의학석사 학위논문

Effect of aspirin, potential P-glycoprotein activator on intestinal absorption of ticagrelor

잠재적 P-glycoprotein 활성제 아스피린이 티카그렐러 장내 흡수에 미치는 영향

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자연과학대학 뇌과학협동과정 전공

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지도교수 노재규

이 논문을 의학석사 학위논문으로 제출함.

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문장섭의 의학석사 학위논문을 인준함.

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Abstract

Effect of aspirin, potential P-glycoprotein activator on intestinal absorption of ticagrelor

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Background: Ticagrelor is a new P_2Y_{12} receptor inhibitor, which is approved for use in patients with acute coronary syndrome in combination with aspirin. However, there is some evidence that high-dose aspirin reduces the effectiveness of ticagrelor. Given the fact that aspirin can enhance intestinal P-glycoprotein (P-gp) expression and ticagrelor is a substrate of P-gp, we hypothesized that concurrent treatment with aspirin may decrease the intestinal absorption of ticagrelor.

Methods and Results: *In vitro* assay using a monolayer of human epithelial colorectal (Caco-2) cells and *in vivo* study using the closed-loop method in rats were performed. Aspirin was administered to Caco-2 cells (0.5 and 1 mmol/l) *in vitro* for 72 hours and to rat intestine (10 mg/kg and 100 mg/kg) *in vivo* for 4 weeks, before administration of ticagrelor. Ticagrelor permeabilization *in vitro* was not affected by aspirin pre-treatment and was neither influenced by the P-gp inhibitor, tariquidar. Furthermore, different doses of aspirin (10 mg/kg and 100 mg/kg) pre-treatment did not affect the absorption of ticagrelor in the rat intestine.

Conclusion: The intestinal absorption of ticagrelor was not affected by enhancement of P-glycoprotein following prolonged aspirin treatment. Further studies will be necessary to clarify how aspirin attenuates the treatment effect of ticagrelor.

Keywords: Aspirin, Ticagrelor, P-glycoprotein, Drug interaction, Intestinal absorption

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Introduction

Patients with acute coronary syndrome (ACS) require treatment using antiplatelet drugs. The current American Heart Association/American College of Cardiology treatment guidelines recommend a P_2Y_{12} receptor inhibitor in combination with aspirin for patients with ACS or patients undergoing percutaneous coronary intervention with stent placement (class 1 recommendation).[1] Clopidogrel is the most widely used P_2Y_{12} receptor inhibitor, and is used as an alternative for, or in combination with aspirin.[2, 3] However, clopidogrel has several limitations. As a prodrug, it requires two-step oxidation by the cytochrome P450 (CYP) enzyme system to produce its active metabolite. These steps result in delay of time to maximal platelet inhibition and give rise to genetic polymorphism.[4, 5] Also, a study reported that the intestinal absorption of clopidogrel may be reduced by prolonged use of aspirin due to the upregulation of multidrug-resistance proteins (MDR1, ABCB1) that encode P-glycoprotein (P-gp).[6]

Recently, a phase 3, randomized, double-blind PLATO trial has demonstrated that ticagrelor is more effective in preventing a composite of cardiovascular death, myocardial infarction, and stroke compared to clopidogrel, without increasing overall major bleeding.[7] Ticagrelor is an orally active reversibly binding inhibitor of the P_2Y_{12} receptor, with a stronger and more rapid antiplatelet effect than clopidogrel.[8] It is also believed to have little interindividual variability and drug-

drug interaction, because hepatic CYP metabolism is not required for its activity.[9] However, the subgroup analysis of PLATO trial discovered that ticagrelor was less effective when it was used with higher doses of aspirin (≥300 mg/day).[10] In our previous work, we demonstrated that prolonged use of aspirin enhanced P-gp in the rat intestine.[6] Therefore, we hypothesized that concurrent treatment with high-dose aspirin may enhance P-gp and thereby decrease the intestinal absorption of ticagrelor which is also known as a P-gp substrate. In the present study, we attempted to verify this hypothesis using an *in vitro* intestinal cell culture method and an *in vivo* in situ closed-loop model.

