ROLE OF NITRIC OXIDE (NO) IN CAPSAICIN MEDIATED ANTI-PLATELET ACTIVITY IN IN VITRO, IN VIVO, EX-VIVO MODEL OF PLATELET AGGREGATION ASSAY AND ARTERIAL THROMBOSIS IN RAT: POTENTIAL THERAPEUTIC TARGET?

MIHIR K. PATEL1, KIRANJ K. CHAUDAGAR1, ANITA A. MEHTA1

Department of Pharmacology, L. M. College of Pharmacy, Navarangpura, Ahmedabad

Received: 06 Feb 2017 Revised and Accepted: 24 Feb 2018

ABSTRACT

Objective: Although recent advances in the treatment of congestive heart disease, mortality among patients’ remains a questionable remark. Therefore, we evaluated the role of capsaicin on in vitro and ex vivo platelet aggregation induced by Adenosine Di-Phosphate (ADP) as well as in in vivo thrombosis models and role of NO, K+

Methods: According to body weight wistar rats were divided into five groups. Group I and Group II was treated with saline and capsaicin (3 mg/kg, i. v), while animals from Group III was treated with N(ω)-nitro-L-arginine methyl ester (L-NAME) (30 mg/kg, i. v) 30 min before administration of capsaicin (3 mg/kg, i. v). Group IV animals were treated with glibenclamide (10 mg/kg, i. v) 30 min before administration of capsaicin (3 mg/kg, i. v). Group V was considered as a positive control and administered clopidogrel (30 mg/kg, p. o). Animals were subjected for in vitro, ex-vivo platelet aggregation assay. ADP (3µM) was utilized as an aggregating agent in these experiments. After these assays; animals of each group were subjected for subaqueous tail bleeding time in a rat model and FeCl3-induced arterial thrombosis model in rats.

Results: In ADP-induced in vitro platelet aggregation, a significant reduction in % platelet aggregation was observed at 50µM (64.35±4.641) and 100µM (52.72±4.192) concentration of capsaicin as compared to vehicle control (85.82±3.716). Capsaicin (3 mg/kg, i. v) also showed a significant reduction (49.53±4.075) in ex-vivo ADP-induced platelet aggregation as compared to vehicle control (89.58±2.057). In FeCl3-induced arterial thrombosis model, Capsaicin (3 mg/kg, i. v) exhibited an increase in time to occlusion in this rodent model and presence of the L-NAME and glibenclamide had inhibited the activity of capsaicin.

Conclusion: In our study, capsaicin (50 µM, 100µM) exhibited potent anti-platelet activity in ADP-induced platelet aggregation, similarly capsaicin exhibited significant anti-platelet action in the ex-vivo study. Moreover, the presence of L-NAME and glibenclamide inhibited the anti-thrombotic and anti-platelet action of capsaicin. Therefore, it was concluded that NO and K+ may be involved in the anti-thrombotic action of capsaicin.

Keywords: Capsaicin, Anti-platelet activity, Glibenclamide, Nitric Oxide, TRPV; channel

INTRODUCTION

Heart attacks and strokes are the most common causes of mortality and morbidity across the world and both represent clinical manifestations of acute arterial thrombosis [1]. Among them, atherosclerosis presents a greater risk to cardiovascular and peripheral vascular system causing mortality in cardiovascular disease patients [2]. Thrombus formation is a key mediator in the development of atherosclerosis [3]. The platelet is believed to play a pivotal role in pathogenesis and progression of atherosclerosis [4]. Previously it was believed that the platelet is having a minor role in this process; however, it is recognized that the platelet places itself as a critical link between thrombus formation, inflammation, and atherosclerosis [5]. At the site of vascular injury, platelets come into contact with subendothelial components and form a plug-like structure to avoid future damage to the endothelium. However, if the injury continues to happen, it will activate the cascade of signaling molecules which will form thrombus at the site of injury. This will lead to life-threatening disease states such as myocardial infarction, atherosclerosis or ischemic stroke [6].

