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Relaxation effect of marmin on guinea pig tracheal smooth muscle via NO-independent mechanisms

Dadang Irfan Husori[1,2], Sugeng Riyanto[3], Agung Endro Nugroho[1]

[1]*Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Jogjakarta 55281, Indonesia*
[2]*Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan ,20155, Indonesia*
[3]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Jogjakarta 55281, Indonesia*

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ABSTRACT

Objective: To investigate the relaxation mechanims of marmin on epithelium of guinea pig isolated trachea smooth muscle (TSM). **Methods:** The study was conducted using *in vitro* isolated-trachea experimental. The guinea pig isolated trachea were incubated in Krebs solution-containing organ bath and supplied with a mixed gas of O_2:CO_2 (95%:5%). **Result:** Removal of tracheal epithelium was associated with significant increases in the potencies of histamine and methacholine to contract guinea pig TSM. The pD_2 value of histamine increased from 6.04±0.08 on epithelial-intact to 6.32±0.06 on epithelial-denuded (P<0.05). The pD_2 value of methacholine also increased from 5.85±0.09 on epithelial-intact to 6.15±0.07 on epithelial-denuded (P<0.05). Marmin exhibited relaxation effects on TSM induced by methacholine (3×10^{-5} mol/L) and histamine (3×10^{-5} mol/L). Inhibition of prostaglandin E2 (PGE_2) through incubation with indomethacin could reduce the relaxation effect of marmin (P<0.05) on methacholine- and histamine-induced contractions. However, no significant differenceswere shown in methylene blue, Nω-nitro-L-arginine (L-NNA) and propranolol-incubated TSM. **Conclusions:** The results suggest that marmin has relaxation effect on TSM which is epithelial-dependent through the release of PGE_2. However, nitric oxide, cGMP and β2-adrenergic-mediated relaxation were not involved.

1. Introduction

Aegle Marmelos Correa (Rutaceae) is one of the potential plants for allergy and asthma therapies. This plant originates from and grows widely in some areas of the Southeast and South Asia countries such as India, Sri Lanka, Indonesia, Malaysia and Vietnam. The leaves, fruit, stems and roots of plants are widely used in traditional medicine for various purposes such as asthma, diarrhoeal, fever, and heart disease[1-4].

Marmin or 7-(6′, 7′-dihydroxyigeranil-oxy) coumarin) belongs to the class of coumarin derivatives. Marmin is found mainly in the bark and roots of *Aegle Marmelos* Correa[5]. Alcoholic extract of *Aegle Marmelos* Correa exhibited a relaxation effect on isolated guinea-pig trachea and ileum through the inhibition of histamine-induced contraction[6]. In the previous study, marmin also showed a protection effect against ethanol-induced gastric irritation, and an inhibitory effect on the gastric motility. Marmin exhibited a relaxation effect on isolated guinea-pig ileum induced by acetylcholine and histamine[7]. Marmin isolated from *Citruss hasaku* showed a spasmolytic activity on isolated guinea-pig intestine induced by $BaCl_2$[8].

Airway epithelial has important function as a barrier and in the regulatory processes on modulation of smooth muscle contraction[9]. Airways epithelium removal in several animal species increase smooth muscle sensitivity to spasmogen stimulation such as histamine, acetylcholine, 5-hydroxytriptamine, leukotrienes C4 and D4 and isoprenalin[10,11].

Epithelium has protection effects on the airway smooth muscle through the ability to release relaxation agents (epithelium-derived relaxant factors/EpDRFs) such as nitric oxide (NO) and prostaglandin E2 (PGE_2)[10-12]. This research aimed to study the contribution of epithelium on the relaxation effect of marmin isolated from *Aegle Marmelos* Correa on guinea-pig isolated tracheal smooth muscle (TSM).

