Review

Effects of garlic on platelet biochemistry and physiology

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Increased platelet aggregation plays a significant role in the aetiology of cardiovascular disease, and is complex involving multiple mechanisms. On platelet activation, there is a transient increase in free cytoplasmic calcium (Ca^{2+}), thromboxane A_2 generation, and the activation of the fibrinogen receptor GPIIb/IIIa. Other modulators are also involved in platelet aggregation and include lipoxygenase metabolites, protein kinase C, cyclic adenosine monophosphate (cAMP), cyclic guanine monophosphate (cGMP) and nitric oxide (NO). Garlic is reported to prevent cardiovascular disease by multiple effects, one of which is the inhibition of platelet aggregation and its ability to do this has been extensively investigated *in vitro*, however, *in vivo* studies are limited. *In vitro* studies indicate that garlic prevents inhibition of platelet aggregation by inhibiting cyclooxygenase activity and thus thromboxane A2 formation, by suppressing mobilization of intraplatelet Ca^{2+}, and by increasing levels of cAMP and cGMP. Garlic also displays strong antioxidant properties and activates nitric oxide synthase (NOS), leading to an increase in platelet-derived NO. It can also interact directly with the GPIIb/IIIa receptors, thus reducing the ability of platelets to bind to fibrinogen. It is concluded that garlic inhibits platelet aggregation by multiple mechanisms and may have a role in preventing cardiovascular disease.

Keywords: Aggregation / Cardiovascular disease / Garlic / Platelets / Thrombosis

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1 Introduction

There has been an increase in awareness and usage of all forms of alternative medical therapies often referred to as complementary medicine (CAM). These include herbs, spices and nutraceuticals, and the last two decades have seen an international resurgence of these medicinal herbs around the world due to an increase interest in "greening and environmental" issues, enhanced interest in diets and natural products to maintain general health [1]. Many bioactive compounds in food have been demonstrated to play a role either in delaying or in preventing chronic diseases such as cardiovascular disease and cancer [2]. Herb and natural supplement use is widespread, for example, in the USA adult population, more than half of all users have stated that herbs and natural products were important to their health and well-being [3] and approximately 68% of adolescents have used one or more of the complementary therapies [4]. In the UK, it has been reported that National Health Service patients are increasingly seeking CAM and have indicated that their health problems have improved on its usage [5] and a recent survey has confirmed the high prevalence of CAM usage both in children and adolescents in a UK-based population [6]. This study reported that the most common medicinal types of CAM were non-prescribed vitamins, minerals and herbal therapies. The major increase in the usage of CAM is largely in patients affected by cardiovascular disease [7, 8], especially the usage of herbs, spices and nutraceuticals such as garlic [9–12]. However, there is a lack of herbal supplement characterization as reported in many published randomized controlled trials [13]. Due to the potential for adverse reactions or drug interactions, and

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Abbreviations: ADP, adenosine diphosphate; **CAM,** complementary medicine; **COX**, cyclooxygenase; **cAMP**, cyclic adenosine monophosphate; **cGMP**, cyclic guanosine monophosphate; **Ca^{2+}**, ionized calcium; **DAG**, diacylglycerol; **ecNOS**, endothelial constitutive nitric oxide synthase; **iNOS**, inducible nitric oxide synthase; **IP_3**, inositol 1,4,5-triphosphate; **ncNOS**, neuronal constitutive nitric oxide synthase; **NO**, nitric oxide; **NOS**, nitric oxide synthase; **PGD_2**, prostaglandin D_2; **PGE_1**, prostaglandin E_1; **PGE_2**, prostaglandin E_2; **PGH_2**, prostaglandin H_2; **PGI_2**, prostacyclin; **PI3-kinase**, phosphoinositide 3-kinase; **PLA_2**, phospholipase A_2; **PLC**, phospholipase C; **TGF-β**, transforming growth factor beta; **TXA_2**, thromboxane A_2; **TXB_2**, thromboxane B_2; **T2DM**, type II diabetes mellitus

increasing regulatory control, studies involving the mechanisms of action of herbal products including garlic are been increasingly undertaken.

