Cytotechnology **40:** 93–98, 2002.

Analysis of the mechanism of the tight-junctional permeability increase by capsaicin treatment on the intestinal Caco-2 cells

Junkyu Han[1], Hiroko Isoda[2*] & Takaaki Maekawa[1]
[1] *Institute of Agricultural and Forest Engineering, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki, Japan;*
[2] *National Institute for Environmental Studies, Onogawa 16-2, Tsukuba, Ibaraki, Japan*
(Author for correspondence; E-mail: hkisoda@gc5.so-net.ne.jp)*

Received 10 May 2002; accepted in revised form 1 October 2002

Key words: actin, capsaicin, HSP47, permeability, PKC, tight junction

Abstract

In a previous experiment (Isoda et al., 2001), we showed that the tight-junctional (TJ) permeability increase in Caco-2 cells during capsaicin exposure was through binding of the capsaicin molecule to a capsaicin receptor-like protein. In the present study, we examined how actin, which modulates TJ permeability, is influenced by capsaicin. We showed that after treatment of the Caco-2 cells with capsaicin, the volume of F-actin decreased. Moreover, we also examined protein kinase C (PKC) and heat shock protein 47 (HSP47), which act as probable second messengers in causing TJ permeability increase. We showed that after capsaicin treatment, HSP47 was activated. However, PKC activity was the same in both control and treatment setups. These results suggest that, while PKC is not involved, it is highly possible that HSP47 plays a role in TJ permeability increase in intestinal Caco-2 cells exposed to capsaicin.

Abbreviations: BSA – bovine serum albumin; CHO cells – Chinese Hamster Ovary cells; DMEM – Dulbecco's modified eagle's medium; ER – endoplasmic reticulum; F-actin – filamentous actin; G-actin – globular actin; HSP47 – heat shock protein; LDH – lactic dehydrogenase; PBS – phosphate-buffered saline; PKC – protein kinase C; TER – transepithelial electrical resistance.

Introduction

In our previous study (Isoda et al., 2001), the influence of capsaicin processing on human intestinal cell line Caco-2 was examined by measuring transepithelial electrical resistance (TER). The permeability of the Caco-2 cell monolayer by measuring the TER value is known to be correlated with a change in the paracellular permeability of the cell monolayer (Hashimoto et al., 1997). There was an increase in permeability at high concentration (200 to 500 μM) of capsaicin, and the effect was inhibited by pretreatment of capsazepine, which is a competitive antagonist of the vanilloid receptor 1 (VR1). We also determined the expression of the VR1-like protein on Caco-2 cells in a time-dependent manner by western blotting using VR1 antiserum. These results show that the permeability increase by capsaicin was through binding of capsaicin to VR1-like protein of Caco-2 cells. In these consecutive phenomenons, LDH (lactic dehydrogenase) activity as well as changes in the intracellular Ca^{2+} were determined to know whether or not capsaicin affected TER activity through influence on the tight junction. LDH activity and the increase in the intracellular Ca^{2+} were considerably high at capsaicin concentration of 200 μM. However, there were no remarkably high LDH activity in Caco-2 cells at concentrations above 300 and 400 μM of capsaicin. These results suggest that the decrease in TER value during capsaicin treatment was not due to cell monolayer damage, but rather, to the reversible opening of a paracellular route.

In this study, possible mechanisms involved in tight-junctional (TJ) permeability increase by cap-

saicin were investigated. Tight junctions play a critical role in epithelial cell biology by forming a selectively permeable barrier in the spaces between epithelial cells and maintaining the cell surface compositional asymmetry, which is characteristic of this cell type. The TJ has been thoroughly characterized morphologically and physiologically; however, only in the last decade has the molecular configuration begun to be understood. The TJ contains at least nine peripheral and three integral membrane proteins, and several of these have been implicated in tumor suppression, growth regulation and signal transduction (Yap et al., 1998). TJ permeability increase is modulated through actin, which is a 43 KDa protein, and the majority of the isotype heterogeneity is located at the amino-terminal 30 amino acid. The amino-terminus of globular-actin (G-actin) is located at the periphery of the double-helix in filamentous-actin (F-actin). G-actin readily polymerizes under physiological conditions to form F-actin with the concomitant hydrolysis of ATP (Holmes et al., 1990; Rayment et al., 1993).

