1 and VCAM-1 gel band with an imaging analyzer (n = 4). TNF-α-activated ICAM-1 and VCAM-1 expression was significantly prevented by AT1R knockdown [ICAM-1: scrambled siRNA, TNF-α (+) vs. AT1R siRNA, TNF-α (+): 0.40 ± 0.04 vs. 0.20 ± 0.03-fold of control, P < 0.05; VCAM-1: scrambled siRNA, TNF-α (+) vs. AT1R siRNA, TNF-α (+): 0.40 ± 0.01 vs. 0.30 ± 0.03-fold of control, P < 0.05].

Candesartan Did Not Inhibit TNF-α-activated Phosphorylation of p38 MAPK Nor TNF-α-activated Phosphorylation and Degradation of Ikβ in SVEC or MJC-1 Cells

To determine the mechanism by which ARB inhibits MadCAM-1 expression, we examined the effect of candesartan on TNF-α-activated phosphorylation of p38 MAPK and of Ikβ, which play key roles in TNF-α induction of MadCAM-1. SVEC and MJC-1 cells were pretreated with or without candesartan (50 μM) for 1 h and then stimulated with TNF-α (20 ng/ml) for the times indicated in Fig. 3. TNF-α-induced transient phosphorylation of p38 MAPK and of Ikβ,
but phosphorylation of these proteins was not influenced by candesartan in either SVEC (Fig. 3A) or MJC-1 (Fig. 3B) cells. TNF-α-induced IκB degradation was also unaffected by candesartan treatment (Fig. 3, A and B).

Candesartan Prevented TNF-α-induced NF-κB Activation by Inhibition of NF-κB Translocation to the Nucleus

We further analyzed the mechanism by which candesartan inhibits MAdCAM-1 expression by determination of its potential effect on the translocation of NF-κB that is induced by activation of IκB and that plays an important role in the regulation of MAdCAM-1 expression. SVEC were pretreated with or without 50 μM candesartan for 1 h and then stimulated with TNF-α (20 ng/ml) for 1 h. NF-κB cellular localization was analyzed by immunofluorescence microscopy and by Western blotting of the whole cell and of the nuclear compartment. Following TNF-α stimulation, the expression of intranuclear NF-κB was increased, although no change in the total cellular level of NF-κB protein was detected by Western blotting analysis. These results indicated that TNF-α stimulation induced NF-κB translocation to the nucleus. Candesartan had significantly blocked the increase of intranuclear expression of NF-κB (Fig. 4A). By immunofluorescence analysis, NF-κB was observed to localize in the cytoplasm under resting conditions, but to translocate into the nucleus following cellular activation with TNF-α. Candesartan completely blocked this translocation of NF-κB to the nucleus (Fig. 4B).

DSS-induced Colitis Was Ameliorated in AT1R−/− Mice

Body weight loss, disease activity, and colon shortening were lower in AT1R−/− than in wild-type mice. Our previous in vitro results suggested that AT1R blockage might be a new therapeutic approach for treatment of IBD. To confirm that this was the case, we compared DSS-induced colitis in AT1R−/− and wild-type mice. Symptoms of acute colitis developed in the mice following 5 days treatment with 2% DSS, with diarrhea being observed first, followed by rectal bleeding and severe weight loss. It is important to note that there was no difference in the consumption of drinking water between wild-type and AT1R−/− mice. The disease activity index, a combinatorial index of body weight loss, stool blood, and stool consistency (15), was significantly lower in AT1R−/− than in wild-type mice (Fig. 5A). The change in body weight induced by DSS, which is presented as the percent change from the baseline value of day 0, was significantly ameliorated in AT1R−/− mice compared with wild-type mice (Fig. 5B). Concomitant with these changes, DSS treatment induced a significant decrease in colon length in both wild-type and AT1R−/− mice. Colon shortening was significantly lower in the AT1R−/− mice (81.3 ± 2.6 mm) than in the wild-type mice (71.9 ± 5.4 mm).

Fig. 6. Effect of AT1R-gene knockout on colon damage in the mouse DSS-colitis model. A: colon damage in control (a) and in 5-day DSS-treated wild-type (b) and AT1R−/− (c) mice was assessed histologically. Differences in mucosal thickness, infiltration of inflammatory cells, and crypt damage were observed between DSS-treated wild-type and control mice. The DSS-induced colonic changes were much less in AT1R−/− than in wild-type mice. In AT1R−/− mice, both the inflammation score (B) and the crypt damage score (C) were significantly lower than those of wild-type mice. The values are means ± SE. **P < 0.01 and *P < 0.05 compared with wild DSS mice. ##P < 0.01 compared with control mice.
Histological damage was milder in AT1R−−/− than in wild-type DSS-treated mice. The effect of DSS on the colon was further examined in AT1R−−/− and wild-type mice by histological assessment of tissue damage. H&E staining of the colon of wild-type mice at day 5 of DSS treatment showed thicker mucosa, greater infiltration of inflammatory cells, and a higher level of crypt damage compared with non-DSS-treated mice (control). In contrast, the colon of AT1R−−/− mice appeared less damaged and inflamed, showing only mild evidence of crypt damage and inflammatory cell infiltration (Fig. 6A). In AT1R−−/− mice, both the inflammation and the crypt damage scores were significantly lower than those of wild-type mice (Fig. 6, B and C).

