

## 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  $\mu$ g/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  $\mu\text{m}$  in pore size, 12 mm in diameter Transwell insert (Costar<sup>®</sup>, Corning, NY, USA) at a density of  $2 \times 10^5$  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<sup>®</sup>-ERS potentiometer (Millipore, MA, USA) before seeding. After 12 days, cells treated with captopril (0-500  $\mu\text{M}$ ) for 30 min, followed by  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ ) for another 4 hr. The TEER value was obtained by multiplying the measured electrical resistance ( $\Omega$ ) with the surface area of the monolayer ( $\text{cm}^2$ ).

### Immunofluorescent staining measurement

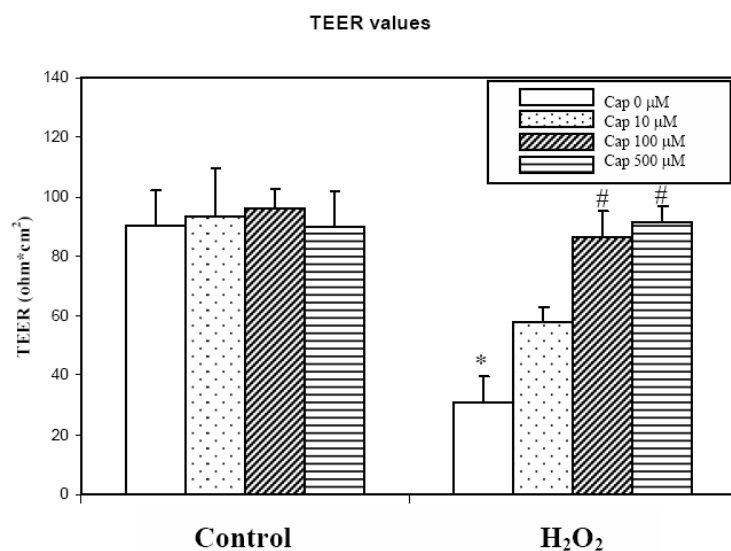
ECV304 cells on Transwell inserts were treated with captopril (100  $\mu\text{M}$ ) for 30 min, followed by  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ ) for 4 hr. The localization of tight junction proteins were visualized by an immunofluorescent staining method (5) with the use of fluorescence microscopy (BX-FLA, Olympus, Tokyo, Japan) at the absorbance of 490 nm.

### Statistical analysis

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

### Results

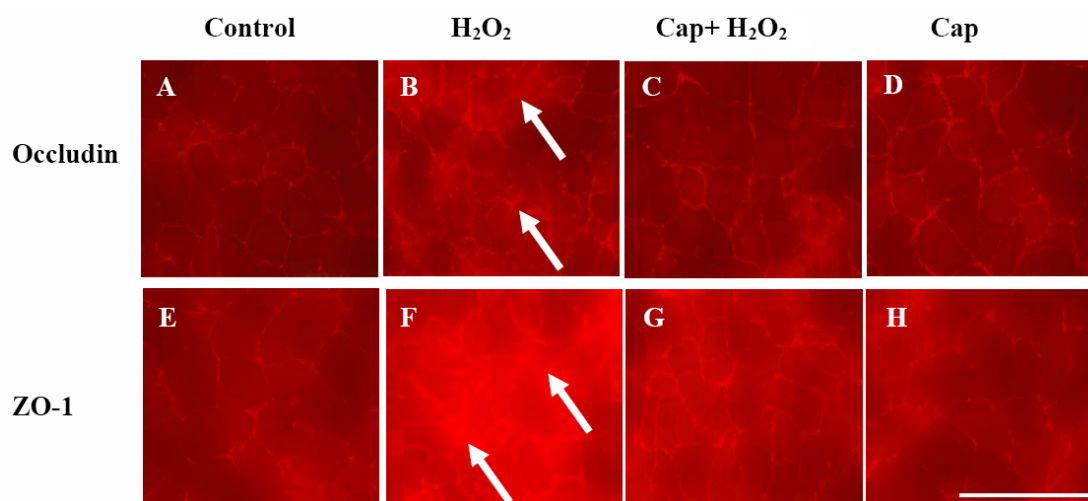
As shown in Fig 1,  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ ) significantly decreased TEER values approximately by 70%, whereas captopril (up to 500  $\mu\text{M}$  in this study) had no effect on the TEER values. Pretreatment the cells with captopril for 30 min prior to  $\text{H}_2\text{O}_2$  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  $\mu\text{M}$ .



**Figure 1** Effects of captopril (cap) on TEER values of ECV304 monolayers treated with  $\text{H}_2\text{O}_2$  200  $\mu\text{M}$ . Captopril was added at concentrations ranging from 0-500  $\mu\text{M}$  for 30 min prior to addition of  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ ) for 4 hr. Each bar represents the mean  $\pm$  SEM with \* $p < 0.05$  compared with control and # $p < 0.05$  compared with  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ ) 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  $\mu\text{M}$ ) for 30 min could

prevent the H<sub>2</sub>O<sub>2</sub>-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<sub>2</sub>O<sub>2</sub>. Occludin and ZO-1 as control (A, E), H<sub>2</sub>O<sub>2</sub> (B, F), captopril plus H<sub>2</sub>O<sub>2</sub> (C, G) and captopril only (D, H) are shown. Bar 100  $\mu$ m.

### Discussion & Conclusion

This study was the first to demonstrate the protective effect of captopril against H<sub>2</sub>O<sub>2</sub>-mediated tight junction disruption. Treatment the ECV304 monolayers with H<sub>2</sub>O<sub>2</sub> at 200  $\mu$ M 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<sub>2</sub>O<sub>2</sub>, 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub>-mediated tight junction disruption would be in need for further investigation.

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