Elevated plasma 5-hydroxytryptamine induced by nicotine consumption responsible for increased risk for myocardial infarction and ischemic stroke

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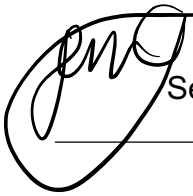

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ABSRACT

The use of tobacco products is directly linked to the increased risk of developing several life threatening complications, like myocardial infarction (MI) and ischemic stroke (IS). MI and IS are both caused by spontaneous clot formation within the blood vessels, leading to the occlusion of arteries supplying nutrient rich blood to either cardiac muscle or cerebral tissue. An important molecule used for platelet-platelet signaling during clot formation is 5-hydroxytryptamine (5-HT). Previous studies have shown that sympathetic nervous system (SNS) stimulation, induced by nicotine, results in elevated concentrations of 5-HT within the blood, but the link between this increase and the increased risk for MI and IS has yet to be made. Therefore, we hypothesize that elevated concentrations of 5-HT stimulated by nicotine, induce a hyperactive platelet phenotype in smokers resulting in the increased risk for MI and IS. To test this hypothesis, we incubated isolated platelets from nonsmoker's in a high concentration cocktail of 5-HT and other known platelet signaling molecules released after SNS stimulation, to see if these elevated concentrations were inducing the hyperactive platelet activity observed in smokers. Incubation of nonsmoker's platelets in the cocktail resulted in more platelets aggregating together after stimulation with collagen than platelets from nonsmokers without addition of the cocktail. Next we looked to block individual receptors on the platelets known to be used for platelet-platelet signaling during clot formation. After blocking these receptors, we were able to reduce the effect of the cocktail on nonsmoker's platelets. Complete negation of the cocktails effect on the magnitude of the aggregation cascade on nonsmoker's platelets was only achieved when all three receptors were blocked at the same time indicating that the receptors are working independently of each other.

Interestingly a time dependent relationship between the cocktail and triple block was observed indicating that platelets exposed to the cocktail first aggregate at a higher magnitude even in the presence of the triple block than when the platelets were exposed to the triple block first. These finding have implication in potential therapies or screenings for high risk MI or IS patients as reduction of reactivity of platelets to clotting factors within the blood vessels could reduce the chance of both MI and IS from occurring.

INTRODUCTION

Blood is responsible for regulating temperature, delivering oxygen to muscle for cellular respiration, removal of carbon dioxide and lactic acid from tissues, delivering glucose and other vital sugars to the brain, transporting communicatory hormones, regulation of body pH, and circulation of leucocytes throughout the body (Hamasaki, N. & Yamamoto, M., 2000) (Kolka, C. M. & Bergman, R. N., 2012). The capacity for blood to self-repair damage done to the blood vessels is its most critical function. Clotting is the process in which several components within the blood transform from a flowing liquid to a solid to prevent hypovolemic shock (Hartwig, J. & Desisto, M., 1991). Although this is vital to our survival, if triggered improperly, our blood's ability to clot can become problematic. Thrombosis, a stationary blood clot formed on the wall of a blood vessel that partially or completely occludes arteries, or embolism, a free flowing blood clot that travels around the circulatory system until trapped by constricting capillaries, are both examples of spontaneous clot formation that can lead to the blockage of blood flow to an organ or tissue (Linden, M. & Jackson, D., 2010). Formation of these spontaneous clots within the blood vessels often leads to tissue death due to lack of oxygen or nutrients.

Myocardial Infarction and Ischemic Stroke

Myocardial infarction (MI) and ischemic stroke (IS) are both medical conditions that are caused by spontaneous clot formation leading to the occlusion of cardiac or cerebral arteries, respectively. When these arteries are occluded by clots, oxygen rich blood can no longer reach either cardiac muscle or cerebral tissue. Loss of blood flood to these tissues will cause these highly specialized cells to die. There are several known lifestyle choices that have been shown to increase the chances both MI and IS, notably among these is cigarette smoking. Around 15% of the American population smokes cigarettes, an activity shown to increase the risk of heart attack, stroke, cancer, high blood pressure, and chronic obstructive pulmonary disease (COPD) by up to 2 to 4 times (Center for Disease Control (CDC), 2016). Unfortunately, the root cause of why cigarettes increase the risk for both heart attack and stroke is still unknown.

The CDC reported that in 2014 of the 2,626,418 deaths in the United States the leading cause was heart disease claiming 614,348 lives or 23.4% of all recorded deaths (Kochanek, K. D. et. al., 2016). Heart disease is defined by the Mayo Clinic as any condition that effects the health or function of the heart. It can include problems such as coronary blood vessel damage or blockage, heart rhythm problems, or heart defects from birth. This umbrella term can account for many different problems but when related to cigarette smoking it usually is caused by coronary artery damage or blockage. A damaged or blocked coronary artery will lead to death of cardiac muscle and is often referred to as a myocardial infarction (MI), or heart attack. It is estimated that a third of the total deaths, 204,780 deaths, caused by heart disease were directly linked to smoking related complications (Mozaffarian, D. et. al., 2014).

Another major cause of death and debilitation in the United States is cerebrovascular disease or ischemic stroke (IS). IS, similarly to MI, is caused by a blockage of a cerebral artery, causing cessation of blood flow to certain parts of the brain. This blockage can lead to permanent neuron death, long lasting physical and mental disabilities, or death. In 2014 the CDC estimates that 133,103 (5.1%) deaths were caused by stroke (Kochanek, K. D. et. al., 2016). Although studies have shown smoking can increase the risk for these life threatening ailments the underlying reason behind why is still unknown. The active ingredient – nicotine – found in cigarettes and all other tobacco products has been implicated in smoker's increased risk for MI and IS. This is believed to be because of smoking's effect on its user's blood physiology, specifically on their platelets.

Platelet Formation by Megakaryocytes

Platelets are, in their simplest definition, small anucleated cellular fragments of much larger cells called megakaryocytes (MKs). Before becoming a platelet, the cellular bodies, organelles, fats and granules of each platelet were within the cytoplasm of MKs. MKs are larger cells (50-100 μm) that grow in the bone marrow and make up only about 0.01% of all nucleated cells found inside the bone marrow (Nakeff, A., & Maat, B., 1974). To produce platelets each megakaryocyte must go through a distinct growth period, known as endomitotic synchronous replication (Deutsch, V. & Tomer, A., 2006).

In order for MKs to become are these large platelet generating cells they must first grow within the bone marrow. Like all other cells within the bone marrow, MKs originate from hematopoietic stem cells (HSCs). Thrombopoietin (TPO) is a key cellular signal sent from both the kidney and liver to the bone marrow that influences HSC differentiation into MKs. (Kuter, D. J., et al., 1994 & de Sauvage, et al., 1994). The first step of MK

development is to become polyploid or contain more than one set of homologous chromosomes. TPO targets the c-Mpl receptor on the surface of MKs causing them to begin a process known as endomitosis (Abraham, R. & Basser, R., 1997). During this key MK growth period, cytoskeletal proteins in the MKs shuttle platelet proteins and fats throughout the cytoplasm in preparation for the next step in platelet formation. This two pronged approach to stimulate endomitosis and maturation allows for rapid MK growth and development resulting in a dramatic increase in the MKs volume as it prepares to expel platelets into the blood.

Mature MKs will migrate to the sinusoidal wall of the bone marrow in preparation for the next step in platelet production. At this step MKs will expel long processes, called proplatelets, into the sinusoidal blood vessels (Schulze, H. et al. 2006). As a proplatelet is being expelled, cytoskeletal proteins shuttle key organelles into each proplatelet ensuring that they contain the proper platelet proteins, lipids, and other vital organelles. Proplatelets acts as moving assembly lines that flow through the blood and extend bidirectionally, budding off platelets at each end (Machlus, K. R. et al., 2013). This extension is driven by the cytoskeletal protein β1- Tubulin, which is responsible for cytoplasmic extension as well as proper organelle and fat transportation for platelet assembly within the proplatelet tips (Lecine, P. et al., 2000). F-actin, found within the proplatelets, is another key filament that helps with platelet organelle organization as well as bifurcation of proplatelet tips as they begin to split into fully formed platelets. The released platelet is known as a terminal platelet, or simply platelet, and will cease further divisions. The terminal size of platelets varies from person to person and can have an effect of reactivity of platelets within a person. Studies have shown that people who have

larger terminal platelets sizes have a higher prothrombotic potential (Bath, P. M., & Butterworth, R. J., 1996). These platelets will flow throughout the blood stream and have two critical functions; regulation of blood 5-hydroxytrptamine (5-HT) concentrations, and clot formation in response to damaged blood vessels.