2 Garlic

Garlic (*Allium sativum)* has been used for centuries both as a flavouring agent, traditional medicine, and a functional food to enhance physical and mental health. It appears in the Egyptian *Codex Ebers*, a 3500-year-old document as a treatment for heart disorders, tumours, worms, bites, and other ailments and thus has acquired a prominent position in the folklore of many cultures as a formidable prophylactic and therapeutic medicinal agent [14]. The composition of garlic is complex; majority is composed of water whilst the bulk dry weight consists of fructose-containing carbohydrates, followed by sulphur compounds, protein, fibre and free amino acids [1]. Some of the other compounds present in garlic in significant amounts are saponins, phosphorous, potassium, sulphur, zinc, moderate levels of selenium and Vitamins A and C, and low levels of calcium, magnesium, sodium, iron, manganese and B-complex vitamins. It also has a high phenolic content [15] and nearly all the compounds present in it are water-soluble (97%) with a small amount of oil-soluble compounds (0.15–0.7%). The characteristic flavour and odour of garlic is due to its organosulphur compounds, which are also responsible for most of its potent pharmacological activity [16], and the majority of investigations on garlic's active principles have focussed on these compounds.

Due to its increasing popularity, a wide variety of garlic supplements have become commercially available and some of the common preparations which have been investigated for their biochemical properties are: raw garlic, garlic powder tablets, oil of steam distilled garlic, oil of oil-macerated garlic, ether-extracted oil of garlic and aged garlic extract; all these preparations differ in their constituents [17].

Garlic has strong antioxidant properties [10, 18] and is reported to prevent cancer [19, 20] and age-related diseases [1]. However, the most widely reported effect of garlic is its ability to either prevent or reduce cardiovascular disease [1, 21–23]. This disease is complex and multifactorial, and is characterised by elevated serum lipids, increased plasma fibrinogen and coagulation factors, oxidative modification of LDL, hypertension, alterations in glucose metabolism and smoking [24]. In addition, increased platelet activation/aggregation and thrombus formation also play a significant role in the aetiology of cardiovascular disease [25, 26].

3 Biochemistry and physiology of platelets

Platelets are anuclear cells derived from bone marrow megakaryocytes and have a life span of approximately 8–10 days [27]. They contain a plasma membrane and many organelles such as mitochondria, lysosomes, peroxisomes and glycogen and storage granules; their cytoskeleton is complex and consists of microtubules and a dense tubular system [25]. The cytoplasmic side of the plasma membrane contain phospholipids, which can serve as substrates for phospholipases, and the intrinsic membrane glycoproteins (GPIIb/IIIa) extrude through the plasma membrane and act as platelet receptors responding to activating and inhibiting agents (Fig. 1). The enzymes involved in prostaglandin synthesis are present within the dense tubular system where majority of the calcium is also sequestered. The storage granules contain proteins such as platelet factor 4, β-thromboglobulin, platelet-derived growth factor, fibrinogen, fibronectin and von Willebrand factor, serotonin, ADP and calcium are stored within these dense granules [25].

The resting platelets are of discoid shape, and once activated become spherical and extend long spiky pseudopods which adhere to each other leading to the formation of the so-called haemostatic plug. Platelets can be activated by physiological and non-physiological molecules, and once activated lead to shape change, aggregation, stimulation of secretory processes and finally the liberation of arachidonic acid, which is rapidly converted to prostaglandin and lipoxygenase products. The activated platelets release mediators such as transforming growth factor-beta (TGF-

