# ผลของแคปโทพริลในการปกป้องโครงสร้างและการทำงานของไทต์จังชัน จากภาวะเครียดออกซิเดชันในเซลล์ อีซีวี 304

นางสาวนภาภร ถนอมลิขิต

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ปีการศึกษา 2552

# PROTECTIVE EFFECTS OF CAPTOPRIL ON OXIDATIVE STRESS-INDUCED DISRUPTION OF TIGHT JUNCTION STRUCTURE AND FUNCTION IN ECV304 CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology
Department of Pharmacology and Physiology
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| Thesis Title | PROTECTIVE EFFECTS OF CAPTOPRIL ON OXIDATIVE STRESS-INDUCED DISRUPTION OF TIGHT JUNCTION STRUCTURE AND FUNCTION IN ECV304 CELLS |
| By | Miss Napaporn Thanomlikhit |
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| Thesis Advisor | Assistant Professor Suree Jianmongkol, Ph.D. |

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นภาภร ถนอมลิขิต : ผลของแคปโทพริลในการปกป้องโครงสร้างและการทำงานของไทต์จังชันจากภาวะเครียดออกซิเดชันในเซลล์ อีซีวี 304. (PROTECTIVE EFFECTS OF CAPTOPRIL ON OXIDATIVE STRESS-INDUCED DISRUPTION OF TIGHT JUNCTION STRUCTURE AND FUNCTION IN ECV304 CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ภญ.ดร. สุรีย์ เจียรณ์มงคล, 88 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของยาแคปโทพริล ในการป้องกันการซึมผ่านที่เพิ่มขึ้นของชั้นเยื่อบุผิวเมื่อถูกเหนี่ยวนำให้เสียหายจากไฮโดรเจนเปอร์ออกไซด์ (200 μM $H_2O_2$ เป็นเวลา 4 ชั่วโมง) โดยใช้แบบจำลองเซลล์ อีซีวี 304 ซึ่งเพาะเลี้ยงไว้เป็นเวลา 2 วัน หรือ 12 วัน โดย $H_2O_2$ ในความเข้มข้นดังกล่าว ไม่มีผลต่อการตายของเซลล์จากการตรวจวัดด้วยวิธี MTT assay ในเซลล์ อีซีวี 304 ที่เพาะเลี้ยงเซลล์เป็นเวลา 12 วัน พบว่า มีค่า transepithelial electrical resistance (TEER) เพิ่มขึ้น และการเคลื่อนที่ของ phenol red ผ่านช่องว่างระหว่างเซลล์ลดลง แสดงถึงการมีอยู่ของไทต์จังชันที่ทำหน้าที่ได้อย่างสมบูรณ์ $H_2O_2$ มีผลต่อการทำหน้าที่ของไทต์จังชันโดยทำให้ค่า TEER ลดลง และการเคลื่อนที่ผ่านของ phenol red เพิ่มมากขึ้น นอกจากนี้ เมื่อตรวจดูเซลล์ด้วยวิธี immunofluorescence แล้ว พบความผิดปกติในการจัดเรียงตัวของโปรตีน occludin และ ZO-1 บริเวณขอบเซลล์ แม้ว่าปริมาณของโปรตีนทั้ง 2 ชนิดซึ่งวัดโดยวิธี western blot analysis ยังคงอยู่ในระดับปกติ ส่วนเซลล์ อีซีวี 304 ที่เพาะเลี้ยงเป็นเวลา 2 วันนั้น การทำหน้าที่ของไทต์จังชันยังไม่สมบูรณ์ และเมื่อได้รับ $H_2O_2$ พบความผิดปกติในการจัดเรียงตัวของโปรตีนทั้ง 2 ชนิดเช่นกัน และยังมีผลทำให้ปริมาณของ occludin ลดลงอีกด้วย การให้แคปโทพริล (100 μM เป็นเวลา 30 นาที) ก่อนให้ $H_2O_2$ พบว่า แคปโทพริลสามารถรักษาการทำหน้าที่ของไทต์จังชันในการเป็นตัวกั้นไว้ได้ โดยแคปโทพริลมีผลขัดขวางความผิดปกติในการจัดเรียงตัวและการลดลงของปริมาณโปรตีนซึ่งเป็นองค์ประกอบของไทต์จังชันที่เกิดจาก $H_2O_2$ ได้ ทั้งนี้เป็นไปได้ว่าการออกฤทธิ์ของแคปโทพริลในการรักษาความคงตัวของไทต์จังชันอาจเกี่ยวข้องกับการทำงานของ ERK1/2 pathway

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS : CAPTOPRIL / TIGHT JUNCTION / OXIDATIVE STRESS / ECV304 CELLS / HYDROGEN PEROXIDE

NAPAPORN THANOMLIKHIT : PROTECTIVE EFFECTS OF CAPTOPRIL ON OXIDATIVE STRESS-INDUCED DISRUPTION OF TIGHT JUNCTION STRUCTURE AND FUNCTION IN ECV304 CELLS. THESIS ADVISOR : ASST. PROF. SUREE JIANMONGKOL, Ph.D., 88 pp.

The objective of this study was to investigate the protective effects of captopril on oxidative stress-induced hyperpermeability of epithelial barriers, using the in vitro model of ECV304 cell monolayers grown for either 2 days or 12 days after seeding. Treatment the monolayers with $H_2O_2$ (200 μM for 4 h) had no effects on cell viability as measured by MTT assay. In 12 days after seeding, the ECV304 monolayers developed high transepithelial electrical resistance (TEER) value and restrictiveness against paracellular movement of phenol red, suggesting the formation of tight junction complexes. Exposure to $H_2O_2$ resulted in reduction of TEER and phenol red hyperpermeability. In addition, immunofluorescence visualization indicated the disruptive pattern of occludin and ZO-1 at the cell borders. However, the amount of occludin and ZO-1 proteins remained normal with the western blot analysis. In 2 days after seeding, the barrier function of ECV304 was not fully developed. Treatment of $H_2O_2$ during this period caused disruptive pattern of immunofluorescence staining of occludin and ZO-1 along with the decreased amount of occludin protein. Pretreatment the monolayers with captopril (100 μM for 30 min) prior to $H_2O_2$ could preserve the barrier restrictive functions. Regardless of the period, captopril was able to abolish the oxidative damages on localization and amount of tight junction proteins. The results also suggested the involvement of ERK1/2 pathway in tight junction stability.

Department : Pharmacology and Physiology Student's Signature .....

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# CONTENTS

Page

# LIST OF TABLES

# LIST OF FIGURES

Figure Page

# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| ACE | = | angiotensin converting enzyme |
| ANOVA | = | analysis of variance |
| ATP | = | adenosine triphosphate |
| BBB | = | blood brain barrier |
| BBMVEC | = | bovine brain microvascular endothelial cell |
| BMVEC | = | brain-derived microvascular endothelial cells |
| BSA | = | bovine serum albumin |
| Caco-2 | = | human colon adenocarcinoma |
| c-AMP | = | cyclic adenosine monophosphate |
| cm | = | centimeter |
| DMEM | = | Dulbecco's Modified Eagle's Medium |
| DMSO | = | dimetyl sulphoxide |
| et al. | = | et alii, and other people |
| e.g. | = | exempli gratia (for example) |
| ECV304 | = | human urinary bladder carcinoma cell line |
| EDTA | = | ethylenediaminetetraacetic acid |
| ERK | = | extracellularly regulated kinase |
| ERS | = | electrical resistance system |
| FBS | = | fetal bovine serum |
| g | = | gram |
| GTP | = | guanosine 5'-triphosphate |
| h | = | hour |
| $H_2O_2$ | = | hydrogen peroxide |
| HGF | = | hepatocyte growth factor |
| HIV | = | human immunodeficiency virus |
| HUVEC | = | human umbilical vein endothelial cell |
| HRP | = | horseradish peroxidase |
| IBD | = | inflammatory bowel disease |

| | | |
|---|---|---|
| JAM | = | junctional adhesion molecule |
| JNKs | = | c-Jun N-terminal kinases |
| kDa | = | kiloDalton |
| L | = | liter |
| LDH | = | lactate dehydrogenase |
| LDL | = | low-density lipoprotein |
| LLC-PK1 | = | kidney proximal tubule cell line |
| MAGUKs | = | membrane associated guanylate kinase-like proteins |
| MAP kinase | = | mitogen activated protein kinase |
| MDCK | = | Madin-Darby canine kidney cell line |
| mg | = | milligram |
| min | = | minute |
| ml | = | milliliter |
| mM | = | millimolar |
| mmol | = | millimole |
| MTT | = | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| MW | = | molecular weight |
| MWF | = | Munich Wistar Fromter |
| NO | = | nitric oxide |
| OD | = | optical density |
| $P_{app}$ | = | apparent permeability coefficient |
| PBS | = | phosphate buffered saline |
| PEG | = | polyethylene glycol |
| PG | = | phosphatidylglycerol |
| PKA | = | protein kinase A |
| PKC | = | protein kinase C |
| PVDF | = | polyvinylidine fluoride |
| ROS | = | reactive oxygen species |

| | | |
|---|---|---|
| rpm | = | round per minute |
| sec | = | second |
| SDS | = | sodium dodecyl sulfate |
| SEM | = | standard error of mean |
| T24 | = | human bladder cell line |
| TBS | = | tris buffered saline |
| TBST | = | tris buffered saline, tween 20 |
| TEER | = | transepithelial electrical resistance |
| µg | = | microgram |
| µm | = | micrometer |
| µM | = | micromolar |
| µmol | = | micromole |
| VEGF | = | vascular endothelial growth factor |
| ZO | = | zonular occludens |
| ZONAB | = | ZO-1-associated nucleic acid binding proteins |

# CHAPTER I

# INTRODUCTION

Formation of tight junctions between adjacent cells allows epithelial and endothelial monolayers to act as protective barriers against blood-borne xenobiotics and pathogens. The tight junction complexes can be found in either epithelial or endothelial cells that line up in several organ systems including renal, gastrointestinal tract, urinary bladder, vascular system, and central nervous system. These structures create a rate-limiting restrictiveness to paracellular transport through epithelial and endothelial barriers into the organ systems. Disruption of tight junction complexes has been linked to a variety of pathological conditions such as stroke, HIV encephalitis, Alzheimer's disease, multiple sclerosis, bacterial meningitis, ischemic insult, cancers, inflammatory bowel disease (IBD) and proteinuria (Huber *et al*., 2001; Gonzales-Mariscal *et al*., 2008).

Oxidative stress is one of the major underlying causes of tight junction disruption. Oxidative assaults could damage the tight junction structure through alteration of expression, localization and assembly of tight junction proteins (Jiang *et al*., 1998; Stelwagen *et al*., 1999; Antonetti *et al*., 2002; Lee *et al*., 2004). It has been reported that reactive oxygen species (ROS) disturbed the epithelial barrier function by destabilizing tight junction complexes in various tissues including renal, gastrointestinal tract and urinary bladder (Buse *et al*., 1995; Blum *et al*., 1997; Bolton *et al*., 1998; Harhaj *et al*., 2002). In bovine brain microvascular endothelial cells (BBMVECs), oxidative stress increased expression of tight junction protein occludin and altered rearrangement of occludin and zonular occludens (ZO)-1. Consequently, the endothelial monolayers lose their paracellular restrictiveness with the reduction of electrical resistance, as evidenced by an increased phenol red transport along with the reduced transepithelial electrical resistance (TEER) values (Lee *et al*., 2004). A number of studies suggested that activation of mitogen activated protein kinase (MAP kinase) pathway by oxidative stress resulted in phosphorylation of occludin and ZO-1 at serine and tyrosine residues, leading to abnormal arrangement of occludin and its dissociation from ZO-1 in

the tight junction complex. In particular, the phosphorylated form of extracellularly regulated kinase 1 and 2 (ERK1/2) was well correlated with oxidative stress-mediated hyperpermeability and tight junction disruption in endothelial monolayers (Kevil *et al*., 2000; Fischer *et al*., 2005; Radhakrishna, 2008).

Angiotensin converting enzyme inhibitors (ACE inhibitors) has been known as a group of medication for treatment of hypertension, chronic heart failure and renal diseases. These compounds exert its actions by inhibition of angiotensin converting enzyme (ACE) in renin-angiotensin system, leading to less production of angiotensin II. Beyond its blood pressure lowering effect, ACE inhibitors have been reported its clinical benefits in several pathological states including decrease stroke incidence and cardiovascular death as well as prevention of proteinuria (Chrysant, 2007; Schrader *et al*., 2007). In stroke-prone hypertensive rats, ACE inhibitors could prevent imminent cerebral edema, and reduced manifestation (Blezer *et al*., 1998). Although the underlying mechanisms of its beneficiary actions are still unclear, ACE inhibitors certainly have roles in protecting the endothelial functions (Antony *et al*., 1996). Several hypothesis have been proposed for its endothelial protective capability including antioxidant activities, anti-apoptotic actions (Cavanage *et al*., 1995, 2000; Benzie and Tomlinson, 1998; Chen *et al*., 2008), and its abilities to increase production of bradykinin and nitric oxide (NO) (Scribner *et al*., 2003).

The capability of epithelium and endothelium monolayers to function as protective barrier largely depends upon the integrity of tight junction complexes. A few studies suggested the protective capabilities of ACE inhibitors on functions and integrity of tight junction complexes. For example, ACE inhibitor (lisinopril) was able to prevent proteinuria spontaneously developed in the aging process of male Munich Wistar Fromter (MWF) rats. This effect was associated with the redistribution of ZO-1 at the glomerular basement membrane (Macconi *et al*., 2000). The protective effects of ACE inhibitor on either epithelial or endothelial functions may be related to its activation on enzymes in MAP kinases signaling pathway (Hu *et al*., 2007). In spontaneously hypertensive rats, captopril was able to prevent the vascular remodeling and reduce left ventricular weight-to-body weight ratios along with decrease in systolic blood pressure (Hu *et al*., 2007). These protective effects of captopril were related to its inhibition of

ERK1/2 activation. However, the abilities of ACE inhibitors in protecting structure and function of tight junction complexes are still unclear, and need further investigation.

This study aimed to demonstrate the protective activity of ACE inhibitors, in particular captopril, against oxidative stress-induced tight junction disruption in ECV304 cell lines. It was possible that captopril could prevent the loss of expression of tight junction proteins occludin and ZO-1 as well as their assembly and stabilization. In addition, these actions could be resulted from alteration of MAP kinase activities.

## Objectives

The specific objectives of this study were:

1. To investigate the protective effect of captopril against hydrogen peroxide ($H_2O_2$)-mediated disruption of tight junctions. Captopril might be able to protect the loss of expression and localization of tight junction proteins from oxidative assaults, and maintain integrity and function of tight junction complexes in ECV304 cells.
2. To examine an involvement of ERK1/2 in the protective actions of captopril on the structure and function of tight junction complexes.

## Scope of study

The present study investigated the protective effects of captopril on $H_2O_2$-mediated the disruption of tight junction complexes in ECV304 cells, using Western blot and immunofluorescence analysis. The integrity of tight junction was evaluated functionally by measurement of paracellular permeability and TEER. In this study, the non-lethal concentration of $H_2O_2$ was used to induce tight junction disruption, which was determined by functional analysis as well as an alteration of expression and localization of occludin and ZO-1. The protective effects of captopril were determined by pretreatment the ECV304 monolayers for 30 min prior to $H_2O_2$. Furthermore, an activation of ERK1/2 was also investigated as a potential protective mechanism of captopril against $H_2O_2$-mediated tight junction disruption.

# CHAPTER II

## LITERATURE REVIEW

Epithelial monolayers are the frontier between the internal organ and the surrounding environment constituted with the content of an internal cavity or duct. These monolayers become the restrictive barriers for the flux of ions, water and molecules. There are two ways for solutes transport across the monolayers, including the transcellular pathway and the paracellular pathway (Figure 1). In the transcellular pathway, the solute needs to be hydrophobic in order to diffuse through plasma membrane or alternatively with the use of specific transport mechanisms such as ion channels, protein transporters, or co-transporters. In the paracellular pathway, solute goes through the intercellular space between adjacent cells, which can be restrictive due to the special structures including tight junction, adherens junction, desmosome junction and gap junction. The tight junction complexes are formed between the apical membrane of adjacent cells and most tightness structure for regulated transport of substances (Gonzalez-Mariscal *et al*., 2008).

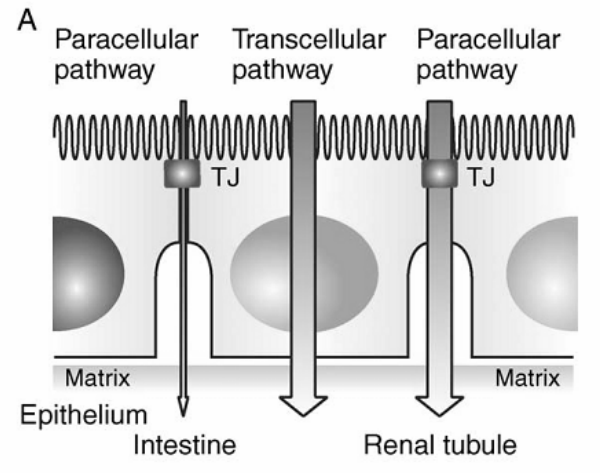
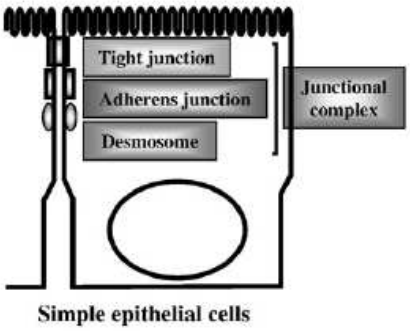



**Figure 1**: Epithelial structure and two pathways through membrane in simple epithelial monolayer (Miyoshi and Takai, 2005).

## Tight junctions

Tight junctions are the apical most constituent of the junctional complexes in vertebrate epithelium and endothelium cells that line up to surfaces of many tissues within the gastrointestinal, respiratory and urinary tracts (Figure 2A). Tight junctions create a rate-limiting barrier to paracellular diffusion of substances epithelium monolayers. Hence, tight junctions act as semipermeable barriers to the paracellular transport of ions, solutes and water. Moreover, tight junctions work as a fence that limits the movement of lipids and proteins within the plasma membrane between the apical and the basal surface. Recently, tight junctions were found to involve in the control of cell differentiation, proliferation, cell polarity and gene expression (Figure 2B) (Chiba *et al*., 2008). These functions of tight junctions are critical for epithelium cells to establish distinct tissue compartments within the body and maintain homeostasis (Chiba *et al*., 2008; Gonzalez-Mariscal *et al*., 2008). However, many physiological and pathophysiological situations require exchange of solutes and water between compartments. This demands requires the tight junction being able to rapidly regulate barrier function in response to appropriate stimuli (Yu and Turner, 2008)

A




B

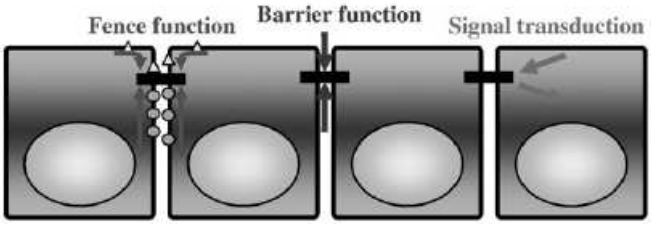



**Figure 2**: Apical junctional complex and tight junctions.

(A) Schematic drawing of the junctional complex.