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Methods

Cell culture and drug treatment

Multidrug efflux pumps of the human colon carcinoma cell line (Caco-2 cells) express similar profiles to those of human intestinal cells.[11] Therefore, Caco-2 cells that are cultured as a confluent monolayer are widely used in studying drug transport across the human intestinal epithelium, including various antiplatelet agents.[6, 12] Caco-2 cell culture was performed as described previously.[6] In brief, Caco-2 cells were obtained from Korean Cell Link Bank (Seoul, Korea) and were cultured in 100 mm plastic dishes in a humidified incubator at 37℃ with 5% CO2/95% air. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum, glutamine (2 mmol/l), streptomycin (100 μg/ml), and penicillin (100 U/ml) was used. Caco-2 cells were seeded at a density of 5 x 10^5 cells per 75 cm^2 in a culturing flask and were sub-cultured whenever they showed 80% confluence. The culture medium was changed every other day. All cell lines were grown as monolayer cultures, but only up to 20 passages. The first step of our study was to investigate the influence of aspirin on ticagrelor permeabilization in monolayer Caco-2 cells. Aspirin (Sigma, St Louis, MO) was dissolved in 0.1 mol/l Tris-HCl. To avoid acidification of the medium, the solution was buffered with Tris base, making the final pH equal to that of the control, DMEM. Aspirin was administered at doses of 0.5 and 1 mmol/l to the apical side of the well, every 24

hour for a total of 72 hours. After pre-treatment of aspirin, ticagrelor (supplied by AstraZeneca) was administered in 2% Dimethyl sulfoxide (DMSO)/Phosphate buffered saline (PBS) at a dose of 1 mmol/l to the apical side of the well (Figure 1a). In the tariquidar treatment group, tariquidar (XR9576, MedKoo, Chapel Hill, NC) was diluted in HBSS at a dose of 40 nmol/l and was administered to the apical side of the well 30 minutes prior to addition of ticagrelor.

Measurement of ticagrelor concentration in transport experiment

Apical-to-basolateral transport was determined by drawing 50 µl aliquots from the basolateral side of the well at the indicated times (10, 20, 30, 60, 90, and 120 minutes after ticagrelor administration). Quantification of ticagrelor was done by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The standard 2% DMSO/HBSS buffer sample was spiked with succeeding dilutions of ticagrelor solution from 1 to 5000 ng/ml. Fifty µl of internal standard solution (Chlorzoxazone 200 ng/ml in 50% methanol) and 600 µl of ethyl acetate were added to the 50 µl aliquot. After vortexing for 30 seconds, the sample was centrifuged at 13000 rpm for 5 minutes. The organic layer was then transferred to an eppendorf tube and evaporated in a SpeedVac concentrator for 1 hour. It was dissolved with mobile phase and injected into the column. Separation was performed on a Gemini C18 column (2.0 x 10 mm, 5 µm; Phenomenex, Torrance, CA) set at 25℃. The mobile phase was 10:90 (v/v) 10 mM ammonium acetate :

acetonitrile at a flow rate of 0.35 ml/minute. The concentration of ticagrelor was calculated from the peak area using the previously calibrated scale.

In vivo experiment

Twelve week-old male Sprague-Dawley rats weighing 220-250 g (Orientbio, Seoul, Korea) were used in this study. All procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital, accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. The closed-loop model was used to measure the absorption of ticagrelor in the rat intestine (Figure 1b).[6] Saline (estimated human dose 40 ml/60 kg) or aspirin (10 mg/kg or 100 mg/kg) was orally administered to nonfasted rats in a volume of 1 ml (n=3-5) every day. After 4 weeks of aspirin pre-treatment, the rats were anesthetized with inhalational isoflurane and a midline abdominal incision was made to expose the intestines. The intestines were divided into three segments (duodenum, jejunum, and ileum), each of which was 10 cm long. The intestinal contents were washed out with pre-warmed (37℃) isotonic phosphate-buffered saline, and both ends of each segment were ligated. After this procedure, 1 ml of ticagrelor (10 mmol/l) in 2% DMSO/PBS was introduced into each of the segments. The intestinal segments were placed within the body for 20 minutes, with a heating lamp to maintain the temperature of the preparation at 37℃. After 20 minutes, the luminal solution in the each segment was collected and the loop was rinsed with 9