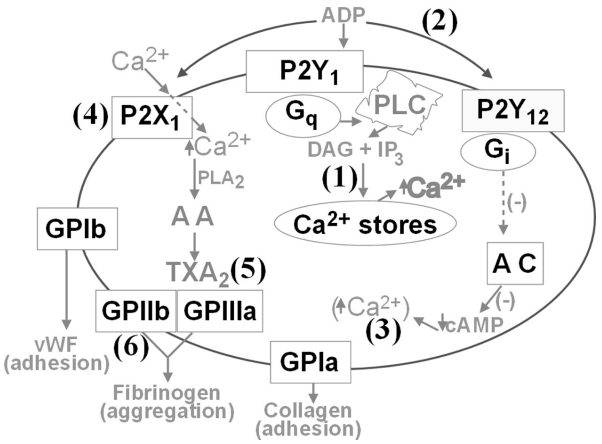

Figure 1. Major pathways of platelet activation/aggregation. Activation of the platelets takes place by the binding of ADP to its receptors, $P2Y_1$, $P2Y_{12}$, and $P2X_1$. Binding to the $P2Y_1$ receptor stimulates PLC, which in turn leads to the formation of DAG and IP_3 and results in the release of Ca^{2+} from intracellular stores (1). Binding to the $P2Y_{12}$ receptor (2) leads to the inhibition (–) of the adenylyl cyclise (AC) which decreases (↓) cAMP levels and leads to a further increase (↑) in Ca^{2+} (3). Finally, the binding of ADP to the $P2X_1$ receptor results in an influx of Ca^{2+} into the platelets (4) leading to shape change and aggregation. The sustained level of Ca^{2+} activates PLA_2, which leads to the generation of TXA_2 via arachidonic acid (AA) (5). On platelet activation, the intrinsic glycoprotein fibrinogen receptor GPIIb/IIIa also becomes exposed on the plasma membrane further assisting in the aggregation process (6).

β), interleukin 1 beta (IL-1β), prostaglandin E_2 (PGE_2), thromboxane A_2 (TXA_2), and CD40 ligand (CD40L) into the circulation [28]. The plasma membrane of the platelets contains receptors to which agonists such as adenosine diphosphate (ADP), collagen, and thrombin can bind and induce platelet aggregation. For example, ADP activates platelets through three purinergic receptors, namely $P2Y_1$, $P2Y_{12}$ and $P2X_1$ (Fig. 1). Transduction of the ADP signal involves a transient rise in free cytoplasmic calcium due to the mobilization of internal stores, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) and a concomitant inhibition of adenylyl cyclase activity [29], all these lead to a change in shape of the platelets and finally aggregation (Fig. 1. steps 1, 2, 3). The $P2Y_1$ receptor is a Galpha q-coupled G-protein receptor and is important for platelet shape change, aggregation, TXA_2 generation, procoagulant activity, adhesion to immobilised fibrinogen and thrombus formation (Fig. 1, step 1). The $P2Y_{12}$ is very similar to the $P2Y_1$ receptor and signals through a Galpha i-coupled G-protein receptor and is important for potentiation of platelet activation mediated by other physiological agonists such as collagen, von Willebrand factor and thromboxane A_2 (Fig. 1, step 2). The $P2X_1$ receptor is an ion channel, and once activated causes an influx of calcium (Ca^{2+}). However, this does not lead to platelet aggregation, but it does cause a shape change and aids in the activation process of other agonists (Fig. 1, step 4) [30]. The sustained level of Ca^{2+} activates PLA_2, which leads to the generation of TXA_2, a potent vasoconstrictor and inhibitor of platelet aggregation via arachidonic acid (Fig. 1, step 5). The fibrinogen receptors GPIIb/IIIa are membrane receptor glycoproteins and are present in an inactive form in resting platelets, and on stimulation by an agonist become activated forming platelet aggregates by binding to the adjacent fibrinogen receptors (Fig. 1, step 6).

There are other modulators of platelet function also present such as lipoxygenase metabolites, protein kinase C, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). The cyclooxygenase (COX) converts arachidonic acid to prostaglandin G_2, followed by a peroxidation reaction to prostaglandin H_2 (PGH_2) in the platelets. This endoperoxide is then converted to one of a series of potential prostanoid products, depending on the location of the enzyme. In the platelets, PGH_2 can be converted into the potent platelet agonist and vasoconstrictor TXA_2 by thromboxane synthetase and within the vascular endothelium, PGH_2 is converted into the potent anti-aggregatory and vasodilating agent prostacyclin (PGI_2) by prostacyclin synthetase [31]. The formation of TXA_2 in platelets induces its aggregation, which is accompanied by the platelet-release reaction whereby serotonin and other granule components are released from platelet stores. TXA_2 also induces a rise in the concentration of Ca^{2+} in the platelet cytosol and a decrease in platelet cAMP formation by inhibiting adenylyl cyclase (Fig. 1). Prostaglandin I_2 binds to specific receptors on the surface of the platelets and stimulates adenylyl cyclase and the resulting increase in platelet cAMP leads to calcium re-uptake by the dense granular system and thereby inhibits platelet activation, platelet granulation secretion and thus platelet aggregation [32]. Vascular endothelial cells and platelets also possess nitric oxide (NO) which stimulates guanylyl cyclase to form cGMP, which results in relaxation and vasodilation of vascular smooth muscle cells. In addition, NO prevents adhesion and aggregation of platelets, and it possesses anti-inflammatory, antiproliferative, and antimigratory effects on leukocytes, endothelial cells, and vascular smooth muscle cells, thus offering protection from atherosclerosis [33, 34].