HSP (heat shock protein) 47 is a novel 47 KDa stress glycoprotein and its expression is highly tissue- and cell-specific. It is likewise restricted to most phenotypically altered collagen-producing cells. Prior to secretion, procollagen molecules are correctly folded to triple helices in the endoplasmic reticulum (ER) where HSP47 specifically associates with procollagen during its folding and/or modification processes and is thought to function as a collagen-specific molecular chaperone (Mohammed et al., 2000; Koide et al., 1999). It was demonstrated that the synthesis of HSP47 paralleled that of types I and IV collagen (Nagata et al., 1986, 1996). In recent studies, the expression of HSP47 was shown to be associated with increased staining of collagen types I, II and IV in an experimental model of interstitial fibrosis of the kidney (Cheng et al., 1998; Moriyama et al., 1998). The expression of collagen-binding HSP47 with various proteins implicated in phenotypic modulation (actin filaments and vimentin) and fibrosis (type I and type III collagens) was examined in control and cisplatin-treated kidneys (Razzaque et al., 1999).

Protein kinase C (PKC), one of the most important proteins in signal transduction, is involved in cell proliferation. It was suggested that PKC is connected with the F-actin cytoskeleton (Savala et al., 1998). Further, it has reported that PKC activation was accompanied with the translocation of this enzyme from the cytosol to the plasma membrane (Hashimoto et al., 1997). From these results, it is assumed that PKC is involved with tight-junctional permeability increase.

In this study, we investigated the possibility that capsaicin triggers cytoskeletal reorganization of the actin filaments and activation of HSP47 and PKC, which are implicated in cytoskeletal reorganization of the actin filaments. It is done to help elucidate the pathway by which capsaicin increases TJ permeability.

Materials and methods

Materials

Capsaicin, Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin MDCB302, 4-Methlyumbellifery β-D-galactoside (M1633) and fetal calf serum (FCS) were obtained from Sigma (U.S.A.). Non-essential amino acid was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). Bovine serum albumin (BSA), kanamycin, L-glutamine, rhodamine-phalloidin (R-415) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The Pep Tag Protein Assay Kit and lysis buffer were obtained from Promega (U.S.A.).

Cell culture

The Caco-2 cells were maintained in DMEM supplemented with 10% FCS, 1% penicillin-streptomycin, and 1% non-essential amino acids. They were incubated in an atmosphere of 5% CO_2 at 37 °C. The cells were passaged at a split ratio of 4 to 8 every 3 or 4 days. For determination of volume of F-actin, cells were seeded on a chamber slide (LabTek, U.S.A.) at a density of 2×10^5 cells per slide. For the PKC assay, cells were seeded on Petri plates at a density of 1×10^6 cells per well.

The HSP47-transformed Chinese Hamster Ovary cells (CHO cells) were maintained in MDCB302 (5.5 g L^{-1}) and DMEM (4.75 g L^{-1}) supplemented with L-glutamine (0.3 g L^{-1}), $NaHCO_3$ (1.27 g L^{-1}), kanamycin (0.1 g L^{-1}), and incubated in an atmosphere of 5% CO_2 at 37 °C. The cells were passaged at a split ratio of 4 to 8 every 3 or 4 days. For determination of the HSP47 promoter activated by capsaicin, cells were seeded onto 96-well plates at a density of 1×10^4 cells/well.

Determination of cellular F-actin

The relative content of F-actin was determined by a fluorescent phalloidin-binding assay (Dadabay et al., 1991; Hashimoto et al., 1997). Caco-2 cell monolayers that had been incubated on the chamber slide for 3 days were rinsed with phosphate-buffered saline (PBS) (–) and then incubated for 0, 30 and 60 min with or without capsaicin (300 or 400 μM). After incubation, the cell monolayers were fixed with acetone/methanol (1:1), and then the actin was stained for 20 min with rhodamine-phalloidin diluted 10-times with PBS (–). Stained cells were extracted with 2 ml methanol and the fluorescence intensity of the extract was measured using a Fluoroscan Ascent FL (Labsystems) fluorescence spectrophotometer with excitation-emission wavelengths of 545–578 nm.