The levels of TNF-α and MCP-1 mRNA in the colon were lower in AT1R−−/− than in wild-type DSS-treated mice. To investigate the role of AT1R in mucosal immunity, TNF-α and MCP-1 mRNA levels in the colon were quantified by real-time RT-PCR. The mRNA levels of the proinflammatory Th1 cytokine TNF-α and the chemokine MCP-1 were significantly elevated in DSS-treated mice compared with control mice (Fig. 7, A and B, respectively). However, the levels of TNF-α and MCP-1 mRNA were significantly lower in AT1R−−/− than in wild-type mice (Fig. 7, A and B, respectively). These results indicate that AT1R is involved in upregulation of a proinflammatory cytokine and a chemokine in the inflamed colon and that AT1R−−/− mice are resistant to DSS-induced colonic damage.

The expression of MAdCAM-1 in the colon is attenuated in AT1R−−/− DSS-treated mice. We next determined whether the in vitro inhibition of MAdCAM-1 expression that we observed following AT1R blockage could also be observed in vivo. We, therefore, evaluated the expression of MAdCAM-1 in DSS-induced inflammatory colon by Western blotting analysis and real-time RT-PCR. MAdCAM-1 protein expression was detected by Western blot analysis and quantified by analyzing the density of the scanned MAdCAM-1 gel band with an imaging analyzer. In AT1R−−/− mice, the colonic expression of MAdCAM-1 at both the protein and the mRNA level was significantly lower than that of wild-type mice (Fig. 8). Therefore, the combined in vitro and in vivo results indicate that AT1R blockage induces resistance to DSS induction of colonic inflammation by inhibition of MAdCAM-1 expression.

DSS-induced Colitis Was Ameliorated in ARB-treated Mice

Then, we investigated whether the blockage of AT1R by using ARB might be a new therapeutic approach for treatment of IBD or not. We further compared DSS-induced colitis in candesartan-treated mice and nontreated mice. Symptoms of acute colitis developed in the mice following 5 days treatment with 2% DSS. Disease activity index was significantly lower in candesartan-treated mice than in nontreated mice (Fig. 9A). The change in body weight induced by DSS, which is presented as the percent change from the baseline value of day 0, was significantly ameliorated in candesartan-treated mice compared with nontreated mice (Fig. 9B).

**DISCUSSION**

This is the first report concerning the relationship between AT1R and the expression of MAdCAM-1, one of the most important pathogenic molecules in IBD. We have shown that AT1R blockage prevents induction of colitis by regulation of the endothelial expression of MAdCAM-1 via inhibition of NF-κB activation.

It has been reported that ANG II activates NF-κB through nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase-derived ROS and that this activation induces ICAM-1 and VCAM-1 expression (30, 49). In preliminary experiments, ANG II induced ICAM-1 and VCAM-1, but not MAdCAM-1 expression (data not shown). This result means that the expression of MAdCAM-1 is regulated by a different pathway than that by which ICAM-1 and VCAM-1 are regulated. This possibility is supported by our laboratory’s previous report that TNF-α-induced MAdCAM-1 expression is controlled by cytochrome P-450-derived ROS, not by NADPH oxidase-derived ROS (45). We, therefore, focused our study on the relationship between AT1R and MAdCAM-1 expression, since it was a potentially novel mechanism for the regulation of MAdCAM-1 expression.

In this experiment, we used two vascular endothelial cell lines, because MAdCAM-1 is preferentially expressed on only endothelial cells in intestinal mucosa, submucosa, and Peyer’s
patches (6). Most experimental studies on MAdCAM-1 have been reported using a brain-derived endothelioma cell line (bEnd.3) and SVEC, because MAdCAM-1 is inducible in these cell lines. We used SVEC (35, 43, 45) in our experiment concerning MAdCAM-1. However, we thought that SVEC was not sufficient to study the colitis, because it is the cell line derived from axillary lymph node. Therefore, we have established a colon endothelial cell line, MJC-1 (2), in which MAdCAM-1 is also inducible.

Interestingly, we showed in this study that blockage of AT1R by using the ARB, candesartan, or by AT1R gene knockdown controlled not only TNF-α-induced-ICAM-1 and VCAM-1 expression, but also the expression of MAdCAM-1 by SVEC and MJC-1 cells. Moreover, our findings suggest that the NF-κB pathway is more critical for MAdCAM-1 expression than the p38 MAPK pathway.