4 Platelets and cardiovascular disease

There are many risk factors associated with cardiovascular disease such as hypercholesterolemia, increase in LDL oxidation, hypertension, diabetes mellitus and smoking [24, 25]. There is also ample evidence, which points to platelets playing a pivotal role in the aetiology of cardiovascular disease. Atherosclerosis is an inflammatory disease in which leukocytes interact with structurally intact but dysfunctional endothelium of the arteries and is mediated, in part by the progressive accumulation of cholesterol within the walls of the arteries [35]. Platelets bind to the leukocytes and promote their recruitment to the endothelium whilst releasing pro-inflammatory and pro-thrombotic factors, which result in the hyperplasia of the intima-medial layer of the vessel and the development of an atherosclerotic plaque. Increased LDL oxidation also acts as a pro-aggregant and enhances adhesion of platelets to the endothelial cells by several signalling pathways [36–39] and produces mediators responsible for the regulation of inflammation. It has been reported that the platelets from cardiovascular disease patients have elevated TXA_2 levels and offer reduced responsiveness to the PGI_2 produced by endothelial cells [28]. The size of the platelet is also important and an increased platelet size has been linked to atherosclerosis, hypertension and diabetes, and may be partly responsible for the hyperactive platelets observed in these diseases [40].There is also a direct relationship between hypertension and increased platelet activity [41, 42] since β-thromboglobulin (a marker of platelet activation) has been reported to be higher in untreated individuals suffering from hypertension [43]. Type II diabetes mellitus (T2DM) is a risk factor for developing cardiovascular disease and platelets from patients with T2DM display increased activation when compared to platelets from non-diabetic subjects when comparing their tendency to adhere to an extra-cellular matrix [44]. Increased platelet size has also been linked to T2DM and larger platelets have enhanced sensitivity to ADP, a key platelet-activating molecule, and rapidly form aggregates [40, 45]. The platelet fibrinogen receptor, glyco-

protein GPIIb/IIIa is important for stabilising clot formation, and is up-regulated in myocardial infarction and T2DM [46]. The platelets from individuals with T2DM and cardiovascular disease have increased levels of bioactivators and show a reduction in the responsiveness to mediators that normally dampen activation. In addition, platelets from diabetic patients are less sensitive to the effects of PGI_2 and NO, both of which inhibit platelet aggregation [47, 48]. Cigarette smoking is also strongly correlated with cardiovascular disease and smokers have increased oxidative stress [18] and also display increased platelet aggregation and adhesiveness, and increased fibrinogen levels [49]. Hence, it is clear that platelets play a pivotal role in the aetiology of cardiovascular disease, for further details please see references [25, 26, 28, 37].