(B) Functions of tight junctions (Chiba *et al*., 2008).

**Components of tight junctions**

Tight junctions are composed of transmembrane and cytoplasmic proteins that linked to an actin-based cytoskeleton. These tight junctions consist of three transmembrane proteins including occludin, claudin and junctional adhesion molecule (JAM) and cytoplasmic proteins including ZO family (ZO-1, ZO-2, ZO-3), cingulin, AF6 and 7H6 (Huber *et al*., 2001) (Figure 3). Their arrangement allows the tight junction to form a seal while remaining capable of rapid modulation and regulation.

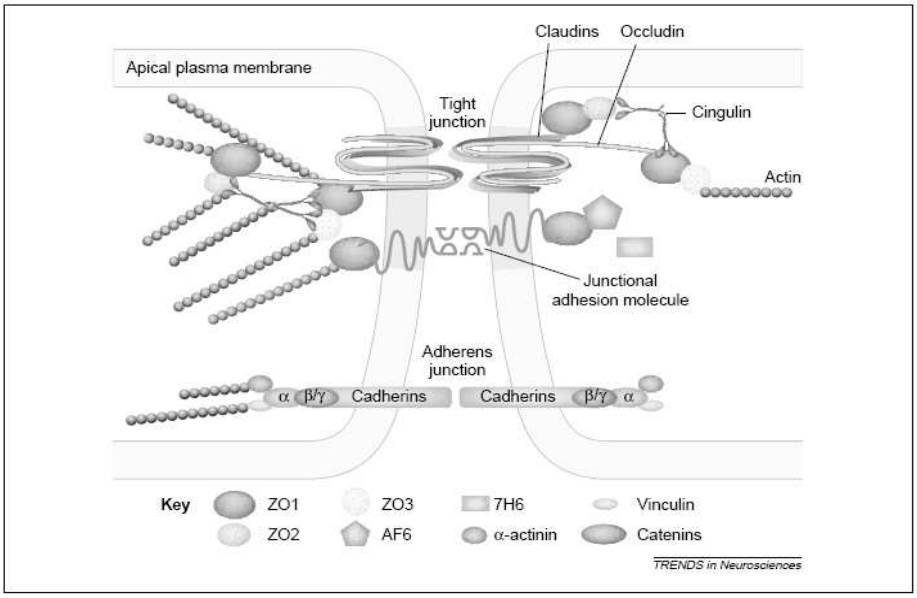



**Figure 3**: Proposed interactions of the major proteins associated with tight junction. Transmembrane proteins (occludins, claudins and JAMs) and cytoplasmic accessory proteins (ZO-1, ZO-2, ZO-3, AF-6 and 7H6) (Huber *et al*., 2001).

Occludin was the first identified component of tight junction complexes. Occludin is an approximately 65-kDa tetraspan membrane protein with two extracellular loops, a long COOH-terminal cytoplasmic domain, and a short $NH_2$-terminal cytoplasmic domain. The two extracellular loops of occludin and claudin originating from neighboring cells form the paracellular barrier of tight junction. The cytoplasmic domains of occludin is directly associated with ZO proteins (Ballabh *et al*., 2004). The cytoplasmic domain of occludin is highly phosphorylated when localized within the tight junction. The phosphorylation of occludin regulates tight junction permeability in G-protein dependent manner (Wolburg and Lippoldt, 2002). The external loops, the transmembrane and the COOH-terminal cytoplasmic domains of occludin are important for the regulation of paracellular permeability (Wolburg and Lippoldt, 2002). The $NH_2$-terminal cytoplasmic domain of occludin regulates transepithelial migration of neutrophils of which process is independent of the transepithelial resistance and the paracellular permeability (Huber *et al*., 2001).

ZO family was a group of cytoplasmic proteins which localize close to the tight junction membrane. ZO family contains three members, including ZO-1 (220 kDa), ZO-2 (160 kDa), and ZO-3 (130 kDa). These proteins belong to a family of proteins known as MAGUKs (membrane associated guanylate kinase-like proteins), which serve as recognition proteins for tight junction placement and as a support structure for signal transduction proteins (Haskins *et al*., 1998). ZO proteins are scaffolds that establish numerous protein-protein interactions. Several proteins involve in the cluster at the tight junction including various kinases, phosphatases, small G proteins and nuclear and transcription factors (Gonzalez-Mariscal *et al*., 2008). These proteins interact with each other and with other anchor membrane proteins such as occludin, claudins and JAMs, and actin cytoskeleton. ZO-1 concentrates at the nucleus when the cells are subjected to adverse conditions such as chemical stress or mechanical injury, or when the cells are cultured at a sparse density (Gonzalez-Mariscal *et al*., 2008). It has been reported that ZO-1 associates with proteins involving in the regulation of gene transcription and cell proliferation such as the transcription factors ZO-1-associated nucleic acid binding proteins (ZONAB) (Balda *et al*., 2003), Jun and Fos (Betanzos *et al*., 2004). Furthermore,

phosphorylation of ZO-1 was important for junction regulation (Tsukamoto and Nigam, 1999).

Occludin and ZO-1 was structural components of the tight junctions that seal the paracellular spaces between the cells and contribute to the epithelial or endothelial barrier function. Their alterations could indicate the function and integrity of tight junction as well as the barrier functional status. It has been suggested that protein phosphorylation plays an important role in tight junction assembly and function as well as the expression of tight junction proteins ZO-1 and occludin (Balda *et al*., 1991; Stuart and Nigam, 1995). Furthermore, the consequences of the phosphorylation and dephosphorylation of tight junction proteins can be influenced by oxidative stress, growth factors, cytokines, drugs, and hormones and by which amino acid residues where phosphorylation occurs (serine, threonine and tyrosine). For example, oxidative stress-induced hyperpermeability is associated with tyrosine phosphorylation of occludin and ZO-1, resulting in redistribution of occludin and ZO-1 from the intercellular junction (Rao *et al*., 2002). The exposure of kidney proximal tubule cell line (LLC-PK1) monolayers to interferon-$\alpha$ resulted in a dose- and time-dependent decrease in TEER values and increase in tyrosine phosphorylation of junctional protein occludin and occludin overexpression (Lechner *et al*., 1999). $H_2O_2$, hydrocortisone, prolactin, and unsaturated fatty acids enhanced malfunction of the tight junction barrier by increase of the occludin expression in endothelial and epithelial cells (Jiang *et al*., 1998; Stelwagen *et al*., 1999; Antonetti *et al*., 2002; Lee *et al*., 2004). Moreover, cytokines and growth factors such as tumor necrosis factor-$\alpha$, interferon-$\gamma$ increased permeability and mediated the discontinuous circumference of epithelial and endothelial cells upon visualization with immunofluorescence staining of ZO-1 and/or occludin (Buse *et al*., 1995; Blum *et al*., 1997; Bolton *et al*., 1998; Harhaj *et al*., 2002). Other stimuli including hepatocyte growth factor, vascular endothelial growth factor (VEGF), and histamine increased permeability in vascular endothelial cells through downregulation of occludin and/or ZO-1 proteins expression (Gardner *et al*., 1996; Antonetti *et al*., 1998; Jiang *et al*., 1999). Clearly, tight junctions are regulated by a diverse groups of extracellular stimuli that initiated certain intracellular signaling cascades. However, mechanistic links of signaling pathways to enhanced permeability have not been extensively elucidated.

## Signaling pathways associated with tight junction

There are two principle types of signal transduction processes associated with tight junction. One involves the signaling pathways from the cell interior towards tight junction in order to guide their assembly and to regulate paracellular permeability. The other is the signals transmitted from tight junction to the cell interior in order to modulate gene expression, cell proliferation, and differentiation (Matter and Balda, 2003). The complete pathways of signal transduction have not been fully understood. Multiple signaling pathways and proteins have been implicated in the regulation of tight junction assembly including calcium, protein kinase C (PKC), protein kinase A (PKA), G protein, calmodulin, cyclic adenosine monophosphate (c-AMP), and phospholipase C (Balda *et al*., 1991; Izumi *et al*., 1998; Mullin *et al*., 1998).

The assembly and regulation of tight junctions involve with several signaling pathways, especially enzymes in the MAP kinase family. Activation of these enzymes has been reported to be responsible for phosphorylation of numerous proteins in the cytoplasm and the nucleus including occludin and ZO-1.

## The MAP kinase signaling pathway

MAP kinases are Ser/Thr protein kinases that respond to extracellular stimuli such as growth factors and stress (Chang and Karin, 2001). They involve in the regulation of various cellular activities including gene expression, mitosis, differentiation and apoptosis (Johnson and Lapadat, 2002). The four district groups of MAP kinases are extracellular regulated protein kinase (ERK) 5, ERK1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNKs) and p38 kinases. The activation of MAPK signaling pathway by oxidative stress can be triggered of small guanosine 5'-triphosphate (GTP)-binding proteins (Small G protein including Ras and Rac protein). MAPK activity are regulated through three-kinase cascades composed of a step, which transmits the signal through a mediator kinase (MEKKKs), MEKKs, MEKs to MAPKs level of the cascades (Figure 4). Many MAP kinases activate specific effector kinases, MAPK-activated protein kinases (MAPKAPKs) and are inactivated by MAPK phosphatases (Chang and Karin, 2001).

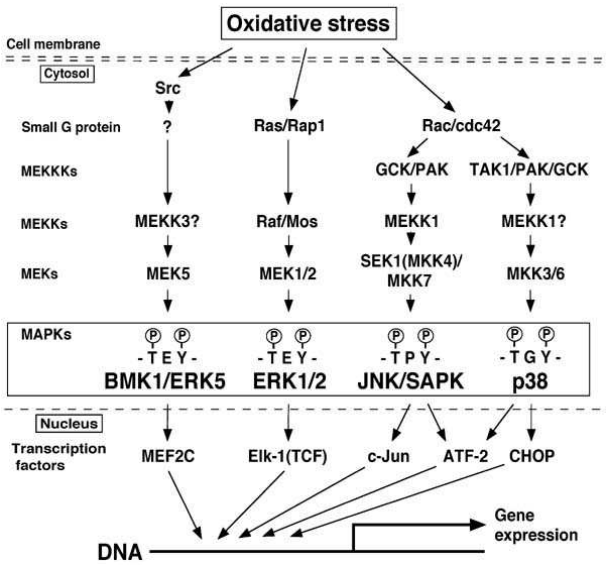



**Figure 4.** The MAP kinase signaling pathways that respond to oxidative stress.

The oxidative stress effects on gene expression possibly through activation of MAP kinase pathways. The activation of ERK5 has been involved with both growth factors and stress stimuli, leading to cell proliferation although its signaling pathway was yet unclear (Gonzalez-Mariscal *et al*., 2008). The stress-induced activation of JNKs and p38 has been linked to small G protein subtype Rac, which are involved in cell differentiation and apoptosis (Gonzalez-Mariscal *et al*., 2008). The activation of ERK1/2 coupled with small G protein subtype Ras to Raf signaling pathway has been affected the paracellular transport by altering junctional protein expression (Chen *et al*., 2000; Lan *et al*., 2004). Consequently, the molecular composition within tight junction complexes could be disturbed. It has been reported that various conditions lead to the activation of the ERK1/2 pathways, followed by a loss in tight junction assembly (Kevil *et al*., 2000; Fischer *et al*., 2005). Those conditions included cellular transfection with Ras or Raf, alcohol, oxidative stress, cytokine and hepatocyte growth factor (HGF) (Wang *et al*., 2004).

Oxidative stress is one of the major causes of hyperpermeability of endothelial barriers. The oxidative damages correlate to an activation of MAP kinase pathways especially ERK1/2 kinase, resulting in disarrangement of occludin (Kevil *et al*., 2000). Application of the specific ERK1/2 inhibitor (PD98059) could prevent an increase of permeability and occludin redistribution (Kevil *et al*., 2000; Fischer *et al*., 2005). In Madin-Darby canine kidney cell line (MDCK) transfected with an activated Ras mutant,

the activation of ERK1/2 increased significantly. Concurrently, these cells exhibited 6-fold increase in the transepithelial permeability of mannitol coupled with disappearance of ZO-1 from cell borders (Chen *et al*., 2000). Thus, activation of Ras is associated with the disassembly of tight junctions and enhanced permeability. In the MDCK cells (MDCK II), the HGF-mediated activation of ERK1/2 resulted in downregulation of claudin-2 and increase of TEER, while the treatment of the MDCK cells (MDCK I) with an ERK1/2 inhibitor induced expression of claudin-2, and decreased the TEER values by 20-fold (Lipschutz *et al*., 2005). Taken together, these data supported the role of ERK1/2 in the oxidative stress-mediated junctional damage.

Raf is likely a downstream mediator of Ras signaling that regulates tight junctions function. Expression of constitutively active Raf in epithelial cells decreased the expression of occludin, disrupted junctional protein and localization of actin cytoskeleton. Activation of Raf, induced the cells to become stratified instead of forming monolayers, and conferred anchorage-independent growth in soft agarose (Li and Mrsny, 2000). Ectopic expression of occludin in these cells reversed all of the effects induced by Raf-1 (Li and Mrsny, 2000). Raf-mediated transcriptional down-regulation of occludin was likely mediated by ERK1/2 since treatment of cells expressing constitutively active Raf with the MEK inhibitor PD98059 (Li and Mrsny, 2000). Thus, Ras/Raf/ERK1/2 signaling pathway mediates tight junction hyperpermeability in epithelial cells (Harhaj and Antonetti, 2004).

## Oxidative Stress

Oxidative stress is a state of imbalance between free radical production and the ability of the organism to defend against free radicals. A free radical is any chemical species that contain one or more unpaired electron, usually making it more reactive than the corresponding non-radical. This may be due to its ability to act as electron acceptors and essentially “steal” electrons from other molecules. The loss of electrons is called oxidative process. The free radicals are commonly referred as oxidizing agents. The most common cellular free radicals are hydroxyl radical ($OH^{\circ}$), superoxide anion ($O_2^{\circ -}$), peroxynitrite ($ONOO^-$) and nitric oxide ($NO^{\circ}$) (Jenner, 1996; Simonian and Coyle, 1996). Other molecules including $H_2O_2$ can generate free radical species through various chemical reactions. Oxidative stress is attributed to the aging process and to the pathogenesis of several degenerative diseases such as Alzheimer’s disease, Parkinson’s disease, seizure, stroke, cerebral ischemia/reperfusion injuries and trauma.

In this study, $H_2O_2$ has been employed to induce the oxidative stress and disruption of tight junction. In general, $H_2O_2$ is a byproduct of normal cellular oxidative metabolism and can be found in human plasma at micromolar concentrations (Lacy *et al*., 1998; Raimondi *et al*., 2000; Liu and Zweier, 2001). $H_2O_2$ has been implicated in the pathophysiology of a variety of cardiovascular diseases including hypertension, myocardial ischemia and atherosclerosis (Hearse *et al*., 1993; Ross, 1993; Lacy *et al*., 1998). However, there is a growing evidence to support the function of $H_2O_2$ as an inter- and intra-cellular signaling molecule. $H_2O_2$ is freely compatible with water with ability to readily cross cell membranes. $H_2O_2$ may undergo transformations into highly reactive hydroxyl radicals such as superoxide in the presence of transition metals (particularly iron and copper) (Halliwell and Gutteridege, 1990). Hence, $H_2O_2$ could produce oxidative damage to macromolecules at the plasma membrane as well as those in cytoplasm and nucleus of the cells (Halliwell and Gutteridege, 1987; Cochrane, 1991).

**Oxidative stress mediated disruption of tight junction**

Oxidative stress affects expression, localization and assembly of tight junction proteins. In epithelial cells, $H_2O_2$ causes an impairment of tight junction complexes which relates to phosphorylation of occludin and ZO-1 and the dissociation of these junctional proteins from the actin cytoskeleton (Rao *et al*., 2002; Kale *et al*., 2003). In the in vitro model of BBMVECs, $H_2O_2$ upregulated expression of occludin and caused the rearrangement of occludin and ZO-1 at tight junctions. These effects well correlated with increased paracellular permeability and reduction of TEER values (Lee *et al*., 2004). In lung endothelial cells, $H_2O_2$ was a key player responsible for the hypoxic injury through an activation of ERK1/2 (Parinandi *et al*., 2003). High concentration of $H_2O_2$ increased endothelial cell permeability which could relate to several signaling pathways including small G protein Rho (Hippenstiel *et al*., 2003). Furthermore, $H_2O_2$ triggered intracellular events, leading to the phosphorylation of occludin and loss of its interaction with actin cytoskeleton. These processes could be protected with VEGF through the inhibition of ERK1/2 activity (Basuroy *et al*., 2006). In brain-derived microvascular endothelial cells (BMVEC), $H_2O_2$ is main inducer leading to the intracellular release of $Ca^{2+}$ followed by activation of the ERK1/2 MAP kinase (Fischer *et al*., 2005). Moreover, in human umbilical vein endothelial cell (HUVEC), oxidative stress-mediated increased endothelial solute permeability involved the loss of endothelial tight junction integrity through increased ERK1/2 activation (Kevil *et al*., 2000).

**Angiotensin converting enzyme inhibitors (ACE inhibitors)**

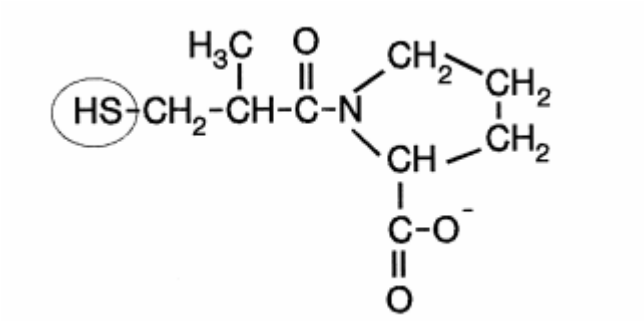
ACE inhibitors effectively reduce the activation of renin angiotensin system and produce vasodilation by inhibiting the formation of angiotensin II. This vasoconstrictor is formed by the proteolytic action of renin (released by the kidneys) and ACE. Renin converts circulating angiotensinogen into angiotensin I. Angiotensin I is then converted to angiotensin II by ACE. ACE also breaks down bradykinin (a vasodilator substance). Therefore, ACE inhibitors increase bradykinin levels, which can contribute to its vasodilator action. The increase in bradykinin is also believed to be responsible for a troublesome side effect of ACE inhibitors was a dry cough.

In addition to their blood pressure-lowering effects, ACE inhibitors reduce the risk of myocardial infraction, stroke, and cardiovascular death (Lonn, 2002). ACE inhibitors have been shown to reduce atherosclerosis in vivo models (rat models) through several protective mechanisms, including antiproliferative effects, reduction of blood pressure and low-density lipoprotein (LDL) oxidation, inhibitory effects on platelet activation, modulation of proinflammatory signals, reduction of macrophage accumulation and improvement of endothelial dysfunction (Scribner *et al*., 2003).

Captopril, the first ACE inhibitor marketed, is still in widespread use today. Although newer ACE inhibitors such as enalapril, benazepril, fosinopril, lisinopril, moexipril, quinapril and ramipril differ from captopril in terms of pharmacokinetics and metabolism, all the ACE inhibitors have similar overall effects on blocking the formation of angiotensin II. Currently, ACE inhibitors are used for treatment of hypertension, myocardial infarction, and congestive heart failure as well as for preservation of kidney function in diabetic nephropathy.

