Tzu Chi Medical Journal 2020; 32(2): 154–161

Original Article

Mechanisms of the antiplatelet and analgesic effects of dextromethorphan and its metabolites

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Submission : 20-Feb-2019
Revision : 27-Mar-2019
Acceptance : 29-Apr-2019
Web Publication : 05-Sep-2019

Abstract

Objective: In the present study, we investigated the effects of dextromethorphan (DM) and its metabolites, including dextrorphan (LK2), 3-methoxymorphinan (LK3), and 3-hydroxymorphinan (LK4), on platelet aggregation *in vitro* and the inflammatory pain caused by carrageenan in rats, and their underlying mechanisms. **Materials and Methods:** Rabbit platelets were pretreated with DM or its metabolites to assess their effects on platelet aggregation and related target mediators. In addition, the analgesic activity and the underlying mechanisms of DM and LK3 were investigated in a carrageenan-evoked thermal hyperalgesia rat model. **Results:** The inhibitory potency of DM and its metabolites on platelet aggregation induced by arachidonic acid or collagen was LK3> DM > LK4>> LK2 as demonstrated by the half-maximal inhibitory concentration values. Moreover, the mechanisms of the antiplatelet effect of DM and LK3 may involve the inhibition of intracellular calcium mobilization, expression of platelet surface glycoprotein IIb/IIIa, the formation of thromboxane B_2, and elevation of platelet membrane fluidity. DM and LK3 also exhibited analgesic effects on carrageenan-evoked thermal hyperalgesia by suppressing the production of pro-inflammatory cytokines, nitric oxide, prostaglandin E_2, and neutrophil infiltration in inflammatory sites. **Conclusion:** DM and its metabolites, especially LK3, exhibit both antiplatelet and analgesic effects, and may, therefore, potentially ameliorate platelet hyperactivity and inflammatory-related diseases.

Keywords: *Carrageenan, Dextromethorphan, Inflammatory pain, Metabolites, Platelet aggregation*

Introduction

Dextromethorphan (DM), an uncompetitive N-methyl-D-aspartate receptor antagonist and sigma-1 agonist, is widely used as a cough suppressant and lacks addictive property. Moreover, additional targets and actions of DM are reported to account for its pharmacodynamics and therapeutic effects [1]. In *in vivo* conditions, DM can be metabolized to active metabolites, including dextrorphan (LK2), 3-methoxymorphinan (LK3), and 3-hydroxymorphinan (LK4), which are mainly catalyzed by cytochrome P450 2D and cytochrome P4503A, which are responsible for drug metabolism through catalysis of the oxidative biotransformation of most drugs [2].

Platelet aggregation is a complex process mediated by several mechanisms such as the activation of glycoprotein IIb/IIIa (GPIIb/IIIa) complex, increased mobilization of intracellular Ca^{2+}, and the imbalance between prostacyclin and thromboxane A_2 (TXA_2) [3]. An increase in platelet cytosolic Ca^{2+} concentration through its secretion by intracellular calcium stores and/or calcium influx has been regarded as a critical factor triggering platelet activation [4]. It is considered that the increased platelet activation observed frequently in various cardiovascular diseases may play a key role in the initiation and development of atherothrombosis [5]. Moreover, activation of platelets and platelet-derived growth factors has been reported to promote neointimal proliferation and restenosis after balloon injury [6]. Therefore, inhibition of platelet hyperactivity by targeting these platelet activating mediators is a promising strategy to prevent and attenuate thrombosis and related vascular disorders. A recent study demonstrated that DM treatment may significantly attenuate restenosis or inflammation, and thereby improve the outcome

Access this article online
Quick Response Code:
Website: www.tcmjmed.com
DOI: 10.4103/tcmj.tcmj_48_19

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How to cite this article: Su WL, Weng YY, Huang WH, Shui HA, Chou TC. Mechanisms of the antiplatelet and analgesic effects of dextromethorphan and its metabolites. Tzu Chi Med J 2020;32(2):154-61.

of percutaneous coronary intervention with bare-metal stent implantation [7]. Thus, DM may have the potential to modulate platelet-regulated responses. However, the effects of DM and its metabolites on platelet activation remain unknown.

Notably, DM can inhibit lipopolysaccharide-induced adhesion molecule expression in human endothelial cells by suppressing extracellular signal-regulated kinase, Protein Kinase B, and nuclear factor-κB (NF-κB) signaling pathways [8]. Moreover, Group A *Streptococcus* infection-induced inflammation was attenuated by DM through the reduction of reactive oxygen species (ROS) and nitric oxide (NO) production [9]. Similarly, it has been reported that administration of LK3 greatly improved the characteristic deleterious features of endotoxemia in rats through inhibition of inflammatory responses and ROS formation [10]. These findings strongly support the existence of anti-inflammatory activity for DM and its active metabolites. Carrageenan-evoked thermal hyperalgesia and paw edema mainly due to the overproduction of pro-inflammatory cytokines and induction of cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) is a widely used animal model for studying inflammatory pain [11-13]. To date, whether DM and its metabolites attenuate carrageenan-induced hyperalgesia has not been reported.