5 Effects of garlic on the biochemistry and physiology of platelets

5.1 *In vitro* effects

Garlic has multiple effects in the prevention of cardiovascular disease and its antiplatelet properties are the most widely studied and established (Table 1). Most of these studies have been performed *in vitro*, some in animal models and a relatively few in humans [23]. Chloroform/acetone extracts of fresh garlic have been shown to inhibit cyclooxygenase activity directly in cell-free assays, with the acetone extract being more effective [50]. The aqueous extracts of garlic inhibit platelet aggregation induced by ADP, epinephrine, collagen and arachidonic acid in a dose-dependent manner *in vitro* [51]. The effect of aqueous extracts of raw and boiled garlic have also been investigated *in vitro* on the collagen-induced platelet aggregation using rabbit and human platelet-rich plasma [52]. The concentration required for 50% inhibition of the platelet aggregation was estimated at 6.6 mg/mL plasma and raw garlic was found to be more potent in inhibiting platelet aggregation compared to boiled garlic, furthermore, raw garlic was found to inhibit COX activity in a non-competitive, dose-dependent and irreversible manner [53]. It has also been shown that pre-treatment of rabbits with an aqueous extract of garlic at a dose of 500 mg/kg significantly inhibited thromboxane- B_2 (TXB_2) synthesis and protected against thrombocytopenia induced by collagen or arachidonic acid [54]. Thus, garlic inhibits thrombin-induced platelet synthesis of TXB_2 in a dose- and time-dependent manner, suggesting that garlic may need to be taken frequently in order to achieve its beneficial effects in the prevention of thrombosis [55]. In support of this, aqueous extracts of fresh garlic but not boiled garlic have been shown to inhibit prostaglandin synthesis in the ovine ureter [56].

The constituents of garlic responsible for the inhibition of platelet aggregation via the COX pathway have also been investigated. Garlic is rich in sulphur compounds and it has been established that these compounds have strong antithrombotic activities [57]. Ajoene, a constituent of essential oil of garlic has been shown to inhibit platelet aggregation both *in vitro* and *in vivo* [58, 59] and one of the mechanisms by which ajoene does this is by altering metabolism of arachidonic acid [60]. Besides ajoene, other compounds with antiaggregatory properties have been identified in garlic and include polysulphides [14, 61] and another garlic constituent, sodium 2-propenyl thiosulphate. The latter is reported to inhibit platelet aggregation by inhibiting COX activity in canine platelets [62] and inhibition of this enzyme has also been observed by garlic in an animal model [63].

Table 1. Summary of the mechanisms involved in the inhibition of platelet aggregation by garlic

Inhibition of cyclooxygenase activity
Suppression of Ca^{2+} mobilization within platelets
Increase in cAMP
Increase in availability of NO levels
Increase in antioxidant levels
Interaction and inhibition of exposure of GPIIb/IIIa to its fibrinogen receptor

Calcium plays a pivotal role in various aspects of platelet activation, shape change, secretion and eventually aggregation [64, 65]. Stimulation of human platelets with various agonists elevates Ca^{2+} in two ways, first by releasing Ca^{2+} from intracellular stores and secondly by facilitating the entry of Ca^{2+} through plasma- membrane channels (Fig. 1). Agonists interact with receptors coupled to phospholipase C (PLC) via G-proteins contained within the plasma membrane, leading to the formation of DAG and IP_3, which then stimulates the release of Ca^{2+} from intracellular stores (Fig. 1, step 1). The emptying of the Ca^{2+} stores regulates Ca^{2+}-conducting channels within the plasma membrane, which initiates the influx of external Ca^{2+} into the platelet cytosol. This sustained level of Ca^{2+} activates phospholipase A_2 (PLA_2), leading to the formation of TXA_2 and the subsequent release of secretory granules to complete the aggregatory process (Fig. 1, step5) [64, 65]. Aqueous extract of garlic has been shown to inhibit platelet aggregation induced by the calcium ionophore A23187, suggesting that the antiaggregation effect of garlic may be related to intraplatelet mobilization of Ca^{2+} [66]. Garlic also inhibits platelet aggregation induced by epinephrine and this implies that garlic does this by inhibiting the uptake of Ca^{2+} into platelets thereby lowering cytosolic Ca^{2+} concentrations [66]. Allicin, a constituent of garlic oil has also been reported to inhibit A23187-stimulated human neutrophil lysosomal enzyme release [67]. Similarly, ajoene has been shown to inhibit platelet aggregation induced by A23187 and reduced amounts of thromboxane and 12-hydroxyeicosatetraenoic acid was produced in ajoene-treated platelets compared to control platelets [60]. It has also been reported that N-ethylmaleimide causes the disaggregation of both ADP-