ml isotonic 2-(N-morpholino)ethanesulfonic acid buffer to obtain a total solution volume of 10 ml. The remaining amount of ticagrelor in the intestinal lumen was determined by LC-MS/MS to estimate the amount of unabsorbed ticagrelor. The standard ticagrelor solution was succeedingly diluted with 1:9 (v/v) MES/DMSO solution to make the concentration of ticagrelor in the range of 1 to 100 μg/ml. A 20 μl aliquot was mixed with DMSO 180 μl. Twenty μl of the mixed solution was added to 1000 μl of acetonitrile. After vortexing for 30 seconds, the sample was centrifuged at 13000 rpm for 5 minutes. Fifty μl of the upper layer was transferred to an eppendorf tube and 500 μl of chlorzoxazone was added to 50 ng/ml acetonitrile. The upper layer was injected onto the column. Separation was performed on a Gemini C18 column (2.0 x 10 mm, 5 μm; Phenomenex, Torrance, CA, USA) set at 25℃. The mobile phase was 10:90 (v/v) 10 mM ammonium acetate : acetonitrile at a flow rate of 0.35 ml/minute. The concentration of the ticagrelor was calculated from the peak area using the previously calibrated scale.

Statistical analysis

All data in this study are presented as mean values±SD. Statistical significance was evaluated using the Mann-Whitney U test. SPSS 18.0 was used for statistical analyses and $P<0.05$ was considered statistically significant.

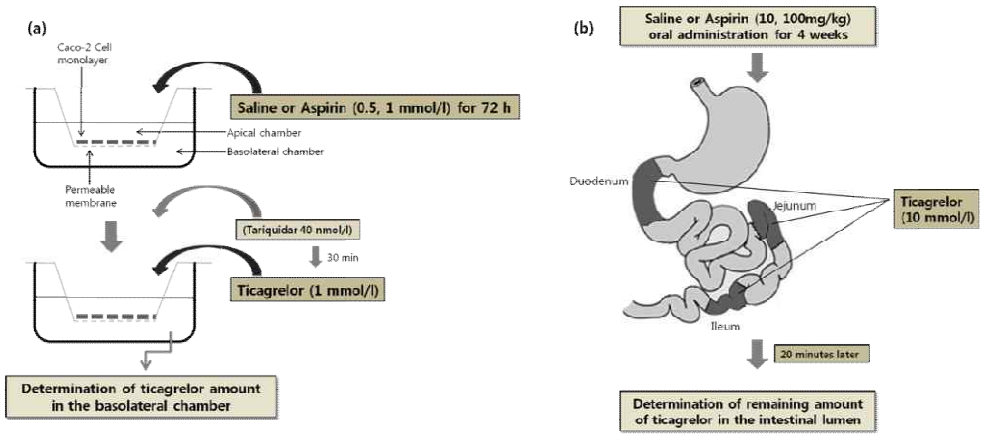

Figure 1. Experimental design of the studies. (a) An experimental design for *in vitro* assay. Saline or aspirin (0.5 mmol/l or 1 mmol/l) is administered to the apical side chamber starting on the third day after seeding Caco-2 cells and is maintained for 72 hours. Subsequently, ticagrelor (1mmol/l) is administered to the apical side chamber, and the amount of transported ticagrelor in the basolateral chamber is evaluated at the indicated times (10, 20, 30, 60, 90, and 120 minutes after ticagrelor administration).

Results

In vitro absorptive permeabilization of ticagrelor across Caco-2 monolayers is not affected by aspirin pre-treatment

Caco-2 cells were incubated with saline or two different doses of aspirin (0.5 and 1 mmol/l) for 72 hours. Serial culture media from the basolateral site of the Transwell culture plate were obtained after administration of ticagrelor on the apical site. Ticagrelor concentration was measured from the obtained culture media. The time courses of absorptive apical-to-basolateral transport in each group of different aspirin pre-treatment doses are displayed in Figure 2. There was no appreciable difference in the absorptive flux of ticagrelor between the three different pre-treatment groups. Although there was a subtle tendency for ticagrelor to be better absorbed more the pre-treatment aspirin dose increased, it was not significantly different (Mann-Whitney U-test).

