THE ROLE OF FREE FATTY ACIDS AS THE FINAL ARBITER IN DETERMINING THE MAGNITUDE OF CLOPIDOGREL RESISTANCE

The use of customized relational databases with the capacity to rapidly mine data and generate new teachings for the anti-platelet drug clopidogrel

PRELIMINARY DRAFT

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SUMMARY AND OVERVIEW

Clopidogrel is a second-generation thienopyridine that inhibits platelet aggregation. When launched as the branded drug Plavix (Bristol-Myers Squibb/Sanofi) its use had a major impact upon the management of patients with acute coronary syndromes (ACS), cerebrovascular accident (stroke), transient ischaemic attack (TIA) and in patients undergoing percutaneous coronary interventions (PCI). Clopidogrel is used in order to prevent the formation of a thrombus (clot) in vulnerable patients. However, a significant proportion of patients who receive clopidogrel remain at risk for subsequent death, myocardial infarction (MI), stent thrombosis, and stroke because of insufficient clopidogrel-induced platelet inhibition.

Clopidogrel is a prodrug and consequently before it can exert its anti-platelet activity, it must be metabolized by the liver into its active form. The mechanism that has generally been accepted for the conversion of clopidogrel into a biologically-active thiol metabolite is a two-step cytochrome P450 (CYP450) enzyme-dependent process. CYP2C19 has been reported to occupy a key role in catalyzing both steps of this bioactivation. Once formed, the active metabolite of clopidogrel inhibits platelet aggregation by irreversibly blocking the binding of ADP to the platelet P2Y12 receptor. A high variability of response to clopidogrel as measured by platelet function tests has been reported among patients who have undergone percutaneous coronary intervention (PCI), and patients who have been identified as low responders appear to be at higher risk for recurrent cardiovascular events. A common genetic variant within the CYP2C19 gene, the CYP2C19*2 loss-of-function polymorphism, has been shown to be associated with an attenuated response to clopidogrel and a worse clinical outcome in patients undergoing coronary stenting.

However, in one analysis the CYP2C19*2 genotype was able to explain only 12% of the variation in response to clopidogrel, and this was in a uniquely homogenous and healthy Caucasian-derived North American population taking no other medications. The same investigators also found that although increased age, body mass index (BMI), triglyceride levels and decreased levels of high-density lipoprotein (HDL) cholesterol are predictors of poorer clopidogrel response, when these factors are combined they account for less than 10% of the variation.

These observations, together with other analyses support an emerging consensus for the existence of other more significant undiscovered factors that explain the variation in clopidogrel response; and, given that CYP2C19, CYP3A4 and paraoxonase-1 (PON-1) loss-of function alleles do not enable an adequate explanation, then an independent reappraisal

and review of this serious problem is warranted. Following a near-exhaustive analysis of the available literature using customized relational databases with the capacity to rapidly mine data (see www.mccormackpharma.com for details of these methods), this document presents the role of free fatty acids as the final arbiter in determining the magnitude of clopidogrel resistance.

By way of introduction, the terms “fats” and “fatty acids” are often used interchangeably. However, fatty acids are actually subunits of fats. Most of the common fats that we eat and which are stored in our bodies exist as a combination of a fatty acid linked to glycerol. Thus, most people have encountered the term monoglyceride which refers to a fat molecule that consists of one fatty acid plus glycerol. Two fatty acids plus glycerol or three fatty acids plus glycerol are described as diglyceride and triglyceride, respectively. In reality, it is triglycerides that constitute the major glyceride in both food and our body. Fatty acids are also incorporated in all cell membranes as compounds known as phospholipids.

When fats are broken down by a metabolic reaction that separates the fatty acid from glycerol, in a physiological context the fatty acid is known as a “free fatty acid”. The concentration of free fatty acid that circulates within plasma is in a dynamic equilibrium with albumin which is the major plasma protein. Indeed, within plasma the level of unbound free fatty acid is tightly regulated, and greater than 99.9% of free fatty acids are non-covalently bound to plasma proteins which means that albumin acts as a reservoir for the transfer of free fatty acids into cells. Moreover, transport of free fatty acids is tightly regulated not just within plasma but also within cells where they are translocated throughout the cytosol by proteins such as fatty acid-binding proteins.

Free fatty acids have important roles not only as energy-storing molecules but also as messengers and mediators in regulating numerous cellular functions. Regulation of the concentration of free fatty acids is critical in order to ensure optimum functioning of cells. Importantly, when free fatty acid levels are too high then these molecules can be extremely toxic. Indeed, as this document shows, while free fatty acids have numerous roles in regulating normal platelet function, when levels of free fatty acids exceed a certain threshold, platelets can become dysfunctional and display an increased tendency to aggregate. In such a context, free fatty acids become pro-aggregatory. Moreover, this document shows that because free fatty acids mediate and modulate numerous signalling pathways within the platelet, when the level of free fatty acids is too high then the effects of treatment with anti-platelet drugs, notably clopidogrel may be severely compromised. Importantly,

in some patients, even normal levels of free fatty acids have the potential to interact with the anti-platelet action of clopidogrel.

Because the effects of free fatty acids upon several signalling processes within platelets are reversible, then in the presence of high concentrations of free fatty acids the law of mass action dictates that the dynamic equilibrium is shifted in a direction that tends to reduce the "pressure" from the high concentration of free fatty acids. And, as I show, this invariably renders the platelet hyper-responsive to the effects of agonists such as ADP, and resistant to the effects of clopidogrel, especially in patients with CYP2C19 loss-of-function polymorphisms.

(Interestingly, and while beyond the scope of this document, there are grounds to believe that the pro-aggregatory effects of free fatty acids may represent a conserved adaptive mechanism from the time when the human species was challenged by the harsh environment of the Ice Ages. During that period it has been argued that vitamin C deficiency (scurvy) may have decimated the human species largely as the result of internal haemorrhage. In the absence of adequate amounts of vitamin C, humans cannot adequately synthesize collagen and elastin, and as result blood vessels become fragile and leak. Some humans may have adapted through the acquisition of free fatty acid-dependent mechanisms that augmented the responsiveness of platelets to environmental and physiological stimuli with an enhanced tendency to aggregate and accumulate at the sites of internal bleeding thereby enabling leaks to be plugged.)

Over Forty years before I started this project, Professor John C Hoak and coworkers at the University of Iowa College of Medicine (Nature 1970 228(5278) 1330-1332) undertook an investigation in order to determine whether exposure of human platelets to albumin-bound FAAs, in concentrations that are elevated but which can occur in physiological conditions, might influence platelet function. They studied the influence of the molar ratio of free fatty acids to albumin upon the response of platelets to ADP. They observed that with an increasing free fatty acid to albumin molar ratio, the degree and rate of aggregation induced by a standard amount of ADP increased. From their observations they conclude…

"We interpret these results to indicate that exposure to a high concentration of fatty acid may alter the reactivity of the platelet and thereby enhance its responsiveness to ADP on (sic) to other agents which influence platelet function in vivo. The principal effect of an increase in the FFA/albumin molar ratio was to increase the FFA content of the platelet. A dynamic equilibrium state may exist between the binding of FFA by albumin and the uptake of FFA by platelets and

other cellular elements. Acute lipid mobilization causing high plasma FFA concentrations or disease states in which the albumin content or FFA binding capacity are reduced might produce a transient state in which excessive amounts of FFA associate with platelets. In this condition the platelet may develop a propensity toward aggregation which could lead to the formation of thrombi."

Starting in 2011, I undertook to explain the basis for these early observations by Hoak and his coworkers. My methods included the use of customised relational database-searching algorithms that have the capacity to rapidly mine data and identify previously-unknown relationships (further details of these methods are available at www.mccormackpharma.com). Using these proprietary tools I explored the relationships between free fatty acids and platelet reactivity and aggregation, and attempted to understand how such relationships could impact upon the actions of clopidogrel as a P2Y12 receptor antagonist. At that time, the primary objective of this project was to provide healthcare professionals and all stakeholders with new teachings about clopidogrel and clopidogrel resistance that would enable enhanced patient management. And as a commercially-driven project these new teachings from McCormack Pharma would result in new revenue for the client by endorsing the client's commitment to patient welfare and medical education, especially within those countries where clopidogrel was available or becoming available as a non-patented generic formulation.

During McCormack Pharma's preliminary data selection (a fundamental prerequisite prior to running the relational searches) an important clue to the relationship between free fatty acids and clopiogrel resistance was provided by the pioneering and systematic studies of Gianni Guidetti and coworkers at the University of Pavia in Italy. Using human platelets they provided the breakthrough discovery that concomitant stimulation of the P2Y12 receptor through the addition of ADP in the presence of the selective P2Y1 receptor antagonist MRS2179 (in order to observe the effects of ADP upon the P2Y12 receptor without confounding from any ADP-bound P2Y1 receptors) significantly potentiated the effect of the diacyglycerol (DAG) analogue sn-1,2-oleodioctanoylglycerol (DiC8) on platelet aggregation. They also observed that pharmacological inhibition of the enzyme DAG kinase (DAGK) restores the agonist-induced phosphorylation of protein kinase C (PKC) substrates prevented by antagonists of the P2Y12 receptor and, in parallel, allows platelets to undergo aggregation. Most importantly, even P2Y12 antagonist-promoted inhibition of Rap1 activation was overcome by pharmacological inhibition of DAGK. These observations are critically important because they show for the first time that signalling by the ADP-stimulated P2Y12 receptor impacts upon, and converges with

signalling within the PKC-DAG-DAGK axis, and that in this way it is possible, albeit under laboratory conditions to overcome pharmacological blockade of the P2Y12 receptor.

We know that DAGK is a signalling regulator that switches off the platelet-activating effects of the messenger molecule DAG by phosphorylating it. What is new however is the observation by Guidetti and coworkers that by using the cell-permeable DAGK inhibitor R59949 (3-{2-[4-(bis-(4-fluorophenyl)methylene]piperidin-1-yl)ethyl}-2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone) blockade of the P2Y12 receptor pool by P2Y12 receptor antagonists can be bypassed/surmounted and that platelet aggregation can be restored.

Why are these findings so important to the research that I first started in 2011?

As I report within this document, some of the endogenous actions of free fatty acids mimic the laboratory interventions of Guidetti and coworkers. That is, above a certain concentration threshold it is not unreasonable to propose that by mimicking the experimental paradigm of Guidetti and coworkers, free fatty acids can overcome the effects of P2Y12 receptor antagonists.

Excitingly, the findings by Guidetti and coworkers reveal the intriguing prospect that even at normal physiological concentrations of free fatty acids, an increase in loading dose of clopidogrel may be of limited value in patients with loss-of-function CYP2C19 alleles. This is because in addition to their finding that inhibition of DAGK can restore platelet aggregation, they also show that stimulation of the P2Y12 receptor by ADP potentiated phosphorylation of pleckstrin (the main platelet protein substrate for PKC) induced by exogenous DiC8 at all the doses analysed. And, using autoradiography combined with densiometric analysis they observed the concomitant inhibition of DiC8 conversion to the inactive phosphorylated form DiC8-PA in intact platelets upon stimulation with ADP. This effect of ADP in ^{32}P-labeled cells was dose-dependent and was prevented by a P2Y12 receptor antagonist but not by the P2Y1 receptor antagonist MRS2179, indicating that it was mediated by activation of the P2Y12 receptor. Restating these outcomes it appears that by an unknown mechanism, signalling by ADP-occupied P2Y12 receptors inhibits the activity of DAGK or exerts some control over the PKC-DAG-DAGK axis by limiting the conversion of endogenous DAG to the inactive phosphorylated form of DAG. The result is an accumulation of the signalling molecule DAG. Given that free fatty acids also regulate DAGK activity, that they induce the accumulation of DAG and potentiate the effects of DAG in activating platelets, then there is the prospect for a significant interaction between agonist-stimulated P2Y12 receptors and

free fatty acids. That is, in those patients with loss-of-function CYP2C19 alleles it is likely that even a normal physiological level of FFAs will amplify the output from unblocked, but ADP-occupied functional P2Y12 receptors and diminish the effects of an increase in loading dose of clopidogrel (that may be administered following CYP2C19 genetic testing), unless the increased loading dose achieved 100% blockade of the P2Y12 receptor pool.

The findings by Helena Haberstock-Debic and coworkers (J Pharmacol Exp Ther 2011 339(1) 54-61) are equally exciting. These workers show that an inducible pool of P2Y12 receptors exists on human platelets and that internalized P2Y12 receptors can be mobilized to the platelet surface after strong agonist stimulation. Since, as this document demonstrates, FFAs certainly qualify as "strong agonists" albeit by numerous non-receptor-mediated pathways, then the induction of an inducible pool of P2Y12 receptors may compromise even further the capacity of clopidogrel to adequately inhibit surface P2Y12 receptors especially in patients with loss-of-function CYP2C19 alleles.