**Captopril**

Captopril is a specific competitive inhibitor of ACE. Captopril is a white to off-white crystalline powder that may have a slight sulfurous odor; it is soluble in water (approximately 160 mg/mL), methanol, and ethanol and sparingly soluble in chloroform and ethyl acetate. The adverse drug reaction profile of captopril is similar to other ACE inhibitors, with cough being the most common adverse drug reaction. However, captopril is also commonly associated with rash and taste disturbances (metallic or loss of taste), which are attributed to the unique sulfhydryl moiety. This sulfhydryl was shown effective scavengers of nonsuperoxide free radicals and also scavenged the other toxic oxygen species hydrogen peroxide and singlet oxygen and inhibited microsomal lipid peroxidation (Chopra *et al*., 1992) (Figure 5).



(2S)-1-[(2S)-2-methyl-3-sulfanylpropanoyl] pyrrolidine-2-carboxylic acid

**Structural formula** $C_9H_{15}NO_3S$ **Molecular weight** 217.29 g/mol

**Figure 5.** Chemical structure of captopril.

Captopril was reported to attenuate oxidative stress-induced endothelial cell apoptosis via p38 MAP kinase inhibition (Yu *et al*., 2006). Several hypothesis of its action include free radical scavenger, antioxidative activity (Cavanage *et al*., 1995, 2000; Benzie and Tomlinson, 1998; Ravati *et al*., 1999; Chen *et al*., 2008) and also increased production of bradykinin and NO (Scribner *et al*., 2003). Among ACE inhibitors, only captopril has been shown the antioxidant activity, which may be due to its thiol moiety in its structure (Benzie and Tomlinson, 1998).

In addition to its blood pressure-lowering effect, captopril has been shown to prevent or reduce urinary protein excretion, glomerular injury, and deterioration of renal function in either experimental animals or in insulin-dependent diabetic patients with nephropathy (Hommel *et al*., 1986; Parving *et al*., 1988; Remuzzi *et al*., 1990). Moreover, certain ACE inhibitors, lisinopril could prevent imminent and reduce manifest cerebral edema in stroke-prone hypertensive rats (Blezer *et al*., 1998). They also have renoprotecitve effects which prevented proteinuria and glomerular redistribution of the protein ZO-1 in MWF rat (Macconi *et al*., 2000). In addition, captopril significantly decreased systolic blood pressure, reduced left ventricular weight-to-body weight ratios, and prevented vascular remodeling in mesenteric arteries in spontaneously hypertensive rats through mechanisms involving decreased Ras expression and phosphorylation of ERK1/2 (Hu *et al*., 2007). The captopril had affected on phosphorylation of ERK1/2 and the tight junction disruption by $H_2O_2$ involved activation of ERK1/2. The captopril might protect tight junction disruption from $H_2O_2$ which correlated ERK1/2 MAP kinase pathway. However, there are no reports of the protective effects of captopril or other ACE inhibitors on tight junction disruption.

In this study, captopril was hypothesized to protect oxidative stress-induced tight junction disruption through mechanisms involving alteration of expression and assembly of tight junction proteins, in particular occludin and ZO-1. These effects might relate to its actions on ERK1/2, a kinase in MAP kinase signaling pathways. The study was carried on in the vitro model of ECV304 cells monolayers.

# CHAPTER III

# MATERIALS AND METHODS

## Materials

### Chemicals

The following chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA); Acrylamide, Bradford reagent, Bovine serum albumin (BSA), Captopril, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Ethylenediamine tetraacetic acid (EDTA), Glycine, Glycerol, N',N'-methylendiacylamide, Phenol red, Protease inhibitor (aprotinin), Penicillin G sodium, Secondary anti-rabbit Cy-3 conjugated, Sodium phosphate dibasic anhydrous, Streptomycin sulfate, Triton X-100, 0.04% Trypan blue, Trypsin and Tween 20.

The following chemicals were purchased from Merck (Darmstadt, Germany); Hydrogen peroxide, Methanol, Pefabloc, Potassium chloride, Potassium dihydrogen phosphate, Skim milk powder, Sodium bicarbonate, Sodium chloride, Sodium hydroxide and Tris-(hydroxymethyl) aminomethane.

Acetic acid and Dimetyl sulphoxide (DMSO) were purchased from Labscan (Gliwice, Ireland).

Developer replenishes, Fixer replenishes and Film X-ray blue were purchased from Kodak (Rochester, NY, USA).

Fetal bovine serum was purchased from Hyclone (Waltham, MA, USA).

M199 medium and M199-free phenol red medium were purchased from Gibco Life Technologies (Grand Island, NY, USA).

Page Ruler™ Prestained Protein Ladder was purchased from Fermentus life Science (Hanover, MD, USA).

Polyvinylidine difluoride (PVDF) transfer membranes were purchased from Pall Gelman Laboratory (Pensacola, FL, USA).

Secondary goat anti-mouse IgG (H&L) horseradish peroxidase (HRP) was purchased from KPL (Gaithersburg, MD, USA).

Super signal West Pico chemilluminescent substrate and secondary goat anti-rabbit IgG (H&L) HRP were purchased from Pierce Biotechnology (Rockford, IL, USA).

The mouse monoclonal anti-β–actin was purchased from Cell Signaling technology (Beverly, MD, USA).

The rabbit monoclonal anti-ZO-1 and anti-occludin were purchased from Zymed (South San Francisco, CA, USA).

The rabbit polyclonal anti-ERK1/2 and anti-phosphorylated ERK1/2 were purchased from Calbiochem (San Diego, CA, USA).

All other chemicals and solvents used throughout this study were commercially available reagents or analytical grade reagent.

## Experimental instruments

The following instruments were used in this study

1. 12-well, 24-well, 96-well cell culture plate, Transwell® insert, Tissue culture flasks, Sterilization filtration membranes 0.22 μm (cellulose acetate membrane) were purchase from Corning, NY, USA
2. Inverted microscope: Axiovert 135, Zeiss, Konstanz, Germany
3. Fluorescence microplate reader: Anthos Labtec HT2 version 1.21E, Anthos Labtec Inst., Salzburg, Austria
4. Milicell®-electrical resistance system (ERS) potentiometer: Millipore, MA, USA
5. Mini-PROTEAN® 3 Electrophoresis Cell and Mini Trans-Blot Electrophoretic Transfer cell: Bio-Rad, CA, USA
6. Fluorescence microscope: BX-FLA, Olympus, Tokyo, Japan
7. Refrigerated centrifuge: Z 383K, Hermle Labortechink, Burladingen, Germany
8. Humidified carbon dioxide incubator: Forma Scientific, OH, USA
9. Vortex mixer: model K550-GE, Scientific Industries, NY, USA

## Methods

### Cell culture

The ECV304 cell line was obtained from Cell Lines Service (Eppelheim, Germany, Lot No. 600560-2).

The ECV304 cell line is human urinary bladder carcinoma cell line. This cell line is derivative of human bladder cell line (T24) (Takahashi *et al*., 1990). The morphology of cultured cells was observed by an inverted microscope with phase contrast optics in combination with microcomputer-assisted image capture system (Pinnacle 8, Pinnacle system, Germany) (Figure 6).

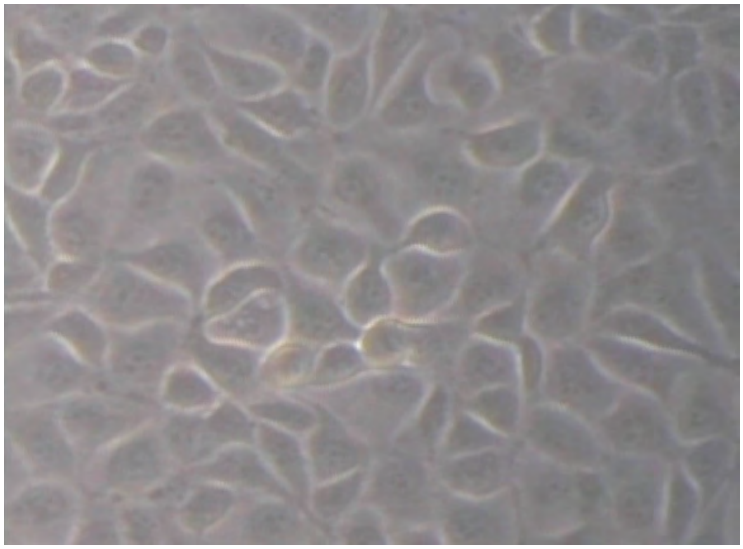


**Figure 6:** The morphology of ECV304 cells under normal culture condition (5 days after seeding). Magnification: x40.

**Maintenance of ECV304 cells**

The cells were grown in the M199 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified incubator with 95% relative humidity maintaining at 37°C and 5% $CO_2$.

The cells were routinely seeded at initial density of $3x10^5$ cells per 15 ml in 75-$cm^2$ tissue culture flasks. The medium was replaced every 48 h. The cells were subcultured at no more than 80% confluency (approximately 3-5 days after seeding). The cell monolayer was washed with phosphate buffered saline (PBS) (5 ml), and detached from the culture flask by incubating with 0.25% trypsin in 1mM EDTA solution (2 ml/75 $cm^2$) for 4 min at 37°C. At the end of incubation period, the medium (4ml) was added to stop the action of trypsin. Then, the cell suspension was dispersed and centrifuged at 1,200 rpm for 4 min at 4°C. The supernatant was discarded. The pellet was resuspended in the fresh M199 medium containing 10% FBS and 1% penicillin/streptomycin for further experiment. The cells were seeded at density $1.3x10^5$ cells/$cm^2$ each well for experiments.

The study was divided into 3 parts as follows:

1. Determination of the effects of $H_2O_2$ and captopril on ECV304 cells viability.
2. Induction of tight junction disruption in the ECV304 model by $H_2O_2$.
3. Determination of the protective effects of captopril on the $H_2O_2$-induced tight junction disruption and its involvement with ERK1/2 activity.

## Methods

### 1. Cell viability test with MTT assay

The cell viability was assessed by MTT assay. ECV304 cells were cultured in 96-well culture plates at the seeding density $1.3x10^5$ cells/$cm^2$. After 48 h, the cells were treated with either captopril and/or $H_2O_2$ at certain conditions as follows.

1. Treatment with $H_2O_2$ at the concentration ranging from 0 to 500 μM at various incubation times of 0.5, 1, 2 and 4 h.
2. Treatment with captopril at the concentration ranging from 0 to 500 μM for 24 h.
3. Treatment with captopril for 30 min prior followed by $H_2O_2$ for 4 h.

### The MTT assay

The objective of this assay was determined the non-lethal concentration of captopril and $H_2O_2$. The MTT assay has been widely chosen as an indirect method for determination of cell viability. The principle of this method is based on the reduction of yellow water-soluble tetrazolium dye to a purple water-insoluble formazan product by active mitochondria dehydrogenase of the living cells (Figure 7) (Mosmann, 1983). Then, the water-insoluble MTT-formazan crystals were dissolved in DMSO and read the absorbance at 570 nm (Freshney, 2005).

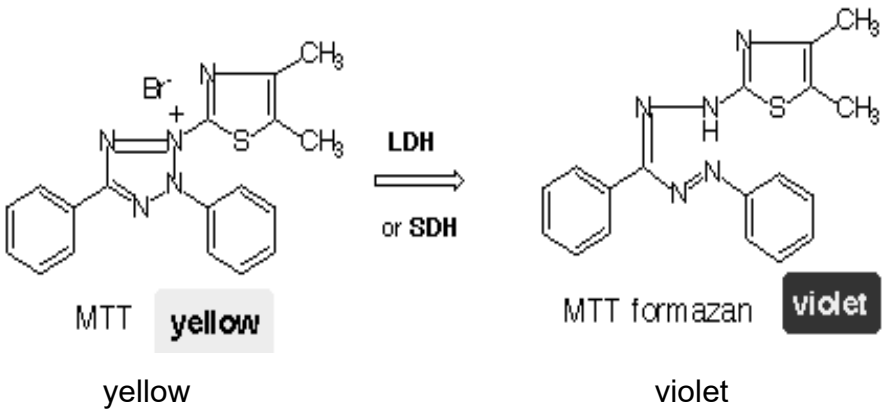



**Figure 7:** Molecular structure of MTT and their reaction product.

In this study, the assay was initiated by replacement of the medium with 100 µl MTT solution (0.4% mg/ml) in each well. Then, the cells were incubated for 4 h in a 95% relative humidity at 37°C and 5% $CO_2$ atmosphere. At the end of incubation, the MTT solution was replaced by 100 µl DMSO to dissolve MTT-formazan crystals. The optical density (OD) was read in a microplate reader at an absorbance of 570 nm, using a reference wavelength of 620 nm (Takahashi and Abe, 2002).

**2. Validation of the ECV304 cell model for $H_2O_2$-induced tight junction disruption**

The ECV304 cell lines grown for either 2 days or 12 days after seeding were used in this study of $H_2O_2$-induced tight junction disruption.

2.1 Tight junction in ECV304 monolayers grown for 12 days after seeding

ECV304 cells were seeded onto Transwell® inserts (Corning, USA (Appendix C)) with 0.4 μm in pore size, 12 mm in diameter at a density $1.3x10^5$ cells/$cm^2$. Then, the insert was immersed in the culture medium, of which the volume was adjusted to 0.5 ml in the upper side and to 1.5 ml in the lower side. Cells were grown under the 95% relative humidity maintain at 37°C and 5% $CO_2$. The medium was replaced and measured for TEER values every 3 days until reaching steady state values (approximately 12 days). Then, 200 μM $H_2O_2$ was added in the upper side and incubated for 4 h. After the end of incubation, cells were visualized by immunofluorescence staining for the presence of tight junction proteins occludin and ZO-1. In addition, assay of phenol red was applied to evaluate paracellular permeability which would be restrictive in the presence of tight junction.

2.2 Tight junction in ECV304 monolayers grown for 2 days after seeding

ECV304 cells were seeded onto Transwell® inserts at a density $1.3x10^5$ cells/$cm^2$ and grown for 2 days. Then, the monolayers were visualized by immunofluorescence staining methods as mentioned above. At this early stage, the TEER values was still in the rising phase, suggesting the absence of “tight” bridging between adjacent cells. The formation of tight junction structure was in an early phase.

**3. The functional studies of tight junction complexes**

In order to evaluate the functional tight junctions of ECV304 monolayers, measurements of TEER and paracellular permeability were performed as follows.

**3.1 Measurement of TEER**

Generally, ECV304 cells monolayers with effective tight junction display high TEER values, ranging between 50 and 100 $\Omega \cdot cm^2$ after 14 days in culture (Kramer *et al*., 2002).

In this study, ECV304 cells were seeded onto Transwell® inserts at a density $1.3x10^5$ cells/$cm^2$. The ERS potentiometer had chopstick electrodes with the short electrode being inserted in the apical side whereas the longer electrode being placed in the basolateral side.

TEER values of ECV304 monolayers is estimated as follows:

$$TEER_{monolayer} = (TEER_{total} - TEER_{membrane}) \times A$$

where $TEER_{monolayer}$ = TEER of ECV304-monolayers at the specified time ($\Omega \cdot cm^2$)

$TEER_{total}$ = Total TEER measured at the specified time

$TEER_{membrane}$ = TEER of the polycarbonate membrane measured before cell seeding

A = area of Transwell® insert ($cm^2$)

### 3.2 Measurement of paracellular permeability

The paracellular permeability could be determined by the paracellular movement of phenol red across the cell monolayers. Phenol red has been suitably used in this method because it hardly permeates through plasma membrane (Martel *et al*., 2003).

In this study, ECV304 cells were seeded onto Transwell® inserts at a density of $1.3x10^5$ cells/$cm^2$ and cultured in complete M199-free phenol red for 12 days after seeding prior to each experiment. Then, captopril (10, 100 or 500 μM) was added into the Transwell® inserts and incubated for 30 min. After the incubation period, captopril was replaced with medium that containing 50 μM phenol red and/or 200 μM $H_2O_2$ in the apical side. Meanwhile, the complete M199-free phenol red (1.5 ml) was added to the basolateral side. Subsequently, a 100 μl of basolateral medium was taken hourly to quantify the extent of phenol red spectrophotometrically at 450 nm.

Phenol red transport (from apical to basolateral side) was monitored for a period of 4 h. The apparent permeability coefficient ($P_{app}$) of phenol red was calculated as follows (Martin, 1993):

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{C_0 A}$$

where $P_{app}$ = apparent permeability coefficient (cm/sec)

dQ/dt = the amount of phenol red appearing in the receiver compartment as a function of time (nmol/sec)

A = the surface area across which the transport occurred ($cm^2$)

$C_0$ = the initial concentration in the donor compartment (μM)

**4. Determination of protein expression**

The expression of tight junction proteins at cell borders was visualized with immunofluorescence staining technique. In addition, western blotting analysis was also performed to determine the expression of specific proteins.

**4.1. Immunofluorescence microscope**

The localization of tight junction proteins was visualized by immunofluorescence staining technique (Chen *et al*., 2000; Lee *et al*., 2004). In this study, ECV304 cells were seeded onto Transwell® inserts at a density $1.3x10^5$ cells/$cm^2$. On the day of an experiment, cells were treated with 100 μM captopril for 30 min prior to 200 μM $H_2O_2$. After 4 h-incubation period, the cells were washed three times with PBS, and then fixed with cold ethanol/acetic acid (95:5) solution at -20°C for 10 min. Then, the cells were permeabilized with 0.05% Triton X-100 at room temperature for 10 min, followed by blocking with 3% BSA in PBS for 30 min. Next, the cells were immunostained with primary antibody (either occludin or ZO-1 (1:200)) in blocking solution for 1 h, followed by treatment with Cy3 conjugated secondary antibodies (1:400) in blocking solution for 30 min in the dark. Then, the cells were washed and visualized with an immunofluorescence microscopy at excitation wavelength 490 nm and emission wavelength 570 nm.

**4.2 Western blot analysis**

The expression of tight junction proteins and ERK1/2 was determined by western blot analysis. ECV304 cells were seeded onto Transwell® inserts at a density $1.3x10^5$ cells/$cm^2$. On the day of an experiment, cells were treated with 100 μM captopril for 30 min prior to 200 μM $H_2O_2$. After 4 h, the cells were washed with PBS, and extracted for proteins with iced-cold lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM Pefabloc) and protease inhibitor (Aprotinin) for 30 min. After centrifugation at 10,000 rpm for 10 min at 4°C, the supernatant was collected for further protein determination. The protein concentration was determined with Bradford method (Bradford, 1976). The samples were mixed with sample buffer (60 mM Tris-HCl pH 6.8, 25% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue), boiled at 95°C for 5 min and stored at -20°C until used.

In order to determine the expression of occludin and ZO-1, equal amounts of protein samples (30 μg/lane) were loaded onto 9% and 8% SDS-polyacrylamide gel electrophoresis and separated under constant voltage of 180 V for 45 min. After gel electrophoresis, protein bands in the gel were electrotransferred to PVDF membrane under constant voltage of 60 V for 90 min. The membranes were blocked in 5% skimmed milk in TBST (pH 7.5, 10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20) for 60 min at room temperature. Then, the membranes were incubated with specific primary antibodies at the dilution of 1:2000 for rabbit monoclonal anti-occludin, 1:1000 for rabbit monoclonal anti-ZO-1 and 1:500 for mouse anti-β-actin, and incubated for 2 h at 4°C. After washing for 7 min with TBST three times, the membranes were incubated with the corresponding HRP-conjugated secondary antibody, goat anti-rabbit immunoglobulin (IgG) (1:2000) in TBST, for 60 min at room temperature. Subsequently, the membranes were washed as described above and incubated with an enhanced chemiluminescence detection reagent, SuperSignal® chemiluminescent substrate for 5 min. The immunoblots were exposed on X-ray films at dark room. Actin bands were monitored on the same blot to verify the consistency of protein loading. Films were scanned and the band intensity of each group was compare to that of control.