Upon platelet formation at the site of vascular injury a series of cascade initates which mainly involves three phases. Phase I, Phase II and Phase III name as the initial phase, extension phase and stabilization phase respectively. The initial phase involves attachment of platelets to the exposed sub-endothelial layer following vascular injury and a monolayer of activated cells is formed. This activated monolayer further recruits more additional platelets to construct another strong layer during extension phase [7]. In order to restrict the recently formed thrombus to the initial injury site, regulation of platelet aggregation is the process to modulate a balance between activation and inhibition of signalling pathway of platelet [8]. Any defect in regulation of platelet activation or aggregation can cause arterial thrombosis, the major manifestation of atherosclerosis which triggers myocardial infarction and stroke. Nitric oxide (NO) is an endogenous gas present in endothelial cell of vascular endothelium, which is believed to play a predominant role in the regulation of platelet aggregation [9]. Endothelial NO responsible for maintenance of basal vascular tone and blood flow, and thereby regulation of blood pressure due to vasodilatory action [10].

A substantial amount of research has been carried out to elucidate the role of NO in platelet aggregation and found that NO can inhibit platelet activation or aggregation in vitro and in vivo [11]. The mechanism by which inhibition of platelet aggregation turns out by NO is caused by the cGMP-dependent pathway. Soluble guanylyl-cyclase (sGC) is an enzyme responsible for the production of cyclic guanosine monophosphate (cGMP), and NO is involved in activation of sGC which further lead to activation of cGMP-dependent protein kinase (PKG) via a cGMP-dependent pathway. This activated PKG caused inhibition of platelet activation via phosphorylating TxA2 receptors and thus inhibits its aggregating action on platelet. Further details include inhibition of influx of Ca2+ and other cations via activating sarcoplasmic reticulum ATPase (SERCA) [12]. PKG is also involved in blockage of the release of Ca2+ cation from the sarcoplasmic reticulum via inhibition of
Animals aggrading effects of collagen in mouse platelet [10]. However, a level of intracellular cytosolic Ca\textsuperscript{2+} during the entire study period, the animals were monitored for growth and health status to be certain that they were healthy.

Various cascades of pathways are being targeted in medical therapies to develop anti-platelet agents. These include therapies aimed at inhibiting T\textsubscript{xA}, ADP, GPIIb/IIIa, thrombin, collagen. Although major therapeutics is available in the area of thrombosis disease prevalence is a cause of concern for healthcare industries in the world. Hence, we aimed to identify the role of capsaicin in platelet aggregation and mechanism of it. Capsaicin is a pungent photochemical present in peppers responsible for spiciness [17]. Capsaicin is a potent agonist of TRPV\textsubscript{1} channel distributed throughout the body. TRPV\textsubscript{1} channel plays a very important role in heat sensation [18]. Nitric oxide (NO) plays a pivotal role in pain sensation via activation of TRPV\textsubscript{1} channel [19].

A number of few reports suggest that capsaicin has potential to inhibit platelet activation. Capsaicin was found to inhibit the aggregating effects of collagen in mouse platelet [20]. However, a significant amount of data spars regarding the detailed mechanism of action of capsaicin in platelet inhibitory action. To our best of knowledge, it has not been studied the role of capsaicin with respect to NO in platelet aggregation. Therefore, current investigation aimed to identify the role of capsaicin in platelet aggregation and elucidate the role of NO, K\textsubscript{ATP} in capsaicin mediated anti-platelet activity.

MATERIALS AND METHODS

Animals

Albino Wistar rats (150–200 g) were housed in a climate-controlled room (temperature 22±1 °C; relative humidity 55±5%) on a 12 h (light–dark) cycle. Animals had access to a standard pellet diet (Certified Amrut brand rodent feed; Prahair Agro Industries, Pune, India) and filtered tap water ad libitum. All experiments were carried out with strict adherence to ethical guidelines and were conducted according to the protocol (LCP/M/Gology/14/10) approved by the Institutional Animal Ethics Committee (IAEC), and according to Indian norms set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, New Delhi, India). In addition, the animals were cared for in accordance with the Guide to the Care and Use of Experimental Animals (Vol. 1, 2\textsuperscript{nd} ed., 1993, and Vol. 2, 1984) throughout the study. Throughout the entire study period, the animals were monitored for growth and health status to be certain that they were healthy.