In vitro absorptive permeabilization of ticagrelor across Caco-2 monolayers is not affected by P-gp inhibitor

Tariquidar was given to Caco-2 cells 30 minutes prior to ticagrelor treatment. Pre-treatment conditions of saline or two different doses of aspirin (0.5, and 1 mmol/l) were evaluated. The concentration of permeabilized ticagrelor did not differ significantly between conditions with and without tariquidar, irrespective to the

pre-treatment aspirin dose (Figure 3.). Only the concentration of ticagrelor at 30 minutes with tariquidar supplement was significantly lower than that of the tariquidar-free group in condition with saline pre-treatment.

In vivo ticagrelor absorption is not affected by aspirin pre-treatment

Higher doses of aspirin and ticagrelor were used for *in vivo* evaluation. Ticagrelor absorption was not significantly different across various regions (duodenum, jejunum, and ileum) of the rat intestine. Four-week pre-treatment of aspirin with two different doses (10 mg/kg and 100 mg/kg) did not greatly influence the absorption of ticagrelor in the rat intestine, when compared to the control (saline pre-treatment) group (Figure 4).

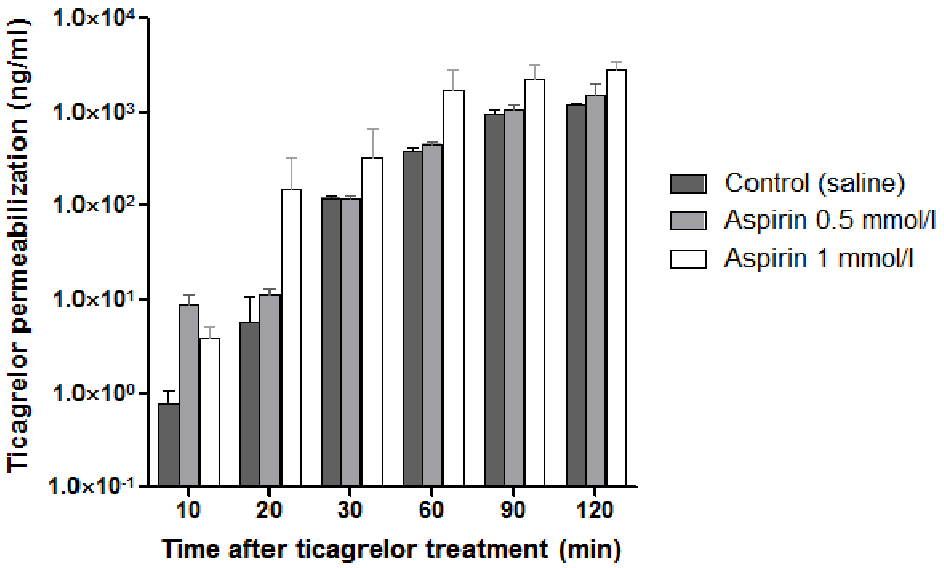

Figure 2. Effect of aspirin pre-treatment on *in vitro* ticagrelor permeabilization. Permeabilization of ticagrelor in Caco-2 cells is evaluated by measuring ticagrelor concentrations in the basolateral chamber over a time course of 120 minutes after loading with 1 mmol/l ticagrelor. Aspirin is pre-treated for 72 hours with two different doses (0.5 mmol/l and 1 mmol/l), and the same procedure is repeated. Each value represents the mean ± SD (n=3-4 per group), and no significant differences are demonstrated among the groups, as determined by the Mann-Whitney U test.