Platelet Function: Regulator of Blood 5-HT

Although it is a common belief that platelets have a singular function of clotting, they also play an important role in blood homeostasis. A plethora of hormones and chemicals are transported in the blood every hour and not all of them reach their target tissue, leaving excess hormones left to circulate throughout the blood. Platelets play a role in regulating blood concentrations of 5-hydroxytrptamine (5-HT). 5-HT is a hormone released by enterochromaffin cells of the intestine and acts as a vasoconstrictor arteries going to intestinal tissues, regulating the

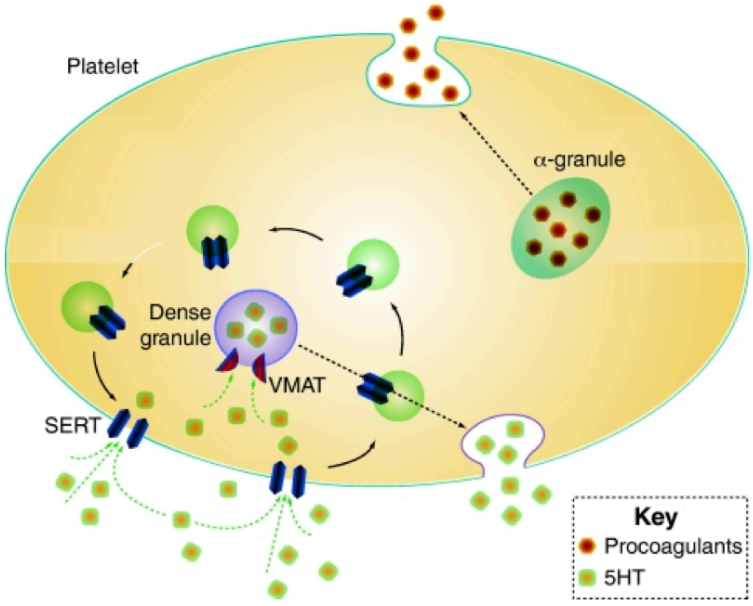


Figure 1. 5-HT uptake and storage within platelet. Extracellular 5-HT being absorbed into platelets via serotonin transporter protein (SERT) and stored in dense granules. α-granules contain other pro-coagulants absorbed or produced within the platelet. Dense and α-granules are expelled upon platelet stimulation to carry on the aggregation cascade. From "Molecular Mechanisms of SERT in Platelets: Regulation of Plasma Serotonin Levels" by Mercado, C. P. & Kilic, F., 2010, *Molecular Interventions*, *10*, 231.

amount of blood flowing to the gastrointestinal tract between digestion (Brenner, B. et al., 2007).

Blood 5-HT levels are maintained by platelets via uptake and storage of the molecule within dense granules (Brenner, B. et. al., 2007). It is important that platelets keep blood 5-HT levels regulated for two reasons. The first is due to 5-HT's vasoconstriction effect. Extracellular 5-HT can effect blood vessel diameter size and helps regulate the amount of blood tissues are receiving (Kamal, L. A. et al., 1984). The second and more significant reason for this regulation is due to 5-HT's use as platelet-platelet communicative hormone used during the clotting process.

To control the levels of extracellular 5-HT, specific proteins on the surface of platelets called serotonin transport proteins (SERT) function to bring extracellular 5-HT into the platelet. Within the platelet, 5-HT is stored and repurposed for platelet-platelet communication in dense-granules (Fig. 1). As 5-HT levels increase in the blood so does the expression of SERT on the platelet membrane (Fig. 2). If a certain threshold of extracellular 5-HT is reached, 5-HT will trigger another pathway by stimulating the 5-HT2A receptor on the surface of platelets (Mercado, C. P., & Kilic, F., 2010). Stimulation of the 5-HT2A receptor will initiate a downstream pathway that will block migration of SERT to the surface of the platelet and trigger the secondary function of the platelet, the clotting process.

Platelet Function: Blood Clotting

The second, and arguably most important, function of the platelet is to form a clot in damaged blood vessels as a response to trauma. Platelet clotting can be triggered by

a number of pro-coagulants; fibrinogen, prothrombin, thromboplastin, collagen, and many others but the one this paper will be focusing on is a collagen stimulated aggregation mechanism due to its abundance within the layers that surround the blood vessels. Blood vessels are lined with 3 distinct epithelial layers wrapped around each other. They are, from outermost to innermost, the tunica adventitia, tunica media, and tunica intima. The layer with the most importance is the innermost layer, the tunica intima divided into 3 separate sections. The outermost section, called the fenestrated layer, consists of mostly elastic fibers and has a perforated appearance. The middle section, the subendothelial layer, is made mostly collagen and elastic fibers. Finally, the innermost section is a smooth simple squamous layer of endothelium cells.

When a traumatic event occurs that leads to damage to the layers surrounding our blood vessels the smooth lining of endothelial cell is inevitably broken. This damage causes bleeding that must be stopped to prevent hypovolemic shock or death. Now that the endothelial layer is broken, collagen and elastic fibers from outer layers are exposed to the inside of the blood vessels. These fibers indicate that a blood vessel has been damaged and triggers our bodies clotting mechanism in an attempt to stop bleeding as quickly as possible (Sugiura, T. et al., 2012). Platelets are designed to begin to aggregate when stimulated by collagen or elastic fibers since that is an indication that the endothelial layer has been damaged or broken. Collagen or elastic fibers stimulate glycoprotein VI (GPVI) and the Fc receptor (FcR) on the platelet and activate the platelet (Cicmil, M. et al., 2000). An activated platelet will expel its dense and α-granules containing pro-coagulants, such as 5-HT, into the blood plasma to propagate the aggregation cascade.

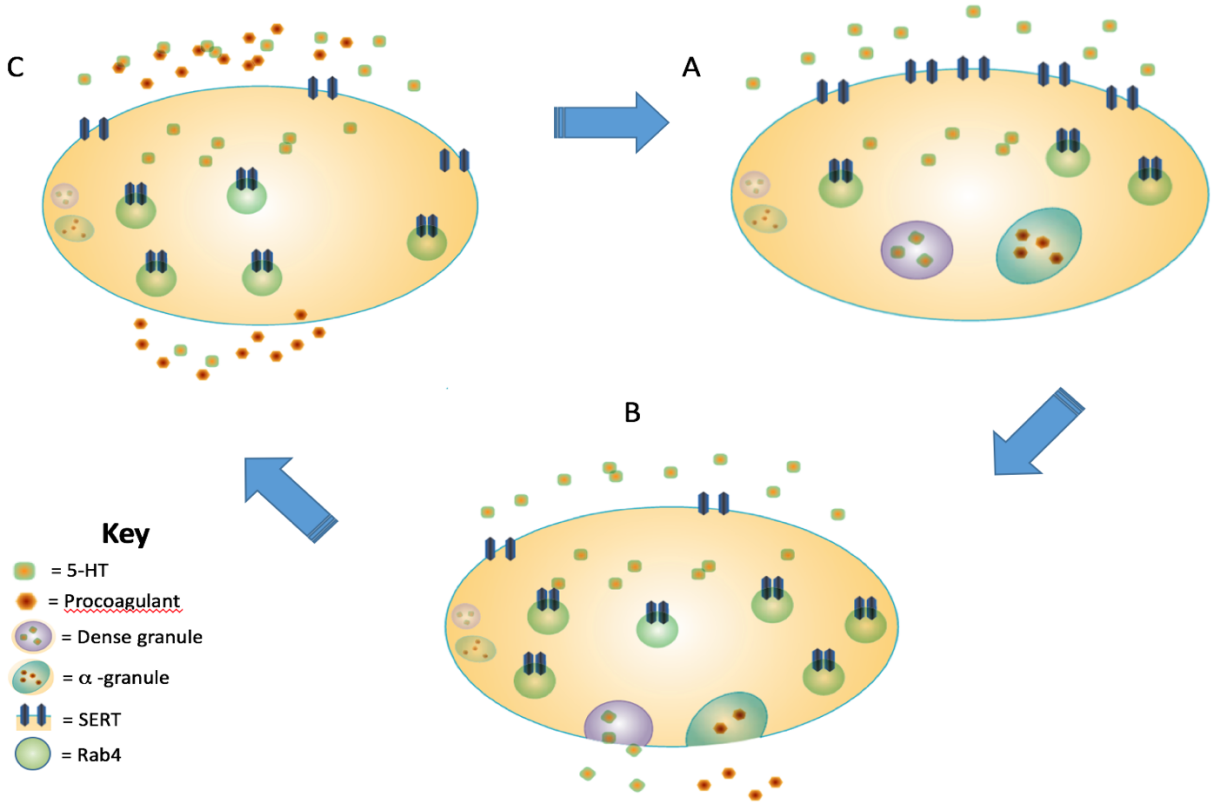


Figure 2. Platelet-platelet signaling during aggregation cascade. A) Plasma 5-HT levels increase and are absorbed into the platelets activating Rab4 intracellularly. B) Activated Rab4 inhibits SERT from reintegrating with the plasma membrane. 5-HT interacts with 5-HT2A receptors stimulating the expulsion of dense and α-granules into the extracellular space. C) Extracellular 5-HT and pro-coagulants diffuse into the surrounding plasma to continue the aggregation cascade. Adapted from "Molecular Mechanisms of SERT in Platelets: Regulation of Plasma Serotonin Levels" by Mercado, C. P. & Kilic, F., 2010, *Molecular Interventions*, *10*, 235.