We also investigated the potential mechanism by which candesartan inhibits NF-κB and found that candesartan did not inhibit TNF-α stimulation of IκB phosphorylation, but did inhibit translocation of NF-κB to the nucleus from the cytoplasm. This effect of candesartan was confirmed by both Western blotting analysis of p65 in nuclear fractions and by an immunofluorescence microscopic analysis of p65 cellular localization. This result is consistent with our laboratory’s previous data that the effect of candesartan on MAdCAM-1 expression is achieved through modulation of the NF-κB pathway and not through the p38 MAPK pathway. NF-κB exists as either a heterodimer or a homodimer, among which...
the p65/p50 is the most ubiquitous heterodimer. In resting cell, NF-κB dimers are sequestered in the cytoplasm through association with inhibitory proteins IkB (51). Upon treatment with NF-κB inducers, such as proinflammatory cytokines, IkB is phosphorylated and degraded through the ubiquitin/proteasome pathway, which eventually leads to the nuclear translocation of NF-κB and binding to the κB site of target genes (13,29). Inhibition of the ubiquitin/proteasome pathway will result in retention of NF-κB in the cytoplasm, even if IkB is phosphorylated. However, we have shown that candesartan had no effect on the degradation of IkB, indicating that candesartan does not act as a proteasome inhibitor. In other words, no inhibitory effect of candesartan was found on the single-mediated IkB phosphorylation and its degradation, a major mechanism of most NF-κB inhibitors (12, 48, 56). In this experiment, candesartan inhibited nuclear translocation of NF-κB, but the detail of its molecular mechanism is not well known, and the exact effect of candesartan on this phenomenon requires further experiments. Furthermore, the nuclear translocation mechanism of NF-κB is still not well known. Recently, it is reported that TNF-α-induced nuclear translocation of NF-κB p50/p65 heterodimer is mediated by importin-α3 and importin-α4 (17). While IkB is bound to NF-κB, IkB inhibits the binding of p50/p65 heterodimer to importin-α3, because IkB masks the nuclear localization signal of NF-κB. But IkB is degraded, importin-α binds to nuclear localization signal of NF-κB p50/p65 heterodimer, and NF-κB is transported into the nucleus. In our experiment, candesartan did not inhibit TNF-α-induced degradation of IkB, but prevented TNF-α-induced NF-κB translocation to the nucleus. So there is a possibility that candesartan prevents the binding of importin-α and NF-κB. On the other hand, it is thought that there are a classical pathway and a nonclassical pathway in NF-κB activation. It is possible that candesartan affects some influence on the nonclassical pathway that also controls the activation of NF-κB. However, we cannot refer its possibility any further, because we have examined only the classical pathway in this experiment.

We employed a model of DSS-induced colitis in mice to assess the potential role of AT1R in gut inflammation. This model system has been shown to have many similarities to UC, including the induction of symptoms such body weight loss, hematochezia, erosion of the colonic mucosa, and shortening of the large intestine (33). In addition, cytokine expression in DSS-induced colitis is similar to that in human IBD (16,24). It has been reported that AT1R is localized in vessel walls, the surface epithelium, crypt bases, and in mesenchymal cells in the lamina propria in human colonic mucosa (22), and that DSS administration to wild-type mice results in upregulation of ANG II and AT1R expression (26). Our study is consistent with and, furthermore, provides a mechanism for, a role for AT1R in DSS-induced colitis. Thus we previously found that cytokine stimulation upregulates AT1R mRNA expression on endothelial cells in vitro (data not shown). Combining this data with previous reports suggested that regulation of AT1R would result in the inhibition of colonic inflammation in the DSS-colitis model. As expected, the AT1R−/− mice exhibited reduced intestinal inflammation compared with wild-type mice. We also showed that AT1R knockout significantly reduced DSS-induced MAdCAM-1 expression in colonic mucosa and inhibited cytokine and chemokine expression. Furthermore, administration of ARB to mice conferred resistance to DSS-induced colitis. These findings suggest that the AT1R is involved in one of the pathogenesis of DSS-induced colitis via the regulation of MAdCAM-1. However, from our additional study to confirm the in vitro result that AT1R blockade inhibits nuclear translocation of NF-κB, we could not detect clear in situ difference of NF-κB to nuclear translocation between DSS-treated wild mice and AT1R−/− mice. To clarify the mechanism, further experiments are necessary.

In summary, we have demonstrated a novel mechanism by which AT1R regulates the expression of MAdCAM-1 by modulation of the nuclear translocation of NF-κB. Moreover, AT1R regulation of MAdCAM-1 expression ameliorates colitis in a mouse colitis model. We conclude that AT1R-regulation of MAdCAM-1 expression potentially provides a promising novel target for the treatment of IBD.

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DISCLOSURES

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