Conventionally, platelet hyperactivity is considered a critical factor causing thrombotic vascular diseases. Notably, several studies have indicated that activated platelets also promote inflammatory responses [14]. In addition to the crucial role of NF-κB in triggering inflammatory responses [15], NF-κB exerts a nongenomic function to activate platelet aggregation and blocking NF-κB activation inhibits platelet activation [16,17]. Thus, inflammation may be another important stimulator of platelet aggregation. Interestingly, platelets can release many inflammatory mediators, including pro-inflammatory cytokines such as interleukin-1 (IL-1), and various chemokines such as platelet factor-4, macrophage inflammatory protein-1α, regulated on activation, normal T cell expressed and secreted (RANTES), and CD40 L, which are present in platelet α-granules [18]. The platelet-derived mediators, in turn, involve the interactions of monocytes, neutrophils, lymphocytes, and endothelium. Accordingly, there is a positive regulating loop between platelet activation and inflammation. Therefore, inhibiting platelet hyperactivity may be an effective strategy to alleviate platelet-mediated thrombotic and inflammatory diseases such as inflammatory pain. In the present study, we examined the effects of DM and its metabolites on platelet aggregation and carrageenan-evoked thermal hyperalgesia and further investigated the mechanisms involved.

Materials and methods

Agents

DM, LK2, LK3, and LK4 were provided by Dr. Wen-Hsin Huang. Collagen (type 1, equine tendon), arachidonic acid (AA), bovine serum albumin, ethylenediaminetetraacetic acid (EDTA) (disodium salt), and Fura-2/AM were purchased from Sigma Chemical Company (St. Louis, MO, USA). Enzyme immunoassay (EIA) kits of cyclic adenosine monophosphate (AMP), cyclic guanosine monophosphate (GMP), thromboxane B_2 (TXB_2), and prostaglandin E_2 (PGE_2) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The paw exudate cytokine concentrations were detected using EIA kits (Genzyme Corporation, Cambridge). The other chemicals and reagents were bought from Sigma Chemical Company.

Platelet aggregation

The animal experiments of this study were approved by the local institutional animal care and use committee (No. 102069). Blood withdrawn from the marginal vein of the rabbit was mixed with EDTA (100 mM, 14:1 v/v), and then centrifuged at 160 × g for 10 min at 25°C to obtain platelet-rich plasma (PRP). The PRP was washed as described previously, and then, the pellet of platelets was collected [19]. The pellet was suspended in tyrode's solution, comprising $CaCl_2$ (1 mM), NaCl (136.8 mM), KCl (2.7 mM), $NaHCO_3$ (11.9 mM), MgCl2 (2.1 mM), NaH_2PO_4 (0.4 mM), glucose (10 mM), and bovine serum albumin (0.35%). The platelet suspension concentration was adjusted to 3.0×10^8 platelets/mL using a Coulter counter (Model ZM). Subsequently, the agents or solvent control (dimethyl sulfoxide, 0.5%, v/v) were preincubated with platelet suspension for 3 min, followed by induction of platelet aggregation with collagen (10 μg/ml) or AA (100 μM) for 6 min. An aggregometer (Model 560; Chrono-Log Corporation, Havertown, PA, USA) was used to turbidimetrically measure platelet aggregation at 37°C with constant stirring at 1000 rpm.

Measurement of cyclic adenosine monophosphate, cyclic guanosine monophosphate, and thromboxane B_2

Various agents or solvent control were incubated with platelet suspension at 37°C for 6 min, and 10 mM EDTA was added to stop the reaction, and the sample was boiled immediately for 3 min. The supernatants were centrifuged at 10,000 × g for 5 min, and the amount of cyclic AMP and cyclic GMP was detected using the respective ELISA kits. Platelets were incubated with vehicle or various agents at 37°C for 3 min followed by the addition of agonists for 6 min and boiling for 3 min to measure TXB_2 formation, a stable metabolite of TXA_2. The amount of TXB_2 in the supernatant was determined using the TXB_2 EIA kit.

Measurement of Ca^{2+} mobilization in platelets

Fura-2/AM was used to measure the intracellular calcium concentration ($[Ca^{2+}]_i$) of platelets, as described previously [16]. Briefly, fura-2-AM (5 μM) was incubated with the platelets (3×10^8 platelets/ml) for 50 min at 37°C in the dark, followed by centrifugation at 500 × g for 10 min. The suspension of pellets in 2 ml of tyrode solution was collected. A fluorescence spectrophotometer (CAF-100; Jasco, Tokyo, Japan) was then used to measure the fluorescence intensity (excitation, 340 nm; emission, 500 nm).

Platelet membrane fluidity

Platelet membrane fluidity was assayed using diphenylhexatriene (DPH)-labeled platelets. The fluorescence emission spectra of platelet membrane were detected using a fluorescence spectrophotometer (excitation, 363 nm; emission, 400–600 nm) [20].

Analysis of platelet surface glycoprotein IIb/IIIa expression

CD41/CD61-fluorescein isothiocyanate incubated with platelet suspension was raised against a platelet GPIIb/IIIa complex, and then, various agents and collagen (10 μg/mL) were added, and the sample was incubated for 10 min. The reaction was ceased by the addition of 500 μL of 1% paraformaldehyde. A flow cytometer (Becton Dickinson, FAC Scan, Heidelberg, Germany) was used to determine the fluorescence intensity of 10,000 platelets per sample.