*Corresponding author: Agung Endro Nugroho, M.Sc., Ph.D. Departement of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara Jogjakarta, Indonesia 55281.
Tel: (0274)543120
Fax : (0274)543120
E-mail : agungendronugroho@yahoo.com; nugroho_ae@ugm.ac.id

2. Materials and methods

2.1. Materials

Marmin isolated from *Aegle Marmelos* Correa was obtained from Prof. Dr. Sugeng Riyanto, M.S., Apt. (Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gadjah Mada University, Jogjakarta). Marmin was prepared by dissolved in DMSO (Merck, Germany) (1% v/v). Krebs solution contains NaCl, KCl, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $NaH_2PO_4 \cdot 2H_2O$, $NaHCO_3$ and glucose. Krebs aerated with O_2 and CO_2 (95:5). Histamine, methacholine (Sigma, USA), *N*ω-nitro-L-arginine (L-NNA) (Sigma-Aldrich, USA), indomethacin (Sigma, USA), methylene blue (Sigma-Aldrich, USA), propranolol (Sigma, USA), and distilled water.

2.2. Animal and tissue preparation

Male guinea-pigs weighing 300-500 g (3-4 months) were housed in a room with controlled temperature and lighting, and allowed free access to chow and tap water. The animal handling protocols of this study were approved in accordance with the guidelines of the animal care of the Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia.

The animals were sacrificed by cervix dislocation. Trachea was dissected out and the connective tissue was gently removed. Subsequently, the rings were cut with a length of 8-9 rings and connected to the transducer. In preparation of denuded tracheal, epithelial layer was removed by gently rubbing the luminal surface with cotton bud. At the end of experimental, arbitrarily selected strips were fixed in 10% formalin for histological examination to confirm the presence and absence of epithelium[13].

2.3. Experimental protocols

2.3.1. Effect of epitheliaum on TSM contraction

Firstly, TSM was equilibrated for 60 min in an organ bath containing 20 mL Krebs buffer at 37 ℃, and stimulated with 3×10^{-5} mol/L agonist (histamine or methacholine). The contractions were recorded by a level transducer (type 368 HSE, Germany) connected to a bridge amplifier (type 336 HSS, Germany) and a recorder (Kipp & Zonen BBD 41, The Netherlands). Subsequently, TSM was washed for 45 min by replacing the Krebs buffer with the new one every 15 min. A series concentration of agonist (histamine and methacholine) was administrated in organ bath containing TSM (epithelium denuded or intact). TSM contractions were recorded until the maximum contraction achieved.

2.3.2. Effects of marmin on epithelium-denuded and -intact tracheas

After equilibration for 60 min with 20 mL Krebs buffer at 37 ℃, the isolated organ was contracted with the agonists (methacholine or histamine). After the organ was washed, and replacement of Krebs buffer solution, the organ was contracted with the agonists (3×10^{-5} mol/L methacholine or histamine). After the maximum contraction was reached, the organ was then incubated with a series concentration of marmin (5-100 μmol/L). The experiments were performed on both epithelium-denuded and -intact tracheas.

2.3.3. Effects of antagonists on relaxation effects of marmin

The epithelium-intact trachea was equilibrated for 60 min with Krebs buffer. The organ was stimulated by 3×10^{-5} mol/L agonist (histamine or methacholine). The organ was washed, and then incubated for 20 min with methylene blue (10^{-4} mol/L), indomethacin (10^{-5} mol/L), 10^{-5} mol/L of L-NNA, or propranolol (3×10^{-5} mol/L). The trachea was contracted with agonists to reach the plateau, and then administrated with a series concentration of marmin (5-100 μmol/L).

Statistical analysis. All data were expressed as mean±SEM. As the previous study, The pD_2 values are derived from the negative logarithm to base 10 of the agonists concentration which cause half maximal response in the form of contraction. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test were used for statistical analyses. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Effect of epithelium on TSM contraction

In the study, contraction response of TSM increased obviously with increasing concentrations of the agonists (histamine and methacholine) either in epithelium-denuded or -intact trachea. Removal of tracheal epithelial shifted the agonist curve to the left (Figure 1). There is a difference to the percentage of contractions by agonists in epithelium-denuded and -intact trachea. The contraction in epithelium-denuded trachea was more potent than this in epithelium-intact trachea. Removal of epithelial layer increased the pD_2 value of histamine from 6.04±0.08 (intact trachea) to 6.32±0.06 (denude trachea) significantly ($P<0.05$). This removal also increased the pD_2 values of methacholine from 5.85±0.09 (intact trachea) to 6.15±0.07 (denude trachea) significantly ($P<0.05$) (Table 1). It indicates that in absence of epithelium increased the potency of agonists to contract TSM.