The effect of captopril on MAP kinase expression was also determined by western blot analysis. Equal amount of protein samples (30 µg/lane) were loaded and separated onto 12% SDS-polyacrylamide gel electrophoresis under constant voltage of 180 V for 45 min. After transferred protein bands in the gel to PVDF membrane, the membrane were blocked with 3%BSA in TBST for 60 min at room temperature. The membranes were incubated with specific primary antibodies at the dilution of 1:1000 for anti-ERK1/2 and anti-phosphorylated ERK1/2 for overnight at 4°C. After incubation period, goat anti-rabbit HRP secondary antibody was applied. Blots were developed using the SuperSignal® chemiluminescent substrate. Three separate experiments were performed for each treatment group.

**Statistical analysis**

All data were expressed as mean±SEM. Data were analyzed using one-way analysis of variance (ANOVA) with Tukey post hoc test for multiple comparisons. Paired T-test was also used for comparison where appropriate. Significance was accepted at $P < 0.05$.

# CHAPTER IV

# RESULTS

## 1. Effects of $H_2O_2$ and captopril on cell viability

In this study, at least 90% of ECV304 cells survived the 4 h-treatment of $H_2O_2$ at the concentration of lesser than 200 μM, as measured by the MTT assay (Figure 8). Captopril at the concentration up to 500 μM had no effects on cell viability after treatment for 24 h (Figure 9). Furthermore in Figure 10, the ECV304 cells treated with captopril prior $H_2O_2$ treatment had no synergistic effect in causing cell death. Pretreatment the ECV304 cells with captopril at the concentration 10, 100, and 500 μM for 30 min prior to 4 h-exposure to 200 μM $H_2O_2$ had no influence on cell survival.

In addition, at the end of TEER measurement on 12 days after seeding, cell survival was assessed with trypan blue staining. The high numbers of blue-stained cells were hardly seen in both the control group and in the $H_2O_2$ treated group. Moreover, general morphology of the cells in both groups was comparable. These results suggested that the cultured period for 12 days after seeding had no significant effect on cell viability in $H_2O_2$ treatment (Appendix B).

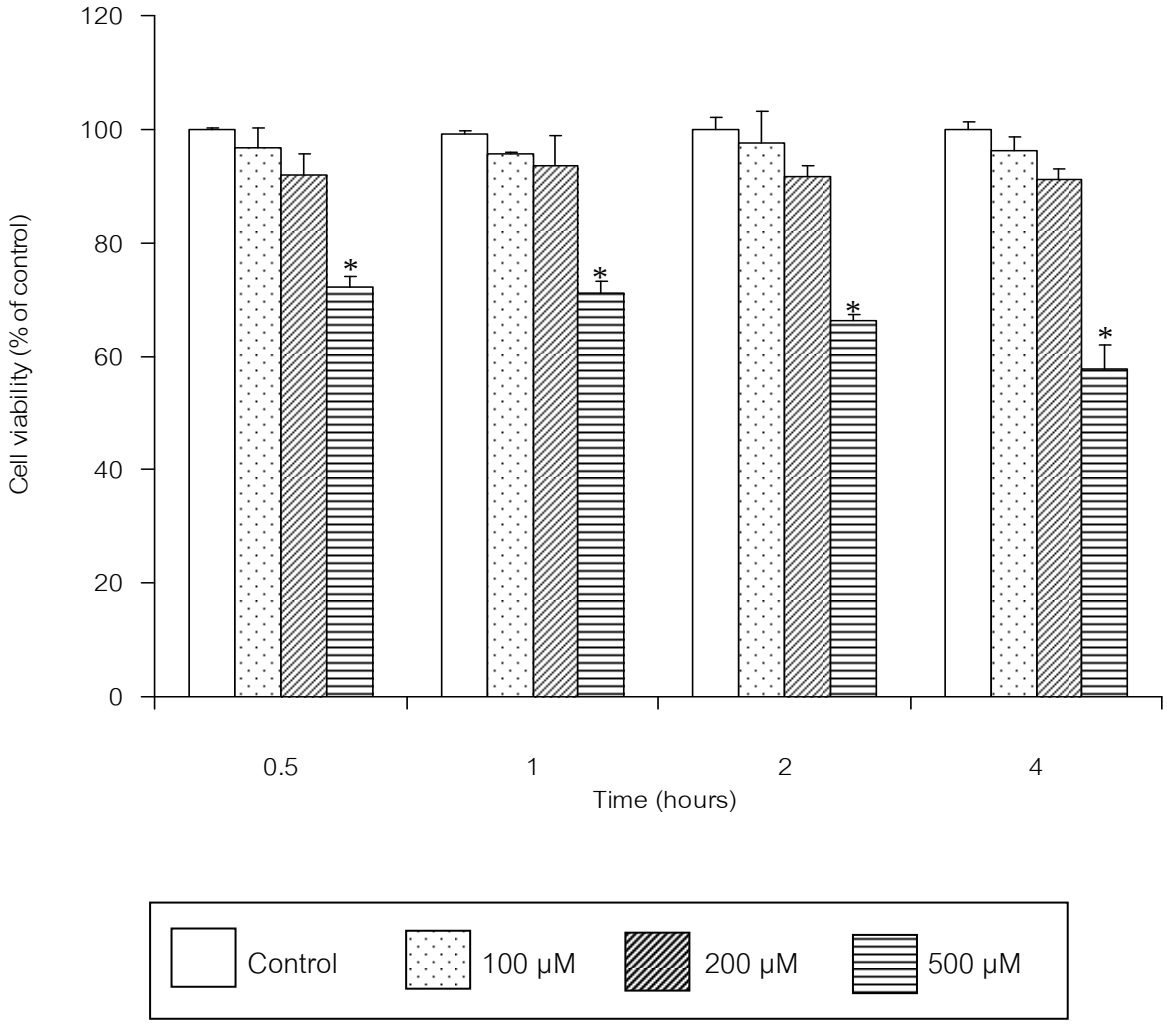



**Figure 8:** Viability of ECV304 cells after $H_2O_2$ treatment. The cell viability was assessed by MTT assay. Data are presented as mean±SEM (N=3).

*indicates significant differences from the control group at $P<0.05$.

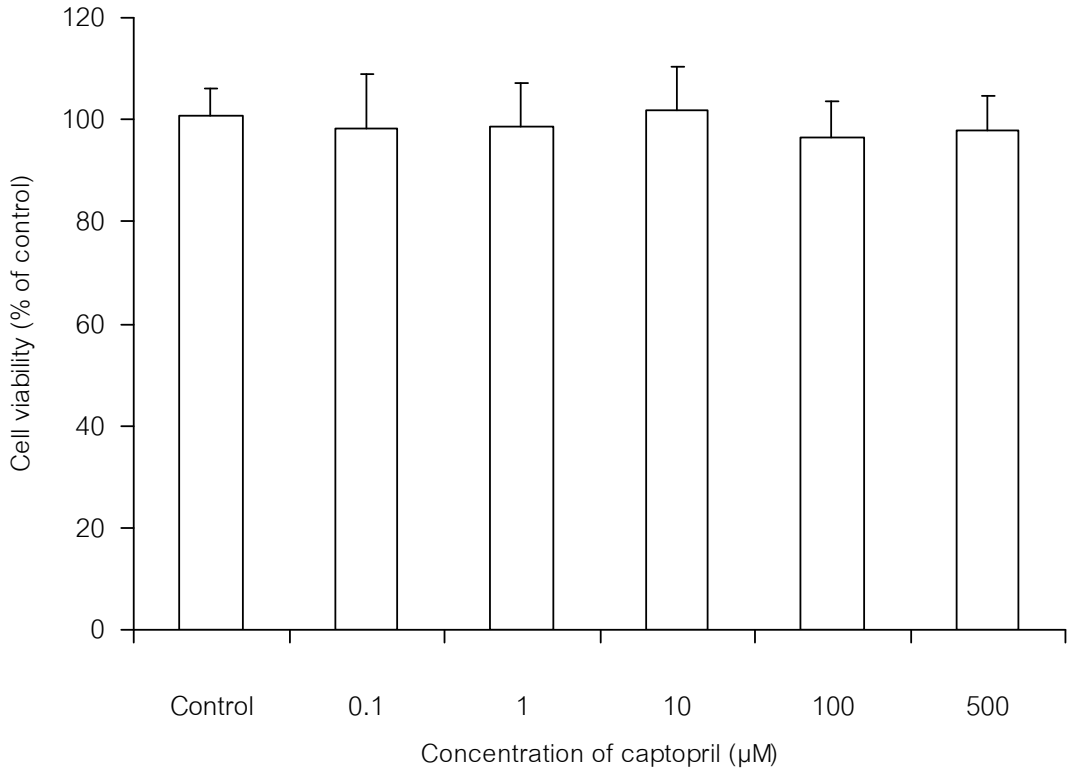



**Figure 9:** Viability of ECV304 cells after treatment with captopril for 24 h. The cell viability was assessed by MTT assay. Data are presented as mean±SEM (N=3).

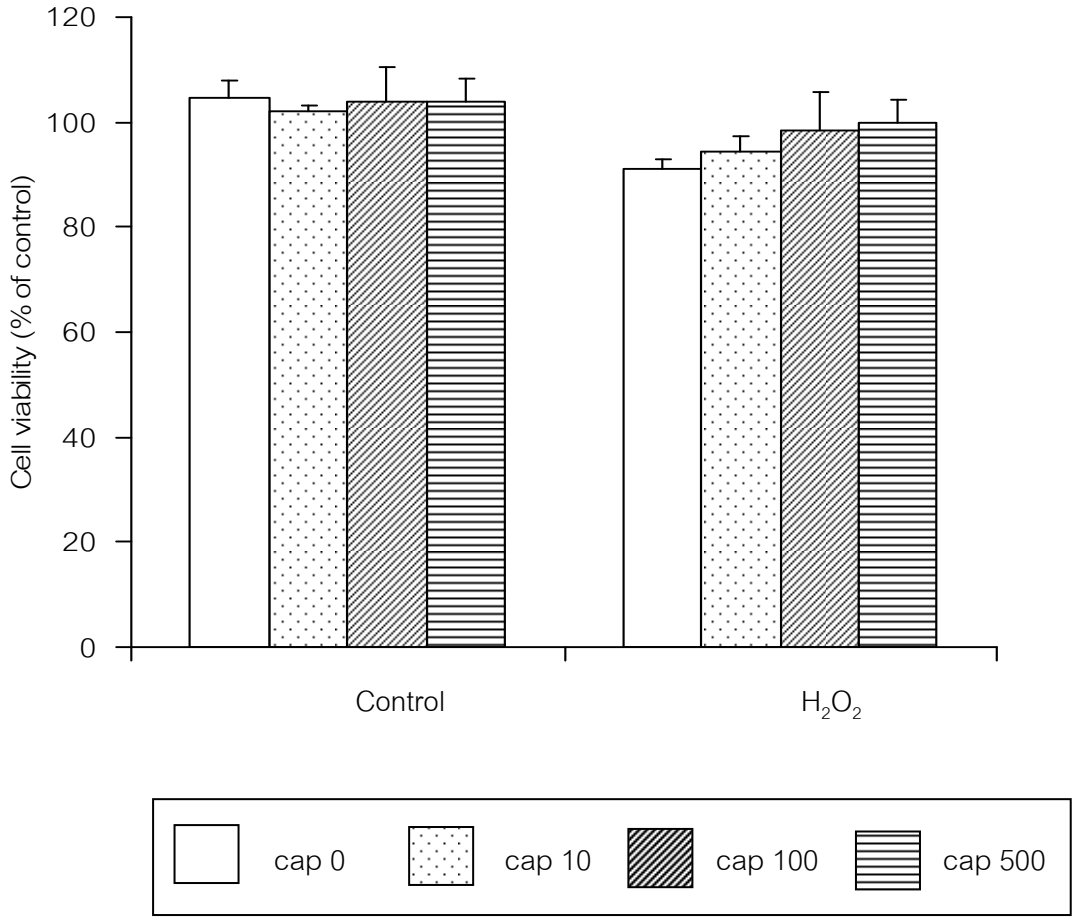



**Figure 10**: Effects of combined captopril with $H_2O_2$ on the viability of ECV304 cells. The cells were treated with captopril 30 min prior to addition of 200 μM $H_2O_2$. After 4 h- incubation period, the cell viabilities were determined with the use of MTT assay. Each bar represents the mean±SEM (N=3).

**2. Expression of tight junction in ECV304 monolayers grown for 12 days after seeding and its disruption by $H_2O_2$**

**2.1 Tight junction in ECV304 monolayers**

Culture of ECV304 monolayers resulted in time-dependent formation of restrictive barriers. As shown in Figure 11, TEER values increased in time-dependent fashion and reached its maximal value on day 9-12. At the plateau state on day 12, the apparent TEER values were 94.03±2.52 $\Omega.cm^2$. In addition, on day 12 the monolayers became restrictive toward paracellular transport, as evidenced by the low phenol red permeability (Table 1). As known, the functional tight junction would tightly restrict the movement of phenol red through the paracellular route of the monolayers. Under the normal condition, ECV304 monolayers expressed the continuous distribution pattern of occludin and ZO-1 proteins at the cell boundaries, as shown in Figure 12A and 12B, respectively. These findings suggested that the functional tight junctions developed in time-dependent manner and the barrier function could be achieved later in at least day 9-12.

### 2.2 Effect of $H_2O_2$ on tight junction disruption

In 12 days after seeding, treatment the ECV304 monolayers with $H_2O_2$ at the concentration of 200 μM for 4 h caused disruption of tight junction between adjacent cells, as evidenced by changes in phenol red permeability, TEER, and immunofluorescence patterns of tight junction proteins. Treatment with $H_2O_2$ caused significant increases in phenol red transport by 13-folds (Table 1) and reductions in TEER values by 3-folds (Table 2). These findings correlated with discontinuous pattern of immunofluorescence staining of occludin and ZO-1 (Figure 12C and 12D).

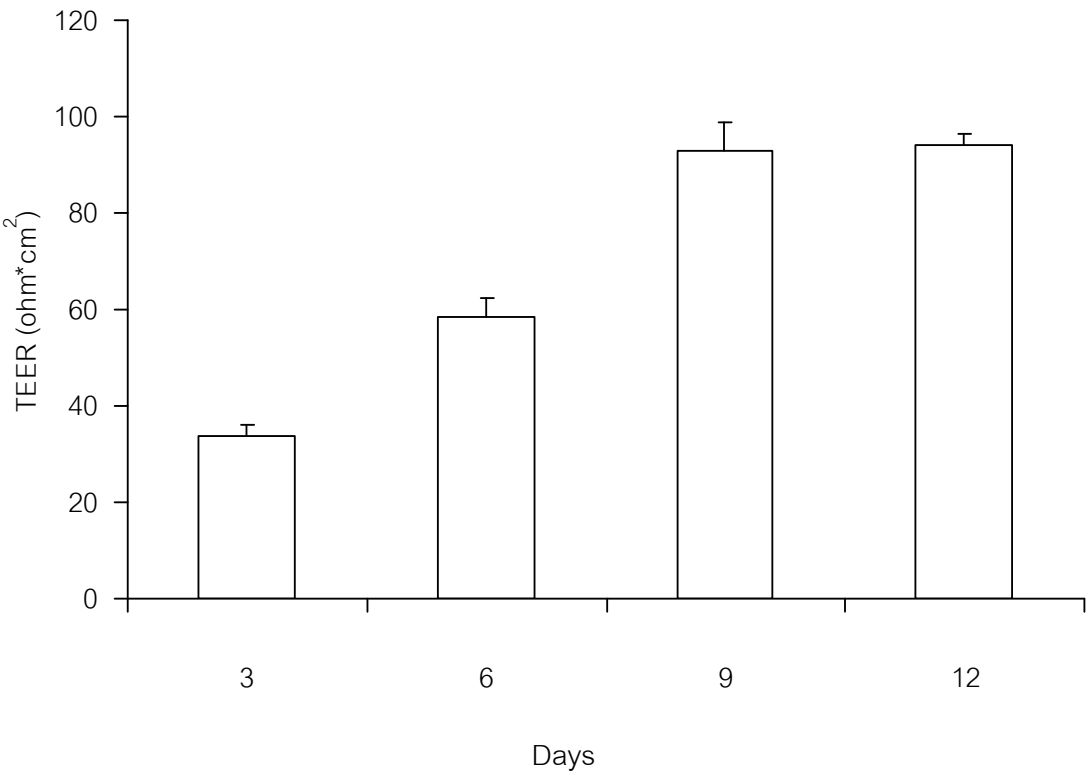



**Figure 11:** Development of the TEER values of ECV304 monolayers. The TEER values increased by 3-fold in 12 days after seeding. The data are expressed as the mean±SEM (N=4).

**Table 1:** The Papp values of ECV304 monolayers on Transwell® inserts 12 days after seeding (N=4).

| Groups | Papp ($x10^{-5}$ cm/sec) |
|---|---|
| Control | 4.41±1.15 |
| $H_2O_2$ | 57.89±3.67* |

*indicates significant differences from the control group at $P<0.05$.

**Table 2:** The TEER values of ECV304 monolayers on Transwell® inserts 12 days after seeding (N=4).

| Groups | TEER values ($\Omega.cm^2$) |
|---|---|
| Control | 90.44±11.68 |
| $H_2O_2$ | 31.22±8.40* |

*indicates significant differences from the control group at $P<0.05$.