Whether the role of FFAs as final arbiter in determining the magnitude of clopidogrel resistance may have evolved as a failsafe mechanism that exists as a means to maintain signalling fidelity in platelet activation is an interesting speculation that has support throughout this document.

Indeed, the regulatory role of free fatty acids within platelets is both ubiquitous and contiguous, and that it is through this document that numerous points of control and convergence become apparent. For example, the gene RASGRP2 codes for a major signalling molecule in platelets, calcium-and-DAG-regulated guanine exchange factor-1 (CalDAG-GEFI). CalDAG-GEFI, also known as RASGRP2 is a guanine nucleotide exchange factor (GEF) that is critical for Ras-like GTPase activation whose target is mainly Rap1 in platelets. Rap1 is one of the most predominant small GTPases in platelets and constitutes a key signalling element that governs platelet activation by directly regulating integrin-mediated aggregation and granule secretion. But there is much about the regulation of CalDAG-GEFI in the platelet that remains unclear. CalDAG-GEFI is found preferentially in the cytoplasm and translocates toward juxtamembrane areas upon cellular stimulation. But the evidence does not support the repeated assertions that now constitute a category error, that DAG regulates RasGRP2 as it does RasGRP1. In fact, the C1 domain sequence of CalDAG-GEFI is atypical and shows only weak affinity for DAG. Consequently, treatment of cells with DAG mimetics has only little effect on the subcellular localization and the activity of CalDAG-GEFI. By contrast, it is well established that CalDAG-GEFI activates Rap1 in response to calcium ionophores. So how

is CalDAG-GEFI translocated within the platelet cytoplasm? Interestingly, it has been proposed that the direct association of the N-terminal domain of CalDAG-GEF1 with F-actin regulates the subcellular localization of CalDAG-GEFI.

Interestingly, our methods reveal that the F-actin-dependent translocation of CalDAG-GEFI is regulated in a concentration-dependent fashion by free fatty acids. The corollary of this new synthesis is that by yet another signalling mechanism, above some concentration threshold free fatty acids may drive the platelet into a hyper-responsive state that surmounts the effects of P2Y12 receptor antagonists.

And so much more.....

PART I

THE ASSOCIATION BETWEEN FREE FATTY ACIDS AND CORONARY ARTERY DISEASE

What are fatty acids?

A fatty acid is a carboxylic acid with a long unbranched aliphatic tail, which is either saturated or unsaturated. Fatty acids without double bonds are known as saturated. Fatty acids that have double bonds are known as unsaturated. The two carbon atoms in the chain that are bound next to either side of the double bond can occur in a *cis* or *trans* configuration. A *cis* configuration means that adjacent hydrogen atoms are on the same side of the double bond. A *trans* configuration, by contrast, means that these hydrogen atoms are bound to *opposite* sides of the double bond. For naturally-occurring FFAs the geometry of the double bond in unsaturated fatty acids is invariably in the *cis* configuration.

What are non-esterified (free) fatty acids?

Non-esterified (unesterified) fatty acids (NEFAs), or free fatty acids (FFAs) represents the fraction of plasma fatty acids that are not esterified to glycerol. They are generally toxic to cells and are maintained at low nanomolar or micromolar concentrations in plasma; these levels can vary greatly depending on the hormonal, metabolic, and nutritional state of the individual. It is generally accepted that approximately 99.9% of FFAs are carried in plasma bound to proteins (unbound FFAs

constitute approximately 0.1% of the total concentration), mainly albumin, which has some 5 to 8 or more binding sites per molecule ranging from high to low affinity. It should be noted however, that some workers have challenged the above estimate of the extent FFAs binding to plasma proteins, claiming that the unbound fraction of FFAs in plasma has been overestimated by an order of magnitude in several studies and that a more realistic estimate is within the region of 0.1-0.01% (Clin Chim Acta 1989 186(1) 31-8). The low concentration of FFAs is maintained in plasma by the competition between the affinity of albumin-binding sites and cell membrane phospholipids for FFAs.

Examples of unsaturated FFAs include palmitoleic acid, oleic acid, elaidic acid, linoleic acid, linoeladic acid, arachidonic acid, eicosapentaenoic acid, docosahexanoic acid, and vaccenic acid. Examples of saturated FFAs include lauric acid, myristic acid, palmitic acid, stearic acid and capric acid. When FFAs occur in the salt form the suffix “ate” is used instead of “ic” and the descriptor “acid” no longer applies (eg sodium stearate, or sometimes just “stearate” is the sodium salt of stearic acid).

Circulating FFAs and coronary heart disease

Elevated FFAs independently predict all-cause and cardiovascular mortality in patients with coronary artery disease

Circulating FFAs mainly originate from lipolysis in the adipose tissue. They contribute to insulin resistance and are elevated in obesity and type 2 diabetes. Recent studies suggest that FFAs also exert negative effects on the vessel wall by triggering endothelial apoptosis and impairing endothelium-dependent vasodilation. The involvement of FFAs in atherosclerosis is supported by observations of an increased risk for cardiovascular disease associated with high levels of FFAs (Arterioscler Thromb Vasc Biol 2000 20(6) 1588-1594; Arteriosclerosis 2002 160(2) 377-384). Elevation of FFAs as observed in myocardial ischemia has been shown to increase the ischemic damage of the myocardium and to be proarrhythmic.

The negative clinical outcome associated with elevated FFAs in MI might be attributed to metabolic alterations like accumulation of toxic intermediates, suppression of glucose use, or mitochondrial dysfunctions. In addition, there are also studies suggesting that altered cardiac FFAs metabolism, especially increased myocardial FFAs uptake, might be related to the development of heart failure. Despite this compelling evidence for a pivotal role of FFAs in cardiovascular disease,

clinical studies addressing the relationship between FFAs and mortality are sparse.

Given the lack of data defining the association between circulating FFAs and cardiovascular mortality, Pilz et al (J Clin Endocrinol Metab 2006 91(7) 2542-2547) undertook an investigation in order to elucidate the relationship between FFAs and mortality in subjects with and without angiographic proven coronary artery disease (CAD).

They showed that in 3,315 study participants who had undergone coronary angiography that plasma levels of FFAs independently predict all-cause and cardiovascular mortality.

Compared with subjects in the lowest quartile (<440 μM) of FFAs, the unadjusted hazard ratios for death at FFA concentrations in the second (440-620 μM), third (630-890 μM), and fourth (>890 μM) quartiles were 1.30 ($P = 0.076$), 1.63 ($P < 0.001$), and 2.75 ($P < 0.001$), respectively. Inclusion of age and gender as covariables decreased these estimates only moderately to 1.14 ($P = 0.393$), 1.36 ($P = 0.036$), and 2.23 ($P < 0.001$), respectively. Thus, FFAs retained their prognostic importance after adjusting for established and emerging cardiovascular risk factors.

Elevated plasma FFAs predict sudden death in patients referred for coronary angiography

Sudden cardiac death (SCD) accounts for approximately 50% of all fatal coronary heart disease events. Patients die unexpectedly and shortly after the onset of a change in clinical status that usually begins with an abrupt loss of consciousness. Ventricular tachyarrhythmias are most often recorded in subjects with SCD but the underlying pathological processes that lead to an electrically unstable myocardium remain to be further clarified. Epidemiological studies have shown that the incidence of SCD is highest in patients already afflicted with CAD but every second SCD occurs in persons of relatively low cardiovascular risk (Eur Heart J 2006 27(17) 2099-2140).

There is growing evidence that FFAs contribute to SCD, and Pilz et al (Eur Heart J 2006 28(22) 2763-2769) in building upon an early study (J Clin Endocrinol Metab 2006 91(7) 2542-2547) examined whether fasting FFA levels predict SCD in 3,315 patients who had undergone coronary angiography.

These workers show that high concentrations of fasting blood FFAs are an independent risk factor for SCD in patients scheduled for coronary angiography. Furthermore, after exclusion of patients with SCD, as

presented above (Eur Heart J 2006 28(22) 2763-2769) high levels of FFAs remained predictive for all-cause and cardiovascular mortality.

These results highlight elevated FFAs as a marker for the identification of subjects at high risk for SCD and other fatal cardiovascular events.

Prognostic value of FFAs in patients with stable heart disease

More recently, Breitling et al (Am J Cardiol 2011 108(4) 508-13) assessed whether serum levels of FFAs are associated with long-term prognosis in subjects with stable coronary heart disease. This observational prospective cohort study included 1,206 participants undergoing inpatient rehabilitation programs after acute MI, acute coronary syndrome, or coronary intervention. Eight-year prognosis (time to a secondary fatal or nonfatal cardiovascular disease event including MI and stroke and time to death from any cause) was examined according to serum FFA quartiles. FFA levels were correlated with established serum markers of cardiovascular risk and strongly related to secondary cardiovascular events and all-cause mortality in age- and gender-adjusted analysis. From this analysis, the investigators conclude that FFAs are closely correlated with cardiovascular risk markers, and in particular, very high levels of FFAs might identify patients with stable coronary heart disease with worse prognoses.

Is there a causal role for FFAs in the pathogenesis of CAD?

Whether high FFAs are a causal factor in the pathogenesis of CAD or whether they are elevated in response to other pathological processes that contribute to fatal cardiovascular events remains to be determined.

Several studies support the hypothesis that FFAs increase cardiac sympathetic activity and are related to ventricular tachyarrhythmias that are the final common event in SCD. FFAs correlate with the frequency of ventricular premature complexes, a risk factor for SCD, and are associated with ventricular fibrillation in subjects with MI. Pro-arrhythmic features of FFAs that may explain their close association with SCD involve damaging of membranes, increases in reactive oxygen species (ROS), and disturbances of ion channels with subsequent instability of the membrane potential of the cardiomyocytes.

A causal role of FFAs in the pathogenesis of SCD is further supported by the fact that FFAs are associated with heart failure that is a significant risk factor for SCD (Eur Heart J 2006 27(17) 2099-2140; EMBO 2005 6(10) 917-921; Lancet 2004 364(9447) 1733-1734). It has been reasoned that FFA concentrations are elevated in patients with heart failure

because lipolysis is stimulated by high levels of catecholamines in these patients (QJM 2006 99(10) 701-709; Lancet 2004 364(9447) 1733-1734).

Furthermore, it was demonstrated that high concentrations of FFAs worsen ventricular function, partially mediated through an increase in mitochondrial uncoupling proteins that impair mitochondrial energy production (see EMBO 2005 6(10) 917-921 for review of the emerging awareness of "uncoupling proteins"). In addition, intralipid/heparin infusions that increase FFA concentrations are associated with raised catecholamine levels and enhanced adrenoreceptor reactivity, therefore suggesting a stimulatory effect of FFAs on the sympathetic nervous system.

Thus, the hypothesis has been proposed of a vicious metabolic cycle, in which catecholamines and natriuretic peptides stimulate an increase in levels of FFAs that causes myocardial dysfunction and an elevation of lipolytic substances (Lancet 2004 364(9447) 1733-1734; J Am Coll Cardiol 2011 58(11) 1119-1125). This relationship between high concentrations of FFAs and heart failure may partially explain the increased risk for SCD in patients with reduced LV function (Eur Heart J 2006 27(17) 2099-2140).

Myocardial infarction, platelet aggregation and FFAs

During MI the concentration of FFAs in plasma is elevated (Lancet 1966 2(7455) 122-7; Lancet 1974 1(7852) 284-89). Following a pilot study in fasting normal human volunteers, Gjesdal et al (Scand J Haematol 1976 17(3) 205-212) undertook an investigation into a possible relationship between platelet aggregation and plasma FFA concentration during MI in patients with suspected MI who were admitted to a coronary care unit. None of these patients had taken aspirin, beta-adrenergic blocking drugs, dipyridamole or oral anticoagulants prior to admission. Hospital employees were used as controls.

In the acute MI group the concentration of plasma FFA (mM) was significantly higher at admission (1.058+/- 0.334 at 3 hours) by comparison with values obtained at 16 hours (0.752+/- 0.297 $p<0.05$) and 24 hours (0.798+/-0.283 $p<0.01$). The percentage of venous reversible platelet aggregates was higher than normal during the first hours of acute MI, and a similar pattern was also found within a subgroup of patients with severe angina pectoris without ECG changes but with an established history of CAD.

In reviewing the findings from patients with MI and severe angina, these investigators propose that in some part, based upon the earlier findings

of Hoak et al (Nature 1970 228(5278) 1330-1332) (presented below) FFAs may reduce the threshold for ADP-induced aggregation or induce aggregation directly.

Circulating FFAs and coronary artery disease

Concluding remarks

The truncated evidence base presented above suggests that there is a greater probability that circulating plasma levels of FFAs will be higher in patients with acute coronary syndrome (ACS) notably CAD, by comparison with levels in healthy individuals. The implications of these findings are several fold: Should plasma levels of FFAs be measured routinely in patients with ACS? How reliable are such measurements given that day-to-day measurements of plasma levels of FAAs show high variability (Eur Heart J 2006 28(22) 2763-2769). What therapies are available for reducing elevated levels of plasma FFAs? Importantly, what are the implications of elevated plasma levels of FFAs upon the management of patients with ACS?