and thrombin-induced platelet aggregation, and that this disaggregation is a result of the removal of calcium ions from the platelet cytosol [68]. We have reported previously that aged garlic extract displays metal chelation properties [10] and could possibly inhibit platelet aggregation by chelating Ca^{2+}. In support of this, a garlic extract has been reported to strongly inhibit calcium binding, and arteriosclerotic nanoplaque formation and size [69]. However, another garlic component, diallyl trisulphide, is reported to inhibit platelet aggregation and Ca^{2+} mobilization in a concentration-dependent manner without increasing intracellular cAMP and cGMP levels and had no effect on TXA_2 production and IP_3 formation [70]. Aged garlic extract and its constituents have been shown to inhibit ADP-induced platelet aggregation by multiple mechanisms and one of these is the suppression of calcium mobilization within the platelets. When human platelets were stimulated with either ADP or A23187, an increase in intraplatelet Ca^{2+} accompanied platelet aggregation. However, this increase in Ca^{2+} was abolished in the presence of aged garlic extract and it is likely that aged garlic extract exerts its inhibitory effect on platelet aggregation either by suppressing the influx of Ca^{2+}, by chelating calcium within the platelets or by altering other intracellular second messengers within the platelets, which are associated with Ca^{2+} mobilization [71, 72]. One can speculate that garlic may inhibit PLA_2, thus reducing levels of lysophosphatidic acid, which causes platelet aggregation and increases intracellular Ca^{2+} (Fig. 1, step 5) [73]. Garlic could also exert its antiaggregatory affects through intracellular signals that inhibit H_2O_2-induced platelet aggregation and the accompanying increase in intracellular Ca^{2+} [74].

Cyclic AMP and cGMP are important negative regulators of platelet function, and many agents exert their inhibitory effects by increasing the intracellular concentrations of either of these since they appear to suppress the early process of platelet activation [75–77]. Platelet and endothelial factors such as PGI_2, prostaglandin D_2 (PGD_2) and prostaglandin E_1 (PGE_1) are all potent endogenous inhibitors of platelet function. The action of these inhibitors are mediated by specific prostanoid cell surface receptors, upon binding this leads to an increase in the levels of intracellular cAMP through G proteins coupled to adenylate cyclase. Cyclic AMP in turn, activates cAMP-dependent protein kinase resulting in the inhibition of platelet aggregation through the decrease of intracellular Ca^{2+} levels via uptake into the dense-tubular system. This then results in the inhibition of most platelet responses, and during platelet aggregation cAMP levels are suppressed by a Gi protein which is coupled to adenylate cyclase (Fig. 1, step 2) [75–77].

Prostaglandin (PGE_1) is able to reverse platelet aggregation and is a potent stimulator of cAMP [78], and has been shown to cause significant inhibition of platelet aggregation up to a concentration of 250 μg/mL when human platelets were stimulated with ADP [71]. Aged garlic extract has also been shown to cause disaggregation of ADP stimulated human platelets in a similar manner, thus it is likely that aged garlic extract does this by increasing cAMP levels [71]. An interesting observation in support of this is that herbs such as alfalfa and nettle inhibit human platelet aggregation, and increase cGMP levels in platelets [79]. Furthermore, C-phycocyanin, a biliprotein isolated from *Spirulina platensis* has been reported to inhibit platelet aggregation by increasing platelet membrane fluidity and cAMP levels through inhibiting cAMP phosphodiesterase [80].

NO regulates platelet activation by inhibiting adhesion and aggregation [81, 82]. It has been shown that both water and alcoholic extracts of garlic activate NOS activity in isolated platelets *in vitro.* Hence, it is likely that activation of calcium-dependent NOS and the subsequent production of NO is one of the mechanisms by which garlic can exert its therapeutic effects [83]. Three kinds of NO synthases, neuronal constitutive NOS (ncNOS), inducible NOS (iNOS) and endothelial constitutive NOS (ecNOS) are responsible for NO biosynthesis. Aged garlic extract is reported to enhance production of NO by activating cNOS but not iNOS, and the arginine contained in the aged garlic extract is not responsible for this [84], hence this is possibly another mechanism by which garlic prevents platelet aggregation. Ajoene and allicin, present in garlic also inhibit the expression of iNOS in activated macrophages [85]. Furthermore, S-allyl-Cysteine, a water soluble component present in aged garlic extract differentially regulates NO production by inhibiting iNOS expression in macrophages whilst increasing NO in endothelial cells [86] hence, it is likely that the platelet-derived NO contributes to the process of platelet disaggregation. In support of this the inhibition of phosphoinositide 3-kinase (PI3-kinase) has been shown to cause platelet disaggregation. The incubation of platelets with PI3-kinase inhibitors leads to a dose-dependent increase in platelet NO and cGMP levels that were temporally related to the period of platelet disaggregation [87].