This results in a rapid increase in extracellular 5-HT levels in the area around the damaged blood vessel and triggers the cascade effect that starts the formation of a clot. As extracellular 5-HT levels increase, it triggers surrounding platelets by stimulating the 5-HT2A receptor on the surface of platelets. The 5-HT2A receptor has a lower affinity for 5-HT then the 5-HT importer protein SERT, and therefore is only triggered when extracellular 5-HT concentrations are relatively high (Fig. 2). 5-HT2A stimulation also activates the transglutaminase within the platelet that activates an inhibitory protein Rab4

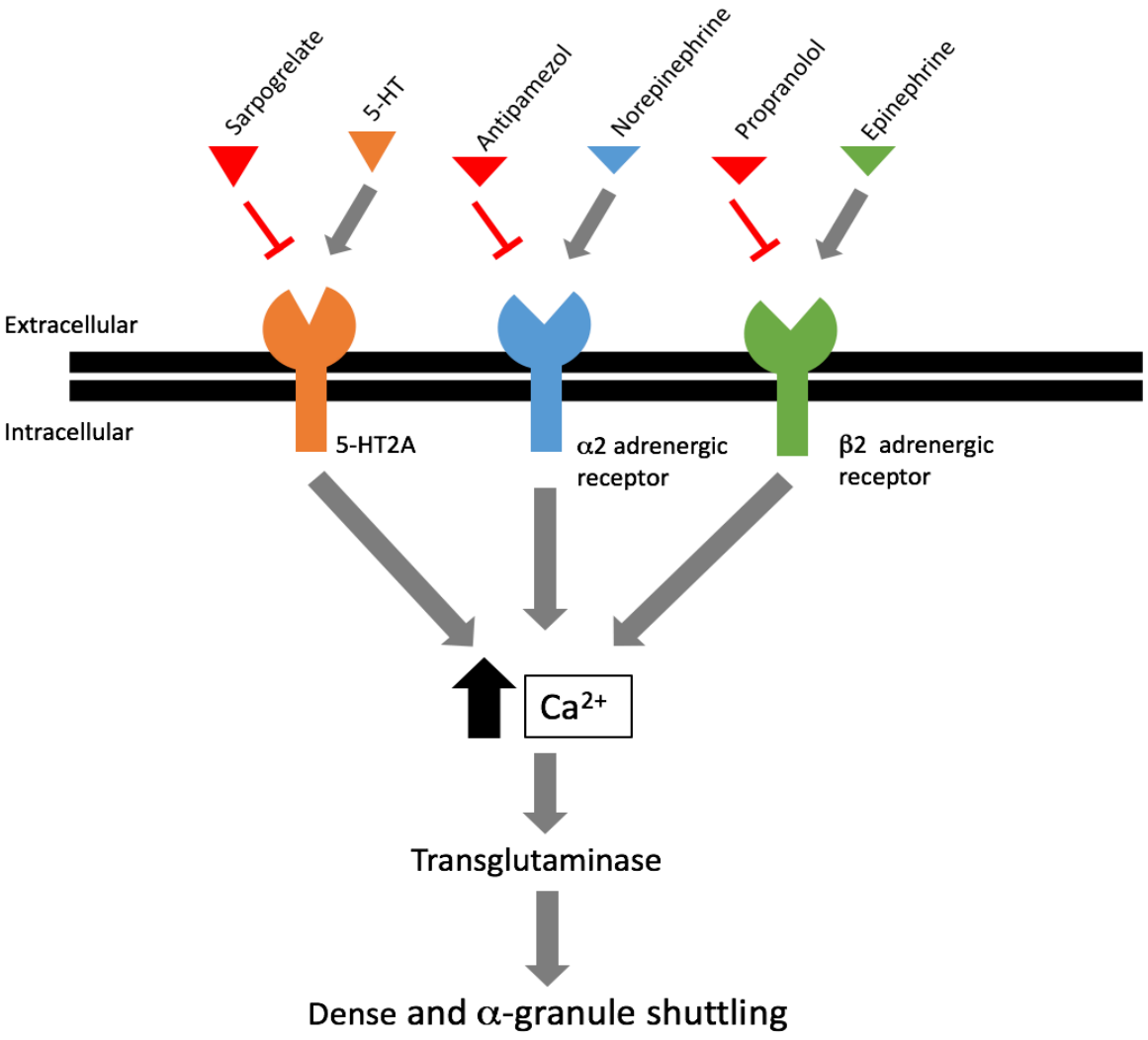


Figure 3. Individual receptor targeted during aggregation cascade. 5-HT2A, α2 adrenergic receptor, and β2 adrenergic receptors within platelet membrane. Upon stimulation with specific pro-coagulant each receptor will independently activate transglutaminase within the platelet by increasing intracellular calcium levels. Activation of transglutaminase will help shuttle dense and α-granules to the platelet membrane to be expelled into the extracellular space. Blockers added to inhibit receptor stimulation, 5-HT2A receptor blocker Sarpogrelate, α2 adrenergic receptor blocker Antipamezol, and β2 adrenergic receptor blocker Propranolol.

(Mercado, C. P., & Kilic, F., 2010). This protein directly inhibits SERT from integrating back with the platelet membrane, reducing the amount of SERT on the platelet membrane (Fig. 2). This switch to decrease the amount of SERT on the surface of platelets increase the chance of a 5-HT2A receptors being stimulated and increases the potential magnitude

of the aggregation cascade. 5-HT stimulation of the 5-HT2A receptor will also activate the platelet causing it to aggregate to the wound site and other activated platelets.

Along with extracellular 5-HT several other hormones, such as epinephrine and norepinephrine, are released by the adrenal glands in response to stress. Both epinephrine and norepinephrine have receptors on the platelet surface that induce activation of the Rab4 protein increasing the chances that a platelet is activated in response to a sudden increase in extracellular 5-HT. Epinephrine targets the β2 receptor on platelet membranes, while norepinephrine targets the α2 receptor (Fig. 3). Downstream of each of these receptors is the same transglutaminase that the 5-HT2A receptor activates that reduces the amount of SERT that can be expressed on the platelets membrane via Rab4 activation. The activated transglutaminase also works to begin shuttling dense and α-granules of recently activated platelets to the membrane to be expelled to continue the aggregation cascade.

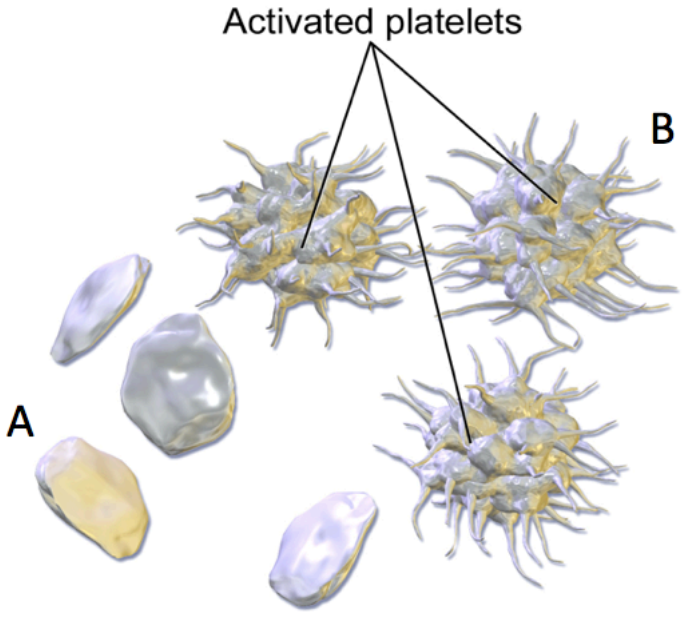


Figure 4. Morphological changes of platelets before and after activation. Inactive platelets (A) undergo morphological change to increase their "stickiness". Active platelets (B) express protruding integrins and glycoproteins that act as anchors for platelet-platelet and platelet-wound aggregation. This morphological change ensures that activated platelets to only interact with other activated platelets and strengthens the clot. Image from "Medical gallery of Blausen Medical 2014". *WikiJournal of Medicine* **1**

Quickly after the activation of a few platelets by collagen fibers in the endothelium thousands of platelets will aggregate together to form the foundation of a blood clot. To quickly and efficiently form the clot, platelets are not only recruiting other platelets to begin the clotting process, but also morphologically change to increase their adhesive nature to ensure the formation of a strong clot. To achieve this transformation, activated platelets will go through physical alterations. There are morphological changes between inactivated and active platelets from rounded non-adhesive cells to a star-like adhesive cells driven by increasing the expression of surface glycoproteins and integrins that aid in platelet-platelet and platelet-surface adhesion at a wound site (Fig. 4). Once a platelet is activated by collagen or another platelet, the expression of integrins $\alpha_{IIb}\beta_3$ or $\alpha_2\beta_1$ is increased on the platelet surface, as well as the adhesive protein P-selectin (Kuwahara, M. et. al., 2002). These integrins act as an extracellular Velcro that will adhere to other activated platelets glycoproteins, adhesive matrices such as collagen, or von Willebrand factor (vWF). The process of clotting is extremely complex and interconnected with our diet, daily exercise, blood pressure, and age. It can be greatly effected by different chemicals or molecules that enter the blood via digestion or inhalation. One notable chemical compound that millions of people willingly introduce into their body daily is nicotine.