Determination of capsaicin-activated HSP47 promoter

HSP47 activity can be determined in HSP47-transformed CHO cells by incubating the latter with the test compound and measuring the enzymatic activity of β-galactosidase. When introduced into a chromosome, the HSP47 plasmid can express β-galactosidase efficiently during stress induction. Experimental CHO cells were transformed by transferring the β-galactosidase gene downstream of the HSP47 promoter while control CHO cells had the β-galactosidase under the control of the SV40 promoter.

Determination of HSP47 activity in cells treated with capsaicin was carried out using HSP47-transformed CHO cells. The latter (1×10^4 cells) were trypsinized and plated onto 96-well plates, and allowed to proliferate for 2 days. After which, the seeding medium was removed and replaced with experimental medium. Capsaicin (300 or 400 μM) was then added and the plates incubated for 15, 30 and 60 min. The cells were then washed twice with PBS (–), and lysed with 50 μl of lysis buffer for 20–30 min at room temperature. The cell lysate was then mixed well by pipetting, and then transferred to 96-well plates at 50 μl/well. After the addition of 100 μl of 4-Methylumbelliferyl β-D-galactoside (0.4 mM), the plates were shielded from light and incubated for 30 min at room temperature. The fluorescence intensity of the extract was then measured with Fluoroscan Ascent FL (Labsystems) at excitation-emission wavelengths of 365–450 nm.

Effect of capsaicin on PKC of Caco-2 cells

Caco-2 cells (1×10^6 cells) were seeded onto Petri plates and cells allowed to proliferate for 3 days until 80% confluent. The seeding medium was then removed and replaced with experimental medium. Capsaicin (300 and 400 μM) was then added and the plates incubated for 15, 30 and 60 min. The cells were then washed twice with equal volumes of PBS (–), collected using a sterile plastic cell scraper and transferred to sterile centrifuge tubes. The cells were then spun at 1770 g for 2 min, followed by removal of PBS (–), addition of 200 μl homogenization solution, and sonication on ice to disrupt the cells. This was followed by centrifugation at 5400 g for 25 min at 4 °C. The supernatant was then used for the protein kinase C assay using the Pep Tag Assay Kit.

Results and discussion

In a previous study (Isoda et al., 2001), we report that capsaicin (200 to 500 μM) increased TJ permeability in Caco-2 cells as shown by TER measurements, whereas the pre-treatment with 10 μM capsazepine (competitive antagonist of the capsaicin receptor) inhibited this effect. In this study, the permeability of Caco-2 cells was increased significantly by capsaicin concentration of 300 and 400 μM in a time-dependent manner (Figure 1). This result showed to be similar to the result in our previous paper and suggests that the rapid decrease of TER (increase in TJ permeability) at 300 and 400 μM of capsaicin after 30 min is not due to cell injury, but to difference in the susceptibility to capsaicin concentration.

Since it has been suggested that cytoskeletal reorganization of actin filaments mediates the increase in TJ permeability (Hashimoto et al., 1997), we examined whether the capsaicin-induced increase in TJ permeability is connected to cytoskeletal reorganization of the actin filaments by determining the amount of cellular F-actin. In Caco-2 cells, the amount of cellular F-actin decreased during capsaicin treatment with concentrations 300 and 400 μM (Figure 1). These capsaicin concentrations likewise increased the TJ permeability in Caco-2 cells. A significant decrease in the amount of F-actin was shown after 60 min of incubation with 300 and 400 μM of capsaicin. This reduction is probably due to F-actin depolymerization into G-actin as a form of cytoskeletal reorganization (Holmes et al., 1990; Rayment et al., 1993).