In closing, a more complete discussion on the association between FFAs and ACS is beyond the scope of this document given that the focus of this document is upon the relationship between FFAs and clopidogrel resistance.

PART II

FREE FATTY ACIDS MODULATE THE ACTIVITY OF HUMAN PLATELETS

Plasma FFAs and platelet activity

Normal volunteers

It has been known for some time that when saturated FFAs are added to platelet-rich plasma or to washed platelets that platelet aggregation occurs (Nature 1963 200(Dec 28th) 1331-2; Nature 1964 202(May 23rd) 765-8). Similarly, early workers also observed that intravenous injection of FFAs into dogs precipitated thrombosis (J Clin Invest 1963 42(June) 860-6).

In humans, increases in plasma concentration of FFAs occur during different states and disorders. An elevated concentration of FFAs is observed in patients with ACS (Q J Med 2011 104(7) 625-627), notably those with acute MI (Lancet 1966 2(7455) 122-7), whereas inhibition of lipolysis during experimental coronary artery occlusion is associated with reduced infarct size (J Clin Invest 1973 52(7) 1770-8). An elevation in plasma concentration of FFAs is also observed in obesity (Circ Cardiovasc Imaging 2012 5(3) 367-375), diabetes mellitus (Nature Clinical Practice Endocrinology & Metabolism 2009 5(3) 150-159), hypertension (Int J Epidemiol 1998 27(5) 808-813) and percutaneous coronary intervention (PCI) (J Invasive Cardiol 2008 20(4) 186-188). Platelet aggregation was studied following intravenous injection of heparin to nine healthy adults. Heparin is known to produce an increase in the concentration of free fatty acids (FFA) within 10 minutes of intravenous injection. A significant correlation was found between the intensity of primary platelet aggregation and changes in plasma levels of FFAs (Am J Hematol 1978 4(1) 17-22). Administration of ACTH will also result in an increase in plasma levels of FFAs following mobilization from adipose tissue (J Lipid Res 1996 37(2) 290-298).

Following the observations of earlier workers that during fasting the plasma FFA concentration rises (J Clin Invest 1956 35(2) 150-154) (in that study plasma FFA concentrations covaried directly with obesity); J Clin Invest 1956 35(2) 206-12), Gjesdal et al (Thrombos Haemostas (Stuttg) 1976 36(2) 325-331) investigated the effects of a fasting-induced increase in plasma FFAs upon platelet function in ten healthy male volunteers who had fasted for seventy-two hours.

Gjesdal and coworkers observed that after fasting the mean plasma concentration of FFAs increased more than two fold to 1.8 mM, and this was associated with an increase in assayed platelet aggregation scores ($p<0.02$). Interestingly, this correlation increased significantly when plasma levels of the saturated FFA, oleic acid were used alone ($p<0.01$). Fasting was also associated with an increase in plasma levels of the small CXC family cytokine platelet factor 4 (PF4) (this cytokine is released from platelet alpha granules) suggesting that platelet reactivity had been enhanced.

In their summary, the investigators propose that fat mobilization during fasting is associated with changes in platelet function and that such changes are caused by an elevation in plasma of the levels of saturated FFAs.

Effects of FFAs upon ADP-induced platelet aggregation

Hoak et et al (Nature 1970 228(5278) 1330-1332) undertook an investigation in an effort to determine whether exposure of human platelets to albumin-bound FAAs, in concentrations that are elevated but which can occur in physiological conditions, might influence platelet function. They studied the influence of the molar ratio of FFA to albumin upon the response of platelets to ADP.

The effects of incubation of platelets with increasing concentrations of albumin-bound palmitate were evaluated initially. With an increasing FFA to albumin molar ratio, the degree and rate of aggregation induced by a standard amount of ADP increased. By contrast with the results for the saturated FFA palmitate, the unsaturated FFAs oleate and linoleate caused little or no enhancement even at the highest molar ratio of six.

The results obtained using the saturated FFA stearate, were similar to those using palmitate. Using FFAs of shorter chain length, less enhancement of aggregation was found with the saturated FFA myristate, which produced "moderate" enhancement of the ADP response, and the FFA laurate which produced "little or no response".

The concluding remarks of Hoak et al are presented below in full without editing. It should be kept in mind that their comments and views were expressed over four decades prior to this new analysis of clopidogrel non-responsiveness/resistance, and almost three decades before the first launch of Plavix.

"We interpret these results to indicate that exposure to a high concentration of fatty acid may alter the reactivity of the platelet and thereby enhance its responsiveness to ADP on to (sic) other agents which influence platelet function in vivo. The principal effect of an increase in the FFA/albumin molar ratio was to increase the FFA content of the platelet. A dynamic equilibrium state may exist between the binding of FFA by albumin and the uptake of FFA by platelets and other cellular elements. Acute lipid mobilization causing high plasma FFA concentrations or disease states in which the albumin content or FFA binding capacity are reduced might produce a transient state in which excessive amounts of FFA associate with platelets. In this condition the platelet may develop a propensity toward aggregation which could lead to the formation of thrombi."

FFAs activate PKC

Several laboratories have shown that *cis*-unsaturated FFAs such as arachidonic and oleic acids activate PKC (Science 1984 224(4649) 622-

625; FEBS Lett 1985 192(2) 189-193; J Biol Chem 1986 261(33) 15424-15429; Biochem Biophys Res commun 1987 145(2) 797-802; Biochem Pharmacol 1988 37(16) 3079-3089; FEBS Lett 1990 276(1-2) 223-226; Biol Chem 1992 267(6) 3605-3612); Trends Biochem Sci 1992 17(10) 414-417; FASEB J 1995 Apr; 9(7):484-496).

Saturated FFAs that have carbon chain lengths of C13 to C18 activate γ- and ε-PKC *in vitro* (J Biochem 1995 117(3) 648-653), and unsaturated FFAs, such as arachidonic and oleic acid, enhance the kinase activity of several PKC subspecies (J Biochem 1988 103(5) 759-765; Biochem Biophys Res Commun 1991 179(3) 1522–1528; Proc Natl Acad Sci USA 1991 88(12) 5149-5153; Biochem J 1992 282(1) 33-39).

And as reviewed and discussed by Khan and colleagues (Cell Signal 1995 7(30 171-184) the second messenger function of FFAs as activators of PKC is achieved by a mechanism(s) that is distinct from activation by DAG.

Synergistic interaction of cis-unsaturated FFAs and DAG

Consonant with the above studies, several workers have reported a synergistic interaction of *cis*-unsaturated FFAs and DAG for the activation of PKC, especially the alpha and beta isoforms, at low Ca2+ concentrations (Proc Natl Acad Sci USA 1991 88(12) 5149-5153; Biochem Biophys Res Commun 1988 154(1) 20-20; Biochem J 1992 282(1) 33-39).

FFAs and DAG synergistically activate human platelets

Siefert's seminal studies

Siefert et al (Biochem Biophys Res Commun 1987 149(2) 762-768) following preliminary studies (not reported here), systematically investigated whether FFAs and DAG interacted together in the activation of human platelets. They used intact human platelets suspended in a saline and glucose buffer with the addition of bovine serum albumin (BSA). FFAs were used within the concentration range of 200-400 μM. Instead of DAG they used the synthetic membrane-permeable DAG mimic, 1-oleoyl-2-acetyl-rac-glycerol (OAG) (5 μM) which substitutes for endogenous DAG in the activation of PKC.

They demonstrated that the unsaturated octadecadienoic FFAs, *cis, cis*-linoleic acid and *trans,trans*-linolelaidic acid, and OAG, concentration-dependently induced activation of gel-filtered human platelets; that is,

aggregation and phosphorylation of 20 kDa and 47 kDa peptides. The investigators state that in agreement with previous studies, thrombin also caused phosphorylation of a 47 kDa peptide which they add is the major substrate of PKC in intact platelets, and that the 20 kDa peptide corresponds to myosin light chain which is also phosphorylated by PKC and myosin light chain kinase.

By contrast, the unsaturated octadecenoic FFAs, *cis*-oleic acid and *trans*-elaidic acid, and the saturated octadecanoic acid, stearic acid were inactive. Octadecadienoic acid-induced platelet activation was suppressed by the PKC inhibitor polymyxin B, but not by the cyclo-oxygenase inhibitor, indomethacin.

Importantly, Siefert and coworkers showed for the first time that DAG-induced activation of platelets was potentiated by the unsaturated octadecadienoic FFAs at non-stimulatory concentrations, suggesting a critical role for DAG in mediating FFA-induced platelet activation. They conclude that their data indicate that unsaturated octadecadienoic acids and DAG synergistically induce platelet activation via PKC. They add that these new findings using intact human platelets support their earlier observations and conclusions derived using purified PKC (Naunyn-Schiedeberg's Arch Pharmacol 1987 335 (Suppl 1) R36; Proc IVth Intern AICR Symposium on "Eicosanoids, Lipid Peroxidation and Cancer" (Nigam S Ed)., Springer-Verlag, Berlin, Heidelberg NY).

FFAs and DAG synergistically activate human platelets

Yoshida's characterization studies

Motivated by the pioneering observations of Siefert (above), Yoshida et al (Proc Natl Acad Sci USA 1992 89(4) 6443-6446) undertook to establish whether in activated human platelets, activation of PKC can be sustained by the synergistic interaction between *cis*-unsaturated FFAs and DAG, particularly when calcium ion concentration returns to basal levels.

When the membrane-permeant DAG analogue, 1-oleoyl-2-acetyl-rac-glycerol (OAG) was added to intact human platelets, a PKC-specific endogenous substrate, a 47-kDa protein, was rapidly phosphorylated. However, once the phosphate was covalently attached to this protein, it was removed quickly by the action of phosphatases, presumably, as the investigators reason because the OAG available for maintaining the active PKC disappeared rapidly as a consequence of hydrolyic degradation catalyzed by non-specific esterases and/or DAG lipase.

Nevertheless, this short-lived protein phosphorylation was greatly enhanced by the addition of linoleic acid; this FFA alone was completely inactive.

By contrast, when platelets were stimulated by the phorbol ester, phorbol 12-myristate 13-acetate (PMA) (a PKC activator) instead of OAG, a 47-kDa protein was similarly phosphorylated, but the radioactive phosphate remained attached to this protein for a longer period of time. The investigators propose that this reflects the slower degradation of PMA with a resultant prolonged activation of PKC.

Linoleic acid also significantly enhanced the PMA-induced phosphorylation of the 47-kDa protein.

In the presence of ionomycin (a potent and selective calcium ionophore agent that acts as a motile calcium ion carrier and enhances calcium influx by direct stimulation of cation entry across biological membranes) and either OAG or PMA, serotonin was released. The addition of linoleic acid to the platelets further enhanced the release reaction.

Under the conditions employed by Yoshida and coworkers however, linoleic acid alone was totally inactive, and as described, phosphorylation of the 47-kDa protein and serotonin release always required the coexistence of either OAG or PMA.

Both phosphorylation of the 47-kDa protein and serotonin release were increased by linoleic acid in a dose-dependent manner up to 50 μM. Above 200 μM, the FFA alone appeared to damage the membrane resulting in lysis of platelets.

The potentiation of the phosphorylation of the 47-kDa protein by linoleic acid described above was also observed for many other naturally-occurring cis-unsaturated FFAs such as oleic, linolenic, eicosapentaenoic, and docosahexaenoic acids. Arachidonic acid was not tested by Yoshida and coworkers because of potential confounding effects of its metabolites such as thromboxane A2. Neither saturated FFAs such as palmitic and stearic acids nor trans-unsaturated FFAs such as elaidic and linolelaidic acids were active in enhancing the protein phosphorylation induced by 1,2-DG.

These characterization and kinetic studies of Yoshida and coworkers using intact human platelets indicate that in their hands, *cis*-unsaturated FFAs act as “enhancer” molecules rather than as second messengers for PKC activation since DAG (whether endogenous, analogue or mimic) is absolutely conditional for the FFA action.

These investigators also report that consistent with their earlier *in vitro* observations (Proc Natl Acad Sci USA 1991 88(12) 5149-5153), in the

presence of DAG, *cis*-unsaturated FFAs appear to increase the sensitivity of PKC activation to calcium ion, rendering the enzyme more active at lower calcium ion concentrations.

FFAs induce the accumulation of DAG within human platelets

Homa et al (Biochem Biophys Res Commun 1980 95(3) 1321-1327) provide one of the earliest reports that exogenous FFAs induce the accumulation of DAG within intact human platelets. They observed that when FFAs particularly those containing double bonds (unsaturated FFAs), were added to whole human platelets pre-labelled with ^{14}C arachidonate, radioactive DAG accumulated within the platelets (the exogenously-added FFAs were not radio-labelled).