Nicotine's Role in Platelet Hyperactivity

The U.S. Food and Drug Administration (FDA) have isolated 93 harmful chemicals within cigarettes including acetaldehyde, formaldehyde, nicotine, and mercury. A prominent threat that cigarette smoking has on its users is the alteration to the blood physiology due to the introduction of these chemicals into the blood. The high felt from

smoking a cigarette is partially induced by a stimulation of the sympathetic nervous system, by nicotine, inducing your fight or flight reflex. When this reflex is stimulated, the body prepares itself for extreme stress and takes several preliminary steps. Some of these include vasoconstriction, increases in blood pressure, and importantly the potential increase in platelet sensitivity to progocgulants.

Nicotine's effect on the body can be felt and observed immediately after smoking a cigarette. As the smoke enters the user's lungs chemicals within the cigarette smoke are able to diffuse into the blood stream, where they stimulate changes in the user's physiology via stimulation of ganglionic transmission within the central nervous system and nicotinic acetylcholine receptors (nAChRs) found both within the central nervous system and on chromaffin cells in the adrenal glands (Mishra, A. et.al., 2015). Stimulation of nAChRs on chromaffin cells in the adrenal glands of smokers has been shown to increase blood epinephrine levels drastically, a key catecholamine in the stimulation of platelet aggregation (Levine, P. H., 1973). The stimulation of nAchRs within the central nervous system results in the activation of the sympathetic nervous system. Originally used to keep us alive in dangerous situations, stimulation of our sympathetic nervous system is now mostly a result of anxiety or daily stress and results in a dramatic change of the bodies physiology.

It is nicotine's ability to stimulate the sympathetic nervous system that may be responsible for some of its dangerous side effects. Stimulation of the sympathetic nervous system leads to increased respiratory rates, elevated blood pressure, elevated heart rate, as well as increased plasma catecholamine levels (Haass, M., & Kübler, W., 1997) (Watts, D., 2006). Studies on canines have shown that after exposure to cigarette smoke

there is an immediate increase in plasma catecholamine levels as well as increased aortic blood pressure (Folts, J. D. et. al., 1982). Prolonged exposure to nicotine due to repeated cigarette smoking may lead to prolonged periods of elevate plasma catecholamine levels. Notably, key catecholamine released by the body after sympathetic nervous system stimulation are 5-HT, epinephrine, and norepinephrine, all vital molecules used in platelet-platelet communication during aggregation (Kamal, L. A. et al., 1984) (Folts, J. D. et. al., 1982). Each of these catecholamine prepares the body for a stressful or potentially dangerous encounters by allowing our platelets to clot together more readily, but prolonged exposure may result in hyperactive platelets resulting in increased risk spontaneous clot formation within the blood vessels.

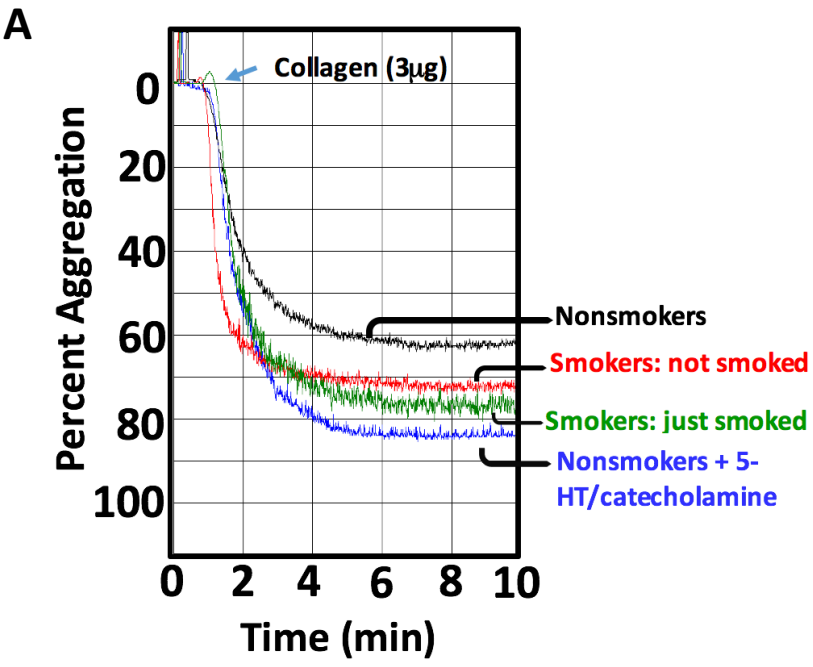
As previously mentioned platelets use and regulate plasma 5-HT levels by absorbing 5-HT released by enterochromaffin cells and use it to aid in the stimulation of the clotting cascade. Notably, chronic cigarette smoking has been shown to increase the amount of 5-HT both in the plasma and within platelets (Sugiura, T. et. al., 2012). The elevated 5-HT, epinephrine, and norepinephrine levels stimulated by nicotine may be priming platelets within smokers to become hypersensitive to vascular damage. This hypersensitivity would result in larger and stronger thrombus formation upon stimulation within the blood vessels, increasing the chance of complete blockage of coronary or cerebral arteries resulting in MI or IS. If elevated levels of 5-HT and catecholamine are causing platelets to aggregate together more readily, we next plan to look at the potential effects of blocking known receptors on the platelets used in the aggregation cascade. By blocking the platelets ability to recruit other platelets we may be able to reduce the risk of spontaneous clot formation within the blood vessels. Overall, we hypothesize that

nicotine's effect on 5-HT and catecholamine concentrations within the blood is influencing platelets to become hyperactive, increasing the chances that they will spontaneously clot together within the blood vessels, increasing the chances of arterial occlusion which would lead to MI and IS.

RESULTS

Model system for studying platelet activation

What makes the platelet clotting such a difficult subject to study are the many different interactions that occur between the blood, platelets, extracellular proteins, and extracellular fibers that can and help induce clotting. The whole process is filled with many positive feedback loops that help induce clotting as quickly and locally as possible to ensure that any vessel damage is repaired as quickly as possible. For this experiment we wanted to use a very basic model for studying platelet adhesion and attempted to remove as many variables as possible. To do this, we isolated platelets from the rest of the blood in a mixture called platelet rich plasma (PRP). This allows us to study individual stimulants of coagulation at a time, reducing the amount of variables we had from subject to subject. By isolating the platelets, we are reducing the aggregative stimulation of enzymes like thrombin or proteins like fibrin or vWF. Our platelets are stimulated with collagen to induce an aggregation cascade. Platelets that are activated by the collagen expel their dense and α-granules increasing the concentration of 5-HT in their surrounding space stimulating other platelets to begin the aggregation process. Platelets will adhere together



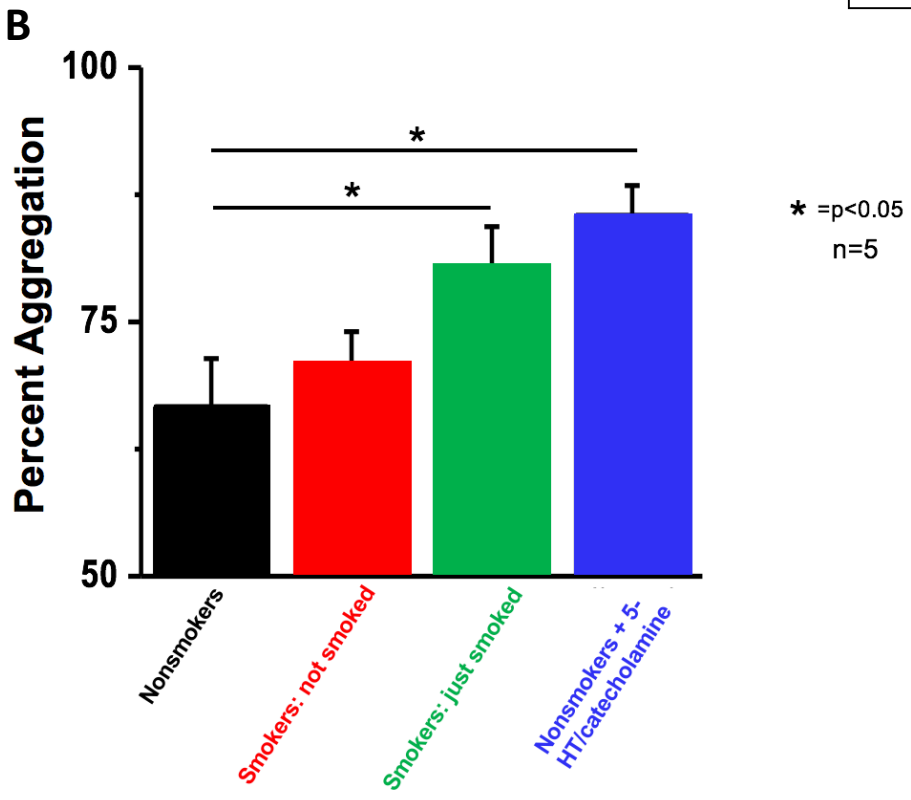


Figure 5. Smokers and nonsmokers platelets with cocktail have increased percent aggregation when compared to nonsmokers platelets. (A) Aggregation curves of platelets from nonsmokers, smokers who had not smoked in 4 hours, smokers immediately after smoking a cigarette, and nonsmokers pretreated with 5-HT/catecholamine cocktail. (B) Multiple aggregation curves generated using light-transmission aggregometer and averaged (n=5). (*) indicate significance in differences for average percent aggregation from two tailed t-test with p value <0.05.