Carrageenan-evoked paw thermal hyperalgesia model

Male Sprague–Dawley rats (age, 8–9 weeks; weight, 200–250 g) were used; the rats were purchased from the National Animal Center (Taipei, Taiwan). The hyperalgesia model was established as described previously [11]. Briefly, λ-carrageenan (2 mg) (100 μL of 2% [wt/vol] in saline) was injected subcutaneously into the plantar surface, through intraplantar injection (i.pl.), of the right hind paws of rats at time 0 (T = 0) to induce local inflammation. Different agent or vehicle (saline) was injected at 30 min before (T = −30) or 135 min after (T = 135) carrageenan injection. Paw withdrawal latency with a nonnoxious heat stimulus was determined at specific time points using 7370 plantar test (Ugo Basile, Comerio, Italy) to assess hyperalgesia. The baseline latencies were assessed 40 min before (T = −40) carrageenan injection. The control group was the vehicle (intraperitoneal [i.p.])-treated and the saline (i.pl.)-injected rats. The rats were sacrificed by exsanguination when the analgesic experiments ended. The hind paws at the level of calcaneus bone were cut and centrifuged at 400 × g for 15 min at 4°C to collect the edema fluid (exudates), which were used to measure paw edema and the levels of cytokines and PGE_2 using the respective EIA kits. A sievers NO analyzer (280 NOA; Sievers, Boulder, CO, USA) was used to measure the nitrate levels (total nitrite and nitrate) in paw exudates.

Measurement of myeloperoxidase activity

Tissues of carrageenan-injected paws were removed and homogenized with ice-cold 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM phosphate buffer (pH 6.0; 5 mL of HTAB per gram of tissue) on ice using a homogenizer (Pro Scientific Inc., Pro Model 200; Monroe, CT, USA), followed by centrifugation at 15,000 × g for 15 min at 4°C. The supernatant was then mixed with assay buffer at a ratio of 1:30 and the absorbance of the solution was read at 460 nm. The assay buffer consisted of 100 mM potassium phosphate (pH 6.0), 0.083 mL of H_2O_2 (30% stock diluted 1:1000), and 0.834 mL of o-dianisidine hydrochloride (10 mg/mL). The myeloperoxidase (MPO) activity was determined and expressed as A460 per minute per milligram of protein.

Statistical analysis

Data are expressed as mean and the standard error. The one-way ANOVA with *post hoc* Bonferroni test was used for statistical analysis. The results were considered significant different at a value of $P < 0.05$.

Results

Effects of dextromethorphan and its metabolites on platelet aggregation

The platelet aggregation induced by collagen or AA was dose-dependently inhibited by DM and its metabolites. The inhibitory potency of these agents on collagen or AA-induced platelet aggregation was LK3>DM>LK4>>LK2 as demonstrated by the values of half-maximal inhibitory concentration [Table 1]. Thus, we focused on investigating the actions of DM and LK3 in the subsequent tests.

Effects of dextromethorphan and LK3 on thromboxane B_2, cyclic guanosine monophosphate, and cyclic adenosine monophosphate production

The formation of TXB_2 was increased markedly after the addition of collagen or AA compared to that in resting platelets. Treatment with DM or LK3 greatly inhibited TXB_2 formation stimulated by collagen but did not have a significant effect on AA-induced TXB_2 formation [Table 2]. However, there was no

Table 1: The half-maximal inhibitory concentration values of dextromethorphan and its biological metabolites on platelet aggregation induced by collagen or arachidonic acid

Inducer	IC_{50} values (μM)			
	DM	LK3	LK4	LK2
AA (100 μM)	281.4±37.1	144.9±12.0	479.6±20.3	>500
Collagen (10 μg/mL)	116.9±32.5	45.6±10.3	131.9±18.0	251.4±1.5

Different concentrations of DM, LK3, LK4, or LK2 were preincubated with platelets at 37°C for 3 min, then AA or collagen was added to induce platelet aggregation. Then, the IC_{50} of various agents on platelet aggregation was measured data was expressed as mean±SEM (n=5). DM: Dextromethorphan, AA: Arachidonic acid, SEM: Standard error of the mean, IC_{50}: Half-maximal inhibitory concentration

Table 2: Effects of dextromethorphan and LK3 on the thromboxane B_2 formation induced by arachidonic acid or collagen in platelets

	TXB_2 (ng/mL) formation induced by	
	AA (100 μM)	Collagen (10 μg/mL)
Resting	5.7±1.1	5.8±1.5
Control	753.3±14.4	502.6±43.4
DM (μM)		
400	587.0±89.6	
300	676.7±13.7	
200	738.5±25.2	348.2±31.6*
100		362.2±33.1*
50		459.5±38.1
LK3 (μM)		
200	611.2±52.2	
150	627.0±97.2	
100	637.7±49.3	433.6±42.9*
50		439.9±43.3*
25		465.2±40.3

DM (50-400 μM) or LK3 (25-200 μM) was preincubated with platelets at 37 for 3 min, then inducer was added for 6 min to trigger TXB_2 formation. The action was terminated by boiling for 3 min and the amounts of TXB_2 were measured by EIA kit. Data was expressed as mean±SEM. *P<0.05 versus compared with collagen-stimulated alone group (n=5). TXB_2: Thromboxane B_2, DM: Dextromethorphan, AA: Arachidonic acid, SEM: Standard error of mean, EIA: Enzyme immunoassay

significant change in cyclic GMP and cyclic AMP production of platelets in the presence of DM or LK3 (data not shown).

