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A CAPE analogue as novel antiplatelet agent efficiently inhibits collagen-induced platelet aggregation

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Objective: Platelet activation plays a pivotal role in the pathogenesis of thrombosis, which can lead to fatal diseases such as myocardial or cerebral infarction, and atherosclerosis. The present study focused on investigating the effect of CAPE-NO_2 against collagen-induced platelet aggregation. *Methods:* Caffeic acid phenethyl ester (CAPE) is an active component in propolis. CAPE-NO_2 is a nitro derivative of CAPE. Its effects on rat platelet aggregation induced by collagen were tested *in vitro* and the potential mechanisms underlying the activities were investigated. *Results:* CAPE-NO_2 significantly inhibited collagen-induced platelet aggregation in a concentration-dependent manner. It also reduced TXB_2 formation and COX-1 activity in collagen-activated platelets. Moreover, CAPE-NO_2 caused an increase in NO production and cGMP levels and attenuated 5-HT release in the collagen-activated platelets. *Conclusion:* These findings suggest that the inhibitory mechanism of CAPE-NO_2 on collagen-induced platelet aggregation might be associated with the down-regulation of TXB_2, COX-1 and 5-HT and the elevation of NO and cGMP production. These indicators are closely related to platelet function. So CAPE-NO_2 may be a promising candidate for the extension of the current spectrum of antiplatelet drugs.

1. Introduction

Caffeic acid phenethyl ester (CAPE) (Fig. 1A) is a natural flavonoid like compound concentrated in honeybee propolis (Rezzani et al. 2005). Along with a variety of beneficial effects, it has been a popular folk medicine through the ages. It has been reported to exhibit numerous bioactive properties including anti-viral (Shvarzbeyn and Huleihel 2011), anti-oxidant (Yilmaz et al. 2004; Okutan et al. 2005; Ozguner et al. 2005; Pekmez et al. 2007), and anti-carcinogenic (Hwang et al. 2006; Onori et al. 2009; Ozturk et al. 2012; Wang et al. 2005). Other studies have shown that CAPE inhibits certain enzyme activities such as lipoxygenases (Ozturk et al. 2012; Song et al. 2002), cyclooxygenase (Chen et al. 2013; Peng et al. 2012), and xanthine oxidase (Ozturk et al. 2012; Kavakli et al. 2010; Mansour and Tawfik 2012). CAPE has potent hepatoprotective effects against CCl_4-induced hepatic damage in mice. This may be caused by its ability to block the bioactivation of CCl_4 by inhibiting CYP2E1 activity in combination with its ability to scavenge free radicals (Lee et al. 2008). It has already been reported that human CCRF-CEM acute lymphoblastic leukemia cells exposed to increasing concentrations of CAPE undergo apoptosis in a time and dose-dependent manner (Avci et al. 2011). CAPE-induced apoptosis at least involves loss of mitochondrial membrane potential which activates caspases (Burgazli et al. 2013; Prasad et al. 2011). Previous studies have demonstrated that CAPE prevents lipid peroxidation induced by ischemia-reperfusion injury in the spinal cord (Akyol et al. 2013), renal tissue (Roso et al. 2012), intestine (Aviello et al. 2010), liver (Saavedra-Lopes et al. 2008), and brain (Altug et al. 2008).

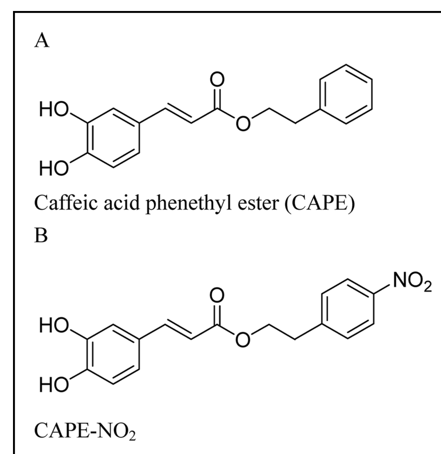

Fig. 1: Chemical structures of CAPE (A), and CAPE-NO_2 (B).