by increasing surface expression of integrins and glycoproteins, but will not form a clot at a specific site. Instead they will form embolism like clots that will float free in the plasma suspension. This model allows us to isolate the effect that increased 5-HT and catecholamine levels have on this one clotting mechanisms and allow us to extrapolate our data and relate back to the larger process of clotting.

Platelets of cigarette smokers aggregate at a greater magnitude after stimulation than nonsmokers.

We first needed to test the effects, if any, that elevated plasma 5-HT concentrations had on platelet sensitivity to collagen, specifically in platelet aggregation and expression of the activation marker P-selectin. Comparison of the magnitude of aggregation cascades of nonsmokers and smoker's platelets as well as detectable levels of surface P-selectin allowed us to determine if elevated 5-HT concentrations had an effect on platelet sensitivity to procoagulants. Isolated platelets from each experimental group were stimulated with a 2:1 collagen/PBS solution and the percentage of platelets transitioning from inactive, to active and aggregated was obtained by a light spectrometry aggregometer (Fig. 5A-B). Ten minutes after stimulation, approximately 62% of the platelets from nonsmokers had aggregated. After the same period of time approximately 72% of the platelets from smokers, after a 4-hour cigarette break, aggregated. When this same collagen stimulation was preformed on the platelets of smokers immediately after smoking this number increased to around 80% platelet aggregation (Fig. 5A-B). P-selectin levels of platelets after stimulation with collagen shows that nonsmokers have a mean of fluorescence of 130 (Fig. 6). Smokers a cigarette have a mean of fluorescence of 225 for surface P-selectin (Fig. 6). This data suggests that the platelets of smokers both before and after a 4-hour cigarette break aggregate at a higher rate when stimulated with collagen than non-smokers platelets and also have increased levels of P-selectin on the surface.

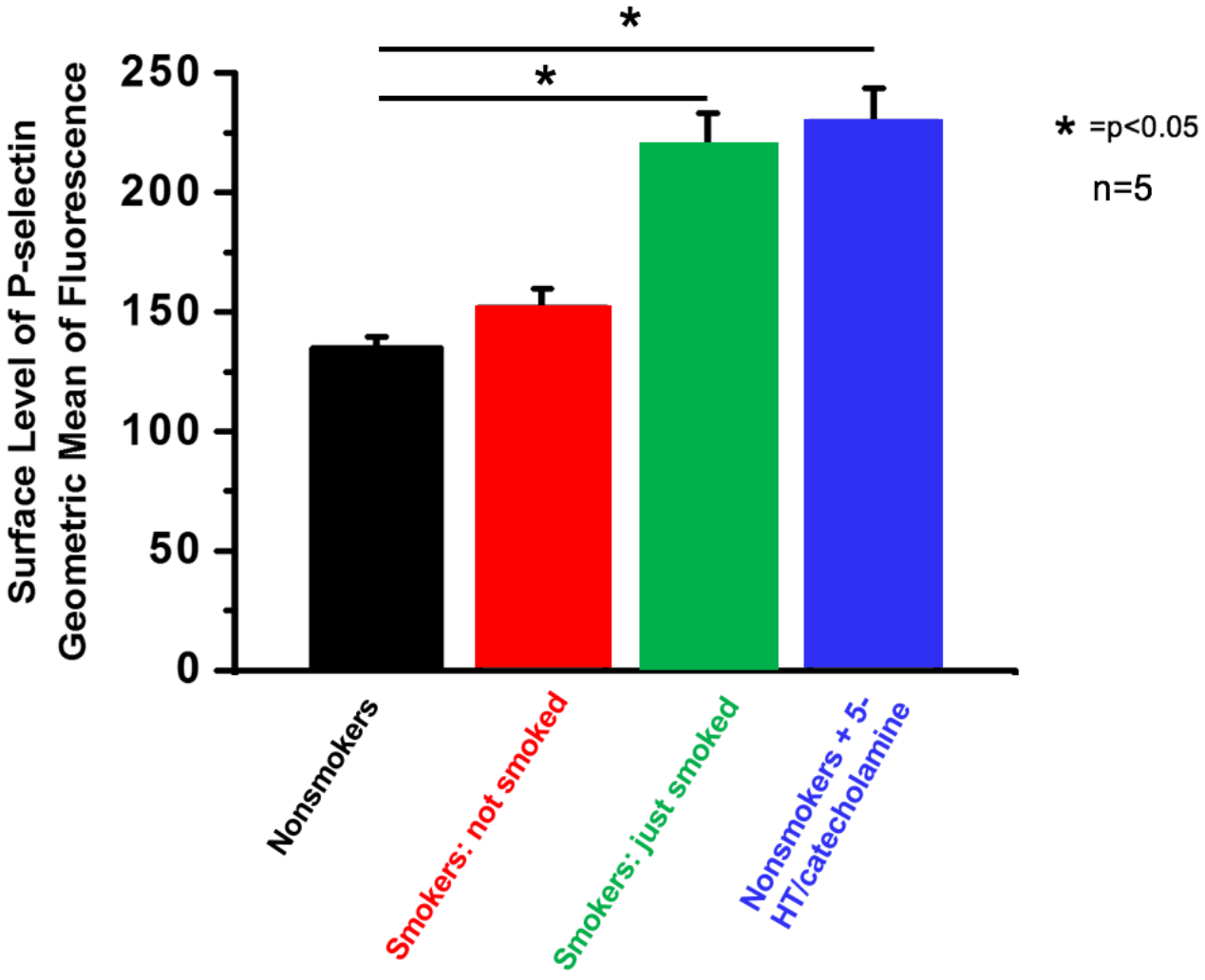


Figure 6. Surface levels of P-selectin in smokers and nonsmokers with cocktail increased when compared to nonsmokers platelets. Flow cytometry assay for membrane surface protein P-selectin of isolated platelets of either nonsmoker with or without 5HT/catecholamine cocktail pretreatment and in smokers after a 4-hour break or immediately after smoking. P-selectin detected using FITC labeled P-selectin antibody. (*) indicate significance in differences in detectable levels of surface P-selectin (n=5) from two tailed t-test with p value <0.05.

Pretreatment of nonsmoker's platelets with 5-HT/catecholamine cocktail induces both an increase in the aggregation cascade and surface P-selectin levels

Next, we tested our hypothesis that elevated blood 5-HT and catecholamine concentrations stimulated by cigarette smoking are partially responsible for the increased platelet aggregation rates observed in smokers. To address this hypothesis, we pre-