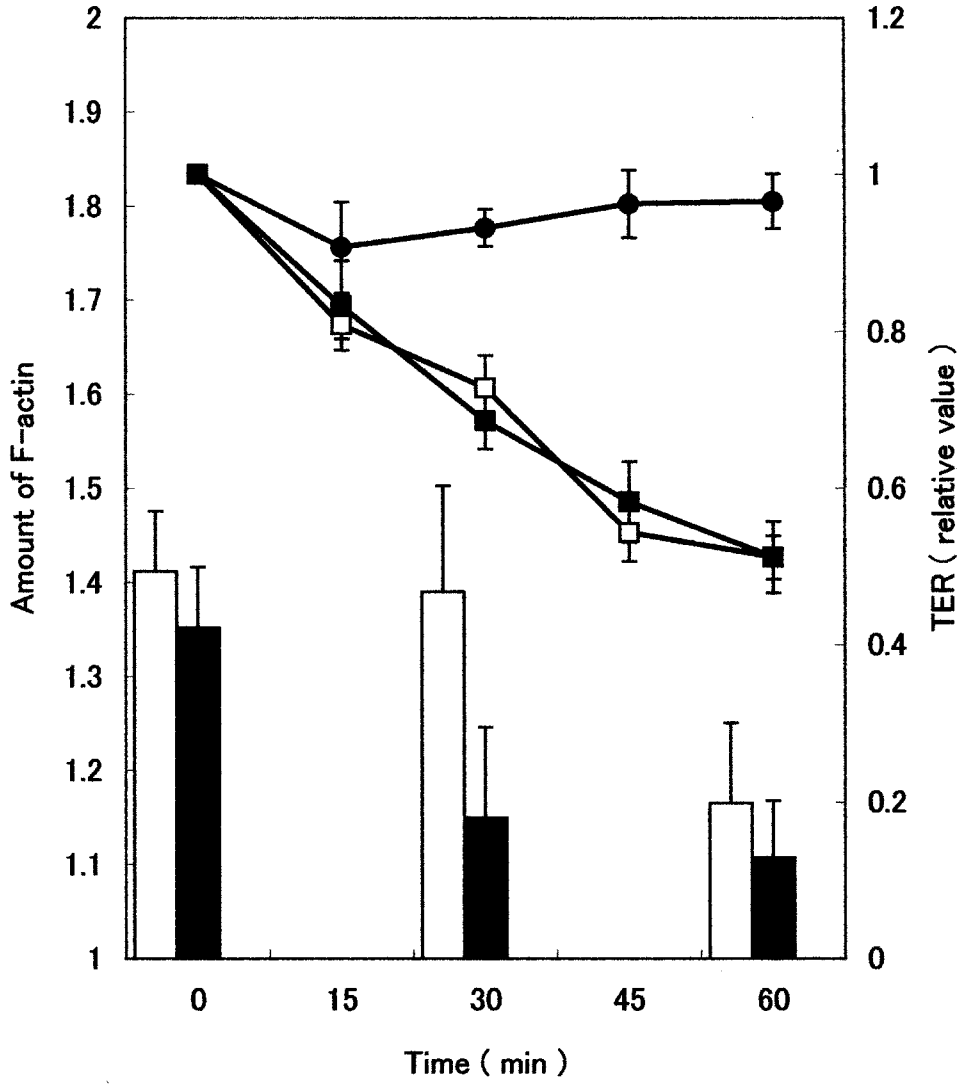

Figure 1. Effect of 300 μM (open bar) and 400 μM (solid bar) capsaicin concentrations on the F-actin of Caco-2 cells as well as on the transepithelial electrical resistance (TER). TER values are presented as relative to the values at zero time. (●) 0 μM; (□) 300 μM capsaicin; (■) 400 μM capsaicin. Standard deviation were <10%.

Other studies suggest that there might be a relation between cytoskeletal reorganization, actin filaments expression, and HSP47 activation. A recent paper, for example, reports that there is an increased expression of actin filaments in phenotypically-altered, HSP47-producing renal cells in chronic diabetic rats (Diange et al., 2002). Another publication shows that collagen-binding HSP47 is associated with increased phenotypic alteration of actin filaments interstitial and epithelial cells (Razzaque et al., 1998). In our case, we supposed that HSP47 activation and cytoskeletal reorganization of the actin filaments in Caco-2 cells are closely related and therefore examined HSP47 activation after capsaicin treatment. In HSP47-transformed CHO cells, the HSP47 promoter activity was increased at capsaicin concentrations of 300 and 400 μM after 15 min, and stayed elevated at capsaicin concentration of 300 μM after 30 and 60 min (Figure 2). Interesting observations were that in the case of 400 μM capsaicin treatment, the HSP47 activity decreased rapidly after 15 min. As following this result, the amount of F-actin in Caco-2 cells decreased rapidly. In contrast, the 300 μM treatment showed only a minimal decrease both the amount of F-actin and HSP47 activity. Cap-