Polyunsaturated and mono-unsaturated FFAs were equally effective and the saturated FFA, palmitate produced the lowest effect. The accumulation of DAG was unrelated to thrombin which by comparison produced only a modest effect. Using (added) doses of FFA within the physiological range, the accumulation of DAG was dose-dependent, with oleate and linoleate for example, each at a total concentration of 300 μM achieving a 63- and 52- fold increase respectively, by comparison with FFA-free controls (in 1% BSA the unbound level of FFA is comparable to that in human plasma (J Lipid Res 1995 36(2) 229)).

The investigators add that they have observed a similar accumulation of radioactivity in lymphocytes that were pre-labelled with ^{14}C arachidonate and then incubated with non-radioactive linoleate (Biochim Biophys Acta 1983 752(2) 315-323).

FFAs induce the accumulation of DAG within an array of cell types

Vascular smooth muscle cells

In the study by Lu and coworkers (Diabetologia 2000 43(9) 1136-1144) primary cultures of porcine carotid vascular smooth muscle cells (VSMCs) were rendered quiescent and exposed to FFAs at concentrations up to 150 μM. FFAs were bound to albumin prior to addition to the culture medium, and the ratios of FFA to albumin yielding final total and unbound concentrations are reported by the investigators as being representative of those seen *in vivo*. DAG accumulation was seen only with saturated FFAs that included myristate, palmitate and stearate. In their conclusions the authors

reason that FFA-induced DAG accumulation could occur in many cell types.

Human hepatocarcinoma cells

In a recent study, using HepG2 human hepatocarcinoma cells, Lee et al (*Metabolism 2010 59(7) 927-934*) investigated the effects of the FFAs, oleate and palmitate, each at a concentration of 500 and 1,000 μM, in 0.5% and 1% BSA, respectively. Whereas palmitate increased DAG accumulation by 300% by comparison with FFA-free controls, oleate had no effect. Protein kinase C (PKC) epsilon was significantly activated in cells treated with palmitate.

It should be noted that the total concentrations of FFAs in this study are supraphysiological, and no estimates of unbound FFA are reported.

Human skeletal muscle cells

Montell et al (Am J Physiol Endocrinol Metab 2001 280(2) E229-37) investigated the metabolic fate of saturated (palmitate or stearate), polyunsaturated (linoleic) or mono-unsaturated (oleate) ^{14}C-FFAs when preincubated with human skeletal muscle primary cultures obtained from patients considered free of muscle disease. Muscle cells were incubated with 0.1 to 0.4 mM BSA and the FFAs diluted in BSA in a molar ratio of 2.5:1 were added within the dose range of 0.05 to 1 mM. In cells incubated with saturated FFAs, the maximal net increase of radioactivity (as measured by ^{14}C) was found in DAG labeling (30-fold and 10-fold for palmitate and stearate, respectively). By contrast, with the unsaturated FFAs, oleate and linoleate, there was only minor incorporation into DAG (approximately 2-fold).

Because it was found that the saturated FFAs specifically promoted the chronic accumulation of DAG, which could translocate and activate PKC, the investigators analyzed the effect of palmitate and oleate on the subcellular distribution of PKC activity.

In control cells incubated in the absence of FFA, the major part of the calcium and lipid-dependent phosphorylation activity was found in the cytosolic fraction. PKC activity distribution was not modified by incubation with oleate. By contrast, in cells incubated with palmitate, there was a two fold increase in the PKC activity present in the membrane fraction at the expense of the activity in the cytosolic fraction, which was decreased.

The investigators conclude that these data indicate that palmitate specifically promotes persistent translocation of PKC activity to the muscle cell membrane.

FFAs may induce DAG accumulation by inhibiting growth factor-induced DAG kinase α activation

Platelets express DAG kinase α

In addition to an FFA-induced *de novo* synthesis of DAG by stepwise acylation with subsequent accumulation (Diabetologia 2000 43(9) 1136-1144; Diabetologia 2001 44(5) 614-620), or activation of PLC by FAA (Biochem Biophys Res 1980 95(3) 1321-1327), Du et al (Biochem J 2001 357 275-282) hypothesized that FFAs induce DAG accumulation by inhibiting a DAG-kinase (DAGK) isoform. DAGK represents a family of enzymes that catalyzes the conversion of DAG to phosphatidic acid (PA) utilizing ATP as a source of phosphate. As both DAG and PA function as bioactive lipid signalling molecules with distinct cellular targets, DAG-kinase therefore occupies an important position, effectively serving as a switch by terminating the signalling of one lipid while simultaneously activating signalling by another. Thus, inhibiting the activity of DAGK can be predicted to result in an accumulation of DAG.

Using primary cultures of vascular smooth muscle cells (VSMCs) Du and coworkers (Biochem J 2001 357 275-282) demonstrated that the DAGK α isoform was the major DAG-kinase activity present (Notably, DAGK α is one of the R59946-sensitive DAG-kinase isoforms present in platelets). PDGF markedly increased the DAGK activity of cultured VSMCs. An inhibitor, selective (but not specific) for the DAGK α isoform, R59949, abolished the growth-factor-induced increase in DAGK activity, but had little effect on basal activity.

The investigators reason that PDGF selectively activates DAGK α. Interestingly, epidermal growth factor (EGF) and α-thrombin stimulated total DAGK activity similarly to PDGF. Activation by epidermal growth factor was sensitive to R59949, again suggesting involvement of DAGK α. However, the α-thrombin-induced activity was unaffected by this agent. Unsaturated FFAs inhibited growth-factor-induced DAGK α activation, but had no effect on basal activity. DAG was observed to accumulate when FFAs were added to VSMCs in the presence of growth factor. These results indicate that in VSMCs inhibition of DAGK α contributes to FFA-induced accumulation of DAG.

It remains to be demonstrated whether growth factors activate DAGK α in human platelets. It should be added that a variety of stimuli activate DAGK activity in an array of cell types (Biochem Biophys Res Commun 1997 232 111-116; 1994 205 1338-1344;1987 144 1025-1030; Biochem Pharmacol 1997 53 1683-1694; Biochem J 1994 300 51-56; J Biol Chem 1996 271 10334-10340; 1989 264 10643-10648; 1989 264 2537-2544). Moreover, it is important to note that HGF is markedly increased in many patients with ACS (Appendix I).

FFA–induced translocation of PKCs in living cells

Arachidonic acid–induced translocation of ε-PKC and oleic acid–induced translocation of α-, βII-, and δ-PKC have been shown by immunoblot analysis (J Cell Sci 1997 110(14) 1625-1634; J Biol Chem 1993 268(7) 5063–5068). However, there is limited understanding of the underlying mechanism of the effects of FFAs on PKC translocation. In an attempt to characterise the physiological involvement of FFAs in PKC signalling pathways, Shirai et al (J Cell Biol 1998 143(2) 511-521; Jpn J Pharmacol 1998 78(4) 411-417) studied the effects of eleven FFAs (arachidonic acid, docasahexaenoic acid, heptadecanoic acid, myristic acid, oleic acid, palmitic acid, pentadecanoic acid, linoleic acid, linolenic acid, stearic acid and tridecanoic acid) on the translocation of γ- and ε-PKC in COS-7 and CHO-K1 cells.

Using the PKC isoenzymes fused with green fluorescent protein (GFP), γ-PKC–GFP and ε-PKC–GFP predominated in the cytoplasm, but only a small amount of γ-PKC–GFP was found in the nucleus. All the FFAs examined by Shirai and coworkers induced the translocation of γ-PKC–GFP from the cytoplasm to the plasma membrane within thirty seconds with a return to the cytoplasm in three minutes, but they had no effect on γ-PKC–GFP in the nucleus. Arachidonic and linoleic acids induced slow translocation of ε-PKC–GFP from the cytoplasm to the perinuclear region, whereas, with the exception of palmitic acid all the other FFAs induced rapid translocation to the plasma membrane. The target site of the slower translocation of ε-PKC–GFP by arachidonic acid was identified as the Golgi network. The critical concentration of FFAs that induced translocation varied among the eleven FFAs tested. In general, a higher concentration was required to induce the translocation of ε-PKC–GFP than that of γ-PKC–GFP, the exceptions being tridecanoic acid, linoleic acid, and arachidonic acid.

Interestingly, arachidonic acid and the diacylglycerol analogue DiC8 had synergistic effects on the translocation of γ-PKC–GFP. Simultaneous application of arachidonic acid (25 μM) and DiC8 (10 μM) elicited a

slow, irreversible translocation of γ-PKC–GFP from the cytoplasm to the plasma membrane after rapid, reversible translocation, but a single application of arachidonic acid or DiC8 at the same concentration induced no translocation.

Spatial convergence between FFAs and P2Y12 upon PKC

Importantly, the FFAs-induced activity and translocation of PKC isoforms maps an additional point of spatial convergence between FFAs-mediated signalling and that of the platelet P2Y12 receptor. Using human platelets, Mundell and coworkers (Mol Pharmacol 2006 70(3) 1132-1142) showed that novel, but not classical isoforms of PKC regulate P2Y12 function and trafficking. Thus, the FFAs-induced increase in activity and translocation of PKC may amplify signalling by ADP-occupied and PKC-regulated P2Y12 receptors in patients with CYP2C19 loss-of-function polymorphisms who are being managed with clopidogrel, given that low levels of the active thiol metabolite will likely result in less than maximal blockade of the P2Y12 receptor pool.

PART III

MODULATION OF DIACYLGLYCEROL-MEDIATED SIGNALLING BY THE P2Y12 RECEPTOR IN HUMAN PLATELETS

Antagonists of the Gi-coupled P2Y12 receptor completely block PKC activation

A new finding

Using human platelets, Guidetti and coworkers (J Biol Chem 2008 283(43) 2895-28805) show that in accord with expectation, blockade of ADP binding to the Gq-coupled P2Y1 receptor abolished protein kinase C (PKC)-mediated protein phosphorylation. Unexpectedly, however, they observed that antagonists of the Gi-coupled P2Y12 receptor also completely prevented PKC activation. From this interesting finding they conclude that stimulation of the Gq-coupled receptor is necessary but not sufficient for ADP to induce PKC activation, and the concomitant stimulation of Gi is also required. And, consistent with what they observed in ADP-stimulated platelets, the P2Y12 antagonists AR-

C69931MX (cangrelor) and 2MeSAMP (2-methylthioadenosine 5′-monophosphate triethylammonium salt) also prevented PKC-mediated protein phosphorylation induced by the binding of the thromboxane A2 analogue U46619.

Pursuing the functional implications of this crosstalk they showed that subthreshold doses of the membrane-permeable PKC activator PMA restored normal PKC activity in both ADP- and U46619-stimulated platelets despite P2Y12 receptor inhibition.

However, under these conditions platelets were still unable to aggregate. Consequently, blockade of the P2Y12 receptor still prevents U46619-induced aggregation even if PKC is normally activated. This indicates that although PKC activity is regulated by Gi, this is not the element of the Gi-dependent pathway that is critical for platelet aggregation. Following this new observation, Guidetti and coworkers provide evidence that Gi targets the PKC upstream regulator diacylglycerol (DAG). They propose that the cross-talk between Gq and Gi most likely occurs at the level of DAG metabolism, which plays a more crucial role than PKC itself in the regulation of platelet aggregation. That is, these workers show that stimulation of the P2Y12 receptor or direct inhibition of diacylglycerol kinase (DAGK) potentiated the effect of the cell-permeable DAG analogue DiC8 on platelet aggregation and pleckstrin phosphorylation, in association with inhibition of its phosphorylation to phosphatidic acid (PA). These results reveal a novel and unexpected role of the G_i-coupled P2Y12 receptor in the regulation of diacylglycerol-mediated events in activated platelets.

Inhibition of DAG kinase surmounts blockade of P2Y12

An additional perspective on clopidogrel resistance starts to emerge

DAG is generated by activated phospholipase C (PLC) but is subsequently phosphorylated to phosphatidic acid (PA) by the enzyme DAG kinase (DAGK), which therefore turns off the effects of DAG as second messenger. Excitingly, Guidetti and coworkers go on to show that pharmacological inhibition of DAGK restores the agonist-induced phosphorylation of PKC substrates prevented by antagonists of the P2Y12 receptor and, in parallel, allows platelets to undergo aggregation. Most importantly, AR-C69931MX-promoted inhibition of Rap1 activation was overcome by pharmacological inhibition of DAGK. Guidetti and coworkers propose that this finding may be explained by the observation that the main nucleotide exchange factor responsible for the activation of Rap1 expressed in platelets is calcium-diacylglycerol

guanine nucleotide exchange factor 1 (CalDAG-GEFI) which is directly activated by DAG independently of PKC and is required for platelet aggregation. However, as discussed Part IV of this document, CalDAG-GEFI has little if any affinity for binding with DAG and consequently, in isolation this explanation is not sufficient

Potentiation of DAG-mediated platelet activation by stimulation of P2Y12 receptor

Importantly, Guidetti and coworkers show for the first time that stimulation of Gi hampers the conversion of exogenous DAG to PA, thus extending and potentiating the effects of DAG on platelet function. A logical corollary of these outcomes is that whereas DAG is generated by PLC activated through the Gq-dependent pathway, its effective accumulation requires inhibition of DAGK activity through Gi.