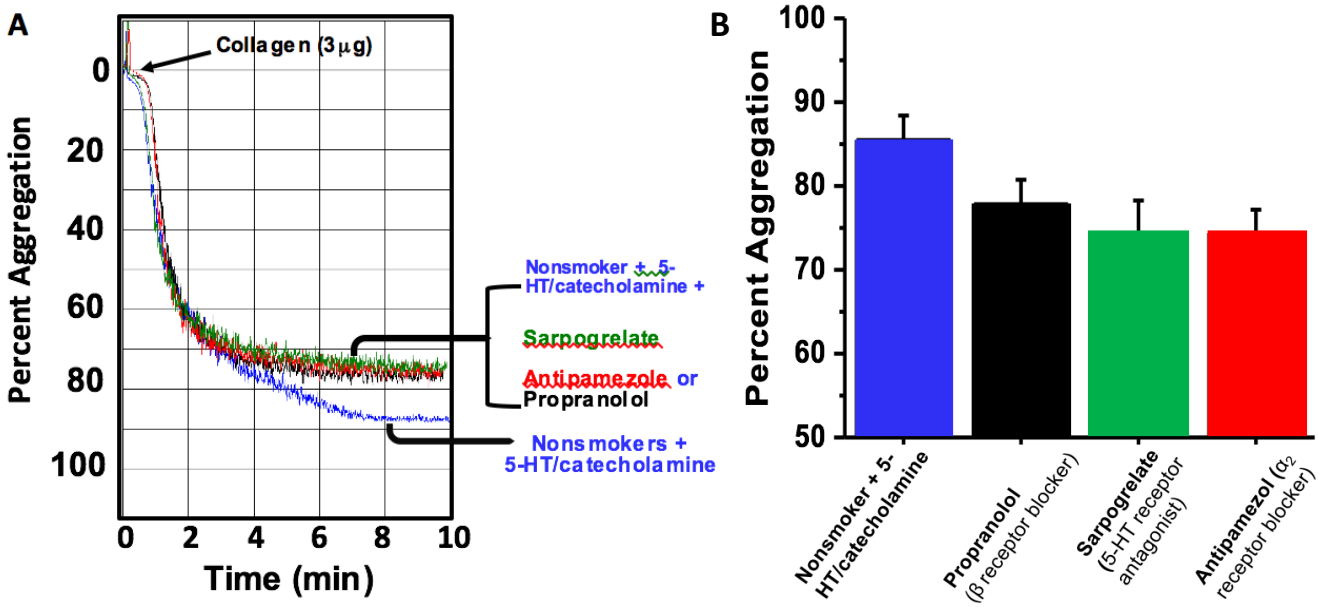


Figure 7. Individually blocking receptors used in platelet-platelet aggregation cascade reduce effect of cocktail on nonsmokers platelets. (A) Comparison of aggregation curves of isolated platelets from a nonsmoker pretreated with 5-HT/catecholamine cocktail with one or no receptor blockers. Three different receptor blockers were used, Sarpogrelate (5-HT receptor antagonist), Antipamezole (α_2 adrenergic receptor blocker), and Propranolol (β adrenergic receptor blocker). (B) Multiple aggregation curves generated using light-transmission aggregometer and averaged (n=4).

treated non-smoker's platelets with a 5-HT/catecholamine cocktail that mimicked the physiological conditions immediately after smoking. After a 15-minute incubation time, the platelets were stimulated with the 2:1 collagen/PBS mixture; we observed an increased aggregation rate of around 84% (Fig. 5A-B). Flow cytometry for platelet activation surface marker P-selectin was preformed to ensure that the increase in percent aggregation was due to an increase in the number of platelets being activated. Flow data demonstrates that both smoking a cigarette and pretreatment with 5-HT/catecholamine cocktail results in a greater mean of fluorescence for P-selectin, approximately >220 when compared to the nonsmoker group with a mean of fluorescence of 130 (Fig. 6). Together, this data suggests that 5-HT and catecholamine levels within the blood influence the magnitude at which platelets will aggregate together when stimulated with collagen.

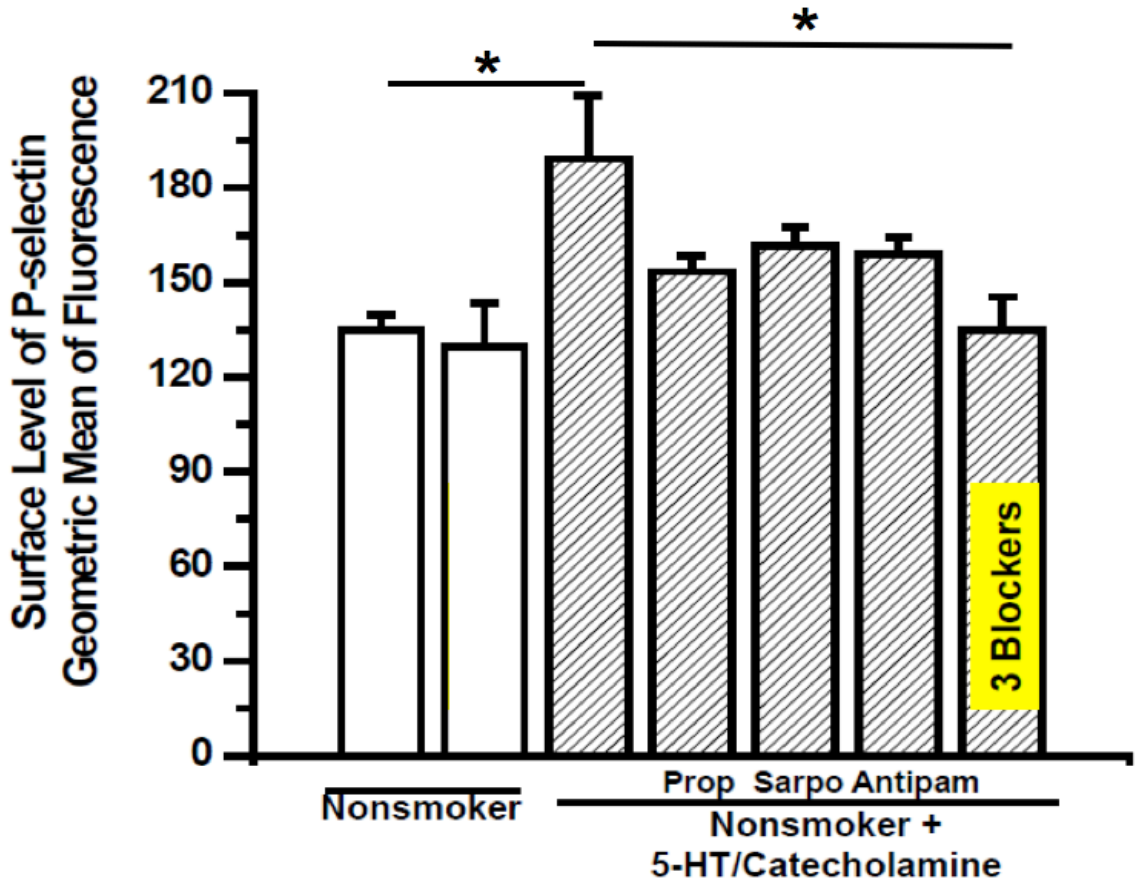


Figure 8. Surface P-selectin levels decreased in nonsmokers with cocktail and triple block when compared to nonsmokers with cocktail. Flow cytometry assay for surface protein P-selectin in isolated nonsmokers platelets pretreated with either an individual blocker or triple block in the presence of the 5-HT/catecholamine cocktail. P-selectin detected using FITC labeled P-selectin antibody. (*) indicate significance in differences in detectable levels of surface P-selectin (n=5) from two tailed t-test with p value <0.05.

Blocking receptors for either 5-HT, epinephrine, or norepinephrine on platelets reduces the effects of cocktail pretreatment of nonsmokers

Given our previous results, we next hypothesized that by blocking receptors for 5-HT and certain catecholamine on the platelets surface we could reduce the effect of the

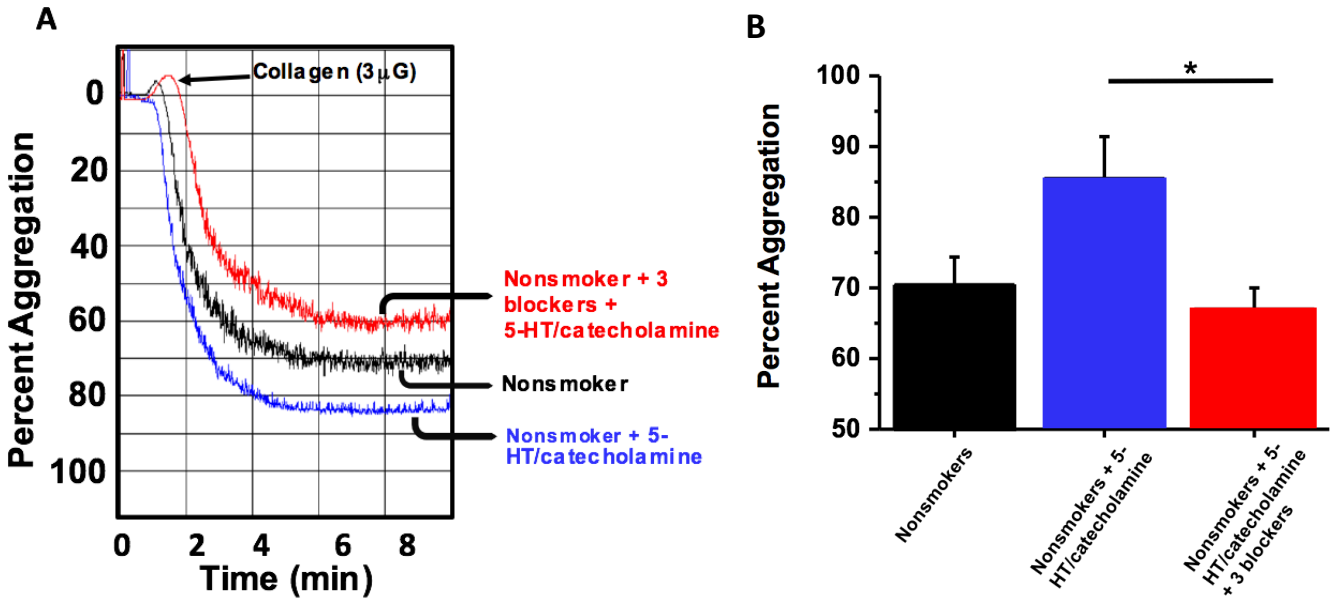


Figure 9. Blocking all 3 receptors at once reverses effect of cocktail on nonsmokers platelets. (A) Comparison of aggregation curves of isolated platelets from nonsmokers treated with 5-HT/catecholamine cocktail in the presence or absence of all three receptor blockers (Sarpogrelate, Antipamezole, and Propanolol). A control group of untreated platelets from nonsmokers was used for comparison. (B) Multiple aggregation curves generated using light-transmission aggregometer and averaged (n=5). (*) indicate significance in differences for average percent aggregation from two tailed t-test with p value <0.05.