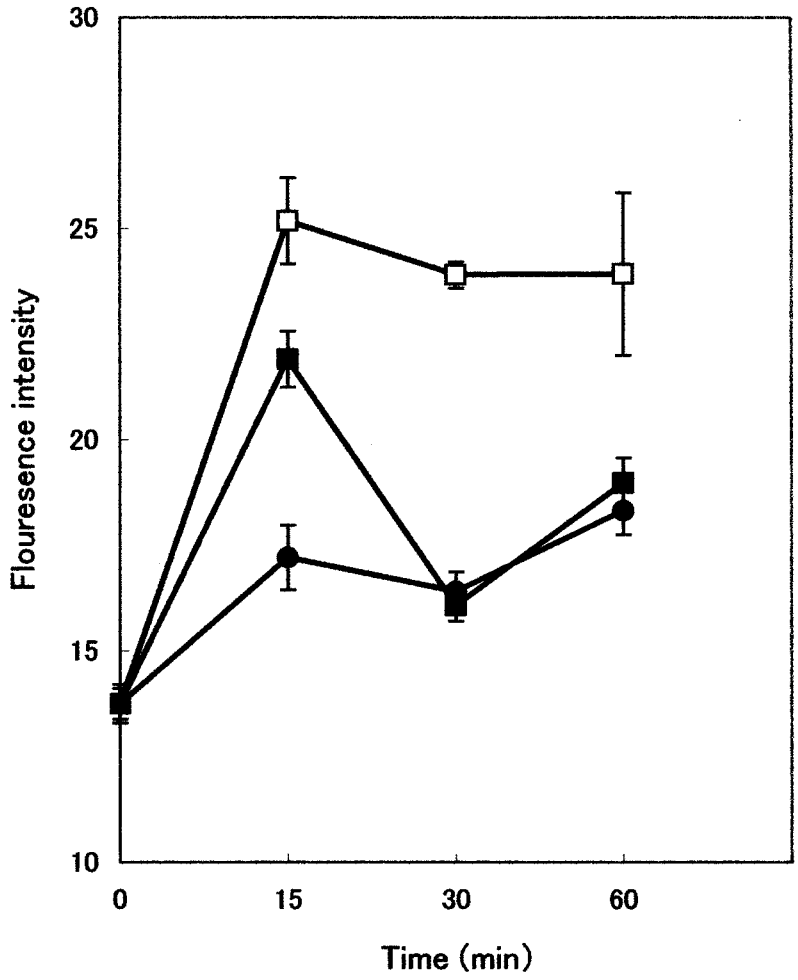

Figure 2. Effect of capsaicin on the HSP47 of HSP47-transformed CHO cells. (●) 0 μM; (□) 300 μM capsaicin; (■) 400 μM capsaicin. Standard deviation were <10%.

saicin did not have any effect on the β-galactosidase activity of non-transformed control cells (data not shown).

From the results that HSP47 and actin filaments were influenced by capsaicin treatment, we suggest that the capsaicin treatment resulting increase in TJ permeability is caused by reorganization of actin filaments. Moreover, we suggest that the actin filaments are themselves affected by HSP47 up and down regulation.

In relation to a report showing that the change in TJ permeability was modulated by PKC in Caco-2 cells (Hashimoto et al., 1997), we examined if PKC is activated by capsaicin treatment. Results showed that capsaicin treatment had no effect on the PKC activity compared to the untreated control (data not shown). This proves that the results obtained were not related to the activity of this kinase, and PKC is not implicated in cytoskeletal reorganization of the actin filaments.

Conclusion

In this study, we showed that 300 and 400 μM capsaicin concentrations reduced the amount of F-actin in Caco-2 cells, which implied cytoskeletal reorganization. Moreover, HSP47, which acts as a molecular chaperone during processing and/or secretion of procollagen, was also activated by capsaicin treatment. The capsaicin concentrations used also increased TJ permeability in Caco-2 cells. Thus, it is suggested that the increase in TJ permeability by capsaicin treatment

occurs due to the cytoskeletal reorganization of the actin filaments, and through HSP47 expression.

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