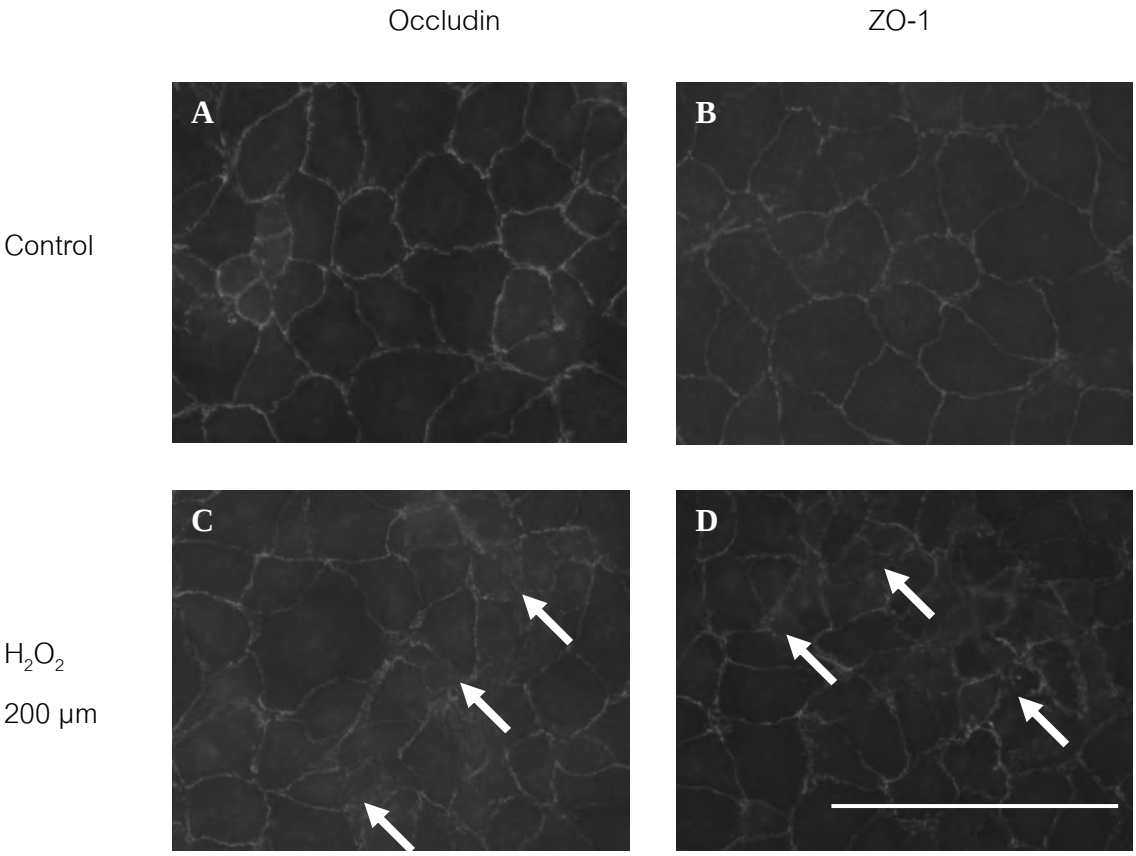



**Figure 12:** Immunofluorescence staining for expression of tight junction proteins occludin and ZO-1 in ECV304 monolayers grown for 12 days after seeding; control group (A, B), $H_2O_2$ treatment group (C, D). The ECV304 monolayers were treated with 200 µM $H_2O_2$ for 4 h. The immunofluorescence was visualized. Note that $H_2O_2$ caused discontinuous pattern of occludin and ZO-1 proteins. Bar 100 µm. Arrows indicated disruption of tight junction proteins pattern.

### 2.3. Protective effects of captopril on $H_2O_2$-induced tight junction disruption

#### 2.3.1 Functional expression of tight junction

At the concentration up to 500 μm, captopril had no influence on tight junction integrity and function, as evidenced by the indifferences in TEER and phenol red transport from the control group (Figure 13 and 14). Pre-treatment the cells monolayers with captopril for 30 min was able to prevent the hyperpermeability and reduction of TEER values induced by 4 h-exposure to 200 μM $H_2O_2$. As shown in Figure 13 and 14, the protective effect of captopril was concentration dependent. At the concentration of 100 μM, captopril completely abolished the oxidative assaults of $H_2O_2$ on the barriers function of ECV304 monolayers.

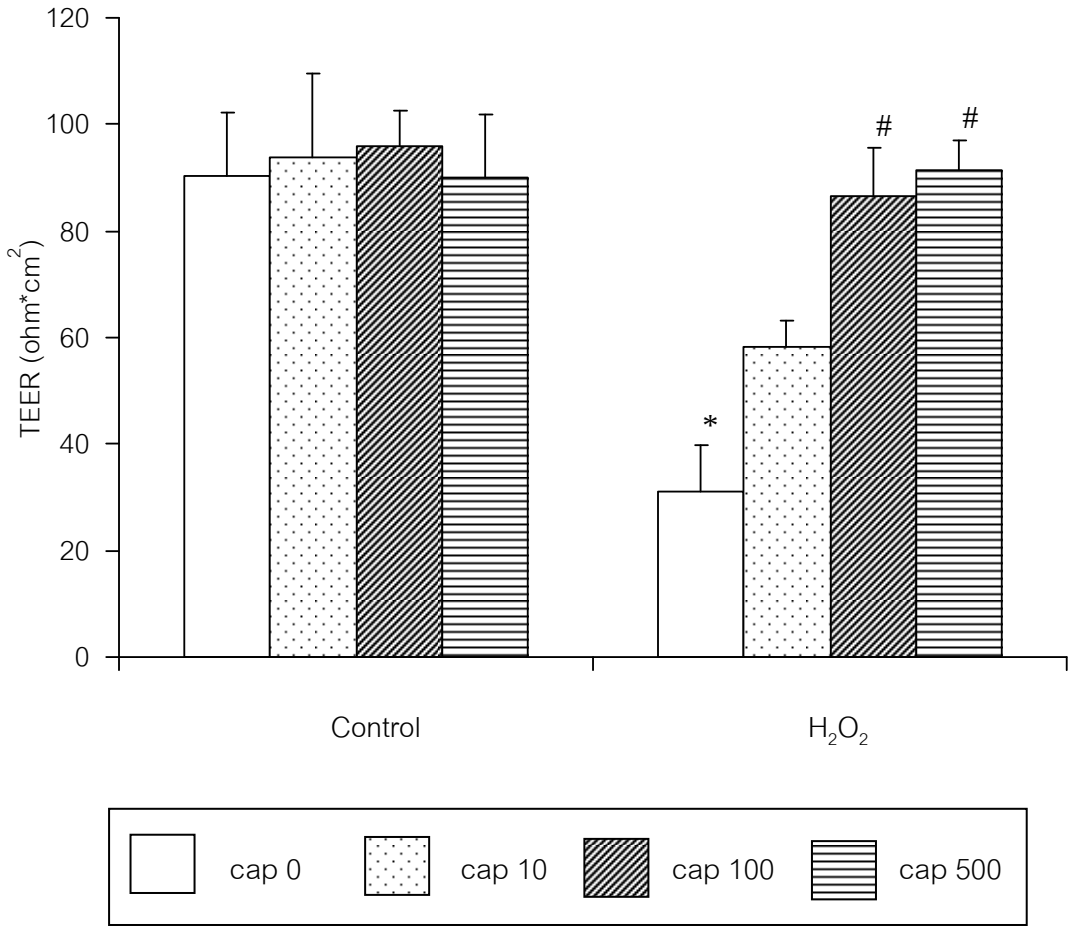



**Figure 13:** Effects of captopril on TEER values of the ECV304 monolayers in the model of $H_2O_2$-induced tight junction disruption. Captopril (10, 100, and 500 μM) was treated for 30 min prior to treatment with 200 μM $H_2O_2$ for 4 h. Each bar represents the mean±SEM (N=4).

*****$P$<0.05 indicates significant differences from control; and #$P$<0.05 indicates significant differences from $H_2O_2$ alone.

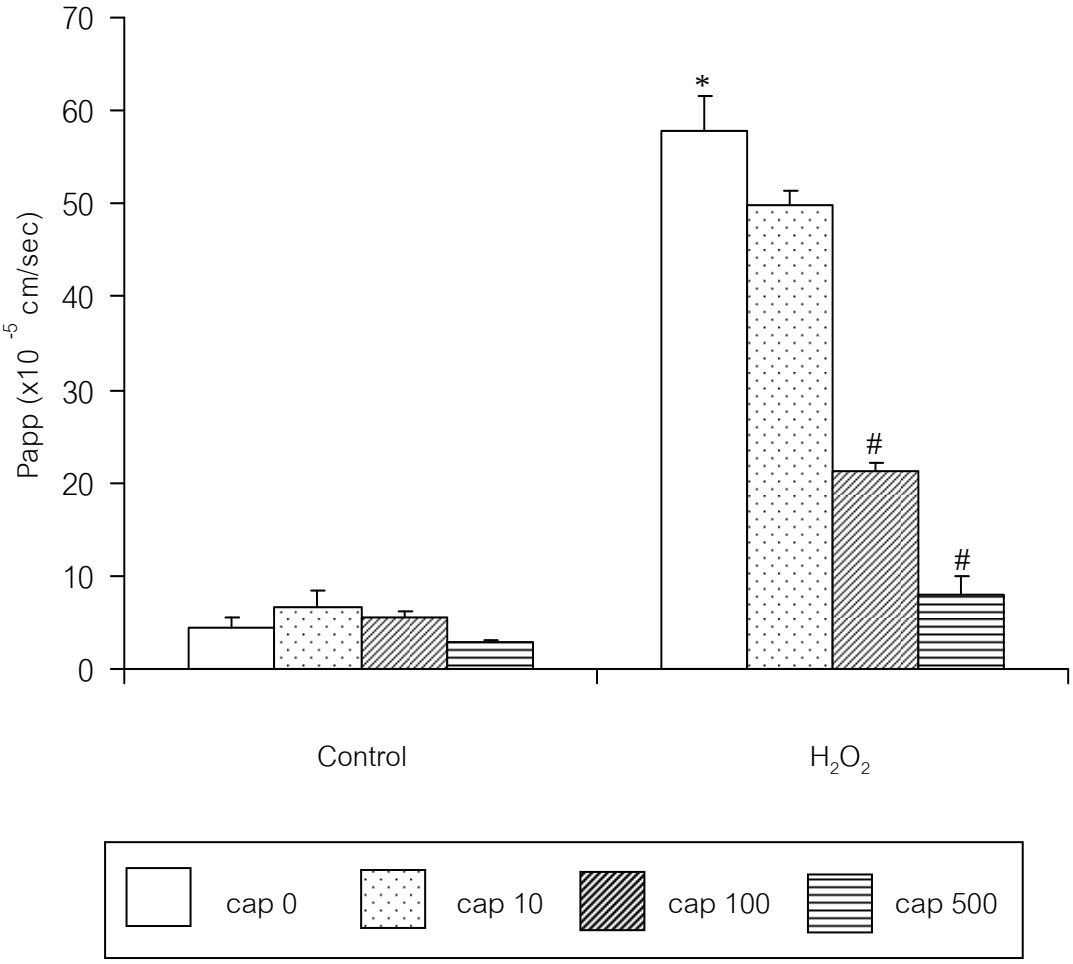



**Figure 14:** Paracellular permeability of ECV304 cells monolayers, as measured by phenol red transport. Cell monolayers grown on Transwell® inserts were treated with/without captopril for 30 min prior 200 μM $H_2O_2$ treatment for 4 h. The apical-to-basolateral flux of phenol red was measured. Each bar represents the mean±SEM (N=4).

*****$P$<0.05 indicates significant differences from control; and #$P$<0.05 indicates significant differences from $H_2O_2$ alone.

### 2.3.2 Localization and expression of tight junction proteins

In order to evaluate the involvement of occludin and ZO-1 in $H_2O_2$-mediated the loss the barrier function, the localization and expression of these tight junction proteins were determined by the immunofluorescence microscopy and western blot analysis.

***Immunofluorescence analysis*** Under the normal condition, ECV304 monolayers expressed the continuous distribution pattern of occludin and ZO-1 proteins at the cell boundaries, as shown in Figure 15A and 15B, respectively. Captopril had no influence on the expression and localization of these proteins at the cell borders, as shown in Figure 15C and 15D. By contrast, $H_2O_2$ at the concentration of 200 μM apparently disrupted the distribution pattern as seen by a discontinuous pattern of the immunofluorescence staining at the cell borders in Figure 15E and 15F. Treatment the monolayers with 100 μM captopril for 30 min prior to $H_2O_2$ completely abolished the effects of $H_2O_2$ on the circumferent expression and localization of occludin and ZO-1, as shown in Figure 15G and 15H.

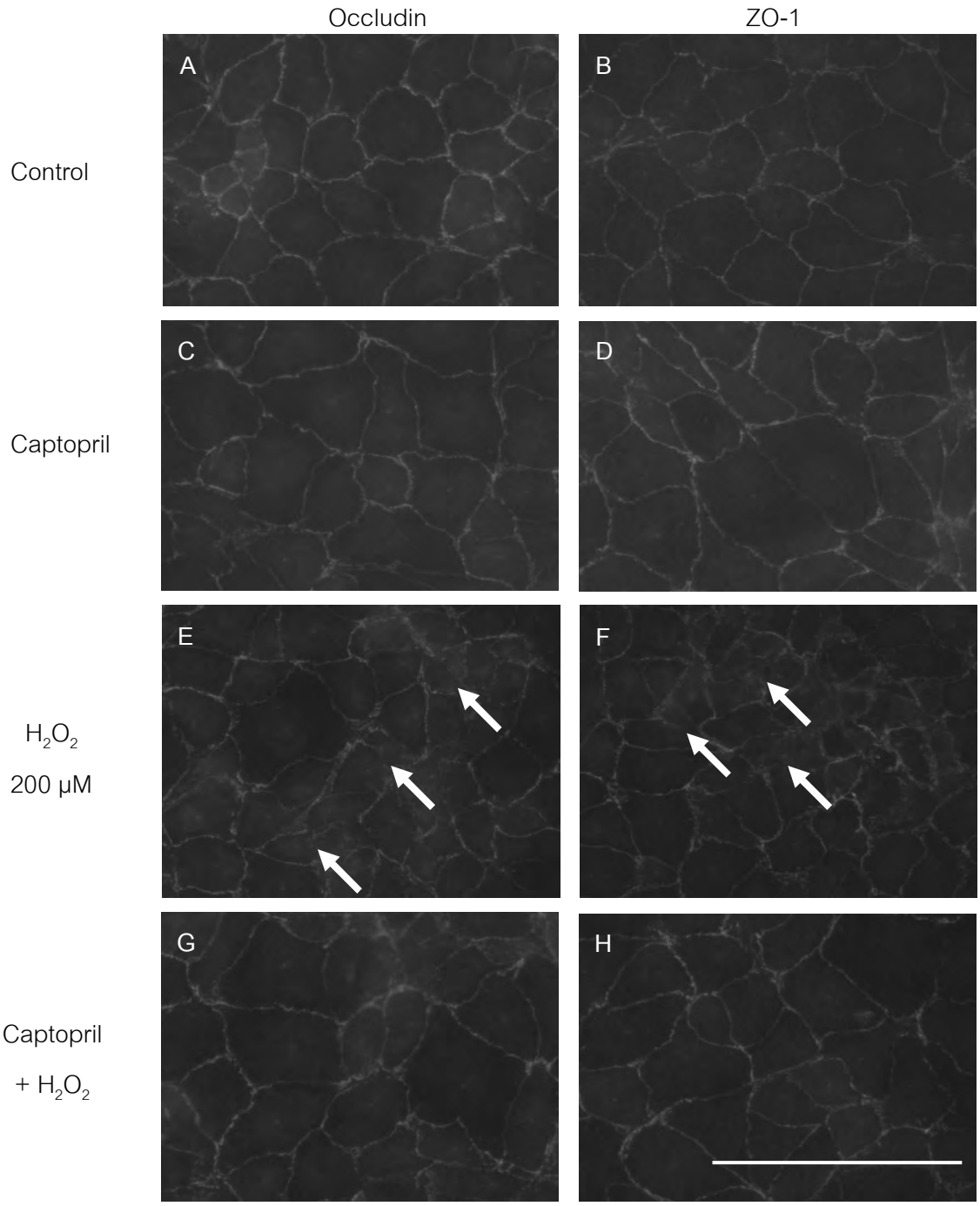



**Figure 15:** Immunofluorescence staining for tight junction proteins occludin (left panel) and ZO-1 (right panel) of the ECV304 monolayers grown for 12 days after seeding. The cells were included control groups (A, B), captopril alone groups (C, D), $H_2O_2$ alone groups (E, F) and captopril prior $H_2O_2$-treated groups (G, H). Bar 100 µm. Arrows indicated disruption of tight junction proteins pattern.

***Western blot analysis*** By immunoblotting, alterations in amount of tight junction proteins including occludin and ZO-1 were determined. The specific antibody for occludin, ZO-1 and actin recognized its targets at the expected molecular weight in ECV304 cells homogenate. The occludin, ZO-1 and actin bands were detected at 65, 220 and 42 kDa, respectively.

As shown in Figure 16, treatment the ECV304 cells grown for 12 days after seeding with either 200 μM $H_2O_2$ for 4 h or 100 μM captopril for 30 min had no significant effect on protein amounts of the occludin and ZO-1 proteins. The protein amounts of occludin and ZO-1 were comparable in all treated groups. Hence, the losses of tight junction proteins were not involved in the $H_2O_2$-induced barrier leakage of ECV304 monolayers.

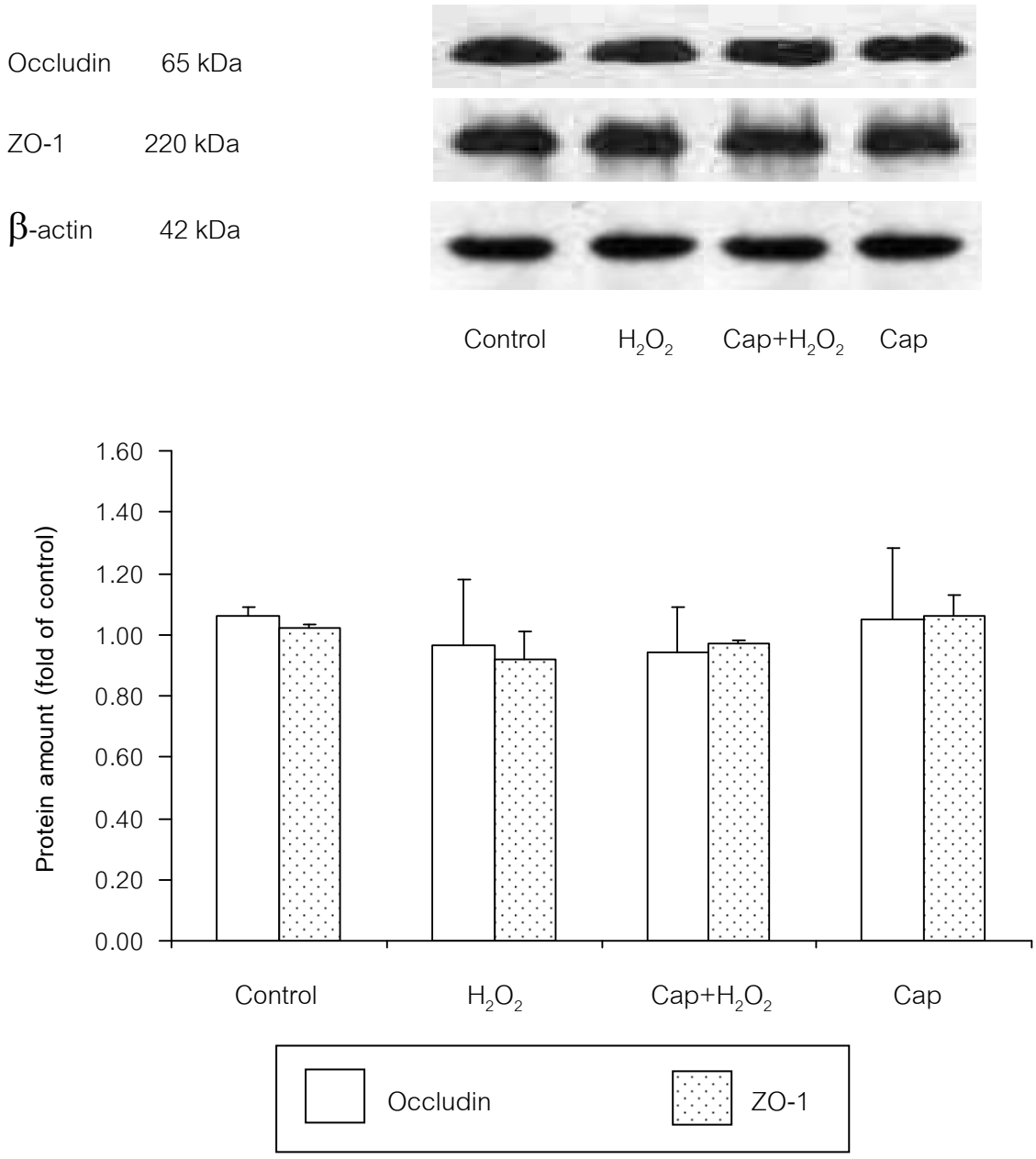
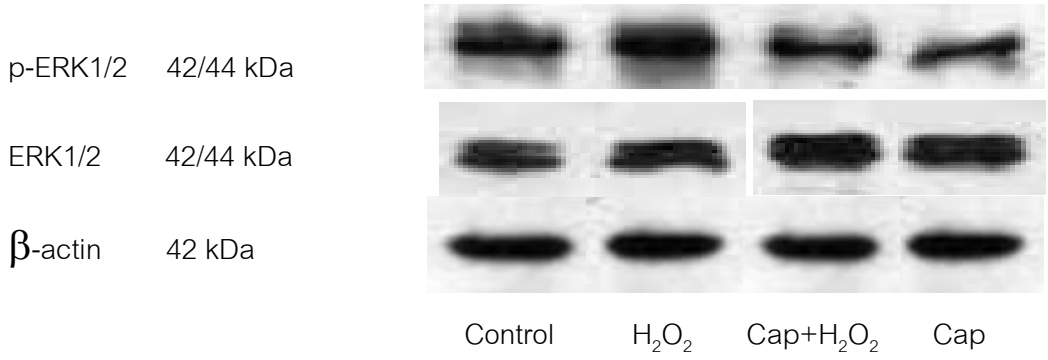



**Figure 16:** The protein amounts of occludin and ZO-1 in ECV304 monolayers grown for 12 days after seeding as determined by western blot analysis. Cell lysated were run on 9% and 8% SDS-PAGE. Densitometrical analysis of immunoblots of occludin and ZO-1 were performed and expressed as fold-increases relative to control group.