cocktail pre-treatment. Specific receptors known to play a role in 5-HT and catecholamine platelet-platelet signaling were targeted and blocked using known receptor blockers (Fig. 3). The 5-HT receptor antagonist Sarpogrelate, α_2 adrenergic receptor blocker Antipamezole, or β adrenergic receptor blocker Propanolol were individually administered to the platelets of non-smokers during the 5-HT/catecholamine cocktail incubation period. After the 15-minute incubation, platelets were stimulated with collagen and percent aggregation was measured via light aggregometer. Without any blockers platelets pretreated with 5-HT and catecholamine cocktail had a percent aggregation of approximately 86% (Fig. 7A-B). Addition of any of the three receptor blockers reduced platelet percent aggregation to less than 77% (Fig. 7A-B). Mean of fluorescence for surface P-selectin was also reduced when individual blockers were administered to

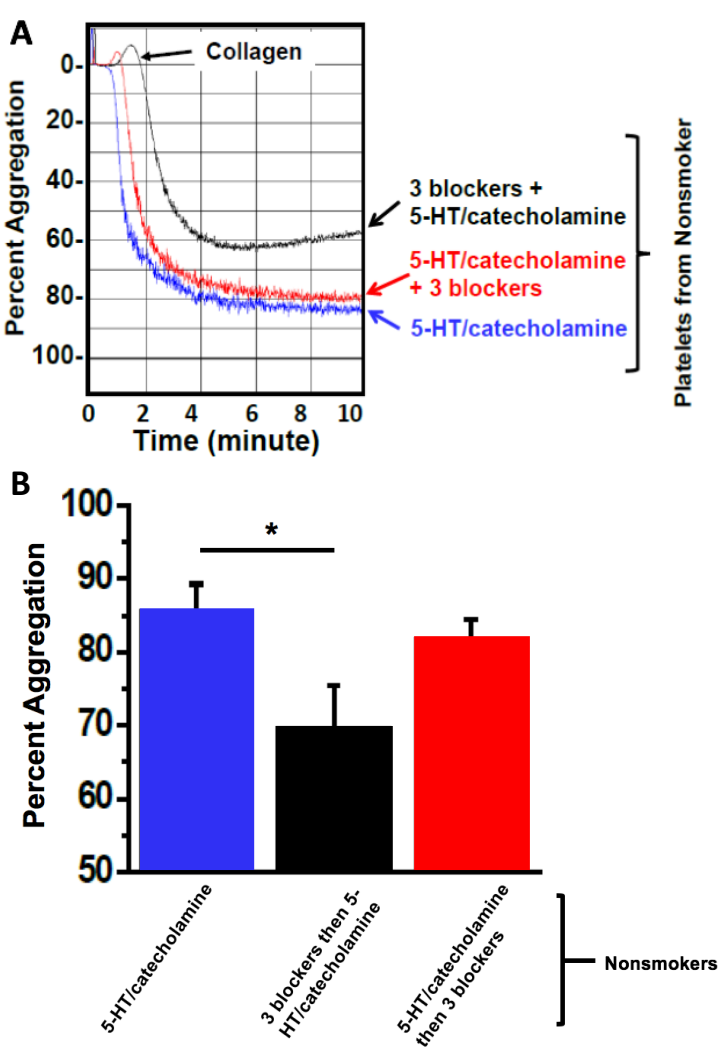


Figure 10. Efficacy of triple block dependent on time administered in relation to increased concentration of 5-HT and catecholamine. (A) Aggregation curves of nonsmokers platelets pretreated with either triple block first then cocktail (black) or cocktail first then triple block (red). (B) Multiple aggregation curves generated using light-transmission aggregometer and averaged (n=5). (*) indicate significance in differences for average percent aggregation from two tailed t-test with p value <0.05. (C) Mean of fluorescence of tagged surface protein P-selectin of all three groups. P-selectin detected using FITC labeled P-selectin antibody. (*) indicate significance in differences in detectable levels of surface P-selectin (n=5) from two tailed t-test with p value <0.05.

approximately <160 when compared to smokers after a cigarette and our nonsmokers with cocktail (Fig. 8). This data suggests and supports previous experiments that increased 5-HT and catecholamine levels in smokers could potentially increase the reactivity of platelets to pro-coagulant stimulants. This data also suggests that multiple receptors on the platelets are work independently for platelet-platelet communication during aggregation.

Addition of the triple block negated the effects of cocktail on nonsmokers platelet

Since blocking a single receptor reduced the effect of the cocktail, we next looked to see the effect that simultaneously blocking all three receptors had on platelet function and aggregation after stimulation. Non-smokers platelets were either pre-treated with the 5-HT/catecholamine cocktail alone or with simultaneous administration of all three receptor blockers. Ten minutes after stimulation, 84% of platelets from non-smokers pretreated with the cocktail alone had aggregated together (Fig. 9A-B). Non-smokers platelets pretreated with both the cocktail and the triple block had significant reduction in aggregation with only 60% of platelets aggregating (Fig. 9A-B). Mean of fluorescence for surface P-selectin for the triple block was also reduced to approximately 125 compared to the mean fluorescence of nonsmokers with the cocktail alone of >220 (Fig. 8). This data suggests that the blockage of all three receptors on the platelets greatly reduces the platelets ability to communicate between each other during the clotting process resulting in a reduction in the magnitude of the aggregation cascade.

Efficacy of triple block inhibiting the effect of the 5-HT/catecholamine cocktail treatment is time dependent

To further investigate the relationship between the receptors, the cocktail, and the blockers we examined the effect that time had on our triple blocks efficacy. To do so, we looked to see whether or not the triple block needed to be administered before the 5-HT/catecholamine cocktail to be effective in reversing its effects. Nonsmokers platelets were incubated with the the triple block then the cocktail or visa versa. After a 10-minute

incubation, our 5-HT/catecholamine cocktail was added to the first group and blockers to the second group. Each group was stimulated with collagen and aggregation was analyzed via aggregometer. Platelets that received the cocktail first and then the triple block had approximately an 80% aggregation (Fig. 10A-B). Platelets that received the triple block first followed by the cocktail had approximately a 60% aggregation (Fig. 10A-B). Flow cytometry data for surface P-selectin shows that addition of the cocktail before the triple block resulted in a mean of fluorescence of approximately 200 (Fig. 10C). Mean fluorescence for surface P-selectin of the platelets is approximately 125 (Fig. 10C). This data suggests that inhibition of these three receptors on platelets before exposure to the cocktail greatly reduce the ability for platelets to communicate with each other after stimulation. Contrarily, addition of the three inhibitors after the cocktail greatly reduces their effect on platelet-platelet communication.

DISCUSSION

Understanding the interactions between nicotine and platelet physiology is vital for the study of the life threatening consequences of smoking, specifically MI and IS. We examined the effects that elevated concentrations of 5-HT had on platelet sensitivity to clotting factors potentially found within the blood vessels. The data suggests that on average smoker's platelets aggregate at a higher magnitude when stimulated with collagen than nonsmokers. Incubation of nonsmoker's platelets with a 5-HT/catecholamine cocktail resulted in an increase in platelet sensitivity to collagen. We also showed that specific receptors on platelets independently stimulated the aggregation cascade of each other. Finally, we showed that when platelet-platelet communication is completely impeded, via triple block, we were able to reverse the effects of elevated 5-

HT and catecholamine. Together this data suggests, as previously hypothesized, that blood concentrations of 5-HT and other catecholamine have a direct effect on the sensitivity of platelets to pro-coagulant stimulants.