Deep vein thrombosis, myocardial infarction, pulmonary embolism, and stroke have been the most frequent cardiovascular events, while intravascular thrombosis has been one of the most prominent causes of morbidity and mortality (Chaireti et al. 2009). Platelets secrete and express a large number of substances that are crucial mediators of both coagulation and inflammation. Platelets respond at the site of vascular injury, where successive phases are involved including an adhesion of glycoprotein Ib (GPIb) and glycoprotein VI (GPVI) to von Willebrand factor and collagen, respectively. The response then triggers the release of granules and thromboxane A_2 (TXA_2) and

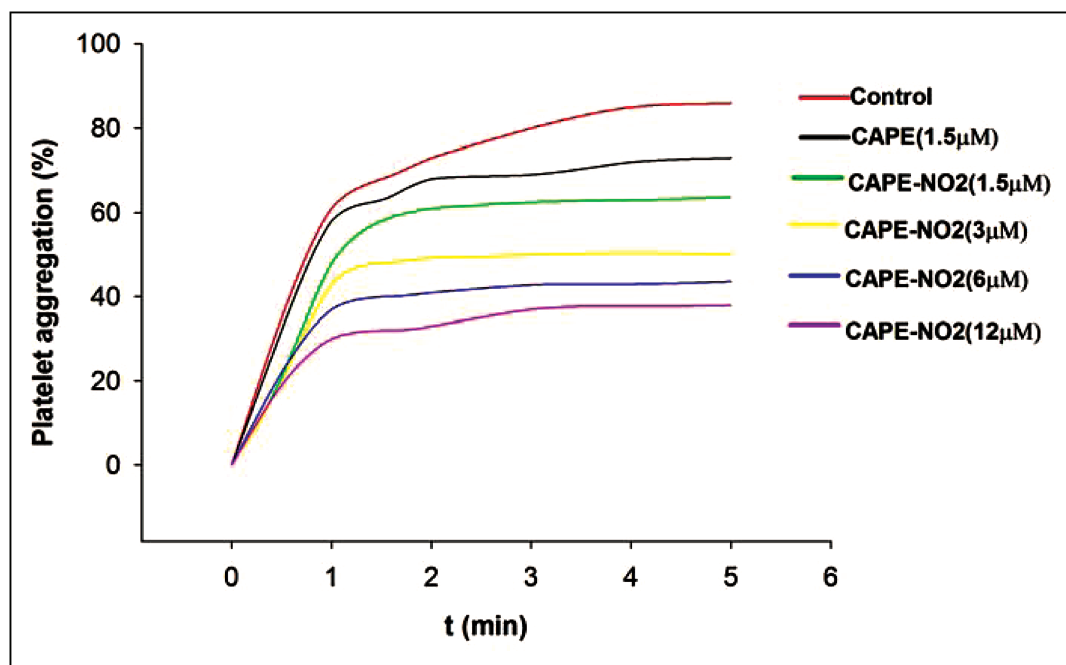

Fig. 2: Inhibitory effects of the CAPE-NO_2 and CAPE on *in vitro* platelet aggregation. The PRP (platelets400 × 10^9/L) was preincubated with isovolumetric solvent control, CAPE-NO_2 or CAPE for 3 min, collagen (4 μg/ml) was then added to trigger platelet aggregation. The antiplatelet aggregatory activity by CAPE-NO_2 was dose-dependent upon collagen-induced platelet aggregation.

the activation of integrin $\alpha_{IIb}\beta_3$, which binds to fibrinogen and allows platelet aggregation (Ruggeri 2002). So platelet aggregation may play a key role in the atherothrombotic process. But the antiplatelet effects of CAPE-NO_2 (Fig. 1B)have not been investigated and no data are available concerning the detailed effects of CAPE-NO_2 in platelet aggregation. In this study, we have investigated the possible inhibitory effects of CAPE-NO_2 on collagen-stimulated platelet aggregation.