Chemicals

Capsaicin, ADP (Adenosine Diphosphate) and Urethane were also purchased from Sigma Chemical, USA and ADP were prepared in phosphate buffer (pH 7.4). FeCl\textsubscript{3} was purchased from Hi-Media, India. Clopidogrel bisulfate was the generous gift from Zyudus Cadila, India. In all experiments, water for injection was utilized for preparation of L-NAMe and clopidogrel whereas capsaicin, glibenclamide was prepared in DMSO (0.5%)+saline.

Preparation of platelet-rich plasma (PRP) and platelet poor plasma (PPP)

Blood was collected into the tubes containing 3.8% trisodium citrate via retro-orbital route under light ether anesthesia from rats. All blood samples were centrifuged at 2000g for 20 min and supernatant platelet rich plasma (PRP) was collected carefully. The remaining amount of blood was centrifuged at 8000g for 20 min and supernatant platelet poor plasma (PPP) was collected with utmost care.

Measurement of platelet aggregation using ADP as an aggregating agent

Platelet aggregation studies were performed on microplate reader in 96-well, flat-bottomed, microtiter plates. A 180-μl volume of PRP was placed in each well, followed by addition of 20 μl of ADP (30μM). For in vitro studies, PRP/PPP was incubated with various concentrations of capsaicin (25, 50 and 100 μM) for 2 min at 37 °C before addition of ADP. Readings were taken every 1- min over a 5- min period at 405-nm wavelength. During the runtime, the plate was incubated at 37 °C and was shaken vigorously in a shaking mode at the maximal speed available. All platelet aggregation studies were performed in triplicate. Change in optical density (OD) was measured by taking OD of buffer as blank.

% Aggregation was calculated using formula:

\[
\% \text{ Aggregation} = \frac{\text{[Initial OD-Final OD]}}{\text{Initial OD}} \times 100
\]

Experimental design for ex vivo platelet aggregation study in rats

On the basis of body weight wistar rats were randomly divided into following groups. Experimental design was given in fig. 1.

Via the retro-orbital route blood samples were collected in tubes containing 3.8% trisodium citrate under anesthesia. PRP/PPP was prepared as described above and samples were subjected to ADP-induced platelet aggregation assay. % Aggregation was calculated in order to estimate the influence of capsaicin in platelet aggregation.

Subaqueous tail bleeding time in rat

Anaesthetized rats were fixed in supine position on a temperature-controlled (37 °C) heating table. After a defined latency period, the tail of the rat was transected with a razor-blade mounted on a self-constructed device at a distance of 4 mm from the tip of the tail. Immediately after transection, the tail was immersed into a bath filled with isotonic saline solution (37 °C). The time until continuous blood flow ceased (s) was measured, with a maximum observation time of 30 min (longer bleeding times were assigned a value of 30 min). Clopidogrel (30 mg/kg, p. o., 120 min pretreatment time) was used as positive control.

FeCl\textsubscript{3}-induced arterial thrombosis model in rats

Rats (n=6) were treated as per given protocol and then subjected to FeCl\textsubscript{3}-induced arterial thrombosis. The fecl\textsubscript{3}-induced chemical injury was used as a model of arterial thrombosis. A midline cervical incision was made on the ventral side of the neck and left carotid artery was isolated. Cannulation of the carotid artery was performed and connected to blood pressure measurement instrument (Biopac Systems, Inc, California, USA for B. P. measurement). A2×3 mm strip of Whatman filter paper No. #1 saturated with 35% (w/v) FeCl\textsubscript{3} was kept on the carotid artery for 5 min. A sudden decrease in B. P. signaling was taken as an indicator of cessation of blood flow as a consequence to thrombus formation. Time to occlusion (TTO) was defined as the time from FeCl\textsubscript{3} application to time of thrombus formation. A cut-off time was fixed at 60 min in case no thrombus formation was observed in drug-treated animals. Assessments of wet thrombus weight were also performed.