In resting platelets, the fibrinogen receptor GPIIb/IIIa is present in an inactive form and becomes activated once stimulation by an agonist has taken place and then forms platelet aggregates by binding to fibrinogen (Fig. 1, step 6). While GPIIb/IIIa inhibitors block fibrinogen-platelet binding, stimulation of other functionally important receptors can still occur. The blocking of the GPIIB/IIIa receptor prevents platelet aggregation and enhances platelet NO release but not activation; however, the subsequent effect on other platelet pathways is unknown [88]. The antiaggregatory effect of the garlic constituent ajoene has been attributed to its direct interaction with the fibrinogen receptor GPIIb/IIIa [89]. It has been shown that ajoene inhibits agonist-induced exposure of fibrinogen receptors, as well as intracellular responses such as the activation of protein kinase C and increases in intracellular Ca^{2+} induced by receptor-dependent agonists [90]. This study also reported that binding of fibrinogen to chymoytypsin-treated platelets is only slightly

inhibited by ajoene and it does not act as a calcium chelator. Ajoene is also reported to inhibit protein tyrosine phosphatase activity in human platelets [91] and inhibits phosphatidylcholine biosynthesis and cell proliferation in *Trypanosoma cruzi* [92]. In contrast, diallyl trisulphide, another component of garlic had no effect on the tyrosine phosphorylation of PLC at concentrations that inhibited platelet aggregation [70]. Garlic contains flavonoids and in support α-napthoflavone , a potent antiplatelet flavonoid is reported to mediate its effects via inhibition of PLC activity and stimulation of cGMP formation [93]. Garlic could be acting by inhibiting 5-hydroxytryptamine or by blocking the TXA_2/PGH_2 receptor [94, 95]. Aged garlic extract has also been shown to reverse platelet aggregation induced by ADP and it is likely that this occurs by aged garlic extract binding to the GPIIb/IIIa receptors on the platelet membrane and changing its conformation such that platelets have a decreased affinity for fibrinogen (Fig. 1, step 6) [71]. GPIIb/IIIa plays a major role in the regulation of platelet aggregation and upon activation by ADP a signalling process is initiated known as 'inside-out' signalling, which causes a conformational change within the receptor. This conformational change increases the affinity of GPIIb/IIIa for its ligand fibrinogen. Upon fibrinogen binding the receptor undergoes further shape changes and through a process termed 'outside-in' signalling results in the amplification of the aggregation process [25], hence aged garlic extract could exert its effects at this level.

Garlic is a strong antioxidant [10, 18,] and dietary supplementation of garlic increases antioxidant levels especially in smokers who also display an increased platelet aggregability and impaired platelet-derived NO levels [96]. Hence, garlic ingestion could inhibit platelet aggregation by increasing antioxidant levels resulting in an increase in the platelet derived NO oxide. Inhibition of PI3-kinase has been shown to cause platelet disaggregation and incubation of platelets with PI3-kinase inhibitors led to a dose dependent increase in platelet NO and cGMP levels that were temporally related to the period of platelet disaggregation [87]. PI3-kinase inhibition also decreases the functional activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and this corresponded to decreased superoxide release. This regulation of endogenous reactive oxygen species in platelets can reverse aggregation [87] and garlic with its strong antioxidant properties [10, 18] could be acting in a similar manner.