2. Investigations and results

2.1. *Effects of CAPE-NO_2 against collagen-induced platelet aggregation*

The antiplatelet-aggregation activity of CAPE-NO_2 (1.5, 3, 6, and 12 μM) was evaluated upon collagen-induced aggregation. As shown in Fig. 2, CAPE-NO_2 suppressed the collagen-induced platelet aggregation at concentrations of 1.5–12 μM, in a concentration-dependent manner. For instance, 1.5, 3, 6 or 12 μM CAPE-NO_2 significantly reduced collagen-induced platelet aggregation to 62 ± 5%, 52 ± 5.1%, 46 ± 4% or 34 ± 4.7%, respectively. Compared with the control group, CAPE-NO_2 inhibited the collagen-induced platelet aggregation by 55% at the concentration of 12 μM (Fig. 2). CAPE inhibited the collagen-induced platelet aggregation by 14% at the concentration of 1.5 μM. These results indicate that CAPE-NO_2 exhibits a stronger antiplatelet-aggregation activity than CAPE.

2.2. *Effects of CAPE-NO_2 on NO production*

The platelet-derived NO-mediated process is known to exert an inhibitory effect on platelet aggregation (Chou et al. 1999). NO production was evaluated indirectly by measuring nitrite/nitrate (NO_x). In the present study, levels of NO were increased significantly in the CAPE-NO_2 and CAPE group compared to the control group. CAPE-NO_2 increased NO level in the PRP in a concentration dependent manner, 12 μM of it showed a significant increase (Fig. 3).

2.3. *Effects of CAPE-NO_2 on cGMP formation*

The platelet-derived cGMP-mediated process is known to exert an inhibitory effect on platelet aggregation. We investigated whether CAPE-NO_2 up-regulated the cellular level of cGMP. The level of cGMP in the control group was about 10.1 ± 0.5 nM. As shown in Fig. 4, CAPE-NO_2 itself dose-dependently increased the cGMP formation in the presence of collagen as compared to the control group. At the same time, CAPE (1.5 μM) increased the cGMP level in rat platelets. In collagen-activated platelets, CAPE-NO_2 (1.5 to 12 μM) progressively increased the cGMP level from 12.4 ± 0.5 to 16.0 ± 0.7 nmol/L in comparison with the control (10.1 ± 0.5 nmol/L).

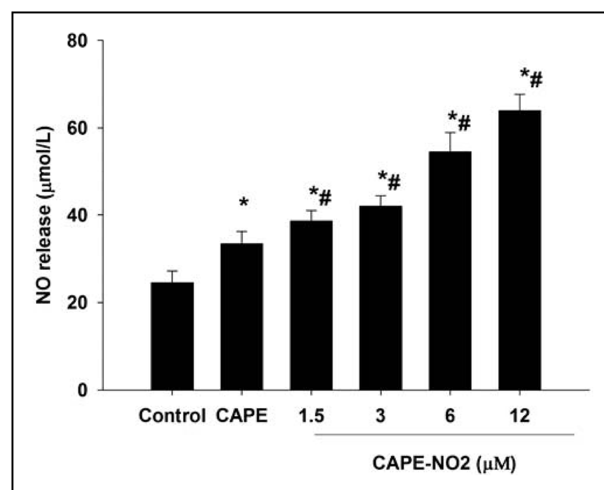

Fig. 3: The effects on NO level in rat platelets. The PRP was pretreated with isovolumetric solvent control, CAPE-NO_2 or CAPE, and then was treated with collagen. Contents of nitrite and nitrate were used to express the NO levels in the supernatant. CAPE (1.5 μM) was added into PRP. Data are expressed as mean ± SD (n = 6). $^*p < 0.05$ compared with control values. $^\#p < 0.05$ compared with the CAPE values.