Effects of dextromethorphan and LK3 on intracellular calcium mobilization of platelets

In Fura-2/AM-loaded platelets, DM or LK3 dose-dependently reduced the rise in $(Ca^{2+})_i$ of platelets evoked by collagen or AA in the presence of external Ca^{2+} (1 mM) [Figure 1], which was similar to that in the Ca^{2+}-free solution (data not shown). Similarly, LK3 exhibited a stronger inhibition on intracellular calcium mobilization than that by DM.

Effects of dextromethorphan and LK3 on platelet membrane fluidity

The platelet membrane fluidity was analyzed in the DPH-labeled platelets. The high fluorescence intensity indicated decreased platelet membrane fluidity with hyperactivity of platelet aggregation. Our data showed that DM or LK3 attenuated the DPH-related fluorescence intensity of platelets in a dose-dependent manner [Figure 2], indicating that DM and LK3 are capable of increasing platelet membrane fluidity.

Effects of dextromethorphan and LK3 on platelet glycoprotein IIb/IIIa expression

As shown in Figure 3, compared to that in only collagen-stimulated platelets, LK3 dose-dependently inhibited collagen-induced GPIIb/IIIa expression on the platelet surface as reflected by the fluorescence intensity. However, DM did not significantly affect the surface GPIIb/IIIa expression in collagen-induced platelets.

Effects of dextromethorphan and LK3 on carrageenan-evoked thermal hyperalgesia and paw edema

Compared to that of the control rats, injection of carrageenan (1 mg/paw, i.pl.) into the right hindpaws of rats evoked thermal hyperalgesia as demonstrated by a marked decrease in paw withdrawal latency. Treatment with various doses of DM [Figure 4a] or LK3 [Figure 4b] at 30 min before injection of carrageenan dose-dependently attenuated the hyperalgesia from 1 to 4 h reflected by a longer paw withdrawal latency than that in the carrageenan-injected alone group. The withdrawal latency of the contralateral left hind paw (no injection of carrageenan in this paw) was still constant at the basal levels throughout the experiment (data not shown). The values of paw withdrawal latency at 4 h of DM and LK3 at the dose of 20 mg/kg were 9.8 ± 1.8 and 15.1 ± 2.2 s, respectively, suggesting that LK3 has a stronger analgesic effect than that of DM. As expected, the paw edema reflected by the volume of paw extrude was reduced by LK3 [Figure 5a]. To examine the therapeutic effect of LK3 on the inflammatory pain, LK3 (10–20 mg/kg, i.p.) was administered at 135 min after carrageenan injection. We found that posttreatment with LK3 also prolonged the paw withdrawal latency compared to that of the carrageenan-injected alone group [Figure 5b].

Effects of LK3 on the production of pro-inflammatory cytokines in the paws

Pretreatment with LK3 (5–20 mg/kg, i.p.) markedly inhibited the carrageenan-induced rise of the production of pro-inflammatory cytokines, including IL-6, tumor necrosis factor-α (TNF-α), IL-1β, and IL-8 in paw exudates at 4 h compared to that of the carrageenan-injected alone group [Figure 6].

Effects of LK3 on the NOx and prostaglandin E2 levels and the myeloperoxidase activity in the paws

The paw exudates were collected to measure the levels of NO_X and PGE_2 at 4 h after carrageenan injection. Pretreatment with LK3 (5–20 mg/kg, i.p.) dose-dependently inhibited the