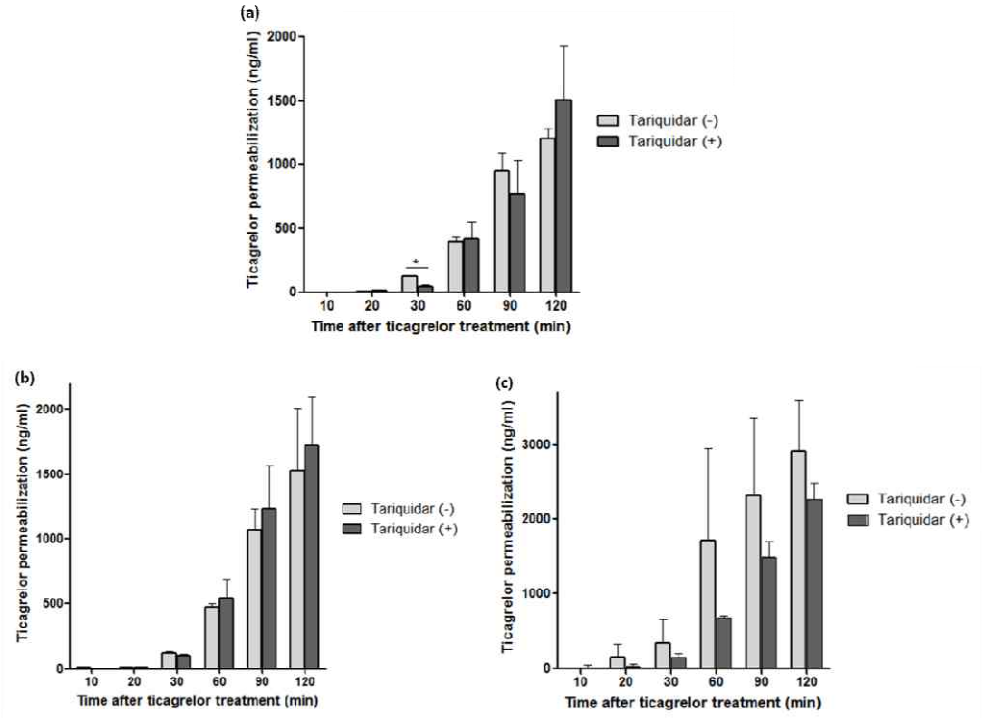

Figure 3. Effect of tariquidar on *in vitro* ticagrelor permeabilization. Tariquidar (40 nmol/l) is given 30 miniutes prior to ticagrelor administration. Each graph compares the ticagrelor concentration in the basolateral chamber when treated with or without tariquidar after pre-treatment of saline (a), 0.5 mmol/l aspirin (b), and 1 mmol/l aspirin (c). After saline pre-treatment, ticagrelor concentration at 30 minutes is significantly lower in the tariquidar group as determined by the Mann-whitney U test. Otherwise, no differences are demonstrated among the groups. Each value represents mean ± SD (n=3-4 per group).

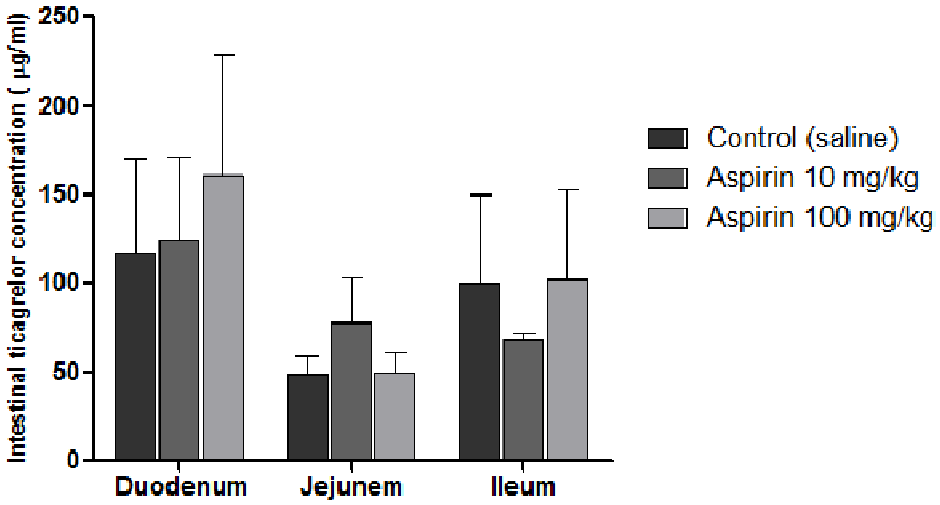

Figure 4. Effect of aspirin pre-treatment on *in vivo* ticagrelor absorption in the rat intestine. Ticagrelor absorption from various regions of the rat intestine is evaluated by the closed-loop method. Concentration of unabsorbed ticagrelor is measured 20 minutes after 1 ml of 10 mmol/l ticagrelor is administered into each of the intestinal segments. No significant differences are shown in intestinal unabsorbed ticagrelor concentrations, regardless of different aspirin pre-treatment doses, as determined by the Mann-Whitney U test. Each value represents mean ± SD (n=3-5 per group).