Using the cell-permeable DAG analogue sn-1,2dioctanoylglycerol (DiC8) Guidetti and coworker investigated the relationship between Gi-dependent signalling and DAG-mediated effects on platelet activation. A weak platelet aggregation was observed upon treatment of platelets with 3.5 μM but not with 2 μM DiC8. Concomitant stimulation of the P2Y12 receptor through the addition of ADP in the presence of the selective P2Y1 receptor antagonist MRS2179 (in order to observe the effects of ADP upon the P2Y12 receptor without confounding from any ADP-bound P2Y1 receptors) significantly potentiated the effect of DiC8 on platelet aggregation. DiC8-induced PKC activation was dose-dependent and occurred in a range of concentrations much lower than those required for aggregation. Consistent with expectation, using immunoblotting they observed that faint pleckstrin phosphorylation detectable at the lowest concentration of DiC8 (100 nM) was markedly potentiated by the DAGK inhibitor R59949. Additionally, thin layer chromatographic analysis of the lipids extracted from ^{32}P-labeled platelets revealed that exogenous DiC8 was actively phosphorylated to DiC8-phosphatidic acid (DiC8-PA) by a DAGK activity in intact cells and that this process was inhibited upon platelet incubation with R59949.

However, remarkably they observed that similarly to the effects of R59949, stimulation of the P2Y12 receptor by ADP in the presence of MRS2179 resulted in potentiation of pleckstrin phosphorylation induced by exogenous DiC8 at all the doses analysed. Additionally, using autoradiography combined with densiometric analysis they observed the concomitant inhibition of DiC8 conversion to DiC8-PA in intact platelets upon stimulation with ADP. This effect of ADP in ^{32}P-labeled cells was dose-dependent and was prevented by AR-C69931MX but not by

MRS2179, indicating that it was mediated by activation of the P2Y12 receptor.

Summary

This elegant study reveals a novel role for Gi in the regulation of PKC activity within human platelets, and points to the crucial role of DAG-metabolizing enzymes, notably DAGK in the regulation of platelet activation. These results support the hypothesis that Gi-dependent signalling potentiates the effect of endogenous DAG by limiting its conversion to PA.

However, despite these seminal observations by Guidetti and coworkers a direct negative modulation of DAGK by Gi in agonist-stimulated platelets remains to be demonstrated.

Importantly, they demonstrate that the need for Gi stimulation to trigger a full platelet response can be bypassed by direct inhibition of DAGK activity by using the cell-permeable DAGK inhibitor R59948. Interestingly, these experimental interventions by Guidetti and coworkers are mimicked by the endogenous effects of FFAs, listed below:

- *FFAs activate PKC*
- *FFAs and DAG synergistically activate PKC*
- *FFAs and DAG synergistically activate human platelets*
- *FFAs induce translocation of PKC to the plasma membrane*
- *FFAs induce the accumulation of DAG within human platelets*
- *FFAs induce DAG accumulation by inhibiting growth factor-induced DAGK* α activation
- *FFAs may regulate a DAG-independent and PKC-dependent translocation of CalDAG-GEFI to the plasma membrane*
- *FFAs mediate the Rac1-dependent and F-actin-dependent translocation of CalDAG-GEFI*

Thus, taken together, the results of Guidetti and coworkers allow the new hypothesis that FFAs, above some concentration threshold can surmount blockade of the platelet P2Y12 receptor pool by targeting the PKC-DAG-DAGK axis. Importantly, these effects of FFAs upon the platelet PKC-DAG-DAGK axis will also converge upon the regulation of PKC activity by the agonist-activated P2Y12 receptor. Consequently, above some concentration threshold FFAs may amplify signalling by ADP-occupied and PKC-regulated P2Y12 receptors in patients with CYP2C19 loss-of-function polymorphisms who are being managed with clopidogrel, given that low levels of the active thiol metabolite will likely result in less than maximal blockade of the P2Y12 receptor pool.

PART IV

CLOPIDOGREL RESISTANCE IS ALSO A FUNCTION OF THE CONCENTRATION-DEPENDENT EFFECTS OF FREE FATTY ACIDS UPON CALDAG-GEFI

What is CalDAG-GEFI (RASGRP2)?

RasGRP2 was originally identified in a genome sequencing project and named HCDC25L (human CDC25-like) (Hum Genet 1997 100(5-6) 611-619). The sequence was rediscovered the following year in differential display experiments aimed at identifying novel brain transcripts and was called CalDAG-GEFI (Proc Natl Acad Sci U S A. 1998 95(22) 13278-13283). This name refers to its presumed mode of regulation by DAG and calcium. The protein was independently rediscovered and renamed RasGRP2 (J Biol Chem 2000 275(41) 32260-32267).

What are RASGRPs?

Ras guanyl (guanine) nucleotide releasing proteins (RasGRPs) are guanyl nucleotide exchange factors (GEFs) that activate Ras and related small GTPases such as Rap.

What are small GTPases?

Small GTPases of the Ras and Rap superfamily are key regulators of diverse cellular and developmental events, including differentiation, cell division, vesicle transport, nuclear assembly, and control of the cytoskeleton. Small GTPases act by a conserved mechanism whereby the

molecule's signalling activities differ when bound to either GTP or GDP; intrinsic GTPase activity (which hydrolyzes GTP to GDP), determines the equilibrium between the GTP-bound and the GDP-bound state. Generally, GTP-bound molecules are active signalling molecules and GDP-bound forms are inactive.

GAPS versus GEFS

Molecules that regulate small GTPases include GTPase-activating proteins (GAPs) which promote the GDP-bound state of the small GTPases by activating the GTPase activity and thus favour the inactive state, and guanyl nucleotide (GTP/GDP) exchange factors (GEFs) which promote the active, GTP-bound state by facilitating the exchange of GDP for GTP. That is, GEFs stimulate GTP loading and this action is antagonized by GAPs that catalyze GTP hydrolysis.

The terms RasGRP2 and CalDAG-GEFI are used interchangeably within this document

RasGRP2 may be expressed in two forms, a standard form and a variant longer form. The existence of the longer form was considered to arise from alternative splicing on the basis of an expressed sequence tag. When ectopically expressed in Chinese Hamster Ovary (COS) cells, the long form possesses a unique N-terminus that enables lipid modification. Clyde-Smith et al (J Biol Chem 2000 275(41) 32260-32267) show that in COS cells this longer alternatively-spliced form incorporates label from both tritiated myristic acid and tritiated palmitic acid. They further show that co-translational myristoylation and post-translational palmitoylation are associated with localization of the longer variant form of RasGRP2 to the plasma membrane. However, the splice sites proposed to generate the longer form are not universally-conserved across species, and importantly the endogenous proteins corresponding to the long form have not been identified (Genes Cancer 2011 2(3) 320-334). Consequently, in this report RasGRP2 and CalDAG-GEFI are used interchangeably and are not meant to include the longer alternatively-spliced form of RasGRP2.

The C1 domain sequence within CalDAG-GEF1 is atypical and shows only weak affinity for DAG

The GEF activity of RasGRPs is provided by two domains within the N-terminal catalytic region: a Ras exchange motif (REM) and a dual-specificity phosphatase CDC25 homology domain. The C-terminal

regulatory region comprises two Ca^{2+}-binding EF hand domains and an atypical C1 domain. Historically, C1 domains were categorized into two families whereby those that bound DAG/phorbol ester were termed "typical," and the more divergent members that failed to bind were termed "atypical" (Protein Sci 1997 6(2) 477-480). Atypical Cl domains retain some hydrophobic residues thought to be required for membrane insertion, and basic residues that may promote anionic phospholipid electrostatic interactions. Unlike RasGRP1 and -3, CalDAG-GEFI is found preferentially in the cytoplasm and translocates toward juxtamembrane areas upon cellular stimulation. It specifically activates Rap1 and Rap2 but lacks Ras exchange activity *in vivo*.

Throughout the literature, RasGRP2 is frequently referred to as Ca1DAG-GEFI since it has a pair of EF hands that are thought to be regulated by Ca^{2+}, and a Cl domain that historically was presumed to be regulated by DAG (Nat Med 2004 10(9) 982-986; Blood 2007 110(10) 3682-3690; J Biol Chem 2004 279(12) 11875-11881; J Exp Med 2007 204(7) 1571-1582). However, the evidence does not support the repeated assertions that DAG regulates RasGRP2 as it does RasGRP1. By contrast, the regulation of RasGRP1 by DAG is an event which is now well established (Mol Biol Cell 2007 18(8) 3156-3168; Nature 2003 424(6949) 694-698; EMBO J 2003 22(13) 3326-3336; Mol Cell Biol 2004 24(8) 3485-3496).

The activation of RasGRP1, -3, and -4 is mediated via the phospholipase C-γ-dependent generation of DAG (Science 1998 280(5366) 1082-1086; J Med Chem 2002 45(4) 853-860; Cancer Res 2001 61(3) 943-949; Mol Pharmacol 2000 57(5) 840-846). This second messenger binds to the C1 domain present in these GEFs and thereby enabling the translocation of each RasGRP to the cell membrane and their subsequent association with their target GTPases (Prog Nucleic Acid Res Mol Biol 2002 71 391-444; J Med Chem 2002 45(4) 853-860). By contrast, RasGRP2 (CalDAG-GEF1) shows a very poor response to DAG and, as a consequence, it does not undergo the characteristic rapid translocation of other RasGRPs to the plasma membrane and endomembranes when cells are treated with DAG agonists (J Biol Chem 2000 275(41) 32260-32267; J Biol Chem 2003 278(35) 33465-33473).

Johnson and coworkers (Biochem J 2007 406(2) 223-226) assessed the capacities of the C1 domains of RasGRP2, RasGRP4α and RasGRP4β to associate with membranes by interacting with DAG. These *in vivo* and *in vitro* experiments demonstrated that the C1 domain of RasGRP4α binds DAG within membranes, although with a moderately reduced affinity compared with the RasGRP1 C1 domain. By contrast, the C1 domains of RasGRP2 (CalDAG-GEFI) and RasGRP4β did not bind DAG, although

they interacted with membrane vesicles enriched in anionic phospholipids in a manner equivalent to that of other C1 domains. From these results, Johnson and coworkers conclude that RasGRP2 and RasGRP4β cannot be regulated directly by DAG binding to their C1 domains, thus disproving the common assumption that all RasGRPs are regulated by membrane translocation driven by a DAG–C1 domain interaction.

FFAs transduce signalling by CalDAG-GEF1

Does the C1 domain of CalDAG-GEF1 recognize different lipid signal transducers?

It was proposed by Johnson and coworkers (Biochem J 2007 406(2) 223-226) and reviewed by Goulding (Membrane translocation of RasGRPs by C1 domains., PhD Thesis., Oct 2008 R E Goulding., The University of British Columbia, Vancouver) that RasGRP2 and RasGRP4β likely underwent opportunistic evolution away from DAG-mediated regulation by gene duplication and acquisition of alternative splicing following the expansion of the RasGRP family.

Johnson and coworkers (Biochem J 2007 406(2) 223-226) tested the hypothesis that the C1 domains of RasGRP2 and RasGRP4β have evolved to recognize different lipid signal transducers, other than DAG. Phosphatidic acid was of particular interest as a ligand because it can be generated from DAG by DAG kinases (DAGKs). However, phosphatidic acid at 5 mol% did not promote vesicle binding of any of the C1 domains, under conditions in which 5 mol% DAG promoted nearly complete binding of the C1 domains of RasGRP1, RasGRP4α and PKCδ. Other signalling lipids with larger headgroups, lysophosphatidic acid and sphingosine 1-phosphate, also failed to promote binding of the C1 domains of RasGRP1, RasGRP2 or RasGRP4β. Ceramide is structurally similar to DAG and has been postulated to be a potential ligand for the non-DAG-binding C1 domains of Raf-1, PKCs and DAG kinases, although a direct interaction with these or any other C1 domain has not been demonstrated. Ceramide at 5 mol% was also ineffective as a ligand for the C1 domains of RasGRP1, RasGRP2 or RasGRP4β.

The FFAs arachidonic acid and oleic acid induce minor increases in membrane binding

FFAs, particularly polyunsaturated species such as arachidonic acid, have been reported to influence the activity of some PKC isoforms (Cell Signal 1995 7(3) 171-184). Arachidonic acid results in a C1b-dependent redistribution of PKCϵ in Chinese-hamster ovary cells (J Biol Chem 2002 277(20) 18037-18045), which implied that it could be a ligand for other C1 domains. Consequently, given these earlier findings it is interesting to report that Johnson and coworkers observed that arachidonic acid and oleic acid both induced a minor increase in membrane binding of the C1 domains of RasGRP1 and RasGRP2; an effect that is likely to be the result of an increased membrane negative charge provided by these anionic lipids.