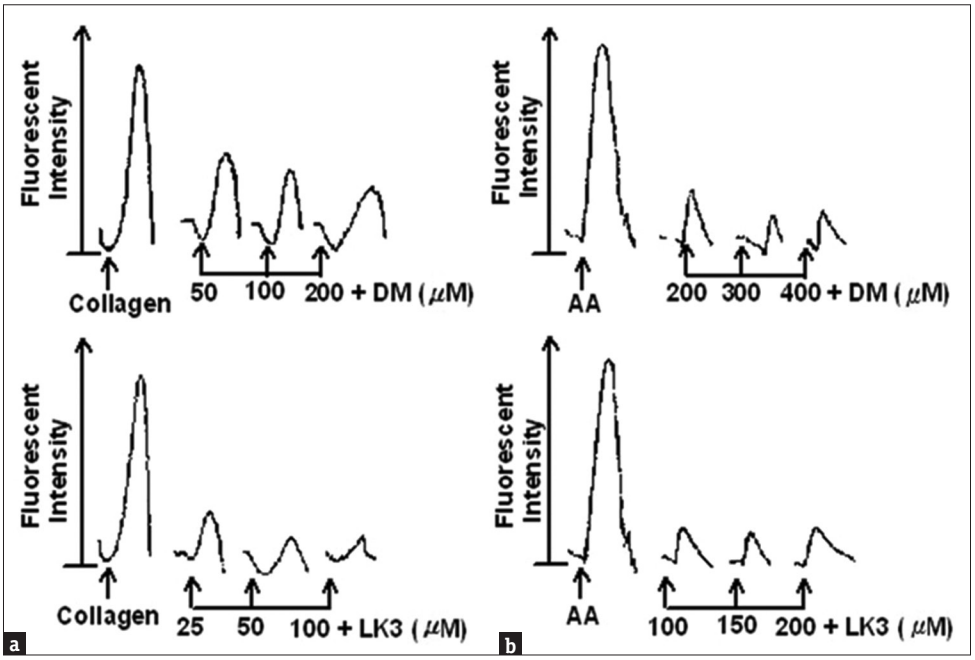

Figure 1: Effects of dextromethorphan and LK3 on the rise of intracellular calcium concentration of Fura2-loaded platelets induced by collagen or AA in the presence of $(Ca^{2+})o$ = 1 mM. The dimethyl sulfoxide (0.5%, control), dextromethorphan or LK3 was incubated with platelets for 3 min followed by the addition of collagen (a: 10 μg/mL) or AA (b: 100 μM)

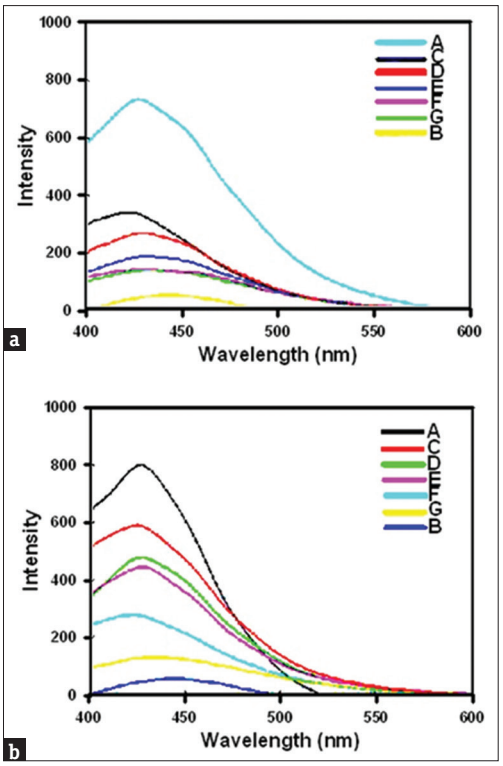

Figure 2: Effects of dextromethorphan and LK3 on platelets membrane fluidity. The fluorescence emission spectra of platelet membrane were measured in diphenylhexatriene-labeled platelets after treatment with various concentrations of dextromethorphan or LK3 for 3 min. In the upper (a), the platelets were treated with dextromethorphan (C = 50, D = 100, E = 200, F = 300, G = 400 uM). A presents as diphenylhexatriene-labeled alone platelets; B represented as diphenylhexatriene-unlabeled platelets. In the bottom (b), the platelets were treated with LK3 (C = 25, D = 50, E = 100, F = 150, G = 200 μM). a: Diphenylhexatriene-labeled alone platelets; b: Diphenylhexatriene-unlabeled platelets