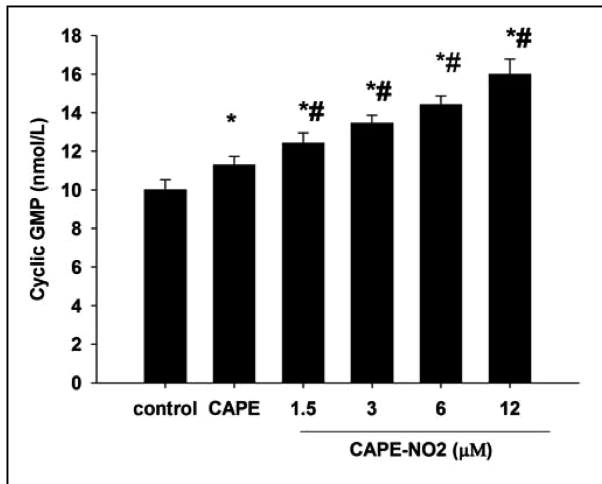

Fig. 4: Effect of $CAPE-NO_2$ on cGMP formation in rat platelets. The PRP was pretreated with isovolumetric solvent control, $CAPE-NO_2$ or CAPE, and then was treated with collagen. The reaction was stopped by the addition of EDTA (5 mM). CAPE (1.5 μM) was added into PRP. Data are expressed as mean ± SD (n = 6). $^{*}p < 0.05$ compared with control values. $^{\#}p < 0.05$ compared with the CAPE values.

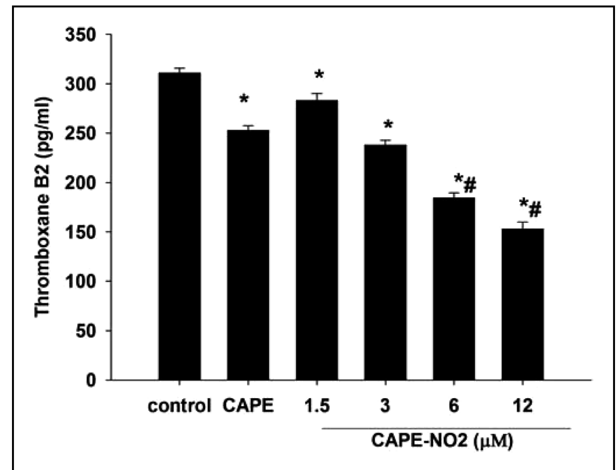

Fig. 5: Effect of $CAPE-NO_2$ on collagen-induced platelet TXB_2 production (n = 6). The PRP was pretreated with isovolumetric solvent control, $CAPE-NO_2$ or CAPE, and then was treated with collagen (4 μg/ml). CAPE (1.5 μM) was added into PRP. The level of platelet TXB_2 production was measured with ELISA. Data are expressed as mean ± SD (n = 6). $^{*}p < 0.05$ compared with control values. $^{\#}p < 0.05$ compared with the CAPE values.

2.4. *Effects of $CAPE-NO_2$ on collagen-induced platelet TXB_2 production*

TXA_2 is a potent stimulus of platelet aggregation. Therefore, we next examined whether $CAPE-NO_2$ blocked the production of TXB_2, the final and stable metabolite of TXA_2, under collagen exposure. Platelet aggregation by collagen is associated with the stimulation of COX-1 and generation of TXB_2 that mediates the activation of platelet thromboxane receptor and full platelet aggregation. $CAPE-NO_2$ markedly inhibited the platelet TXB_2 production. As shown in Fig. 5, $CAPE-NO_2$ suppressed the TXB_2 production by 9.5, 23, 40 and 51% respectively at concentrations of 1.5, 3, 6 and 12 μM. Collagen-induced platelet TXB_2 production was inhibited by 1.5 μM of CAPE with 18% of inhibition.

2.5. *Effects of $CAPE-NO_2$ on COX-1 enzyme activities*

Since the platelet TXB_2 production is regulated by COX-1, it is intriguing to know whether inhibition of platelet TXB_2 production and aggregation by $CAPE-NO_2$ is due to COX-1 inhibition. The AA-induced COX-1 activity was markedly reduced by $CAPE-NO_2$. As shown in Fig. 6, $CAPE-NO_2$ inhibited the COX-1 activities by 20, 36, 45 and 60% at concentrations of 1.5, 3, 6 and 12 μM, respectively. But CAPE inhibited the COX-1 activities by 41% at the concentration of 1.5 μM.