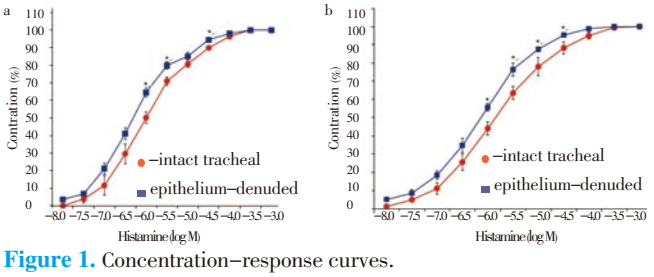

Figure 1. Concentration-response curves.
a histamine; b: methacholine. Data represent mean±SEM, *n*=4.
*Significant difference $P<0.05$ compared to the values of intact group.

Table 1
pD_2 values of histamine and methacholine in epithelium -denuded and -intact tracheal smooth muscle of guinea pig.

Agonist	pD_2 (Mean±SEM, *n*=4)	
	Intact trachea	Denude trachea
Histamine	6.04±0.08	6.32±0.06*
Methacholine	5.85±0.09	6.15±0.07*

*Significant difference $P<0.05$ compared to the values of intact group.

3.2. Effects of marmin on epithelium-denuded and -intact tracheas

Marmin 80 μmol/L showed difference effects on intact and denuded tracheas (P<0.05). The compound relaxed the epithelium-denuded and -intact tracheas by 114.36%±3.11% and 133.18%±5.90%, respectively. Similarly, relaxation effects of marmin 100 μmol/L on epithelium-denuded and -intact tracheas were 124.80%±3.79% and 146.83%±5.66%, respectively. The results indicate that the relaxation effect of marmin on histamine-induced trachea is epithelium-dependent (Figure 2a). Difference of relaxation effect was clearly demonstrated in the study with methacholine. Marmin at 20, 40, 80 and 100 μmol/L contracted the epithelium-intact trachea more potently than the effects on denuded trachea (Figure 2b). It indicates that the relaxation effect of marmin on methacholine-induced trachea is also epithelium-dependent.

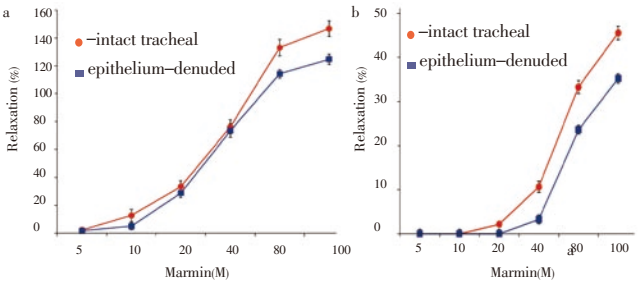

Figure 2. Relaxation effect of marmin.
a: single contraction by histamine; b: single contraction by methacholine of 3×10^{-5} mol/L. Data represent mean±SEM, n=4-9. *Significant difference P<0.05 compared to the values of intact group.

3.3. Effects of antagonists on relaxation effects of marmin

After preincubation with L-NNA 10^{-5} mol/L and methylene blue 10^{-4} mol/L, incubation of epithelium-intact trachea with marmin 100 μmol/L produced relaxation effects by respective 143.71%±4.19% and 141.83%±4.87%. Whereas the relaxation effect of marmin 100 μmol/L in the intact trachea without preincubation (control) was 146.83%±5.66%. There were no significant differences among these values (P>0.05). Initial incubation with propranolol 3×10^{-5} mol/L in the histamine-induced contraction produced a relaxation effect of marmin 100 μmol/L by 142.30%±2.68%. This effect was also no significant different in comparison to the effect in the control group (P>0.05).