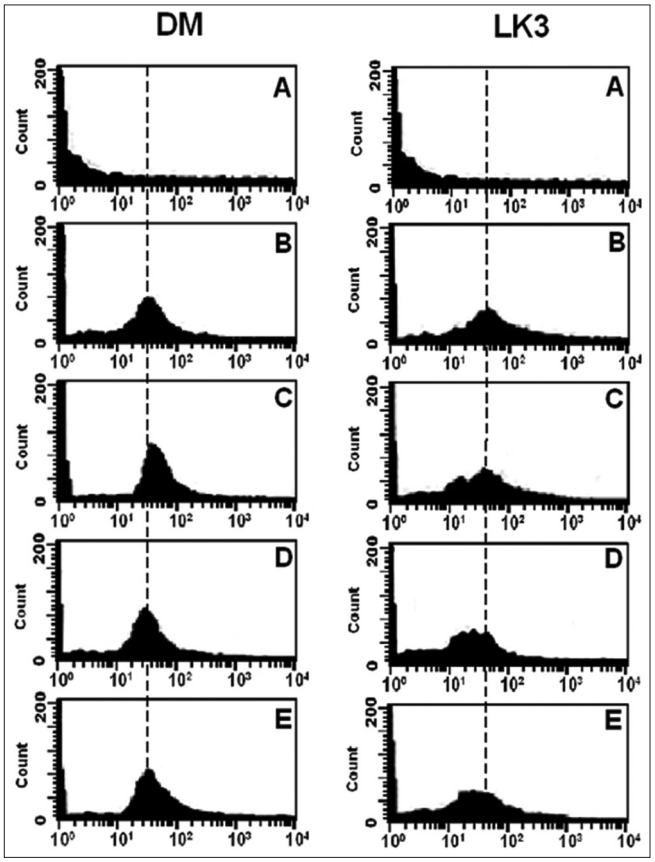

Figure 3: Effects of dextromethorphan and LK3 on the glycoprotein IIb/IIIa expression on platelet membranes. The platelet glycoprotein IIb/IIIa expression evaluated by flow cytometric analysis in the presence of collagen and dextromethorphan (A) or LK3 (B). In the left figure, the A as a solvent control, (B) as the collagen positive control, (C) as dextromethorphan 200 μM, (D) as dextromethorphan 300 μM, (E) as dextromethorphan 400 μM. In the right figure, the (A) as a solvent control, (B) as the collagen positive control, (C) as LK3 100 μM, (D) as LK3 150 μM, (E) as LK3 200 μM

formation of NO_X and PGE_2 in carrageenan-injected paws compared with that in the only carrageenan-injected group [Figure 7]. Furthermore, carrageenan-stimulated elevation in MPO activity in the paws was greatly decreased by LK3 [Figure 7].

Discussion

This is the first study to demonstrate that DM and its active metabolites, especially LK3 exert antiplatelet and analgesic activities. When platelets are activated by collagen, AA is released from membrane phospholipids by the actions of diglyceride lipase and various phospholipase A_2 [21,22]. The AA is then converted to TXA_2, a potent inducer of platelet aggregation, through COX and thromboxane synthase. Addition of DM or LK3 dose-dependently inhibited collagen-induced TXB_2 formation but did not affect AA-induced TXB_2 formation. These findings suggest that DM and LK3-mediated inhibition of collagen-induced TXB_2 formation may be mainly due to the suppression of AA release rather than the direct inhibition of COX and thromboxane synthase activity. Cyclic AMP and cyclic GMP are negative regulators of platelet aggregation, releasing granule contents, and rising intracellular Ca^{2+} mobilization [23,24]. As DM and LK3 have no significant effects on the amounts of cyclic AMP and cyclic GMP in platelets, the antiplatelet activity of DM and LK3 maybe not due to elevation of cyclic AMP and cyclic GMP formation.

In the presence of platelet inducers, the $(Ca^{2+})_i$ of platelets increases markedly, and thereby inducing platelet aggregation [25]. The rise in $(Ca^{2+})_i$ of platelets is largely controlled by calcium influx and calcium release from intracellular calcium stores. The inhibition of calcium mobilization by DM and LK3 with or without extracellular 1 mM $CaCl_2$ was similar, suggesting that the inhibition of cytosolic Ca^{2+} mobilization is mainly through the suppression of calcium release from intracellular calcium stores. Furthermore, DM and LK3-mediated inhibition of Ca^{2+} mobilization may, in turn, suppress phospholipase A_2 activity and the subsequent TXA_2 formation [26].

Alteration in membrane fluidity is considered a factor modulating platelet function. A decrease in platelet membrane fluidity (relatively rigid membrane) results in hyperactivity of platelets to agonists [27]. Treatment with DM or LK3 significantly enhanced platelet membrane fluidity, which may, at least in part, contribute to their antiplatelet effects. The GPIIb/IIIa receptor is responsible for fibrinogen-binding and subsequent platelet aggregation [28]. Agents blocking GPIIb/IIIa receptor activation have been regarded as promising

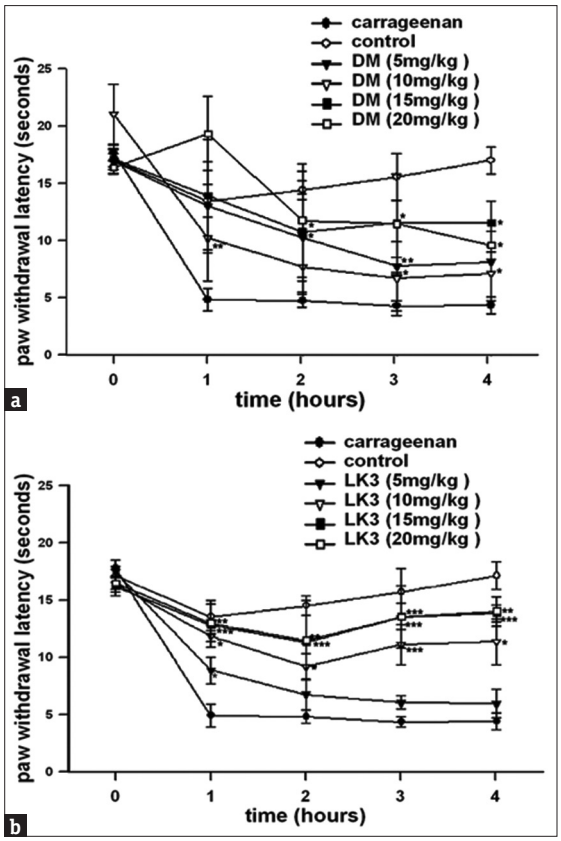

Figure 4: Effects of dextromethorphan and LK3 on carrageenan-evoked paw thermal hyperalgesia. Different doses of dextromethorphan (a) or LK3 (b) (5–20 mg/kg, intraperitoneal) were administered 30 min before carrageenan (1 mg/paw, intra-plantar) injection. Paw-withdrawal latencies were assessed in various groups at specific time. Data was expressed as mean ± standard error of the mean, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus carrageenan-injected group ($n = 5$)