### 2.3.3 An involvement of ERK1/2 pathway in $H_2O_2$-induced tight junction disruption

As shown in Figure 17, $H_2O_2$ caused a significant increase in phosphorylated ERK1/2 (p-ERK1/2). The level of ERK1/2 phosphorylation decreased significantly in the monolayers pretreated with 100 μM captopril for 30 min prior to $H_2O_2$ treatment, comparing to those expressed in the $H_2O_2$ alone group. The results suggested that captopril was able to completely prevent the $H_2O_2$-mediated activation of ERK1/2 in the ECV304 cells.



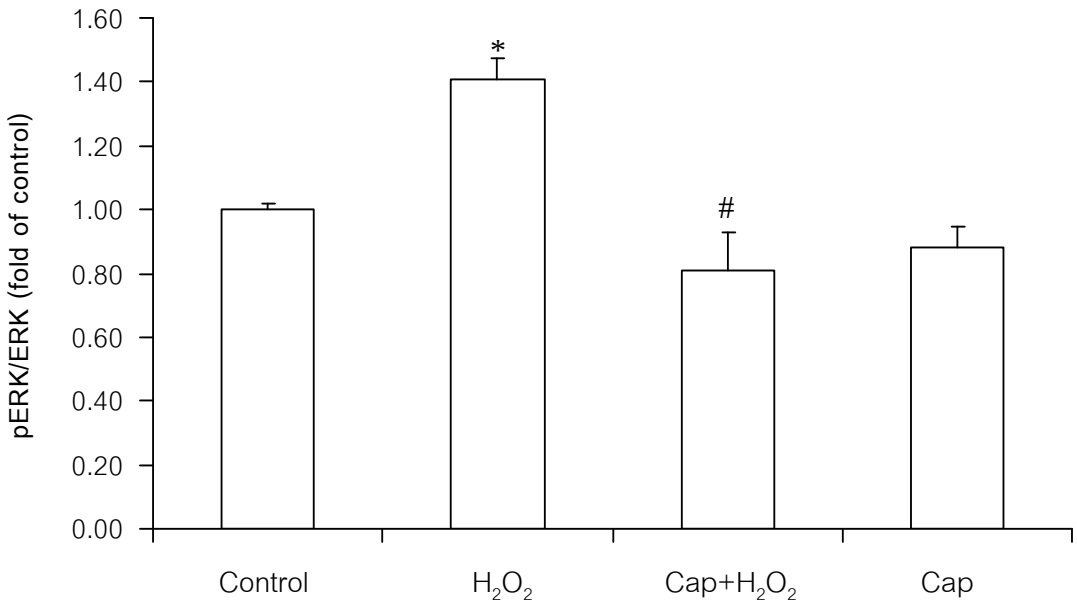




**Figure 17:** The expression of p-ERK1/2 and ERK1/2 in ECV304 monolayers grown for 12 days after seeding as determined by western blot analysis. Cell lysated were run on 12% SDS-PAGE. Densitometrical analysis of immunoblots performed and the results were expressed as the ratio of phoshorylated ERK1/2 to ERK1/2. Each bar represents the mean±SEM (N=3).

*****$P<0.05$ indicates significant differences from control; and #$P<0.05$ indicates significant differences from $H_2O_2$ alone.

**3. Expression of tight junction in ECV304 monolayers grown for 2 days after seeding and its disruption by $H_2O_2$**

**3.1 Tight junction formation and effect of $H_2O_2$**

In this study, the protective effects of captopril on tight junction proteins against $H_2O_2$ treatment in the early phase of tight junction formation were investigated. The barrier function of ECV304 monolayers was not fully developed after grown the cells for 2 days. However, the immunofluorescence visualization revealed the expression of occludin and ZO-1 tight junction proteins at the cell border (Figure 18A and 18B, respectively). These findings suggested that the tight junction proteins expressed at the cell border within 2 days after seeding.

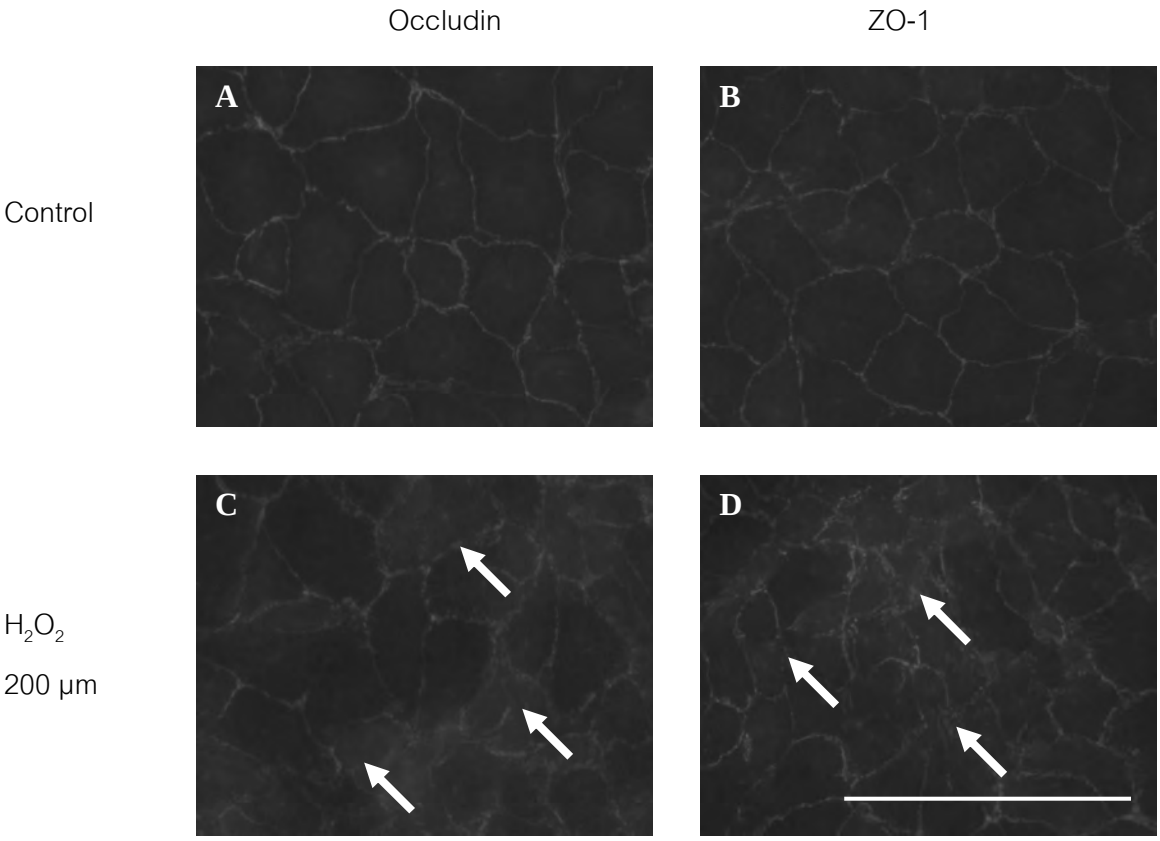



**Figure 18:** Immunofluorescence staining for expression of tight junction proteins occludin and ZO-1 in ECV304 monolayers grown for 2 days after seeding; control group (A, B), $H_2O_2$ treatment group (C, D). The ECV304 monolayers were treated with 200 µM $H_2O_2$ for 4 h. The immunofluorescence was visualized. Note that $H_2O_2$ caused discontinuous pattern of occludin and ZO-1 proteins. Bar 100 µm. Arrows are indicated disruption of tight junction proteins pattern.

### 3.2 Protective effects of captopril on tight junction proteins against $H_2O_2$ treatment

#### 3.2.1 Localization and expression of tight junction proteins

The alteration of occludin and ZO-1 in the ECV304 monolayers grown for 2 days from $H_2O_2$ were determined with the immunofluorescence microscopy and western blot analysis.

***Immunofluorescence analysis*** ECV304 monolayers grown for 2 days after seeding expressed the continuous distribution pattern of occludin and ZO-1 proteins at the cell boundaries, as shown in Figure 19A and 19B, respectively. Captopril had no influence on the localization and expression of these proteins at the cell boundaries, as shown in Figure 19C and 19D. By contrast, 200 μM $H_2O_2$ apparently disrupted the distribution pattern as seen by a discontinuous pattern of the immunofluorescence staining at the cell borders in Figure 19E and 19F. Treatment the monolayers with 100 μM captopril for 30 min prior to $H_2O_2$ completely abolished the effects of $H_2O_2$ on the circumferent expression and localization of occludin and ZO-1, as shown in Figure 19G and 19H.

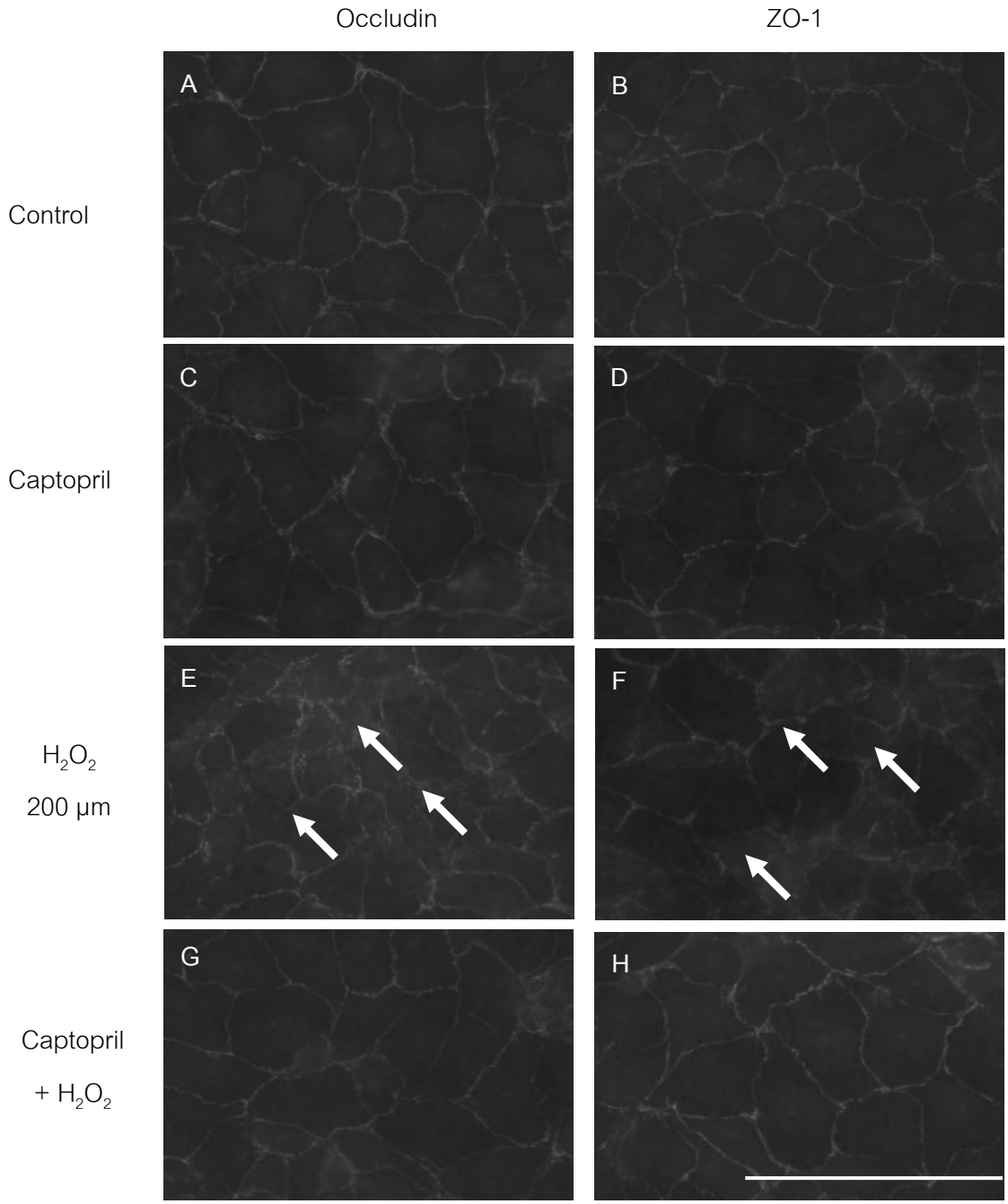



**Figure 19:** Immunofluorescence staining for tight junction proteins occludin (left panel) and ZO-1 (right panel) of the ECV304 monolayers grown for 2 days after seeding. The cells were included control groups (A, B), captopril alone groups (C, D), $H_2O_2$ alone groups (E, F) and captopril prior $H_2O_2$-treated groups (G, H). Bar 100 µm. Arrows indicate disruption of tight junction proteins pattern.

***Western blot analysis*** Alterations in amount of tight junction proteins including occludin and ZO-1 were determined in early stage of tight junction formation. The specific antibodies for occludin, ZO-1 and actin recognized its targets at the expected molecular weight in ECV304 cells homogenate.

As shown in Figure 20, treatment the ECV304 monolayers grown for 2 days after seeding with 200 μM $H_2O_2$ for 4 h had no significant effect on expression of the ZO-1 proteins. In contrast, the amount of occludin significantly decreased by half-folds in $H_2O_2$ treated group. Pretreatment the monolayers with 100 μM captopril for 30 min prior to $H_2O_2$ resulted in an increase the amount of occludin, comparing to the $H_2O_2$ alone group. These findings suggested that $H_2O_2$ could interfere with tight junction assembly through decreased amount of occludin protein. In addition, this effect could be prevented by captopril.

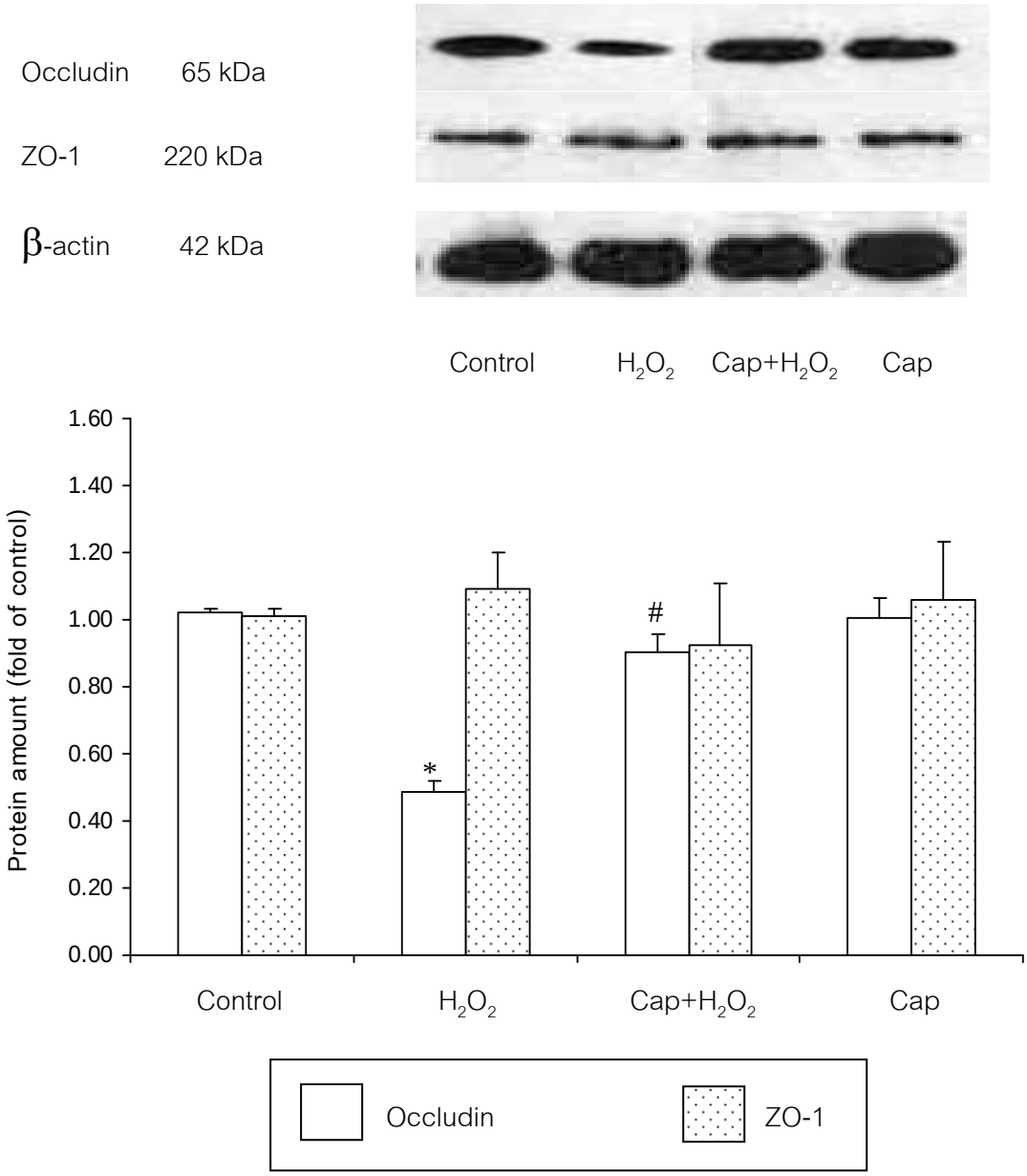



**Figure 20**: The expression of occludin and ZO-1 in ECV304 monolayers grown for 2 days after seeding as determined by western blot analysis. Cell lysated were run on 9% and 8% SDS-PAGE. Densitometrical analysis of immunoblots of occludin and ZO-1 were performed and expressed as fold-increases relative to control groups. Each bar represents the mean±SEM (N=3).