Similar results were also shown on methacholine-induced contraction. After preincubation with L-NNA and methylene blue, relaxation effects of marmin 100 μmol/L on epithelium-intact trachea were 43.83%±1.85% and 44.24%±1.59%, respectively. The relaxation effect of marmin 100 μmol/L in the intact trachea without preincubation was 45.55%±1.54%. In this case, there were no significant differences among these values (P>0.05). After preincubation with propranolol, marmin 100 μmol/L relaxed the methacholine-induced contraction of trachea by 45.65%±1.78%. This effect was no significant different in comparison to this of the control group (P>0.05).

Relaxation effect of marmin on intact trachea preincubated with indomethacin 10^{-5} mol/L was lower than this of control group (without preincubation) (P<0.05) (Figure 3a). The decrease in relaxation effect is due to the inhibition of PGE_2 by indomethacin preincubation. Marmin at 100 μmol/L produced a relaxation effect by 102.94%±1.12% in indomethacin-preincubated trachea. This value was lower than this of control value (without preincubation) (146.83%±5.66%). In methacholine-induced contraction (Figure 3b) incubation with indomethacin also caused a decrease in marmin relaxation effect. Administration of marmin 100 μmol/L to indometachin-incubated trachea produced a relaxation effect by 28.41%±1.04%. This effect was lower than this of control value (without preincubation) (45.55%±1.54%) (P<0.05).

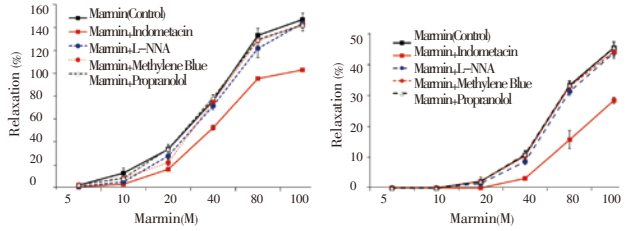

Figure 3. Relaxation effect of marmin on intact tracheal smooth muscle of guinea pig.
a: single contraction by histamine of 3×10^{-5} mol/L; b: single contraction by methacholine of 3×10^{-5} mol/L. Data represent mean±SEM, n=4-9. *Significant difference P<0.05 compared to the values of control group.

4. Discussion

Airway smooth muscle contraction is regulated by histamine-H_1 and muscarinic ACh-M_3 receptors[14]. In the other side, smooth muscle relaxation is regulated by adrenergic nerves through β2-adrenergic receptor and airways epithelial cells through the release of relaxation agents[9].

The administration of the series of methacholine and histamine concentrations (10^{-8}-10^{-3} mol/L) have increased TSM contraction. Histamine triggers the contraction of airway smooth muscle by activation of histamine-H_1 receptor that plays a role in regulating airway smooth muscle contraction[15]. A contraction by methacholine occurs through muscarinic M_3 receptor stimulation[14,16,17]. Subsequently, the activation of histamine-H_1 and muscarinic-M_3 receptors stimulate the activation of PLC, and then enhance the formation of IP_3 and DAG playing a role in increasing the levels of intracellular Ca^{2+}[16,18].

Removal of tracheal epithelium increased the pD_2 values of histamine and methacholine to trigger the contraction of tracheal smooth muscle. The increase of pD_2 occurred as a result of loss of protective function of epithelium as a barrier

between smooth muscle and the environments due to the loss of EpDRF such as NO and PGE_2[10,11,19].

In the study, marmin exhibited relaxation effects on epithelium-intact and -denude tracheas contracted with histamine or methacholine. Relaxation effect of marmin in epithelium-intact trachea was stronger than this in epithelium-denude trachea. These facts indicate that the relaxation effect of marmin on tracheal smooth muscle related to epithelium contribution through EpDRFs release.

Action mechanism of marmin on smooth muscle relaxation did not known certaintly. However, based on previous studies it relates to decreased-intracellular Ca^{2+} levels. Previous study reported that marmin succeeds to inhibit the Ca^{2+} influx in mast cells. Inhibition of Ca^{2+} influx and Ca^{2+} release from calcium stores sarcoplasmic reticulum by marmin will reduce the levels of intracellular Ca^{2+} and inhibit smooth muscle contractions[16,20,21].