2.6. *Effects of $CAPE-NO_2$ on 5-HT release*

5-HT is released from dense granule of activated platelets. $CAPE-NO_2$ caused significant inhibition of platelet 5-HT release. The 5-HT was reduced to 376 ± 8.2, 408 ± 3.2, 435 ± 7.7, and 454 ± 7.64 μg/L in the presence of 12, 6, 3, and 1.5 μM $CAPE-NO_2$, respectively (Fig. 7). $CAPE-NO_2$ inhibited the 5-HT release by 87% at the concentration of 12 μM. The inhibition of the CAPE is 19% at the concentration of 1.5 μM.

3. Discussion

The principal objective of this study was to determine the mechanism involved in the effect of platelet aggregation by $CAPE-NO_2$, a nitro derivative of the CAPE. $CAPE-NO_2$ exerts an effect upon rat platelet aggregation stimulated by collagen, which has not been described previously.

At the concentration of 1.5 μM, CAPE inhibited the collagen-induced platelet aggregation by 14.5% (Fig. 2). CAPE increased NO and cGMP formation, as compared to the control (Fig. 3 and Fig. 4). It also inhibited collagen-induced TXB_2 formation. In addition, AA-induced COX-1 activity was reduced by CAPE. These effects of CAPE are consistent with previous research (Hsiao et al. 2007). Moreover, 5-HT was determined, a key substance released from dense granules when platelets are activated and participates in a positive feedback regulation of platelet activation. CAPE 1.5 μM decreased secretion of 5-HT in PRP induced by collagen at 4 μg/ml (Fig. 7).

$CAPE-NO_2$ inhibited platelet aggregation in a concentration-dependent manner (Fig. 2). At the same time, the levels of NO and cGMP were increased in the platelet by $CAPE-NO_2$ and CAPE in a same dose. In addition, $CAPE-NO_2$ reduced TXB_2 formation, COX-1 activity and 5-HT release. The effect of CAPE was similar with $CAPE-NO_2$ in the low dose of 1.5 μM. Nitric oxide is an important cellular messenger implicated in the regulation of various physiological functions in the cardiovascular, nervous and immune systems. Nitric oxide (NO) is a well-defined inhibitor of platelet activation (Li et al. 2006). NO can be generated from several cellular sources, via one or the other of three nitric-oxide synthases (NOS): neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) (Carrier et al. 2007). Platelets contain eNOS and use circulation L-arginine to generate NO. No is known to inhibit activation of the platelet integrin $\alpha_{IIb}\beta_3$ (Zhou et al. 1995). As shown in Fig. 2, $CAPE-NO_2$ significantly inhibited collagen-induced platelet aggregation. Platelet adhesion to the $\alpha_{IIb}\beta_3$ ligand fibrinogen was inhibited by NO. Therefore, the anti-platelet activity of $CAPE-NO_2$ is possibly due to that NO indirectly interferes with the binding of collagen to its specific receptors (i.e., $\alpha_{IIb}\beta_3$ integrin) (Ruggeri 2002) on the platelet membrane. Platelet cGMP is endogenous negative regulator of platelet aggregation (Park et al. 2004; Homer and Wanstall 2002). The increased level of cGMP participates in activating PKA or PKC and consequently this enzyme phosphorylates its substrate proteins, resulting in negative regulation of platelet aggregation (Cho et al. 2007). PKC is a key enzyme in signal transduction systems involving the breakdown of phospholipids (Sheu et al. 2002). In platelets, PKC plays an important role in the induction of the aggregatory and sceretory responses as well as in preventing a continuous activation of the platelet excitatory signal transducting system (Sheu et al. 2002). Some