Statistical analysis

Results were expressed as means±SEM. Data were analyzed by One-way ANOVA followed by Dunnet's multiple comparison tests. All analysis was performed using graphpad prism software\textsuperscript{\textregistered} version 6.0. P<0.05 was considered to be statistically significant.
RESULTS

Effects of capsaicin on ADP-induced in vitro and ex vivo platelet aggregation and mechanisms

In ADP-induced in vitro platelet aggregation, capsaicin exhibited a significant reduction in % platelet aggregation at 50µM (64.35±6.41%) and 100µM (52.72±4.19%) concentration as compared to vehicle control (85.82±7.16%) (fig. 2). On the other hand, capsaicin (3 mg/kg, i. v) also showed a significant reduction (49.53±4.075) in ex-vivo ADP-induced platelet aggregation as compared to vehicle control (89.38±2.057) (fig. 3). We also explored the mechanism of antiplatelet action of capsaicin by using L-NAME and glibenclamide to identify the role of NO and K+ in capsaicin mediated antiplatelet action. Capsaicin at 3 mg/kg, i. v exhibited a significant reduction in % platelet aggregation (49.53±4.075) as compared to vehicle control (89.38±2.057) (fig. 3). Pretreatment of L-NAME somehow inhibited platelet inhibitory action of capsaicin, while on other hand addition of glibenclamide exhibited inhibition of anti-platelet actions of capsaicin shown in fig. 3.

Moreover, FeCl3-induced arterial thrombosis model was also performed to elucidate the role and mechanism of capsaicin in platelet aggregation. In that, time to occlusion (TTO) was measured as endpoint parameter. Capsaicin (3 mg/kg, i. v.) exhibited an increase in time to occlusion in this rodent model as shown in fig. 5. This action was completely reversed in presence of L-NAME, while the presence of glibenclamide partially inhibited the activity of capsaicin as compared to vehicle control. Reduction in thrombus weight was observed in capsaicin treated group whereas L-NAME and glibenclamide exhibited inhibition of capsaicin activity partially depicted in the graph (fig. 6).

Effects of capsaicin in vivo models of subaqueous tail bleeding time and FeCl3-induced thrombosis in rats and mechanisms involved

Following ex-vivo and in vitro platelet aggregation assay we carried out in vivo model of thrombosis to explore the role of capsaicin in thrombosis and its underlying mechanism. Capsaicin (3 mg/kg, i. v.) (446.3±38.75) showed a significant rise in bleeding time as compared to vehicle control (241.8±17.94). Further, bleeding time for capsaicin (3 mg/kg, i. v.) is significantly lower than positive control clopidogrel (30 mg/kg, p. o.) (fig. 4). Bleeding time is a significant parameter of antiplatelet or anticoagulant activity, therefore it has a major impact on the identification of antiplatelet or anticoagulant effect of the substance. In our study, pretreatment of L-NAME significantly blocked the antiplatelet effect of capsaicin, while on another hand a significant rise in bleeding time was observed with the addition of glibenclamide to capsaicin as compared to vehicle control (fig. 4). However, bleeding time for glibenclamide pretreated group was significantly lower than positive control. Hence, it can be concluded that NO may be involved in the antiplatelet activity of capsaicin.
DISCUSSION