C1 domains bind phospholipid vesicles in proportion to the anionic lipid content

Interestingly, the surface of the PKCδ C1b domain contains basic residues which are positioned to interact with an anionic membrane surface (Cell 1995 81(6) 917-924; J Med Chem 2002 45(4) 853-860pubmed). The RasGRP C1 domains contain these and additional basic residues positioned appropriately for interaction with anionic surfaces, particularly at positions 10 and 32. The RasGRP C1 domains are also bordered at their C-termini by a three- or four-residue basic patch which enhances binding of the RasGRP1 and RasGRP3 C1 domains to PMA/phosphatidylserine micelles (Bioorg Med Chem 2004 12(17) 4575-4583). Johnson and coworkers (Biochem J 2007 406(2) 223-226) have demonstrated that all C1 domains bound phospholipid vesicles in proportion to the anionic lipid content and that this binding is insensitive to the specific headgroup and can occur in the absence of DAG.

In summary, although the proposal that that the C1 domains of RasGRP2 and RasGRP4β have evolved to recognize different lipid signal transducers is speculative, nevertheless it is evident from Goulding's research that functional divergence of RasGRPs has occurred, via both functional divergence of C1 domains and the acquisition (and perhaps loss) of other membrane-localizing domains. As genes duplicate and diversify, domains may retain functions as seen for RasGRP1 and 3 C1 domains, but they may also diversify their functions as seen for the C1 domains of RasGRP4 and RasGRP2. Diversification of C1 domains may occur as a result of the acquisition of new domains, which in turn may eliminate the requirement to retain the original functionality of the C1

domain. For example if RasGRP2 had to be functional at the Golgi apparatus it had to retain DAG binding capability of its C1 domain to achieve this. But once it acquired an alternative mechanism of Golgi localization, by acquisition of the PDZ-binding motif, it was possible for the C1 domain to lose the DAG binding ability and gain the ability to selectively target the plasma membrane via anionic phospholipid binding.

Can FFAs concentration-dependently modulate DAG-independent electrostatic interactions by increasing membrane negative charge?

In the case of a DAG-binding C1 domain, Johnson and coworkers propose that the weak electrostatic interaction with negatively-charged phospholipids may facilitate a two-dimensional search for DAG on the membrane surface, as well as reinforcing the membrane-binding strength of the ligated C1 domain. For the C1 domains of RasGRP2 and RasGRP4β, a DAG-independent electrostatic interaction with membranes is evidently insufficient to dictate strong membrane binding *in vivo* since RasGRP2 and RasGRP4β did not noticeably co-localize with membranes (Membrane translocation of RasGRPs by C1 domains., PhD Thesis., Oct 2008 R E Goulding., The University of British Columbia, Vancouver). However, as Goulding elegantly showed, the RasGRP2 C1 domain can functionally replace the C1 domain within RasGRP1 (Membrane translocation of RasGRPs by C1 domains., PhD Thesis., Oct 2008 R E Goulding., The University of British Columbia, Vancouver). As the C1 domain is essential and sufficient for RasGRP1 membrane localization, leading to RasGTP loading (Mol Cell Biol 1998 18(12) 6995-7008), this implies that the RasGRP2 C1 domain can provide membrane binding to a physiologically significant extent despite its lack of detectable DAG binding and lack of membrane localization observable by microscopy.

Low affinity binding is sufficient to activate Ras

And as Goulding further observed, it appears that the association of RasGRP1 with membranes can be quite weak and yet still be sufficient for Ras activation, as detected by transformation of NIH 3T3 cells. Consequently, the effect of FFAs in inducing minor increases in membrane binding of the C1 domains of RasGRP1 and RasGRP2 may be sufficient for the membrane-specific activation of Rap1 by CalDAG-GEF1 (RasGRP2), as a result of an increased membrane negative charge provided by these anionic lipids as proposed by Johnson and coworkers (Biochem J 2007 406(2) 223-226).

Concentration of FFAs is likely to be a critical determinant in inducing membrane binding of the C1 domain

Moreover, the electrostatic effects of FFAs in inducing membrane binding of the C1 domain will likely be sensitive to the concentration of FFA given that Johnson and coworkers (Biochem J 2007 406(2) 223-226) have demonstrated that all C1 domains bound phospholipid vesicles in proportion to the anionic lipid content and that this binding is insensitive to the specific headgroup and can occur in the absence of DAG.

In addition to the RasGRP2 C1 domain, there are other examples of C1 domains that do not bind DAG and which do not provide microscopy-detectable binding to membranes on their own, but are required for efficient localization to membranes. The Raf-1 C1 domain is required to stabilize membrane binding via the adjacent Ras-binding domain (Mol Biol Cell 2002 13(7) 2323-2333), while the C1 domain of kinase suppressor of Ras (KSR) makes an essential contribution to mediate constitutive localization to internal membranes and is needed for cytokine-induced translocation to the plasma membrane (J Mol Biol 2002 315(3) 435-446).

FFAs induce the accumulation of DAG within human platelets: New insights into a role for DAG in mediating the translocation of RasGRP2?

Goulding (Membrane translocation of RasGRPs by C1 domains., PhD Thesis., Oct 2008 R E Goulding., The University of British Columbia, Vancouver) discusses an alternative mechanism for the apparent DAG-mediated translocation of RasGRP2. That is, as Goulding reasons although some groups have shown that RasGRP2 is regulated by phorbol ester in a number of different contexts (J Biol Chem 2000 275 (41) 32260-322267; Nat Med 2004 10(9) 982-986; J Biol Chem 2001 276(15) 11804-11811; Science 1998 279() 2275-2279) this is unlikely to occur via a phorbol ester-C1 mediated translocation of RasGRP2 to membranes. Instead these results may be explained by DAG activation of PKC enzymes, which when active may phosphorylate and activate RasGRP2 (Biochem Soc Trans 2006 34(5) 858-861), as is the case for RasGRP1 and RasGRP3 (Procl Natl Acad Sci USA 2004 101(47) 16612-16617; Mol Pharmacol 2004 66(1) 76-84; Mol Cell Biol 2005 25(11) 4426-4441; Blood 2003 102(4) 1414-1420; Blood 2005 105(9) 3648-3654).

As Goulding emphasizes, both RasGRP1 and RasGRP3 are phosphorylated by PKC enzymes in a DAG-dependent manner at homologous positions, threonine 184 and threonine 133 respectively.

RasGRP2 has a threonine at the homologous position 134 (Blood 2005 105(9) 3648-3654), and therefore could be similarly regulated (Membrane translocation of RasGRPs by C1 domains., PhD Thesis., Oct 2008 R E Goulding., The University of British Columbia, Vancouver). Previous reports of phorbol ester-induced or PLC-dependent activation of RasGRP2 or 4β, which were interpreted as evidence for direct binding of their C1 domains to phorbol esters or DAG (J Biol Chem 2002 277(28) 25756-25774; J Biol Chem 2000 275(41) 32260-32267; Proc Natl Acad Sci USA 1998 95(22) 13278-13283; J Biol Chem 2001 276(15) 11804-11811; Nat Med 2004 10(9) 982-986; J Biol Chem 2004 279(12) 11875-11881) may instead reflect the DAG or phorbol ester–dependent phosphorylation by PKC enzymes in the activation of these two RasGRPs. Moreover, given the earlier observations by Homa et al (*Biochem Biophys Res Commun 1980 95(3) 1321-1327*) of the dose-dependent effects of FFAs upon DAG accumulation, then this mechanism will be critically dependent upon the concentration of FFAs.

The outcomes of the experiment by Goulding (Membrane translocation of RasGRPs by C1 domains., PhD Thesis., Oct 2008 R E Goulding., The University of British Columbia, Vancouver) with isolated monomeric versus tandem C1 domains indicate that the C1 domain of RasGRP2 can directly bind the plasma membrane in NIH 3T3 cells, but does so with an affinity that is too low to support stable localization of RasGRP2 at the plasma membrane. Other parts of RasGRP2 must cooperate with the C1 domain to enable effective plasma membrane binding, either by modifying the affinity of the C1 domain or by providing additional weak interaction sites. The latter is analogous to the situation with RasGRP1, where stable plasma membrane binding requires cooperativity between the C-terminal PT (plasma membrane-targeting) domain, which specifies plasma membrane targeting, and the C1 domain which provides an additional point of contact with the membrane (Mol Biol Cell 2007 18(8) 3156-3168). Goulding showed that a C1 domain plus C-terminus form of RasGRP2 is sufficient for localization at both the plasma membrane and Golgi apparatus, and that the C-terminus is sufficient for Golgi localization. Since the C1 domain is incapable of localizing at the plasma membrane, this indicates that a region in the C-terminus may stabilize C1 domain-mediated RasGRP2 localization.

In summary, the meticulous efforts of Goulding show that the C1 domain of RasGRP2 is required for full efficiency of Rap1 activation in NIH 3T3 cells, and is also essential for localization of RasGRP2 at the plasma membrane in both NIH 3T3 fibroblasts and Jurkat T-cells. Previous studies have demonstrated that the RasGRP2 C1 domain does not have measurable DAG binding affinity by comparison with the C1 domains of RasGRP1, RasGRP3 and RasGRP4α (Biochem J 2007 406(2) 223-

226; Bioorg Med Chem 2004 12(17) 4575-4583). Furthermore, in NIH 3T3 cells the localization of the RasGRP2 C1 domain is distinct from the localization of DAG-binding C1 domains (Biochem J 2007 406(2) 223-226)). Consequently, it seems unlikely that by itself, DAG production at the plasma membrane is what drives C1-dependent localization of RasGRP2 to the plasma membrane. However, independently of DAG binding, all C1 domains in the RasGRP family have substantial affinity for vesicles enriched in anionic phospholipids (Biochem J 2007 406(2) 223-226). Moreover, the RasGRP C1 domains, like the Raf-1 C1 domain, show no significant preference for phosphatidylserine, phosphatidylcholine, phosphatidylglycerol or phosphatidic acid (Biochem J 2007 406(2) 223-226) and are therefore likely to interact with anionic phospholipids strictly through an electrostatic interaction rather than by specific headgroup recognition (Biochem J 2007 406(2) 223-226). Therefore it is possible that the C1 domain of RasGRP2 binds to membranes by this mechanism; the role of FFAs, especially concentration-dependency, in facilitating this association remains to be clarified.

F-actin-dependent translocation of CalDAG-GEFI promotes regionalized activation of Rap1

Goulding (Membrane translocation of RasGRPs by C1 domains., PhD Thesis., Oct 2008 R E Goulding., The University of British Columbia, Vancouver) also reports that RasGRP2 accumulates at the plasma membrane as a rapid response to the chemokine SDF-lα (stromal cell-derived factor-1alpha) in Jurkat T-cells cells, and that this is completely dependent on the C1 domain. Goulding proposes that this relocalization of RasGRP2 could reflect transient increases in anionic lipids at the plasma membrane, or it might also involve the induction of an as yet unidentified ligand for the Cl domain. Interestingly, using myeloma cells that express the fully-functional chemokine receptor CXCR4 for which SDF-lα is its specific ligand, Sanz-Rodriguez and coworkers (Blood 2001 97(2) 346-351) observed that SDF-lα induced a rapid and transient increase in filamentous-actin (F-actin) polymerization that they propose is subsequent to an SDF-lα-induced activation of Rac1. This raises the interesting speculation that the SDF-lα-induced accumulation of RasGRP2 at the plasma membrane in Jurkat T-cells is mediated by polymerized F-actin.

Notwithstanding the critical role of Ca^{2+} ion as the principal second messenger that regulates CalDAG-GEFI function in platelets, Mariía Caloca and colleagues at the University of Salamanca in Spain (J Biol Chem 2004 279(19) 20435-20446) elegantly show that additionally, it is

the Rac1-dependent direct association of the N-terminal domain of CalDAG-GEFI with F-actin that regulates the subcellular localization of CalDAG-GEFI, thereby enabling regionalized activation of Rap1 within juxtamembrane areas of the cell (these workers use the nonmyristoylatable isoform of RasGRP2 which is synonymous with CalDAG-GEFI).

The cytoskeletal regulator Rac1 mediates the F-actin-dependent translocation of CalDAG-GEFI

The induction of F-actin assembly and polymerization by cytoskeletal regulators such as Vav, Vav2, Dbl, and Rac1 leads to the shift of CalDAG-GEFI from the cytosol to membrane ruffles and its co-localization with F-actin. Treatment of cells with cytoskeletal disrupting drugs abolishes this effect, leading to an abnormal localization of CalDAG-GEFI in cytoplasmic clusters of actin. Specifically, in the hands of Caloca and coworkers (J Biol Chem 2004 279(19) 20435-20446) the use of Rac1 effector mutants indicates that the translocation of CalDAG-GEFI is linked exclusively to actin polymerization and is independent of other pathways such as p21-activated kinase JNK, or superoxide production.