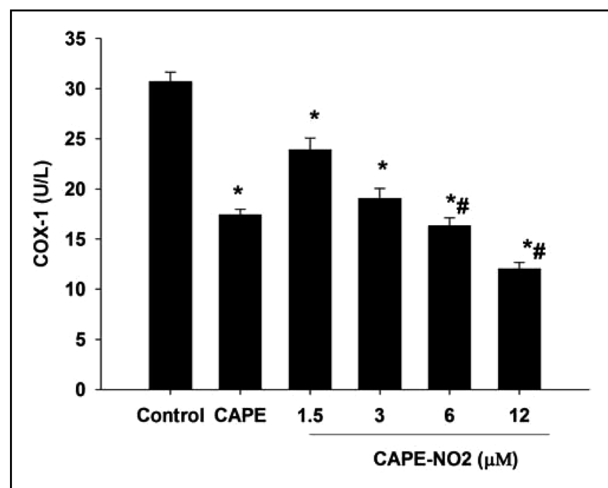

Fig. 6: Effect of different concentrations of $CAPE\text{-}NO_2$ on COX-1 enzyme activity (n = 6). The PRP was pretreated with isovolumetric solvent control, $CAPE\text{-}NO_2$ or CAPE, and then was treated with collagen (4 μg/ml). CAPE (1.5 μM) was added into PRP. Data are expressed as mean ± SD (n = 6). $^{*}p < 0.05$ compared with control values. $^{\#}p < 0.05$ compared with the CAPE values.

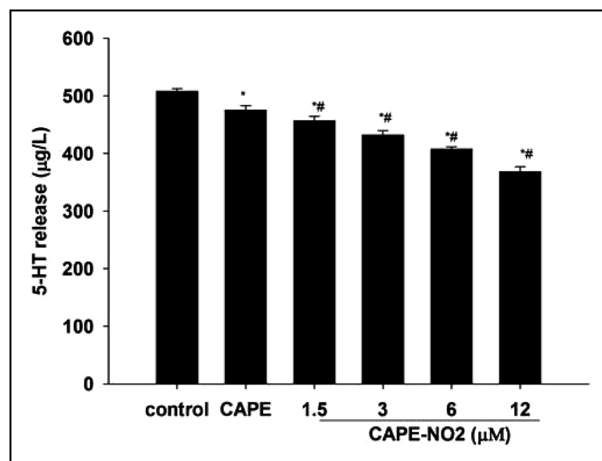

Fig. 7: Effect of different concentrations of $CAPE\text{-}NO_2$ on 5-HT release (n = 6). The PRP was pretreated with isovolumetric solvent control, $CAPE\text{-}NO_2$ or CAPE, and then was treated with collagen (4 μg/ml). CAPE (1.5 μM) was added into PRP. Data are expressed as mean ± SD (n = 6). $^{*}p < 0.05$ compared with control values. $^{\#}p < 0.05$ compared with the CAPE values.

stimulatory pathways in platelets may be blocked by the rise of cGMP. Increased cGMP can negatively affect agonist-induced phosphoinositide breakdown and $[Ca^{2+}]_i$ (McDonald and Murad 1996). An increased level of cGMP suppresses platelet shape change, aggregation, and Ca^{2+} mobilization induced by various agonists (Sage and Rink 1985). The inhibitory effect of $CAPE\text{-}NO_2$ against platelet aggregation seems to be due to the up-regulation level of cGMP and lowering of $[Ca^{2+}]_i$.