Smooth muscle relaxation occurs after the uptake of intracellular Ca^{2+} to the sarcoplasmic reticulum and/or through the efflux of Ca^{2+} into extracellular which lead to decrease the intracellular Ca^{2+}. Subsequently, it can inhibit the formation of calcium-calmodulin complex[16,22]. Marmin able to influence the process of Ca^{2+} uptake into intracellular calcium stores and the process of Ca^{2+} efflux[16,20,21]. Further studies are recommended to explore the action mechanism of marmin on these pathways.

Elucidating the contribution of epithelium on relaxation effect of marmin showed that relaxation effect of marmin on trachea was not changed significantly by preincubated with L-NNA. Reportedly, the epithelial layer functions as a barrier and has ability to release NO that inhibits exogenous acetylcholine-induced contraction[23]. Smooth muscle relaxation by NO can be obtained via cGMP-dependent pathway (NO-GC-cGMP). In the pathway, NO activates the soluble guanylate cyclase that will convert GTP into cGMP. Relaxation is obtained through the activation of protein kinase G (PKG) by cGMP[24-26]. In the study, inhibition of endogenous relaxation effect of cGMP-dependent pathway with methylene blue did not influence the relaxation effect of marmin in epithelium-intact trachea. It indicates that relaxation effect of marmin is NO-independent.

In addition, inhibition of β2-adrenergic receptor with propranolol also did not influence the relaxation effect of marmin in epithelium-intact trachea. It indicates that relaxation effect of marmin is not related to β2-adrenergic receptor activation.

In epithelium-intact trachea, inhibition of PGE_2 production through incubation with indomethacin succeeded to reduce the relaxation effect of marmin on both methacholine- and histamine-induced contractions. These results indicate that the relaxation effect of marmin affected by the release of PGE_2. Inhibition PGE_2 production by indomethacin produced a significant reduction of marmin relaxation effect on trachea smooth muscle. Removal of epithelium resulting in reduce of PGE_2 production is associated with the trachea smooth muscle hyperresponsive[27].

We also found that the relaxation effect of marmin in indomethacin-preincubated intact trachea was significantly lower than the effect in epithelium denuded-trachea (absence of epithelium). This result suggests that indomethacin not only inhibits the production of PGE_2 in epithelial layer but also in smooth muscle. PGE_2 is not only produced by human airway epithelial but also produced by airway smooth muscle cells[28]. The influence of relaxation effects of PGE_2 from epithelium is partial to the relaxation effect of marmin on smooth muscle.

Bronchoprotective action of PGE_2 through indirect inhibition of acetylcholine release in parasympatic nerve terminal and inhibition of histamine release from mast cells[28]. PGE_2 that inhibits bronchoconstriction response from inhaled-allergens can be induced by lowering PGD_2 production[29]. The decrease of PGD_2 production is one mechanism of PGE_2 as a bronchoprotector against early asthma response. Meanwhile, the relaxation effect of PGE_2 is directly obtained through the EP_2 receptor activation. Subsequently, this activation triggers the coupling between the EP_2 receptor with Gs protein, and produces relaxation effect[15].

Relaxation effect of marmin isolated from *Aegle Marmelos* Correa on tracheal smooth muscle function is epithelial-dependent through the release of PGE_2. The study provides a point of consideration when marmin used in condition where there is a damage on tracheal epithelium, such as bronchial asthma triggered by the occurrence of bronchial hyperactivity associated with epithelial damage.

In conclusion, removal of the tracheal epithelial was associated with significant increases in the potencies of histamine and methacholine to contract guinea pig trachea smooth muscle. Marmin exhibited relaxation effecst on guinea-pig TSM induced by methacholine and histamine. The relaxation effect of marmin was epithelial-dependent through the release of PGE_2. However, NO, cGMP and β2-adrenergic-mediated relaxation were not involved. Further research is needed to elucidate the effect of marmin on EpDRF release such as NO and PGE_2. In addition, it is needed to study the effects of marmin on PLC (IP_3 and DAG), PKC, IP_3 receptors and Rho-A to elucidate the detail action mechanisms of marmin.

Conflict of interest statement

We declare that we have no conflict of interest.

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