5.2 *In vivo* studies

A positive effect on the inhibition of platelet aggregation has also been observed in human studies involving garlic supplementation [22, 23, 97]; however, a direct comparison of these studies cannot be made due to the use of different garlic preparations and the length of the clinical trials. Since 1981, there have been 12 major clinical trials involving different preparations of garlic, and all except three have shown garlic to inhibit platelet aggregation. In one trial an *in vitro* effect was observed whilst no change was seen *in vivo* [22, 23]. These trials although showing inhibition of platelet aggregation do not provide any indication of the mechanisms by which garlic inhibits platelet aggregation as this was not investigated in the majority of these studies. One of the mechanisms by which garlic can inhibit platelet aggregation *in vivo* is by reducing the formation of lipoxygenase and thromboxane, and inhibiting phospholipase activity in the platelets (Fig. 1) [22, 98, 99]. It has been reported that ajoene strongly inhibits the metabolism of arachidonic acid by both cyclooxygenase and lipoxygenase pathways thus inhibiting the formation of TXA_2 [60], and these effects may explain, in part, inhibition of platelet aggregation. We have previously shown that dietary supplementation with aged garlic extract significantly inhibited both the total percentage and initial rate of platelet aggregation at concentrations of ADP up to 10 μmol/L. The Michaelis-Menten constant (K_M) for ADP-induced aggregation was approximately doubled after supplementation with aged garlic extract, whereas the maximum rate of aggregation was unaffected. However, no significant changes in plasma TXB_2 and 6-ketoprostaglandin $F_{1\propto}$ concentrations were observed [97]. In contrast, Ali and Thomson [100] have shown that in healthy males aged 40–50 years, 3 g of fresh garlic supplementation for 16 weeks results in about 80% reduction in serum thromboxane together with approximately 20% reduction in serum cholesterol. Dietary supplementation with garlic also increases antioxidant status and thus may increase the platelet-derived NO leading to an increase in the inhibition of platelet aggregation [18, 96, 101]. Increased LDL oxidation can act as a pro-aggregant and enhances adhesion of platelets to the endothelial cells by several signalling pathways [36–39]; hence, an increase in antioxidant status could prevent this. Garlic also has the ability to chelate metals [10] and thus may inhibit platelet aggregation by suppressing Ca^{2+} mobilization within the platelets [71, 72]. In support, aged garlic extract has demonstrated a significant slowing of the accumulation of coronary artery calcification in a randomised, placebo-controlled trial when it was given alongside statins [102]. It is likely that garlic inhibits platelet aggregation *in vivo* by multiple mechanisms discussed above, and clearly more in-depth studies are required in order to elucidate these mechanisms. Our laboratory is currently investigating the effects of aged garlic extract on the GPIIb/IIIa receptors, fibrinogen binding and intracellular levels of platelet Ca^{2+} and cAMP *in vivo.*

6 Summary

There are many factors associated with cardiovascular disease and atherosclerosis, one such factor being an increase

in platelet aggregation, which involves multiple pathways. Evidence from numerous *in vitro* experiments points to the fact that garlic has the ability to inhibit platelet aggregation by multiple mechanisms. Thus, garlic inhibits platelet aggregation by inhibiting cyclooxygenase activity, suppressing Ca^{2+} mobilization within platelets, increasing cAMP levels and availability of platelet NO, increasing antioxidant levels and finally interacting directly with the fibrinogen receptor GPIIb/IIIa and inhibiting its exposure to fibrinogen (Table 1). Majority of studies support the role of garlic in inhibiting platelet aggregation, however, the mechanisms involved have been mainly investigated *in vitro* and have not been fully addressed *in vivo.*

The composition of garlic is complex and studies have used different preparations of garlic hence its not surprising that multiple mechanisms have been observed in its ability to inhibit platelet aggregation. The way forward is to conduct well-designed randomised clinical trials with standardised preparations with clearly defined outcomes, which can provide the answers as to the mechanisms involved in the inhibition of platelet aggregation by garlic. The active constituents of garlic, their bioavailability and drug interactions are important issues, which need to be addressed. Cardiovascular disease is on the rise and garlic consumption is increasing, hence further *in vivo* studies are required to meet the demands of consumers, health practitioners and the regulatory authorities.

Dr Khalid Rahman obtained his PhD in physiological biochemistry in 1984. His main area of research is in the role of natural products, antioxidants, and nutraceuticals, especially garlic in the prevention of cardiovascular disease. His main focus of research currently is in investigating the mechanisms by which garlic inhibits platelet aggregation both *in vitro* and *in vivo.* He holds the position of Reader in Physiological Biochemistry in the School of Biomolecular Sciences, Liverpool John Moores University, UK.

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