Platelets play a crucial role in hemostasis and in the initiation and propagation of thrombus formation. Platelet aggregation is mainly due to induction of TXA_2 production and subsequent activation of TXA_2 receptor on platelets (Chan et al. 2005). Thromboxane A_2 (TXA_2) and prostacyclin I2 (PGI_2) are hydrolysis of arachidonic acid by thromboxane synthetase and prostacyclin synthetase. An increase of TXA_2 will result in the adhension, aggregation and release of platelet. Platelet hyperfunction and abnormality in TXA_2 and PGI_2 equilibrium are closely related to thrombosis (Chen et al. 2012). TXB_2 is the stable product transformed from TXA_2 (Chen et al. 2012). In this study, $CAPE\text{-}NO_2$ diminished collagen-induced TXB_2 production in a dose-dependent manner, indicating that the inhibitory action of $CAPE\text{-}NO_2$ on TXA_2 generation contributes, at least in part, to the mechanism of inhibitory activity of $CAPE\text{-}NO_2$ against platelet aggregation (Seo et al. 2013). $CAPE\text{-}NO_2$ effectively inhibited TXA_2-mediated responses *in vitro* platelet aggregation and secretion. Others have found that certain flavonoids inhibited the generation of metabolites of arachidonic acid by cyclooxygenase and inhibited the Ca^{2+} dependent isoforms of protein kinase C (Liu and Liang 2000). So $CAPE\text{-}NO_2$ may involve these mechanisms on inhibiting platelet aggregation. During platelet activation, AA is released from membrane phospholipids, and is further converted to thromboxane A_2 (TXA_2), a potent inducer of platelet aggregation, through the actions of COX and thromboxane synthase (Jennings 2009). Activity of COX-1 is crucial in platelet activation and inflammatory processes (Jennings 2009; Lee et al. 2003). Platelet thromboxane production is tightly regulated by platelet COX-1 enzyme activities (Jennings 2009). The inhibitory action of $CAPE\text{-}NO_2$ on COX-1 activities contributes to the mechanism that $CAPE\text{-}NO_2$ inhibited collagen-induced platelet aggregation. As platelet aggregation is induced by various agonists and amplified by active substances secreted from granules, like ADP, serotonin and calcium (Rivera et al. 2009; Xiong et al. 2012). Serotonin (5-hydroxytryptamine, 5-HT) is released from at the site of vascular injury including thromboxane A_2. 5-HT activated and aggregated platelets are associated with coronary artery disease and with cardiac events (Vikenes et al. 1999; Golino et al. 1994). During thrombus formation, platelet-derived 5-HT plays an important role in creating a positive feedback cycle on further platelet aggregation followed by release of vasoconstrictor such as thromboxane A_2 (Kihara et al. 2001). Figure 2 showed that $CAPE\text{-}NO_2$ inhibited collagen-induced platelets aggregation in a concentration-dependent manner. $CAPE\text{-}NO_2$ significantly inhibited platelet 5-HT secretion induced by agonist would be expected to contribute further to its antiplatelet effect. The elevated cGMP may inhibit platelet phospholipase A2 and COX enzyme. Subsequently, metabolism of arachidonic acid would be interfered. Thus synthesis of peroxide and TXA_2 will decrease. TXA_2 can promote Ca^{2+} release. The releases of ADP, Ca^{2+} and 5-HT will further result in the platelet aggregation and release action.

In conclusion, $CAPE\text{-}NO_2$ exhibits a potent activity at inhibiting collagen-induced platelet aggregation. These findings suggested that the mechanism of $CAPE\text{-}NO_2$ on antiplatelet aggregation was different. This inhibitory effect of $CAPE\text{-}NO_2$ may involve the following mechanisms: $CAPE\text{-}NO_2$ activates the NO/cGMP pathway and increases NO release and cGMP formation. CAPE also inhibits COX-1enzyme activity resulting in the reduction of TXB_2. Finally, $CAPE\text{-}NO_2$ decreases the 5-HT secretion from the activated platelets. However this study did not rule out the possibility of other mechanisms involving in $CAPE\text{-}NO_2$-inhibited platelet aggregation. $CAPE\text{-}NO_2$ would be of potential therapeutic value for thrombotic disease.

4. Experimental

4.1. Reagents and materials

$CAPE\text{-}NO_2$ and CAPE were synthesized by a procedure already described (Liu et al. 2013). Thromboxane B_2 EIA kit, COX-1 EIA kit and cGMP EIA kit were purchased from Nanjing Jiancheng Bioengineering Institute (China). Dimethyl sulphoxide (DMSO), indomethacin, collagen (type I, bovine Achilles tendon), EDTA, sodium pentobarbital, tritonX-100, sodium citrate and cysteine were obtained from Sigma (Sigma Chemical Company, St. Louis, MO, USA).

4.2. Animals

Sixty male Sprague-Dawley rats (240 ± 20 g) were purchased from the Experimental Animal Center of the Chongqing Medical University,

Chongqing, China (SCXK(YU)2012-0001). The present study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). The animal protocol was approved by the Ethics Committee for Animal Experimentation of the Chongqing Medical University. All rats were maintained in similar polypropylene cages of standard dimensions at a temperature of $25 \pm 1^{o}C$ and had a standard 12 h day/night cycle. The rats were fed with standard diet and water.