in vitro	Ticagrelor concentration (ng/ml)					
	10 min	20 min	30 min	60 min	90 min	120 min
Tariquidar (-)						
Saline (n=4)	0.76±0.31	5.74±5.16	120.75±9.64	391.25±39.25	946.5±140.41	1200±80
Aspirin 0.5 mM (n=3)	8.87±2.28	11.07±2.28	117±12.77	470±31.10	1071±159.26	1536.67±475.96
Aspirin 1 mM (n=3)	3.39±1.05	150.8±183.87	338.67±320.16	1715±1234.21	2323.33±1023.44	2910±387.90
Tariquidar (+)						
Saline (n=4)	0.97±0.60	14.04±5.27	43.93±14.54	416±136.75	768.75±256.32	1507.5±423.66
Aspirin 0.5 mM (n=4)	0.5±0	12.04±7.66	96.35±12.97	539.75±147.76	1230±342.05	1727.5±375.62
Aspirin 1 mM (n=4)	15.83±30.65	30.93±28.34	149.75±48.60	684±26.50	1500±196.13	2265±227.82

in vivo	Ticagrelor concentration (μg/ml)		
	Duodenum	Jejunum	Ileum
Saline (n=3)	116.98±53.30	48.78±10.80	100.45±49.87
Aspirin 10 mg/kg (n=3)	124.73±46.39	78.23±24.81	68.2±4.04
Aspirin 100 mg/kg (n=5)	161.12±67.20	49.62±11.86	101.92±51.09

Table 1. Concentrations of ticagrelor in each group. All data are shown as mean ± standard deviation (SD).

Discussion

Our experimental data provided evidence that ticagrelor absorption in the intestine is not significantly affected by the aspirin combination regimen. The results of absorptive permeabilization in Caco-2 cells demonstrate that the efflux clearance of ticagrelor is not affected by aspirin pre-treatment. When we administered a P-gp inhibitor, tariquidar before ticagrelor treatment to eliminate the effect of enhanced P-gp function following aspirin pre-treatment, no remarkable alterations were observed. Furthermore, the *in vivo* evaluation using the closed-loop model of rat failed to demonstrate a meaningful effect of aspirin pre-treatment on ticagrelor absorption.

Our previous study demonstrated that aspirin in high concentrations significantly increased the expression level of the MDR1 gene and protein and enhanced the function of MDR1 protein in human Caco-2 cells and rat intestine.[6] Clopidogrel absorption is known to be influenced by P-gp,[12] thereby clopidogrel permeabilization was decreased in Caco-2 cells when pre-treated with aspirin.[6] As ticagrelor is also a P-gp substrate, we expected similar decremental permeabilization on concomitant use with aspirin. However, ticagrelor permeabilization was not significantly decreased after pre-treatment of aspirin as well as assumptive enhancement of P-gp function.

One hypothetical explanation of this result is based on the fact that ticagrelor and its active metabolite are P-gp substrates and also weak inhibitors of P-gp.[9, 13] The inhibitory property of ticagrelor may have counterbalanced the enhancing property of aspirin on P-gp. To our knowledge, no data are available on the concomitant use of ticagrelor with potent P-gp inhibitors. We administered a P-gp inhibitor, tariquidar, before ticagrelor treatment to eliminate the effect of enhanced P-gp function following aspirin pre-treatment, and demonstrated insignificant effects. This may imply that the enhanced P-gp function due to aspirin pre-treatment is suppressed by other factors, probably by the P-gp inhibitory role of ticagrelor.

However, we noticed that tariquidar neither had any considerable impact on ticagrelor permeabilization in the saline pre-treatment group. Only the concentration of ticagrelor at 30 minutes with tariquidar supplement was significantly lower than that of the tariquidar-free group, but this single difference could happen by chance rather than it being a meaningful trend. These results lead us to another idea that ticagrelor has alternative major absorptive mechanism in the intestine beside P-gp.