Rho GTPases including Rac1 mediate actin dynamics

The Rho family of GTPases is a family of small (~21 kDa) signalling G proteins, and is a subfamily of the Ras superfamily. The members of the Rho GTPase family have been shown to regulate many aspects of intracellular actin dynamics, and are found in all eukaryotic organisms including yeasts and some plants. Three members of the family have been studied a great deal: Cdc42, Rac1, and RhoA. Identification of the Rho family of GTPases began in the mid-1980s. The first identified Rho member was RhoA, isolated serendipitously in 1985 from a low stringency cDNA screening. Rac1 and Rac2 were next identified in 1989 followed by Cdc42 in 1990.

Regulation of Rac1 activity

The Rho family of small GTPases, the best studied of which are Rho, Rac and Cdc42, have important regulatory roles in diverse cell functions such as motility, growth, and differentiation (Nature Rev Mol Cell Biol 2008 9(9) 690–701; Annu Rev Cell Dev Biol 2005 247–69). Inappropriate activation of Rho GTPases is implicated in uncontrolled cell growth, invasion and metastasis of many types of cancers (FEBS Lett 2008 582(14) 2093–101; Clin Cancer Res 2003 9(7) 2632–41; Oncogene 2001

20(43) 6263–8). GTPases cycle between active, guanosine triphosphate (GTP) bound states and inactive, guanosine diphosphate (GDP) bound states, and the rate at which this cycle occurs is regulated by three classes of proteins: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (Genes Dev 1997 11(18) 2295–322).

The RhoGDI family consists of three members (RhoGDI1, 2 and 3) that bind to Rho family GTPases, extract them from membranes, and sequester them in the cytosol, effectively maintaining them in an inactive state and disallowing effector binding (Trends Cell Biol 2005 15(7) 356–63). The interaction of Rac and related members of the Rho family with GDI to form a stable complex appears to be a critical regulatory point in the activation process of these GTP-binding proteins. The ability of GDI to stabilize both the GDP and GTP states of Rac by inhibiting GDP exchange and GTP hydrolysis, respectively, would appear to maintain the protein in a relatively non-interactive form.

FFAs non-covalently facilitate the Rac1-mediated F-actin polymerization

Following the discovery of Rac1, Chuang and coworkers (J Biol Chem 1993 268(35) 26206-26211) postulated that a mechanism to regulate the association of Rac with (Rho)GDI becomes crucial for allowing Rac to be released so that it can interact with other regulatory molecules and effectors and “stably” translocate to the plasma membrane. Using sucrose gradient equilibrium sedimentation analysis they show that under non-activating conditions all of the Rac1 protein present in human neutrophil cytosol exists as a prenylated protein in the GDP-bound form as a complex with (Rho)GDI. Importantly, they describe the ability of a number of biologically active lipid second messengers to regulate complex formation between Rac1(GDP) and RhoGDI. They conclude that their data suggest possible mechanisms by which members of the Rho family might be regulated by physiological activators of lipid metabolism. Indeed, they observed that at physiologically-relevant concentrations that a number of types of lipid were able to inhibit the action of RhoGDI toward Rac1.

FFAs maintain Rac1 in a free form

Unsaturated FFAs of varying chain lengths from 18-22 carbon atoms were able to almost totally prevent the action of RhoGDI to stabilize both the GDP and GTP states of Rac1 by inhibiting GDP dissociation/exchange and GTP hydrolysis. Thus, the FFAs-mediated

release of Rac1(GDP) from the GDI complex to produce the active GTP-bound form would be a necessary step in order to allow Rac1 to mediate F-actin polymerization and the subsequent association of F-actin with CalDAG-GEFI, resulting in translocation of CalDAG-GEFI from a subcellular level to the plasma membrane and thereby enabling regionalized activation of Rap1 within juxtamembrane areas of the cell.

In support of these early findings by Chuang and coworkers, Ugolev et al at the Sackler School of Medicine in Tel aviv (J Biol Chem 2006 281(28) 19204-19219) showed that exposure of Rac(1 or 2)(GDP).RhoGDI complexes to macrophage membrane liposomes containing four different anionic phospholipids resulted in the dissociation of Rac(1 or 2)(GDP) from Rho GDI complexes.

More recently, Ueyama et al at Kobe University in Japan (J Immunol 2013 191(5) 2560-2569) using RAW264.7 macrophages and HEK293 cells examined how the cytosol/membrane cycles of the RhoGDI–Rac(GDP) complex are regulated by anionic lipids. They showed that the negatively-charged and flexible N terminus (25 residues) of RhoGDI, particularly its second negative amino acid cluster possessing five negatively charged amino acids, is a pivotal regulator in the cytosol/membrane cycles of the Rac(GDP).RhoGDI complex. They also showed that translocation of the Rac(GDP).RhoGDI complex was mediated by an interaction between the polybasic positively-charged motif in the C-terminus of Rac1 and anionic phospholipids, such as phosphatidic acid. Taken together they demonstrated that the membrane targeting/accumulation of the Rac(GDP).RhoGDI complex with subsequent dissociation of Rac(GDP) is regulated by an equilibrium between the binding affinity of RhoGDI for Rac(GDP) and displacement by the electrostatic attraction of the polybasic motif in the C-terminus for anionic lipids. This mechanistic elucidation accords with that of other workers (J Immunol 2005 175(4) 2381 -2390; J Biol Chem 2004 279(16) 16007 -16016; Biochemistry 1996 35(49) 15683-15692; J Biol Chem 1994 269(46) 29024-29031).

The FFA palmitate covalently regulates Rac1 function and induces actin polymerization

The post-translational covalent linking of the 16-carbon FFA palmitate, usually by formation of a thioester bond (thioacylation/thioalkanoylation) to cysteine and less frequently to serine and threonine residues of proteins is a process known as “palmitoylation” (discussed below in more detail). Palmitoylation is reversible, and cycles of palmitoylation–depalmitoylation enable

proteins to transiently associate with membranes, thereby regulating their sorting, localization and function (Cell 2010 141(3) 458-471; Nat Rev Mol Cell Biol 2007 8(1) 74-84; J Cell Biol 2007 176(3) 249-254; Curr Opin Neurobiol 2005 15(5) 527-35).

In 2012, Navarro-Lerida and coworkers (EMBO J 2012 31(3) 534–551) report for the first time that Rac1 can incorporate palmitate at cysteine 178 and that this posttranslational modification targets Rac1 for stabilization at actin cytoskeleton-linked ordered membrane regions. Importantly, non-palmitoylated Rac1 shows decreased GTP loading and cells expressing no Rac1 or a palmitoylated-deficient mutant have an increased content of disordered membrane domains with spreading and migration defects. Taken together, their results show that Rac1 influences membrane organization by stimulating actin polymerization and that this function is dependent upon palmitoylation.

Specifically, Navarro-Lerida and coworkers reason that palmitoylation favours partitioning and stabilization of Rac1 into highly-ordered cholesterol-rich domains of the plasma membrane known as liquid-ordered (L_o) domains. L_o domains modulate the capacity of Rac1 to bind effectors in the plasma membrane that stabilize the GTP-bound form of Rac1 that in turn triggers downstream activation of F-actin polymerization. They add that Rac1-mediated actin polymerization induces the expansion or coalescence of ordered microdomains into larger domains, thus promoting membrane order.

Are L_o domains the missing link in the final targeting of CalDAG-GEFI?

It is interesting to speculate that these L_o domains represent an anionic-rich pool that enables targeting of CalDAG-GEFI through electrostatic attraction within discrete regions of the plasma membrane as originally proposed by Johnson and coworkers (Biochem J 2007 406(2) 223-226) and Goulding (Membrane translocation of RasGRPs by C1 domains., PhD Thesis., Oct 2008 R E Goulding., The University of British Columbia, Vancouver) (see above).

PART V

FFAS COVALENTLY REGULATE BOTH PLATELET ACTIVATION AND PLATELET ACCUMULATION INTO THROMBI

Post-translational modification of proteins by FFAs

Posttranslational modification of proteins is the foundation of intracellular signalling. Without the ability to reversibly modify proteins and lipids, cells would be unable to react to signals received from their environment. Post-translational modification of proteins usually, but not always, occurs after a protein has arrived at the appropriate subcellular location. In certain instances, however, such modifications serve as addresses to correctly target the protein within the cell.

Covalent modification of proteins by FFAs includes N-myristoylation, where myristate is co-translationally attached to proteins at N-terminal glycine residues (J Biol Chem 1991 266(14); Annu Rev Biochem 1997 63 869-914; Annu Rev Biochem 1988 57 69-99) and S-acylation where palmitate and, less frequently, other FFAs are added to cysteine residues (Subcell Biochem 2004 37 217-232; Annu Rev Biochem 2004 73 559-587); more generally, S-acylation is known as S-palmitoylation or just "palmitoylation".

S-acylation by FFAs is concentration-dependent

Palmitoylation is characterized by two unique features. First, the cysteinyl residues to be palmitoylated are not specified by obvious consensus sequences (Subcell Biochem 2004 37 217-232), and second, the process of palmitoylation is reversible (J Biol Chem 1996 271(38) 23594-23600; EMBO J 1987 6(11) 3353-3357). Because the process is reversible then palmitoylation/depalmitoylation has the capacity to regulate cellular functions. However, most importantly, aberrant regulation/signalling may result through a concentration-dependent mass action effect when for example the concentration of FFAs is elevated above a threshold (Am J Physiol Endocrinol Metab 2012 302(11) E1390-E1398; Proc Natl Acad Sci USA 2010 107(51) 22050-22054; J Biol Chem 1993 268(32) 23769-23772; FEBS Lett 2003 540(1-3) 101-105). That is, excess FFA will drive control in a "rightward direction" toward maximal gain associated with increased S-acylation. Interestingly, although palmitate is the preferred acyl chain transferred to proteins by palmitoyl acyltransferases, other acyl chains can also be transferred, and substitution will depend upon availability and

concentrations of other FFAs (J Biol Chem 2001 276(33) 30987-30994; J Biol Chem 2002 277(36) 33032-33040; J Biol Chem 1984 259(8) 5054-5057). Acceptable FFA alternative substrates for palmitoyl acyltransferases include stearate, oleate and arachidonate. Thus, saturated, monounsaturated and polyunsaturated FFAs can all be accommodated.

Protein palmitoylation is necessary for both platelet activation and platelet accumulation

Several platelet proteins undergo palmitoylation. Platelet proteins that are palmitoylated include the G_{α} subunits: α_i, α_q, α_s, α_z, and α_{13} (J Biol Chem 1994 269(7) 4713-4716), SNAP (synaptosomal-associated protein)-23 (Biochem Biophys Res Commun 1999 258(2) 407-410), glycoprotein Ib (CD42) (Blood 1996 87(4) 1377-1384), adenylyl cyclase (FEBS Lett 1995 371(3) 241-244), and P-selectin (J Biol Chem 1993 268(15) 11394-11400). Palmitoylation of the platelet adhesion receptor glycoprotein Ib-V-IX complex has been shown to direct its incorporation into lipid rafts (J Exper Med 2002 196(8) 1057-1066).

The above observations that platelets incorporate labelled palmitate into platelet proteins indicates that protein palmitoylation occurs as a posttranslational modification in mature, resting platelets. However, activation of platelets results in both increased and more rapid labelling of proteins with palmitate (Biochim Biophys Acta 1989 1011(2-3) 134-139). More recently, Sim and coworkers (Arterioscler Thromb Vasc Biol 2007 27(6) 1478-1485) evaluated the role of protein palmitoylation in platelet activation and thrombus formation and show how loss of palmitate reduces membrane-association of proteins involved in platelet function. For example, they found that removal of palmitate (using acyl-protein thioesterase 1) from two proteins, $G_{\alpha q}$ and SNAP-23 involved in SFLLRN (thrombin receptor-activating protein)-induced platelet secretion interferes with their membrane association. Taken together, their results allow the conclusion that platelets possess a protein palmitoylation machinery that is required for both platelet activation and platelet accumulation into thrombi and that inhibition of platelet protein palmitoylation blocks platelet aggregation and granule secretion. Additionally, in a murine model of thrombus formation, Sim and coworkers demonstrate that inhibition of protein palmitoylation markedly inhibits platelet accumulation into thrombi at sites of vascular injury.

In 2011, the same group (Blood 2011 118(13) e62-e73) undertook an extensive program in order to characterize the human platelet

palmitoylome. This global analysis of resting platelets identified more than 1300 proteins, of which 215 met criteria for significance and represent the platelet palmitoylome. This collection includes 51 known palmitoylated proteins, 61 putative palmitoylated proteins identified in other palmitoylation-specific proteomic studies, and 103 new putative palmitoylated proteins. They conclude that out of this extensive platelet palmitoylome approximately 50% of identified palmitoyl proteins mediate signal transduction and transport processes.