4.3. Preparation of rat platelets

Fresh blood was drawn from the abdominal aorta of rats which were anesthetized with sodium pentobarbitone (30 mg/kg i.p.) and collected into vacutainer tubes containing sodium citrate (9:1, v/v). The initial 1–2 ml of blood was discarded to avoid spontaneous platelet activation. The blood was centrifuged for 15 min at 150 × g at room temperature to obtain platelet rich plasma (PRP) and the remaining blood centrifuged for 20 min at 800 × g to get platelet poor plasma (PPP) (Liu et al. 2013).

4.4. Platelet aggregation

The turbidimetric method was applied to measure platelet aggregation, using TYXN-96-multifunctional smart aggregometer (Shanghai General Machinery Research Institute). Platelets (PRP) (400×10^9 platelets/L, 200 μl) were prewarmed to 37 °C for 5 min, and then CAPE and CAPE-NO_2 were added 3 min before the addition of the collagen (4 μg/ml). The time of reaction was not less than 6 min.

4.5. NO determination

NO is unstable. As it is difficult to measure NO in biological specimens, tissue nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as an index of NO production. The method for plasma nitrite and nitrate levels was based on the Griess reaction (Cortas and Wakid 1990). Platelets (PRP) were plated in 96-well plates at a density of 400×10^9 platelets/L, 200 μl and preincubated with CAPE and its analogue (CAPE-NO_2) for 3 min, followed by addition of collagen (4 μg/ml) for 6 min. The supernatants were reacted with the Griess regent at the room temperature for 10 min. The NO_2^- concentration was determined by measuring the absorbance at 550 nm.

4.6. Measurement of cGMP formation

Platelet rich plasma (400×10^9 platelets/L, 200 μl) was preincubated with CAPE and CAPE-NO_2 3 min before the addition of the collagen (4 μg/ml) for 6 min. The reaction was interrupted by the addition of EDTA (5 mM). Cell samples were centrifuged (4000 × g 30 min at 4 °C). Fifty microliters of the supernatant was used to determine the cGMP contents with EIA kits following acetylation of the samples as described by the manufacturer (Mendes-Silverio et al. 2012).

4.7. Thromboxane B_2 (TXB_2) assay

PRP were preincubated with CAPE and its analogue (CAPE-NO_2) for 3 min before the addition of collagen (4 μg/ml). Six minutes after the addition of collagen for 6 min, EDTA (5 mM) and indomethacin (50 μM) were added to the reaction suspensions. The vials were then centrifuged for 5 min. The TXB_2 levels of the supernatants were measured using an ELISA kit according to the manufacturer's protocol.

4.8. Determination of COX-1 activity

The COX-1 activity was measured according to instruction of COX-1 ELISA kit. Briefly, after platelets treated with drugs for 3 min, AA (100 μM) was added, followed by addition of 0.1 N HCl and saturated stannous fluoride solution to stop the reaction (Shih and Chou 2012). The absorbance of samples were measured at 450 nm.

4.9. Platelet release reaction of 5-HT

Platelet (PRP) were incubated at 37 °C for 5 min, and resuspended in Tyrode's buffer containing Ca^{2+} 1mmol/L. CAPE and its analogue (CAPE-NO_2) were added to platelets for 3 min prior to collagen stimulation for 6 min. The reaction was terminated by 100 μl EDTA in ice. Then the platelets were centrifuged 1200 rpm for 5 min. 1.5 ml 0.004% phthalaldehyde solution was added to the supernatant. The samples were on a boiling water bath for 15 min. The content of 5-HT was determined by fluorescence spectrophotometer (excitation light 365 nm, emitted light 480 nm).

4.10. Statistical analysis

All data are presented as mean ± S.D. and are accompanied by the number of observations. Statistical differences between groups were assessed using the SPSS 18.0 software by one-way ANOVA tests. A P value of less than 0.05 was considered to denote statistical significance.

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