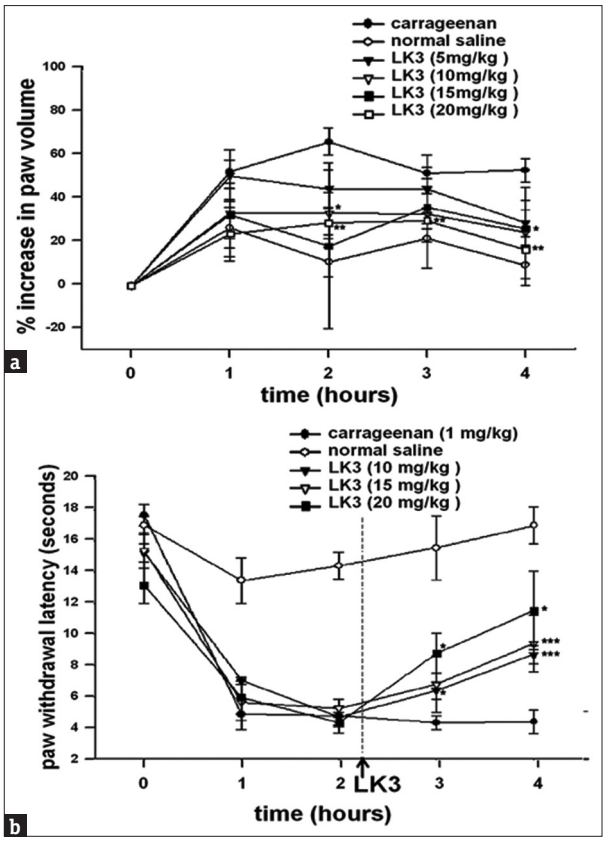

Figure 5: Effects of LK3 on carrageenan-evoked paw thermal hyperalgesia and paw edema. LK3 (5–20 mg/kg, intraperitoneal) was administered 30 min before carrageenan injection. Then, the paw edema was assessed in the carrageenan-injected hind paws of rats at specific time (a). LK3 or vehicle (saline) was injected at 135 min after carrageenan injection and the paw withdrawal latency was measured at specific time points (b). Data was expressed as mean ± standard error of the mean, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus carrageenan-injected group ($n = 5$)

antiplatelet drugs [29]. Interestingly, LK3 but not DM inhibits collagen-induced platelet membrane surface GPIIb/IIIa activation, suggesting that the antiplatelet mechanisms of DM and LK3 may not be the same. However, the reasons remain unclear and may need further investigation.

The previous study has reported that DM can attenuate the neuropathic pain after knee surgery, even when combined with morphine [30,31]. In this study, we further demonstrated that pretreatment with DM or LK3 exerts an analgesic activity on carrageenan-evoked thermal hyperalgesia. In addition, posttreatment with LK3 also attenuated hyperalgesia, indicating that LK3 has preventive and therapeutic effects on inflammatory pain. We then focused on investigating the analgesic mechanisms of LK3. It is well known that overproduction of pro-inflammatory cytokines, NO, and PGE_2 is critical in the pathogenesis of carrageenan-evoked pain and paw edema [11]. As expected, LK3 markedly inhibited the production of pro-inflammatory cytokines such as TNF α, IL-1 β, IL-6, and IL-8 in the paw exudates after carrageenan injection for 4 h.

The process of carrageenan-evoked pain is biphasic [32]. The responses of the initial phase (1 h after carrageenan injection) are associated with the release of histamine and serotonin. The responses in the late phase (4 h after carrageenan injection) are mainly due to the release of pro-inflammatory mediators such as PGE_2 and NO derived from COX-2 and iNOS, respectively, which contribute to the maintenance of inflammatory pain [33,34]. This concept was supported by the fact that blocking COX-2 induction and the subsequent PGE_2 formation or iNOS-derived NO formation greatly ameliorates inflammatory nociception [35]. As expected, carrageenan-induced a marked increase in PGE_2 and nitrate production in rat paws was significantly inhibited by LK3. It has been demonstrated that increased infiltrated neutrophils occurred in damaged tissues can stimulate the production of several pro-inflammatory mediators such as cytokines and ROS [36]. Based on the finding that the enhanced MPO activity, a sign of neutrophil infiltration, in carrageenan-injected paws, was markedly inhibited by LK3, we propose that the inhibition of neutrophil infiltration and regulated inflammatory responses are involved in the actions of LK3. Collectively, the analgesic activity of LK3 is associated with inhibition of the production of pro-inflammatory cytokines, PGE_2, and nitrate, as well as neutrophil infiltration. Accumulating evidence has confirmed that the platelet-mediated release of inflammatory mediators and their interactions with various inflammatory cells and endothelium are important coordinators of thrombotic and inflammatory responses [18]. Thus, it is possible that the