**P*<0.05 indicates significant differences from control; and #*P*<0.05 indicates significant differences from $H_2O_2$ alone.

### 3.2.2 An involvement of ERK1/2 pathway

As shown in Figure 21, $H_2O_2$ caused a significant increase in p-ERK1/2 in ECV304 monolayers growing for 2 days after seeding. The level of ERK1/2 phosphorylation decreased significantly in the monolayers pretreated with 100 μM captopril 30 min prior to $H_2O_2$ treatment, comparing to those expressed in the $H_2O_2$ alone group. The results suggested that captopril was able to completely prevent the $H_2O_2$-mediated activation of ERK1/2 in the ECV304 cells.

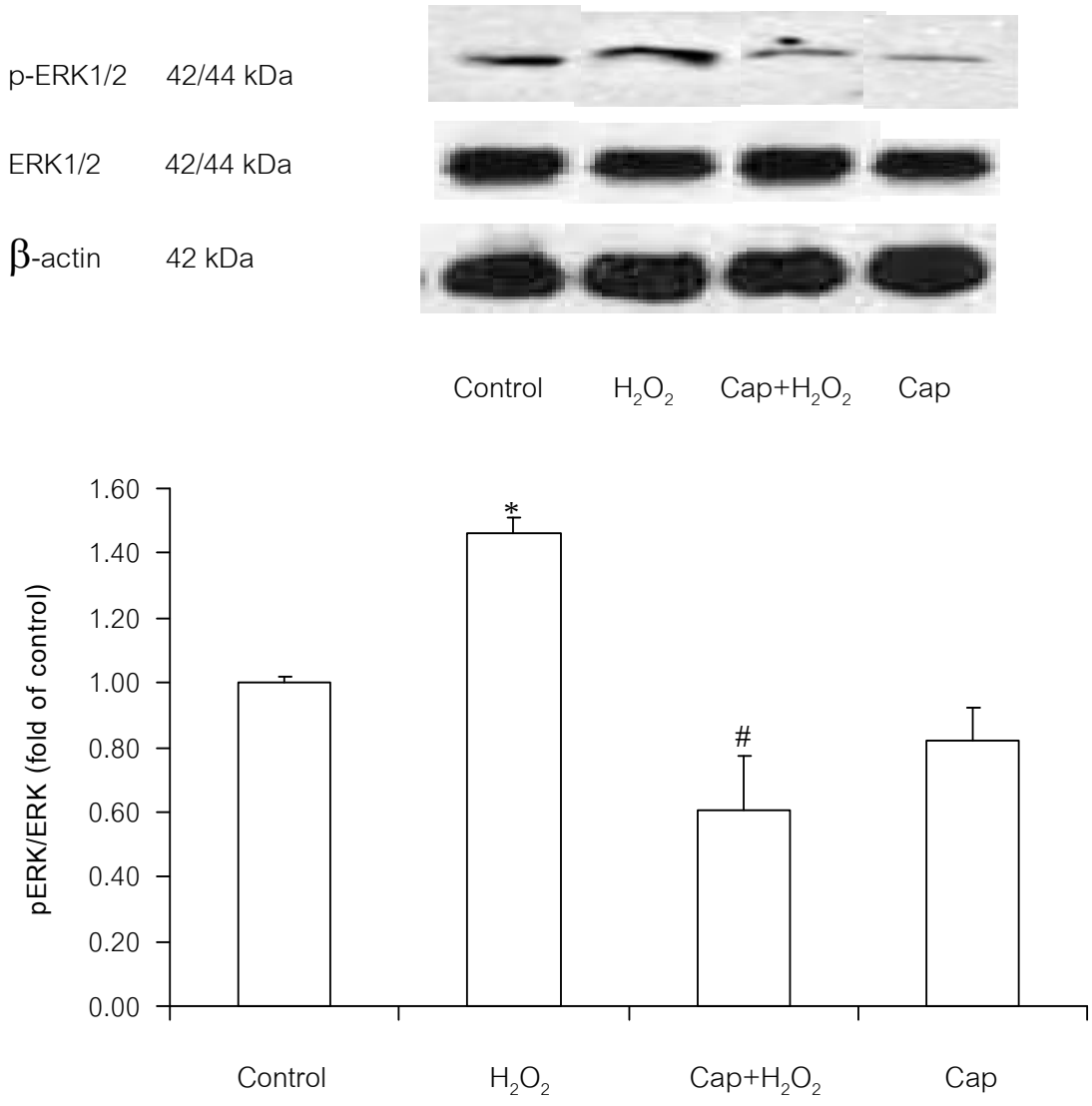



**Figure 21**: The expression of p-ERK1/2 and ERK1/2 in ECV304 monolayers grown for 2 days after seeding as determined by western blot analysis. Cell lysated were run on 12% SDS-PAGE. Densitometrical analysis of immunoblots performed and the results were expressed as the ratio of phoshorylated ERK1/2 to ERK1/2. Each bar represents the mean±SEM (N=3).

*****$P<0.05$ indicates significant differences from control; and #$P<0.05$ indicates significant differences from $H_2O_2$ alone.

# CHAPTER V

# DISCUSSION AND CONCLUSIONS

$H_2O_2$ can cause various oxidative damages to the cells, depending on its concentration and duration of exposure. It attacks a variety of cellular components, including DNA and cellular cytoskeleton. Unless the cells repair properly, these cellular damages can accumulatively lead to cell death (Miura and Roider, 2009). Oxidative stress has been linked to several pathological conditions such as ischemia-reperfusion injury, stroke, endothelial dysfunction, inflammation. This study focused on the oxidative stress-induced tight junction disruption with non-lethal concentration of $H_2O_2$.

Cytotoxicity assays are widely used in in vitro toxicology studies. The lactate dehydrogenase (LDH) leakage assay and MTT assay are the most common employed for the detection of cytotoxicity or cell viability following exposure to substances. The LDH assay is based on the measurement of LDH activity in the extracellular medium. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage (Decker and Lohmann-Matthes, 1988). The MTT assay is cell viability assay often used to determine cytotoxicity following exposure to substances which measured the action of the active mitochondria dehydrogenase in living cells to convert a tetrazolium dye to formazan products in culture medium. In this study, the MTT assay was used to determine the acute cytotoxicity of 200 μM $H_2O_2$ on ECV304 cells. The result suggested that $H_2O_2$ at this concentration had no observable effects on viability of the ECV304 cells after exposure for 4 h. Although this result was in agreement with other reports in literature elsewhere (Kevil *et al*., 2000; Ma *et al*., 2004; Fischer *et al*., 2005; Katsube *et al*., 2007; Miura and Roider, 2009; Chuenkitiyanon *et al*., 2010), the cytotoxicity should be confirmed with LDH assay.

Tight junctions are essential elements in regulating the passage of ions and molecules through the paracellular pathway between adjacent epithelial and/or endothelial cells. Tight junctions are highly dynamic structures whose degree of sealing varies according to external stimuli, physiological and pathological conditions

(Gonzalez-Mariscal *et al.,* 2008). The tight junctions form the barrier to the diffusion of macromolecules across the epithelium or endothelial cells in many tissues such as kidney, bladder, gastrointestinal tract, central nervous system, vascular. The barrier function of tight junctions can be evaluated by measuring the TEER and the flux of tracers including phenol red across the monolayers (Gonzalez-Mariscal *et al*., 2008). There are a number of in vitro cell culture models for studying tight junction. For example, the uses of either primary cell cultures (such as HUVEC, BMVEC, and retinal pigment epithelium cell) or cell lines (such as Caco-2, MDCK, and ECV304) were applied for establishment the model of tight junction assembly (Citi, 1992; Ma *et al*., 2004; Chuenkitiyanon *et al*., 2010). Each model had its own characteristic such as the TEER values which might be different from one another. For example, Caco-2 cells, needed approximately 21 days to achieve formation of functional tight junction (Ma *et al*., 2004). The BMVEC monolayers acquired only 7 days after seeding for the barrier function with the TEER values 70-120 $\Omega.cm^2$ (Fischer *et al*., 2005). On the other hand, MDCK cells monolayers reached the high TEER values of 200-300 $\Omega.cm^2$ in 3 days after seeding (Citi, 1992). Moreover, the ECV304 monolayers reached the TEER values of 95.3±8.36 after seeding for 12 days (Chuenkitiyanon *et al*., 2010).

In this study, ECV304 monolayers were used as the model of $H_2O_2$-induced tight junction disruption. Three major parameters were determined as indicators for functional tight junction development. They included TEER values, paracellular permeability and immunofluorescence staining for tight junction proteins occludin and ZO-1.

The maximal TEER values of the ECV304 monolayers were 90.44±11.68 $\Omega.cm^2$, which could be achieved in 9-12 days after seeding. Although these electrical resistance values were lower than those of Caco-2 (Ma *et al*., 2004) and human corneal epithelial (Wang *et al*., 2004), the numbers were higher than those obtained from BBMVECs which were used as endothelial solute barriers model of blood brain barrier (Lee *et al*., 2004). The high TEER values at 12 days after seeding were correlated with the low phenol red transport across the ECV304 monolayers. These findings suggested that at this stage the functional barrier of the monolayers was fully developed. Moreover, the continuous pattern of immunofluorescence staining suggested that occludin and ZO-1 expressed and localized circumferential at the cell border. Hence, it was likely that

the tight junction structures were formed appropriately and enabled the monolayers to function as the restrictive barrier. It was noteworthy to mention that the immunofluorescence visualization indicated the localization of the proteins, but not their functions or the formation of the tight junction structure. In this study, at 2 days after seeding, occludin and ZO-1 were also visualized at cells circumferences, suggesting the expression and localization of these proteins at the cell borders. It was possible that at this period, the assembly of the tight junction complexes was in the beginning state.

Treatment the monolayers with $H_2O_2$ at non-lethal concentration resulted in significant reduction of TEER values and increased paracellular permeability. These findings suggested that the restrictive barrier collapsed from disruption of tight junction structures. Concurrently, $H_2O_2$ apparently disturbed the distribution pattern of occludin and ZO-1 at the cell border. This evidence strongly supported that $H_2O_2$ could damage the tight junction complexes without causing cell death. Interestingly, $H_2O_2$ had no effects on the amount of tight junction proteins, as determined by the western blot analysis. It was likely that $H_2O_2$ affected the tight junction structures by interfering the localization of tight junction proteins, not its amount level. These findings were in agreement with other reports (Kevil *et al*., 2000; Katsube *et al*., 2007; Miura and Roider, 2009). In several studies, $H_2O_2$ caused hyperpermeability of endothelial monolayers with decreased TEER through disruption of the intercellular junctional localization occludin protein (Kevil *et al*., 2000; Miura and Roider, 2009) or ZO-1 protein (Katsube *et al*., 2007) or disrupted the staining pattern of the both of tight junctions occludin and ZO-1 (Fischer *et al*., 2005; Mayer *et al*., 2001; Lee *et al*., 2004; Chuenkitiyanon *et al*., 2010).

Although the effect of $H_2O_2$ on localization of tight junction proteins was independent of post-seeding period of the monolayer, the effect of $H_2O_2$ on the amount of proteins levels was otherwise. In this study, the susceptibility of the amount level of tight junction proteins toward $H_2O_2$ treatment depended on the post-seeding period of the culture before $H_2O_2$ exposure. As shown in this study, $H_2O_2$ affected the level of occludin amount in the cultures grown after seeding for 2 days, but not in the culture grown for 12 days after seeding. In addition, $H_2O_2$ exposure selectively affected the amount of occludin protein, but the amount level of ZO-1 remained normal. In 2 days after seeding, the tight junction was possibly in the early stage of assembly whereas in

12 days after seeding, the tight junction structure became “tight” bonding. After the tight junction structures were formed, $H_2O_2$ was unable to decreased amount the tight junction protein occludin. In stead, either arrangement or interaction of these proteins in the tight junction structure was readily affected. Hence, occludin which was a transmembrane structural proteins of tight junction was a susceptible target for oxidative assaults especially in the early stage of tight junction assembly. Moreover, oxidative stress could damage the interaction between tight junction proteins either in the “immature” and “mature” tight junction complexes. Consequently, the disarrangement of occludin and ZO-1 was observed.

The effects of oxidative stress on expression of tight junction proteins were demonstrated in a number of in vitro cell culture models (Lee *et al*., 2004; Krizbai *et al*., 2005; Chuenkitiyanon, *et al*., 2010). The expression of occludin increased in BBMVEC treated with $H_2O_2$ for 30 min (Lee *et al*., 2004). On the contrary, the expression of occludin in cerebral endothelial cells decreased after exposure to redox cycling quinone DMNQ (10 μM) for 2 h (Krizbai *et al*., 2005). The expression of occludin and ZO-1 in ECV304 cells decreased after exposure to $H_2O_2$ for 30 min (Chuenkitiyanon, *et al*., 2010). The expression of ZO-1 decreased in Caco-2 treated with TNF-$\alpha$ for 30 min (Ma *et al*., 2004). However, treatment HUVEC with $H_2O_2$ for 30 min did not affect on the expression of both occludin and ZO-1 tight junction proteins (Kevil *et al*., 2000). This discrepancy might relate to cell types, types and degree of oxidative assaults, exposure time, and cultured conditions.

In this study, the different effects of $H_2O_2$ on the amount of occludin suggested that occludin could be one of the primary targets for oxidative assaults during the tight junction formation. Moreover, $H_2O_2$ might also affect the localization of these tight junction proteins. After the tight junction structures were fully developed, $H_2O_2$ had no influence in the amount of tight junction proteins, but certainly affected the localization of these proteins at the cell borders. It was possible that the primary targets of $H_2O_2$ might involve the interaction between proteins in the tight junction complexes in order to maintain the functional integrity. It was reported that $H_2O_2$ disrupted the localization of tight junction proteins occludin and ZO-1 in Caco-2 from the intercellular junctions (Rao *et al*., 2002; Ma *et al*., 2004; Katsube *et al*., 2007). Consequently, the TEER values

decreased. Moreover, it has been reported that disruption of tight junctions was correlated with a loss of occludin but not ZO-1 (Miyoshi and Takai, 2005).

The regulation and maintenance of endothelial and epithelial permeability involved with several processes including receptor activation, secondary messenger mobilization, cytoskeleton and junction associated protein assembly, and junction reorganization (Haselton and Heimark, 1997; Menger *et al*., 1997). Multiple signaling transduction pathways and secondary messengers have been implicated in tight junction biogenesis; for example, G-protein (Denker *et al*., 1996), $Ca^{2+}$ (Ueda and Shah, 1992), PKC (Shasby *et al*., 1988), phospholipase $A_2$ ($PLA_2$) (Goligorsky *et al*., 1993), c-AMP (Ishizaki *et al*., 2003), src kinase (Kevil *et al*., 2001), and MAP kinase (Huot *et al*., 1997; Kevil *et al*., 2001). It has been well established that the effects of $H_2O_2$ on hyperpermeability and reduced TEER values were correlated to the changes of the localization of the tight junction proteins occludin and ZO-1 which mediated through the activation of MAP kinase signaling pathway, in particular ERK1/2 (Kevil *et al*., 2000; Wang *et al*., 2004; Fischer *et al*., 2005). Although some studies demonstrated that the $H_2O_2$-mediated disturbed barrier function in microvascular endothelial cells was regulated by p38 MAPK, but not by ERK or JNK (Usatyuk and Natarajan, 2004). It was reported that exogenous $H_2O_2$ administration could stimulate ERK1/2 activity by increase in ERK1/2 phosphorylation (Kevil *et al*., 2000). In addition, the high levels of phosphorylated ERK1/2 altered the distribution of occludin and ZO-1 (Kevil *et al*., 2000; Wang *et al*., 2004; Fischer *et al*., 2005).

In this study, $H_2O_2$ induced increased ERK1/ERK2 phosphorylation which well correlated to increased solute permeability and decreased in TEER values. In addition, the activation of ERK1/2 was found in the cells treated with $H_2O_2$ regardless of the after seeding period. The oxidative assaults caused the activation of ERK1/2 either in the early phase of tight junction formation (2 days after seeding) or in the functional tight junction (12 days after seeding). It was likely that the increase in ERK1/2 activities might relate to the disrupted-localization and organization of tight junction proteins. Moreover, activation of ERK1/2 might not be responsible for the amount of tight junction proteins.

This study was the first investigation to demonstrate the protective effects of captopril on $H_2O_2$-induced tight junction disruption. Pretreatment the monolayers with

captopril 30 min prior to $H_2O_2$ prevented the TEER reduction and hyperpermeability caused by $H_2O_2$. In addition, the protective effect of captopril was concentration dependent manner and reached the maximal effects at the concentration of 100 μM. Captopril was able to maintain the organization of tight junction structure and localization of proteins either in the early formation of the structure or in the functional tight junction. Moreover, captopril could attenuate the $H_2O_2$-mediated decreased amount of occludin during the tight junction assembly period. The protective action of captopril might involve its ability to suppress the activation of ERK1/2. As shown in this study, the phosphorylated ERK1/2 in the monolayers pretreated with captopril prior to $H_2O_2$ exposure was comparable to those in the control group. Hence, captopril was able to attenuate the increase in ERK1/2 phosphorylation observed in the $H_2O_2$ alone group. This actions were found either in the early formation of tight junction (day 2) or in the functional tight junction (day 12). Although the mechanism of captopril was still unclear, it was possible that captopril contained antioxidant property due to its sulfhydyl group in the molecular structure. It has been reported that captopril was an efficient scavenger of ROS (Aruoma *et al*., 1991; Bagchi *et al*., 1989; Westlin and Mullane, 1988). Furthermore, flavonoid compounds such as quercetin could protect tight junction disruption from $H_2O_2$ (Chuenkitiyanon, *et al*., 2010). As known, flavonoids including quercetin are antioxidant (Ternaux and Portalier, 2002; Dok-Go *et al*., 2003; Heo and Lee, 2004). Hence, captopril could preserve the redox balance or neutralize the oxidative assaults, resulting in its protective effects against $H_2O_2$-induced tight junction disruption (Miura and Roider, 2009). Redox imbalance could induce the oxidative damage of intracellular proteins and nucleotides, leading to the functional damages, and eventually cell death (Miura and Roider, 2009).

In conclusion, the effectiveness of captopril in protecting the barrier function of ECV304 cells monolayers under oxidative stress was proven. The results provide further important information in understanding the therapeutic effect of captopril. Further investigations are needed to elucidate more details, such as redox status of the cells and the involvement of other MAP kinase.

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# APPENDICES

# APPENDIX A

## Data of experimental results

**Table 3**: The viability of cultured ECV304 cells after $H_2O_2$ treatment at 0.5, 1, 2, and 4 h.

| Concentration of $H_2O_2$ (μM) | | % viability | | | |
|---|---|---|---|---|---|
| | | 0.5 h | 1 h | 2 h | 4 h |
| 0 | N1 | 100.00 | 98.61 | 97.57 | 102.31 |
| | N2 | 100.75 | 99.19 | 98.24 | 98.35 |
| | N3 | 99.93 | 99.81 | 104.14 | 99.53 |
| 100 | N1 | 89.99 | 95.74 | 87.45 | 98.85 |
| | N2 | 100.29 | 95.50 | 99.66 | 98.41 |
| | N3 | 99.92 | 96.01 | 105.88 | 91.36 |
| 200 | N1 | 84.59 | 82.99 | 91.72 | 88.99 |
| | N2 | 97.53 | 96.02 | 87.97 | 89.82 |
| | N3 | 93.33 | 101.35 | 95.11 | 94.91 |
| 500 | N1 | 75.56 | 68.56 | 64.89 | 58.19 |
| | N2 | 71.32 | 68.82 | 66.37 | 64.97 |
| | N3 | 69.31 | 75.54 | 67.84 | 49.69 |

Each value represented as mean±SEM of three independent experiments, each performed in triplicate. *$P$<0.05 compare with control cultures.