Antiplatelet activity of capsaicin has been studied in many literatures; however, mechanism of its action remains a controversial. Here, we tried to identify the role of various signaling molecules in antiplatelet activity of capsaicin. It is well-known fact that NO plays an important role in modulation of platelet activity. Now days, platelet aggregation is a prime topic of discussion because of dependency of leading cardiovascular diseases such as myocardial infarction and atherosclerosis on platelet modulation. Transient receptor potential vanilloid receptor 1 (TRPV1), has established its pivotal role in the modulation of pain and inflammatory responses in neurons via multiple chemical and physical stimuli [21]. It is well known fact that capsaicin serves as a potent agonist of TRPV1 and activates these channels [22]. The activation of TRPV1, channels results in increased [Ca2+] level and thereby downstream of signaling cascades activation takes place. Besides its pivotal role in pain modulation, the TRPV1 channels are also found to be playing a major role in regulating the physiological functions of cardiovascular cells and the pathogenesis of cardiovascular diseases [23]. In spite of progress in research of capsaicin and cardiovascular research in last decade, the role and effects of capsaicin on platelet aggregation are not studied well. Several reports states that activation of TRPV1 channels in endothelial cells protects against atherosclerosis, hypertension and stroke via activation of NO [24]. However, role of NO is not elucidated well in platelet function. Hence, we correlated capsaicin with TRPV1 to determine the role of NO using platelet aggregation assay and arterial model of thrombosis.

There are limited reports available suggesting antithrombotic effects of capsaicin and direct role of capsaicin in in vitro thrombosis model has not been studied well. Therefore, we investigated the role of capsaicin in antiplatelet activity and evaluated its mechanism(s). We identified that capsaicin exhibited significant anti-platelet effect at 100μM concentration as compared to vehicle control in in vitro platelet aggregation study. Moreover, we investigated the role of NO and KATP by using L-NAME and glibenclamide respectively in ex vivo and in vivo model of thrombosis. It was observed that capsaicin (3 mg/kg, i. v.) significantly reduced platelet aggregation in in vitro and ex-vivo study and pretreatment of L-NAME and glibenclamide partially blocked anti-platelet effect of capsaicin in similar models. Suggests NO and KATP may have a role in the anti-platelet effect of capsaicin. Going further, we investigated the role of capsaicin in an arterial model of thrombosis and found that capsaicin significantly increased the time to occlude in FeCl3 induced thrombus formation model as compared to vehicle control and interestingly, pretreatment of L-NAME and KATP blocked the antithrombotic action of capsaicin in similar model. Above observations suggest that capsaicin may have antithrombotic activity via NO-cGMP pathway.

Fig. 6: Effects of capsaicin on thrombus wet weight FeCl3-induced thrombosis model in rats. NRM-normal control group, CPS= capsaicin-treated group (3 mg/kg), L-N+CAPS=30 mg/kg L-NAME with 3 mg/kg capsaicin, G+CPS=Glibenclamide 10 mg/kg with 30 mg/kg Capsaicin treated group, CLP=Clopidogrel 30 mg/kg treated group served as positive control; #=p<0.01 vs NRM, @=p<0.05 vs NRM

CONCLUSION

In conclusion, our current investigation also supports previous finding showing capsaicin’s role in platelet aggregation, moreover, we have also investigated the mechanism of antiplatelet activity of capsaicin. We have found that NO plays a predominant role in the anti-platelet action of capsaicin via activation of sGC-cGMP modulatory signaling pathway and shift of anti-thrombotic activity towards KATP channel was also evident from our current investigation result. This may be due to activation of TRPV1 by capsaicin. One previous report stated anti-platelet activity of capsaicin is unrelated to TRPV1, while in our investigation it has been showed that role of TRPV1 in thrombosis of artery via KATP cannot be neglected.

REFERENCES

Mishra Patel carried out the experimental part of the work, performed data analysis and drafted and revised the manuscript. The design of the work and correction of the manuscript was done by the corresponding author Dr. Anita Mehta and Kiranj Chaudagar. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest associated with this study.

Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Baha MJ, Cushman M, et al. American heart association statistics...
Our study was the first to demonstrate that morin hydrate shows in vitro antiplatelet effect and effectively inhibit arterial thrombosis in vivo. In general, dietary intake from the natural sources is likely insufficient for attaining plasma concentrations that can inhibit platelet activation in vivo, however, several nontoxic prophylactic agents such as food products and nutritional supplements is ideal for stopping atherothrombotic events.