Hyperactivity of Smokers Platelets is Potential Link to Increased Risk for MI and IS

Our data show that smoker's platelets aggregate together, and are activated, at a higher rate than nonsmoker's. This hyperactive phenotype found in smokers may be a significant reason for the increased risk for MI and IS in smokers. Larger aggregation cascades will lead to larger and stronger clot formation within blood vessels increasing the probability of arterial occlusion. In both MI and IS patients there is also the problem of completely removing or "busting" the clot that is occluding the arteries. Thrombolysis is key to ensure that blood flow is able to return to the tissues of the occluded arteries (Alexandrov, A. V et al., 2017). Depending on the size and strength of the clot the time it takes to complete thrombolysis, either by the body itself or with medical intervention, can vary from minute to days (Alexandrov, A. V et al., 2001). Our data suggests that smokers will develop larger clots on average than nonsmokers, leading to more aggressive clot formation and increasing the chance of permanent damage to either cardiac muscle or cerebral tissue. Further investigation into the effects that varied plasma 5-HT concentration has on platelet sensitivity could reveal at which point smoking is significantly increases these risks.

Increased 5-HT Concentrations Induce Hyperactivity in Platelets from Nonsmokers

When nonsmoker's platelets were pre-treated with our 5-HT/catecholamine cocktail we were able to induce an aggregation cascade similar to that of the smoker's as

well as an increase in the total number of platelets activated. This data, along with previous research showing that smoking increases the blood concentrations 5-HT, suggest that blood concentrations of these important signaling molecules has an effect on platelet reactivity to clotting factors (Sugiura, T. et al., 2012). The data suggests that as blood concentrations of 5-HT and catecholamine used for platelet-platelet communication during an aggregation cascade are increased, the platelets become more prone to form larger clots when stimulated with a pro-coagulant. Studies show that smokers platelets contain an exponentially larger amount of stored 5-HT than nonsmokers, indicating that smoking is directly effecting the platelets physiology (Racké, K. et al., 1992). By exposing the platelets to increased levels of these signaling molecules, tobacco users are priming their platelets to be hypersensitive to any pro-coagulant that may be within the blood vessel. Future research into elevated 5-HT/catecholamine concentrations could be done on patients who previously experienced MI or IS to see if they too had elevated plasma concentration of 5-HT/catecholamine. If so this information could be used as a predictor of such events.

Blockage of Individual Receptors Reduces Effect of Elevated 5-HT Concentrations

Blocking individual receptors on nonsmoker's platelets for signaling molecules used during the aggregation cascade revealed that our cocktail was in fact stimulating this change in nonsmoker's platelets via the pathway we proposed (Fig. 2). When one of the three key receptors on the surface of the platelets was blocked we observed a decrease in magnitude of the aggregation cascade. The observed decrease of the cocktails efficacy to induce larger aggregation cascades in nonsmoker's platelets suggests that our cocktail was in fact inducing the previously observed change.

Interestingly, we do not see a complete negation of the cocktails effect when we block an individual receptor and we do not observe a complete decrease in the total number of platelets activated after stimulation. This suggests that although each receptor is working to achieve the same goal of platelet aggregation, the mechanisms are independent of each other. Further research into each downstream pathway is needed to fully understand at which point each individual receptor converges. Looking at intracellular calcium levels, an early indicator of activated platelets, and transglutaminase activity, a late stage indicator of activated platelets, could reveal how each receptor is propagating the aggregation cascade (Baldissera, L. et al., 2010) (Chung, S. I. et al., 1974). We hypothesize that each receptor independently works to induce the same mechanism used to shuttle and expel α and dense granules into the plasma. This redundancy could be useful for rapid and local induction of an aggregation cascade at an injury site. Beta-blockers (BBs) are a popular medication prescribed to people who suffer from hypertension, heart disease, MI, and IS but have been shown to be less effective than previously thought in many cases (DiNicolantonio, J. J. et al., 2015). This ineffectiveness of BBs on reducing the mortality of the patients it is prescribed to may be due the redundancy in receptor function on platelets for the aggregation cascade.

Triple Block Completely Reverses Effect of Elevated 5-HT Concentrations

When all three blockers were administered with the cocktail we observed a dramatic decrease in the size of the aggregation cascade. This data suggests that when we block all three receptors used for platelet-platelet communication we are able to effectively reduce the magnitude of the aggregation cascade induce by the addition of collagen. Addition of the three blockers also reduced the total number of platelets

activated to a level similar of nonsmoker's platelets. Blockage of these three receptors indicates that platelet-platelet communication is vital for triggering an aggregation cascade. It also suggests that although platelet-platelet communication was blocked, stimulation with pro-coagulants can still trigger an aggregation response. This may be due to the components of the α-granules, which release a handful of pro-coagulants as well. Stepping back this data suggests that the molecular signals used in an aggregation cascade are not as simple as three molecules but potentially several different molecules all stimulating a similar response.

Clinical Significance of Triple Block in Smokers

In order to test the potential for this triple block to be used in smokers to reduce their chances of MI and IS we looked to see if its effects were time dependent to the elevation of 5-HT/catecholamine concentrations. We observed that when we add our triple block first and allowed the platelets to incubate and then added the cocktail that we produced similar result to the previous experiments. Surprisingly, addition of the triple block after incubating the nonsmoker's platelets in the cocktail reduced the ability of the triple block to decrease the magnitude of the aggregation cascade. We also observed that there were more activated platelets when the triple block was added after the cocktail compared to when the triple block was administered first. Together this data suggests that the time of administration of the triple block influenced the capability of the triple block to reduce the magnitude of the platelet aggregation cascade when 5-HT/catecholamine levels were elevated. Clinical application of the triple block could still be viable since the average platelet is recirculated every 8-10 days (Machlus, K. R. et al., 2013). Therefore, a new platelet could be exposed to a triple block before it comes in contact with elevated

5-HT/catecholamine levels within the blood if smokers were to monitor and regulate smoking and administration of the triple block.

This data, as a whole, suggests that elevated 5-HT and catecholamine concentrations induced by tobacco products are responsible for the increased risk of spontaneous clot formation that leads to the occultation of blood vessels in the heart or brain. Our research focused heavily on the pro-coagulants found within dense granules but further investigations into the contents of α-granules could reveal additional interaction between contents of the blood and platelet sensitivity. α-granules contain more pro-coagulants such as the previously mentioned von Willebrand factor (vWf) an extremely important molecule for clot formation (Blair, P. & Flaumenhaft, R., 2009). The contents of α-granules have also been shown to play a critical role in inflammation within the blood vessel that results in atherosclerosis and may be another component for the increased risk in MI and IS (May, A. E. et al., 2008). Future research needs to be done on smoker's platelets with the triple block to properly determine it would be a viable clinically. Further investigation into how to different concentrations of 5-HT effect the magnitude of the aggregation cascade would also be useful for predicting MI and IS in high risk patients. Development of an assay that can proactively warn high risk patients of an upcoming heart attack or stroke is a potential goal of further research. One of the most important ways to prevent permanent damage for both of these conditions is to reduce the amount of time that the tissues are not receiving oxygenated blood. With this assay we may be able to proactively reduce the mortality rates of these conditions. With this data we have a better understanding of how dynamic platelet-platelet communication is within the blood. MI and IS are life threatening conditions that do not only occur in

smokers and are still not fully predictable or treatable. Currently the best solution for smokers to reduce their risk for both MI and IS is still the cessation of smoking. We hope that this research can be used to enlighten and educates the public not just of the end results and risks of smoking but the mechanisms behind why it is such a detrimental life threat.

MATERIALS & METHODS

Subject Blood Collection: Blood samples were obtained from healthy 20-30 year old male nonsmoking volunteers and from healthy 20-30 year old male daily cigarette smokers volunteers. The study was approved by the University of Arkansas for Medical Sciences (UAMS) IRB. The health status of subjects was determined by their own physicians. Twenty-five ml peripheral venous blood was drawn into a heparin-containing tube by venipuncture from an antecubital vein.

Platelet Isolation: Platelet rich plasma (PRP) was prepared by adding 1/2 volume of Tyrode's HEPES buffer to blood and centrifugation of blood at 1.0 $X10^3$ rpm for 10 minutes. Supernatant plasma containing platelets transferred and spun and 1.0 $x10^6$ for 10 minutes. Platelet poor plasma (PPP) removed leaving remaining PRP for analysis.

Aggregation Assay: For aggregation assays, platelets in plasma were prepared by spinning out and separating red blood cells from the plasma in a Hettich tabletop centrifuge. Platelet counts were normalized (300,000/μL) using a Hemavet 950 (Drew Scientific, Waterbury, CT). The response to collagen (3 μg/ml) as a platelet agonist was monitored by light transmittance (Chrono-log Corp., Havertown, PA).

Flow Cytometry: The level of platelet activation was assessed using FITC labeled P-selectin antibody (Ab) (BD Pharmingen, Cat 553744). Platelets (300,000/μL) were incubated in Ab and at the end of the incubation, 300 μL of 2% formaldehyde in PBS was added to stop the reaction. Samples were gated for single platelets based on forward and side scatter profiles and 20,000 events were recorded and read at the UAMS Flow Cytometry Core Facility with a BD LSRFortessa™.

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