**Table 4**: The viability of cultured ECV304 cells after captopril treatment for 24 h.

| Concentration of captopril (μM) | | % viability |
|---|---|---|
| 0 | N1 | 100.00 |
| | N2 | 89.76 |
| | N3 | 112.67 |
| 0.1 | N1 | 86.21 |
| | N2 | 89.65 |
| | N3 | 119.22 |
| 1 | N1 | 97.23 |
| | N2 | 85.19 |
| | N3 | 114.02 |
| 10 | N1 | 105.60 |
| | N2 | 85.91 |
| | N3 | 114.21 |
| 100 | N1 | 92.02 |
| | N2 | 87.88 |
| | N3 | 110.04 |
| 500 | N1 | 95.20 |
| | N2 | 88.04 |
| | N3 | 110.91 |

Each value represented as mean±SEM of three independent experiments, each performed in triplicate. *$P$<0.05 compare with control cultures.

**Table 5**: The viability of cultured ECV304 cells pre-exposure with captopril for 30 min and followed with 200 μM $H_2O_2$ for 4 h.

| Concentration of captopril (μM) | | % viability | |
|---|---|---|---|
| | | control | add 200 μM $H_2O_2$ |
| 0 | N1 | 100.00 | 88.99 |
| | N2 | 110.92 | 89.82 |
| | N3 | 102.46 | 94.91 |
| 10 | N1 | 103.95 | 89.61 |
| | N2 | 102.36 | 94.34 |
| | N3 | 100.35 | 99.30 |
| 100 | N1 | 116.48 | 112.53 |
| | N2 | 94.70 | 92.69 |
| | N3 | 100.26 | 90.40 |
| 500 | N1 | 95.82 | 108.13 |
| | N2 | 111.25 | 97.92 |
| | N3 | 104.40 | 94.01 |

Each value represented as mean±SEM of three independent experiments, each performed in triplicate. *$P$<0.05 compare with control cultures.

**Table 6:** The TEER values of cultured ECV304 cells pre-exposure with captopril for 30 min and followed with 200 μM $H_2O_2$ for 4 h.

| Concentration of captopril (μM) | | TEER values (ohm*cm$^2$) | |
|---|---|---|---|
| | | control | add 200 μM $H_2O_2$ |
| 0 | N1 | 27.44 | 24.64 |
| | N2 | 51.38 | 52.64 |
| | N3 | 79.94 | 95.62 |
| | N4 | 89.74 | 93.10 |
| 10 | N1 | 45.22 | 32.06 |
| | N2 | 58.1 | 70.98 |
| | N3 | 70.28 | 105.98 |
| | N4 | 94.64 | 99.12 |
| 100 | N1 | 29.12 | 31.08 |
| | N2 | 62.72 | 54.95 |
| | N3 | 87.08 | 107.94 |
| | N4 | 92.96 | 94.36 |
| 500 | N1 | 44.24 | 34.44 |
| | N2 | 64.82 | 50.82 |
| | N3 | 101.92 | 95.62 |
| | N4 | 94.64 | 93.66 |

Each value represented as mean±SEM of four independent experiments, each performed in duplicate. *$P$<0.05 compare with control cultures and $^{\#}P$<0.05 compare with $H_2O_2$ alone.

**Table 7**: The paracellular permeability values of cultured ECV304 cells pre-exposure with captopril for 30 min and followed with 200 μM $H_2O_2$ for 4 h.

| Concentration of captopril (μM) | | Paracellular permeability values ($x10^{-5}$ cm/sec) | |
|---|---|---|---|
| | | control | add 200 μM $H_2O_2$ |
| 0 | N1 | 6.999 | 58.691 |
| | N2 | 3.039 | 52.62 |
| | N3 | 1.999 | 67.977 |
| | N4 | 5.583 | 52.263 |
| 10 | N1 | 3.633 | 48.975 |
| | N2 | 5.758 | 54.036 |
| | N3 | 6.2 | 49.371 |
| | N4 | 11.258 | 46.593 |
| 100 | N1 | 6.143 | 21.905 |
| | N2 | 5.044 | 18.757 |
| | N3 | 3.816 | 22.263 |
| | N4 | 7.205 | 22.296 |
| 500 | N1 | 2.475 | 13.691 |
| | N2 | 3.114 | 7.738 |
| | N3 | 2.58 | 5.012 |
| | N4 | 3.509 | 5.745 |

Each value represented as mean±SEM of four independent experiments, each performed in duplicate. *$P$<0.05 compare with control cultures and $^{\#}P$<0.05 compare with $H_2O_2$ alone.

**Table 8**: The fold increase values of tight junction protein amount and pERK1/2/ERK1/2 in 12 days after seeding.

| Groups | | Occludin | ZO-1 | pERK1/2/ERK1/2 |
|---|---|---|---|---|
| Control | N1 | 1.00 | 1.00 | 1.00 |
| | N2 | 1.05 | 1.04 | 0.98 |
| | N3 | 1.12 | 1.01 | 1.02 |
| 200 μM $H_2O_2$ | N1 | 1.38 | 0.99 | 1.54 |
| | N2 | 0.65 | 0.74 | 1.31 |
| | N3 | 0.86 | 1.03 | 1.37 |
| Captopril + $H_2O_2$ | N1 | 0.99 | 0.98 | 0.87 |
| | N2 | 0.66 | 0.96 | 0.59 |
| | N3 | 1.17 | 0.98 | 0.97 |
| Captopril | N1 | 1.45 | 1.19 | 0.77 |
| | N2 | 0.64 | 0.97 | 0.87 |
| | N3 | 1.06 | 1.02 | 1.00 |

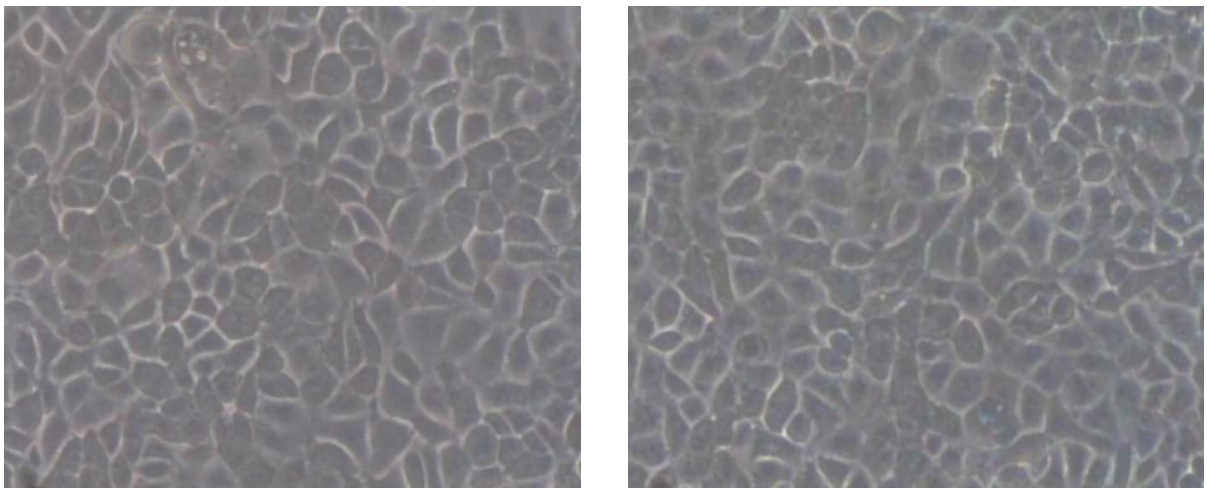
Each value represented as mean±SEM of three independent experiments, each performed in duplicate. *$P$<0.05 compare with control cultures and $^{\#}P$<0.05 compare with $H_2O_2$ alone.

**Table 9**: The fold increase values of tight junction proteins amount and pERK1/2 in 12 days after seeding.

| Groups | | Occludin | ZO-1 | pERK1/2/ERK1/2 |
|---|---|---|---|---|
| Control | N1 | 1.00 | 1.00 | 1.00 |
| | N2 | 1.02 | 0.99 | 0.97 |
| | N3 | 1.05 | 1.04 | 1.03 |
| 200 μM $H_2O_2$ | N1 | 0.45 | 1.19 | 1.56 |
| | N2 | 0.56 | 1.21 | 1.40 |
| | N3 | 0.45 | 0.88 | 1.44 |
| Captopril + $H_2O_2$ | N1 | 0.95 | 1.15 | 0.90 |
| | N2 | 0.79 | 1.06 | 0.58 |
| | N3 | 0.96 | 0.56 | 0.32 |
| Captopril | N1 | 1.08 | 0.71 | 0.94 |
| | N2 | 0.90 | 1.23 | 0.61 |
| | N3 | 1.04 | 1.24 | 0.91 |

Each value represented as mean±SEM of three independent experiments, each performed in duplicate. *$P$<0.05 compare with control cultures and $^{\#}P$<0.05 compare with $H_2O_2$ alone.

# APPENDIX B



Control $H_2O_2$ 200 μM 4 h

**Figure 22.** The morphology of ECV304 cells on transwells after trypan blue test for cell viability (12 days after seeding).

# APPENDIX C

## Photo of Transwell® insert

APPENDIX D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# Protective effects of captopril against hydrogen peroxide-induced disruption of tight junctions in ECV304 monolayers

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***Abstract***

Angiotensin-converting enzyme (ACE) inhibitors demonstrated various beneficial actions on vascular structure and function beyond their blood pressure-lowering effects. In this study, we investigated the protective effect of captopril on hydrogen peroxide ($H_2O_2$)-induced dysfunction of endothelial barriers, using an *in vitro* model of ECV304 cells. Treatment the ECV304 monolayers with $H_2O_2$ for 4 hr at the noncytotoxic concentration of 200 micromolar, resulting in the loss of TEER values and tight junction proteins occludin and zonular occluden (ZO)-1. Pretreatment the cells with captopril for 30 min prior to $H_2O_2$ attenuated the loss of TEER values in concentration dependent manner. The immunofluorescent visualization revealed that captopril prevented the $H_2O_2$-mediated loss of expression and localization of occludin and ZO-1 at the cell borders. Our results suggested that captopril could protect the barrier function of ECV304 monolayers from oxidative stress through preventing the loss of tight junction proteins. Consequently, the integrity of tight junction structure was preserved during oxidative assault.

***Keywords***: captopril, tight junction, oxidative stress, ECV304 cells

## Introduction

Tight junction is a paracellular structural component to restrict the paracellular flux of ions and solutes through the epithelial and endothelial monolayers. Formation of tight junction structure between adjacent cells is attributed to the protective barrier function of endothelial monolayers against blood-borne pathogens. The tight junction is highly dynamic structure, and the degree of "tightness" sealing depends upon external stimuli, physiological and pathological conditions (1). Disturbance of the tight junction is linked to several pathological conditions including stroke, proteinuria, inflammatory bowel disease (2).

Angiotensin-converting enzyme (ACE) inhibitors exerted their actions through interference with the rennin-angiotensin system. In addition, this drug groups proved their beneficial actions in reduction the risk of stroke, myocardial infarction and cardiovascular death which were beyond their blood pressure-lowering effects (3). Moreover, ACE inhibitors was reported to improve endothelial functions (4). In this study, we investigated the plausible action of ACE inhibitors in preventing the disruption of endothelial barriers from oxidative assaults. We evaluated the capability of captopril, a known ACE inhibitors, to preserve the integrity and function of tight junctions that were damaged by treatment with $H_2O_2$ in the in vitro model of ECV304 cells.

## Materials and Methods

### *Culture of ECV304 cells*

The ECV304 cells were maintained in M199 supplemented with 10% FBS, 1% combined penicillin and streptomycin (100 units/100 μg/ml) at 37 °C in an atmosphere of 5% $CO_2$ and 95% relative humidity. Cells were counted and seeded onto polycarbonate

membranes with 0.4 μm in pore size, 12 mm in diameter Transwell insert (Costar®, Corning, NY, USA) at a density of $2x10^5$ cells/transwell for 12 days before experiments.

**Transendothelial electrical resistance (TEER) measurement**

ECV304 cells were counted and seeded onto Transwell inserts. TEER was measured using a Millicell®-ERS potentiometer (Millipore, MA, USA) before seeding. After 12 days, cells treated with captopril (0-500 μM) for 30 min, followed by $H_2O_2$ (200 μM) for another 4 hr. The TEER value was obtained by multiplying the measured electrical resistance (Ω) with the surface area of the monolayer ($cm^2$).

**Immunofluorescent staining measurement**

ECV304 cells on Transwell inserts were treated with captopril (100 μM) for 30 min, followed by $H_2O_2$ (200 μM) for 4 hr. The localization of tight junction proteins were visualized by an immunofluorescent staining method (5) with the use of fluorescence microscopy (BX-FLA, Olympus, Tokyo, Japan) at the absorbance of 490 nm.

**Statistical analysis**

All values were presented as mean ± standard error (SEM). One-way ANOVA followed by the Tukey's test was performed for statistical comparisons, $p<0.05$ was considered significant.

**Results**

As shown in Fig 1, $H_2O_2$ (200 μM) significantly decreased TEER values approximately by 70%, whereas captopril (up to 500 μM in this study) had no effect on the TEER values. Pretreatment the cells with captopril for 30 min prior to $H_2O_2$ could prevent the TEER reduction. Our results demonstrated that the protective effect of captopril was concentration- dependent, with the maximum effect at the concentration of 100 μM.

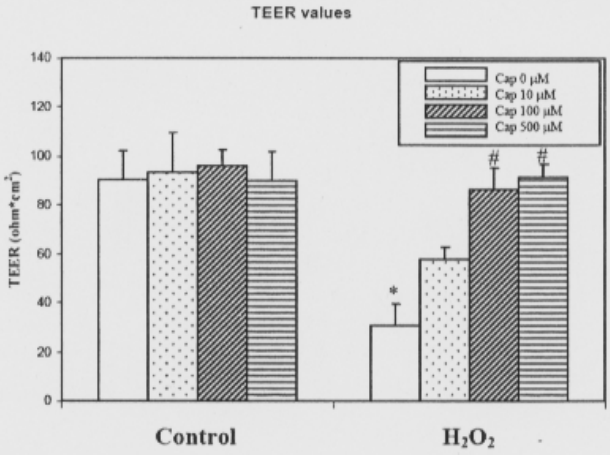


**Figure 1** Effects of captopril (cap) on TEER values of ECV304 monolayers treated with $H_2O_2$ 200 μM. Captopril was added at concentrations ranging from 0-500 μM for 30 min prior to addition of $H_2O_2$ (200 μM) for 4 hr. Each bar represents the mean±SEM with *$p<0.05$ compared with control and #$p<0.05$ compared with $H_2O_2$ only (n=4).

Next, we investigated the integrity of tight junction structure through immunofluorescent visualization of tight junction proteins occludin and ZO-1. Under the normal condition, ECV304 monolayers expressed the continuous distribution pattern of occludin and ZO-1 proteins at the cell boundaries, as shown in Fig 2A and 2E, respectively. Captopril had no influence on the localization of these proteins (Fig 2D and 2H). In this study, treatment of $H_2O_2$ (200 μM) for 4 hr apparently caused dislocalization of occludin and ZO-1 at the cell border as seen in Fig 2B and 2F, respectively. Interestingly, our results revealed that pretreatment the ECV304 monolayers with captopril (100 μM) for 30 min could

prevent the $H_2O_2$-induced disruption of the junctional lining at the cell border, indicating the localization of occludin and ZO-1 remained intact (Fig 2C and 2G).

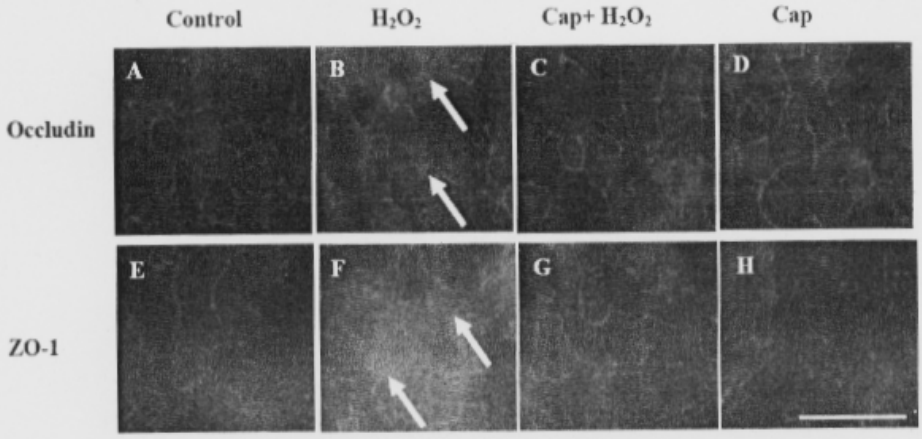


**Figure 2** Immunofluorescent staining of ECV304 monolayers showing tight junction proteins occludin and ZO-1 after incubated with captopril in the presence or absence of $H_2O_2$. Occludin and ZO-1 as control (A, E), $H_2O_2$ (B, F), captopril plus $H_2O_2$ (C, G) and captopril only (D, H) are shown. Bar 100 μm.

**Discussion & Conclusion**

This study was the first to demonstrate the protective effect of captopril against $H_2O_2$-mediated tight junction disruption. Treatment the ECV304 monolayers with $H_2O_2$ at 200 μM for 4 hr directly disrupted expression and localization of tight junction proteins occluding and ZO-1 at the cell borders without any influence on cell viability. Consequently, the loss of junctional proteins compromised the integrity and function of tight junction complexes. This model of oxidative stress induced tight junction disruption was in agreement with others reported in the literature (5). As expected, the TEER values decreased in the presence of $H_2O_2$, indicating the loss of barrier function of the ECV304 monolayers. This dysfunction of ECV304 monolayers as restrictive barrier could be prevented by pretreatment the cells with captopril before $H_2O_2$ exposure. In this study, we demonstrated that captopril was able to retain the expression and localization of occludin and ZO-1 at the cell borders during the oxidative assault. We anticipated that this protective feature of captopril on tight junction disruption might take part in preventing the barrier leakage of endothelium or epithelium monolayers in various pathological conditions such as stroke. Several substances were shown to protect the tight junction damage from oxidative assaults via various mechanisms including alteration of nitric oxide level (6) and inhibition of MAP kinase signaling pathways (7). In addition to its inhibitory effect on ACE, captopril was reported to inhibit the activity of MAP kinases (8). It was possible that the effects of captopril on tight junction proteins might relate to this action. The molecular protective mechanism of captopril against $H_2O_2$-mediated tight junction disruption would be in need for further investigation.

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# VITA

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She has attended and presented poster at the 32$^{nd}$ Pharmacological and Therapeutic Society of Thailand Meeting, March 25-26, 2010 at Thammasat University, Pathumthani, Thailand. The subject presented was protective effects of captopril against hydrogen peroxide-induced disruption of tight junctions in ECV304 monolayers.