Ticagrelor is mainly metabolized by cytochrome P450 (CYP) 3A enzymes. CYP3A4 and CYP3A5 are primarily responsible for the formation of its active metabolite AR-C124910XX.[13] Because of the extensive first-pass metabolism, the mean absolute bioavailability of ticagrelor is only 36% (range 30-42%).[14] A recent study has demonstrated that intestinal CYP3A4 enzyme plays an important role in

the first-pass metabolism of ticagrelor and alteration of the CYP3A4 activity influenced the plasma concentration of ticagrelor. [15, 16] This implies that CYP3A4 enzyme is more important than P-gp in the intestinal absorption of ticagrelor, and explains why concominant aspirin treatment did not influence the ticagrelor concentration.

The *in vivo* data strengthened the above idea. The intestinal concentration of ticagrelor, which reflects unabsorbed ticagrelor did not change meaningfully in the aspirin pre-treatment group compared with the control group (saline pre-treatment group), regardless of aspirin dose. The intestinal concentration of ticagrelor between two different doses of aspirin (10 mg/kg and 100 mg/kg) was also not significantly different. The *in vitro* data and *in vivo* data collectively suggest that enhanced P-gp function following aspirin pre-treatment has a negligible effect on the intestinal absorption of ticagrelor.

When a new drug is introduced into the market, it is very important to evaluate its drug-drug interaction in the aspect of pharmacokinetics and pharmacodynamics. Pharmacokinetic interaction occurs when one drug affects the absorption, distribution or excretion of another drug, and pharmacodynamic interaction happens when changes in drug action occur in the area of the target tissue.[17] Ticagrelor is a new P_2Y_{12} inhibitor, which is currently receiving huge attention for its promising effects in patients with ACS.[7] However, a recent study has shown that there is a clear tendency of ticagrelor to be less effective in patients who were

administered a higher dose of aspirin in advance.[10] Unfortunately, we still do not know why ticagrelor is less effective in the presence a high aspirin maintenance dose. The results of the present study provide evidence that ticagrelor does not have pharmacokinetic interaction with aspirin at the intestinal absorption level. The mechanism of how aspirin attenuates the treatment effect of ticagrelor needs to be elucidated in future studies, especially on the pharmacodynamic aspect.

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요약 (국문 초록)

배경: 티카그렐러는 새로운 P_2Y_{12} 수용체 차단제로, 급성관상동맥 환자에서 아스피린과의 병용 요법이 승인을 받았다. 그러나, 고용량의 아스피린과 함께 투여할 경우, 티카그렐러의 약효가 떨어진다는 일부 증거가 보고된 바 있다. 아스피린이 장내 P-glycoprotein (P-gp)을 활성화시킨다는 점과 티카그렐러가 P-gp의 기질(substrate)이라는 점을 바탕으로, 본 연구에서는 아스피린과 병용 투여시 장내 티카그렐러의 흡수가 감소할 것이라는 가설을 수립하였다.

방법 및 결과: *In vitro* 실험은 인간 대장암세포(Caco-2 cells) 단일막을 이용하였으며, *in vivo* 실험은 랫드에서 폐회로방법(closed-loop method)을 이용하였다. *In vitro*에서는 Caco-2 세포에 아스피린(0.5와 1mmol/l)을 72시간동안 투여한 뒤, *in vivo*에서는 랫드의 장내에 아스피린(10mg/kg와 100mg/kg)을 4주간 투여한 뒤에 티카그렐러를 투약하였다. *In vitro* 실험에서 티카그렐러의 투과성은 아스피린 전처리 혹은 P-gp 억제제인 타리퀴다에 의해 영향을 받지 않았다. 또한, 랫드의 장내 티카그렐러 흡수도 여러 용량의 아스피린(10mg/kg와 100mg/kg) 전처리에 의해 영향을 받지 않았다.

결론: 티카그렐러의 장내 흡수는 아스피린 투여에 따른 P-gp 활성화에 의해 영향을 받지 않았다. 향후 아스피린이 티카그렐러의 치료효과를 약화시키는 기전을 밝히기 위한 연구가 필요할 것이다.

주요어: 아스피린, 티카그렐러, P-glycoprotein, 약물상호작용, 장내 흡수

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