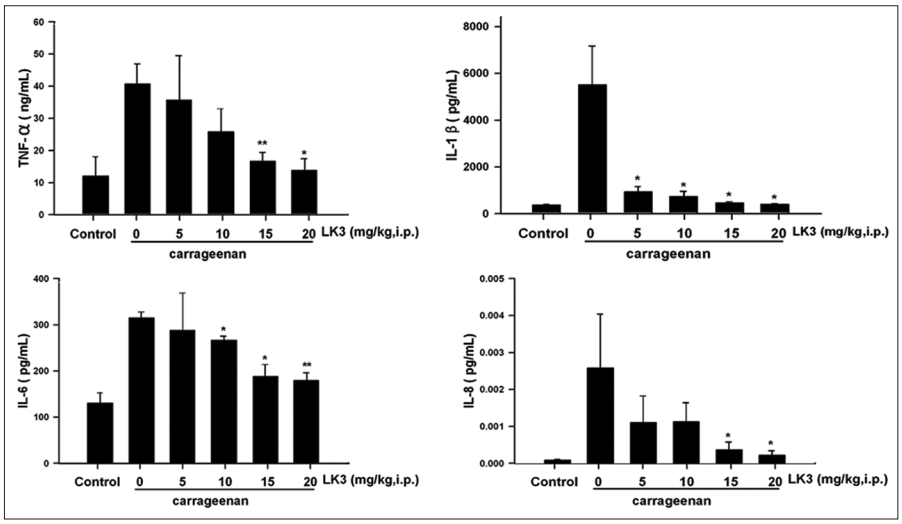

Figure 6: Effects of LK3 on pro-inflammatory cytokine formation in carrageenan-injected paws. LK3 (5–20 mg/kg, intraperitoneal) was administered 30 min before carrageenan injection. The paw exudates were collected for pro-inflammatory cytokine measurement at 4 h after carrageenan injection. The saline (intra-plantar)-injected rats acted as the control group. Data was expressed as mean ± standard error of the mean, $*P < 0.05$, $**P < 0.01$ versus carrageenan-injected alone group ($n = 5$)

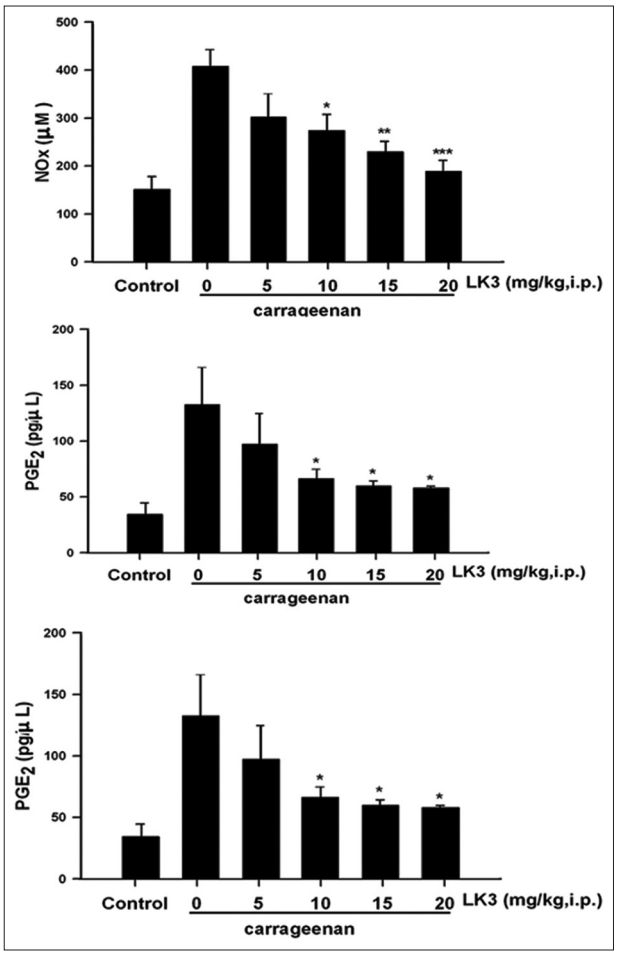

Figure 7: Effects of LK3 on NOx and prostaglandin E_2 formation, and myeloperoxidase activity in carrageenan-injected paws. Different doses of LK3 (5–20 mg/kg, intraperitoneal) was administered 30 min before carrageenan injection. The paw exudates were collected for measurement of NOx and prostaglandin E_2 formation, and myeloperoxidase activity at 4 h after carrageenan injection. The saline (intra-plantar)-injected rats acted as the control group. Data was expressed as mean ± standard error of the mean, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus carrageenan-injected alone group ($n = 5$)

antiplatelet and analgesic effects of DM and LK3 may be regulated mutually.

Conclusion

Taken together, we demonstrated that DM and its metabolites, especially LK3, can inhibit platelet aggregation and carrageenan-evoked thermal hyperalgesia, indicating that DM and LK3 may be potential therapeutic agents for patients with platelet hyperactivity or inflammatory pain-related diseases.

Financial support and sponsorship

This work was partly supported by a grant from Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation (TCRD-TPE-108-45 and TCRD-TPE-107-38).

Conflicts of interest

There are no conflicts of interest.

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