Targeting of Rap2b to lipid rafts is promoted by palmitoylation and is required for efficient protein activation in human platelets

The activation of the small GTPase Rap2b in resting and agonist-stimulated human platelets was investigated by Greco and coworkers (J Thromb Haemost 2004 2(12) 2223-2230). Both thrombin, that stimulates heterotrimeric G-protein-coupled receptors, and the GPVI ligand convulxin, that activates a tyrosine-kinase based signalling pathway, induced the rapid and sustained binding of GTP to Rap2b. Thrombin- and convulxin-induced activation of Rap2b was not dependent on thromboxane A2, did not require the interaction of the protein with the cytoskeleton, and was not regulated by integrin αIIbβ3-dependent outside-in signalling.

P2Y12 receptor mediates the PI-3K-dependent activation of Rap2b

When secreted ADP was neutralized, activation of Rap2b induced by thrombin, but not by convulxin, was significantly reduced. ADP was found to induce the rapid and sustained binding of GTP to Rap2b, and this effect was predominantly mediated by stimulation of the Gi-coupled P2Y12 receptor. Moreover, Rap2b activation induced by thrombin, but not by convulxin, was totally dependent on phosphatidylinositol 3-kinase (PI-3K) activity.

Palmitoylation of Rap2b

Canobbio and coworkers (Cell Signal 2008 20(9) 1662-1670) investigated the localization of Rap proteins to specific microdomains of the plasma membrane called lipid rafts, which implicated in signal transduction. They found that Rap1b was not associated with lipid rafts in resting platelets, and did not translocate to these microdomains in stimulated cells. By contrast, about 20% of Rap2b constitutively associated with lipid rafts, and this percentage did not increase upon platelet stimulation. Rap2b interaction with lipid rafts also occurred in

transfected HEK293T cell. Upon metabolic labelling with tritiated-palmitate, incorporation of the label into Rap2b was observed. Palmitoylation of Rap2b did not occur when Cys176 or Cys177 were mutated to serine, or when the C-terminal CAAX motif was deleted. Contrary to CAAX deletion, Cys176 and Cys177 substitution did not alter the membrane localization of Rap2b, however, relocation of the mutants within lipid rafts was completely prevented. In intact platelets, disruption of Rap2b interaction with lipid rafts obtained by cholesterol depletion caused a significant inhibition of aggregation. Importantly, agonist-induced activation of Rap2b was concomitantly severely impaired. These results demonstrate that Rap2b, but not the more abundant Rap1b, is associated with lipid rafts in human platelets. This interaction is supported by palmitoylation of Rap2b, and is important for a complete agonist-induced activation of this GTPase.

F-actin comes back into focus!

Interestingly, Torti and coworkers (Proc Natl AcAd Sci 1994 91(10) 4239-4243) had previously demonstrated that agonist-induced actin polymerization is necessary for the translocation of Rap2b to the platelet cytoskeleton, suggesting that Rap2b interacts with the newly-formed actin filaments. Comparative immunoblotting revealed that the translocation of Rap2b to the cytoskeleton during platelet aggregation was accompanied by the simultaneous translocation of integrin αIIbβ3. Moreover, the cytoskeleton from aggregated platelets contained Rap2B and αIIbβ3 in comparable amounts. Taken together, Torti and coworkers conclude that these results demonstrate an association of Rap2B with αIIbβ3 and that their translocation to the cytoskeleton in aggregated human platelets are closely-related events.

Convergence of FFAs with P2Y12 receptor signalling

In a broader context, overall these outcomes suggest the convergence of palmitoylated-Rap2b upon the FFA-regulated, Rac1-mediated, F-actin-dependent translocation of CalDAG-GEF1 (see above). The observation that the P2Y12 receptor mediates the PI-3K-dependent activation of Rap2b is interesting since once more we have a point of convergence between FFAs and agonist-stimulated P2Y12 activity. As with many other FFAs-mediated signalling discussed throughout this document this convergence is of special importance in patients with CYP2C19 loss-of-function polymorphisms who are treated with clopidogrel. In patients with CYP2C19 loss-of-function polymorphisms who are being managed with clopidogrel, given that low levels of the active thiol metabolite will

likely result in less than maximal blockade of the P2Y12 receptor pool, then Rap2b palmitoylation will proceed given that ADP-stimulated p2Y12 are conditional for the activation of Rap2B. Conversely, with total blockade of the P2Y12 receptor pool then the rate and magnitude of Rap2b activation and subsequently its contribution to platelet activation, mediated in some part by the simultaneous translocation of integrin αIIbβ3, would be markedly attenuated.

PART VI

HUMAN PLATELETS EXPRESS AN INDUCIBLE POOL OF P2Y12 RECEPTORS THAT CAN EVADE INHIBITION BY CLOPIDOGREL

*A clopidogrel-insensitive inducible pool of **P2Y12** receptors contributes to thrombus formation*

Abstract

Using saturation [^{3}H]2-(methylthio)ADP ([^{3}H]2MeSADP) binding studies, Helena Haberstock-Debic and coworkers at Portola Pharmaceuticals in California (J Pharmacol Exp Ther 2011 339(1) 54-61) demonstrated that platelet stimulation with thrombin and convulxin (mouse) and thrombin receptor activating peptide (TRAP) (human) significantly increased the surface expression of P2Y12 receptors relative to that of resting platelets. Importantly however, they observed that while clopidogrel dose-dependently inhibited ADP-induced aggregation through maximal blockade of surface P2Y12 receptors on resting mouse platelets, achieving complete inhibition at the highest dose (50 mg/kg), it failed to block the inducible pool.

Human Platelets Express an Inducible Pool of $P2Y_{12}$ upon Strong Agonist Stimulation

Following their initial observations using mouse platelets, Haberstock-Debic and coworkers assessed whether an inducible pool of P2Y12 exists in human platelets. Stimulation of human platelets with 5 μM or 20 μM TRAP peptide induced a comparable and significant increase in the expression of P-selectin measured by flow cytometry. Using saturation

radioligand binding they then addressed whether newly- exposed P2Y12 receptors can be detected on unstimulated (control) platelets by comparison with those stimulated with TRAP (5 μM). The increase in P2Y12 receptor number observed upon TRAP stimulation was significant (untreated, 423 ± 28; TRAP-stimulated, 529 ± 28 $p = 0.02$), with no significant change in K_d (the equilibrium constant between ADP and the P2Y12 receptor) observed in control versus stimulated conditions (control, $K_d = 3.6 \pm 1.7$ nM; TRAP-stimulated, $K_d = 5 \pm 1.5$ nM). From these observations they conclude that, similar to mouse platelets, an inducible pool of P2Y12 receptors exists on human platelets and that these internal receptors can be mobilized to the platelet surface after strong agonist stimulation.

Clopidogrel Does Not Completely Block the Inducible Pool of $P2Y_{12}$

To determine whether clopidogrel blocked this inducible pool of P2Y12 receptors, Haberstock-Debic and coworkers next determined the number of P2Y12 receptors on the platelets from mice treated with clopidogrel (using saturation binding studies with [^{3}H]2MeSADP) with and without treatment with 5 nM thrombin. The results of these studies showed that when the all of the surface P2Y12 receptors are blocked after chronic clopidogrel treatment, there is an inducible pool of P2Y12 receptors which are expressed after platelet stimulation that are not inhibited by the clopidogrel active metabolite. Importantly, they show that this inducible pool is fully functional and capable of mediating platelet thrombosis.

There is a discrepancy between inhibition of the P2Y12 receptor and platelet aggregation

Interestingly, Haberstock-Debic and coworkers observed a discrepancy between inhibition of the P2Y12 receptor and platelet aggregation. They report that whereas submaximal doses of clopidogrel (0.5–5 mg/kg) inhibited P2Y12 receptor binding by 65 to 86% on resting platelets, it only inhibited ADP-mediated aggregation by 32 to 57%. From this observation, Haberstock-Debic and coworkers conclude that a limited number of functional receptors is sufficient to provide a sustained aggregation response, suggesting that exposure of a new clopidogrel-insensitive pool of P2Y12 receptors (25–35% increase) could, as they demonstrate, exert a significant impact on thrombosis.

Clinical implications of these new findings by Haberstock-Debic

Although these workers do not fully explore the reasons for the clopidogrel-insensitivity of the inducible pool of P2Y12 receptors, they propose that surface expression of induced P2Y12 receptors will evade blockade by clopidogrel if levels of the active thiol clopidogrel metabolite are declining (in addition to this pharmacokinetic argument, a pharmacodynamic explanation for their observations cannot be excluded). Examining the clinical implications of their findings, Haberstock-Debic and coworkers propose that if, for example a plaque rupture occurs and initiates thrombosis then newly-mobilized P2Y12 receptors may overwhelm the capacity of clopidogrel with a resultant unchecked thrombotic process. As I discuss below, FFAs above some concentration threshold unequivocally represent a "strong thrombogenic stimulus" that in effects is comparable with the definition of "strong agonists" as used by Haberstock-Debic and coworkers.

FFAs represent a strong thrombogenic stimulus with the potential to induce the surface expression of an inducible pool of P2Y12 receptors

Early investigations showed that FFAs affect the coagulation of blood (Br J Exp Path 1955 36(3) 248-253; Clin Sci 1957 16(2) 269-274; Br J Exp Path 1957 38(5) 529-538). In particular, certain FFAs greatly accelerated the formation of thrombi from rat or human blood using the Chandler Loop procedure (Q J Exp Physiol Cogn Med Sci 1961 46 1-7; J Clin Invest 1962 41(6) 1199-1205). Long-chain, saturated FFAs, such as stearic and palmitic shortened the time of thrombus formation, whereas the unsaturated FFAs of similar chain length (oleic, linoleic, linolenic, and arachidonic) did not influence the thrombus formation time. Short-chain, saturated FFAs of chain length less than C12 were likewise without effect.

Following these early investigations, Connor and coworkers (J Clin Invest 1963 42(6) 860-866) observed that massive and extensive thrombosis and sudden death occurred after the intravenous infusion of long-chain (>C14), saturated FFAs into dogs. These effects did not occur when unsaturated FFAs or short-chain, saturated FFAs were given. These more innocuous FFAs did, however, induce some hypercoagulability of the blood as indicated by thrombus formation in a jugular vein segment isolated after the completion of the infusion.

When FFAs-albumin solutions were infused intravenously into rabbits, none died but eight of fifteen given stearic acid-albumin (1400-2100 μM FFA) and four of ten given oleic acid-albumin (1960-2100 μM FFA) developed thrombi in the lungs. The whole-blood silicone clotting time

decreased from 37.8±3 to 23.2±3 min (p< 0.01) in rabbits given stearic acid and from 38.0±2.6 to 23.9±2 min (p< 0.01) in rabbits given oleic acid. None of ten rabbits given a 5% albumin solution (200-400 μM) developed lung thrombi and whole-blood silicone clotting times were not shortened (Arch Pathol 1966 81(2) 136-139).

In rabbits given adrenocorticotropic hormone (ACTH) as a manoeuvre to mobilize endogenous lipid, the mean plasma FFAs increased to 1770 μM at 2 hours and remained elevated at 1269 μM at 5 hours. Toxic signs developed within 1 h and included an increase in respiratory rate followed by weakness, laboured respirations, and decreased alertness. Four of ten rabbits given ACTH died during the experiment. Hearts with thrombi and lungs with pulmonary edema and congestion were common findings at necropsy. Controls did not show any significant increase in FFAs, toxic signs, or pathologic findings (Am J Pathol 1963 43 987-990).

In a clinical setting, Armstrong and coworkers (Can J Surg 1979 22(4) 366-368) measured the plasma concentrations of FFAs before, during and after total hip arthroplasty in seven patients. Each patient underwent examination for deep vein thrombosis with ^{125}iodine leg scanning, impedance plethysmography and contrast venography. Substantially higher concentrations of FFAs were found in the plasma of patients shown to have thrombosis than in those without thrombosis (for a review of the literature on the relationship between FFAs and acute coronary syndrome please see Part I of this document).

An FFAs-inducible pool of P2Y12 receptors will compromise the anti-platelet efficacy of clopidogrel

Taken together, the above findings together with the evidence reviewed in Parts I,II and III of this document support the view that above a certain threshold, FFAs have the capacity to induce thrombosis that can be massive and extensive and result in death. Importantly, this document shows that the effects of FFAS converge upon numerous mediators within signalling pathways induced by activated G protein-coupled receptors (GPCRs) in platelets. Consequently, it is not unreasonable to propose that FFAs in common with the diverse agonists studied by Haberstock-Debic and coworkers (J Pharmacol Exp Ther 2011 339(1) 54-61) will induce the surface expression of an intracellular inducible pool of P2Y12 receptors. The implications of this new proposal are considerable, especially in patients with raised levels of FFAs who are managed with clopidogrel.

As discussed above, Haberstock-Debic and coworkers observed a discrepancy between inhibition of the P2Y12 receptor and platelet

aggregation. They report that whereas submaximal doses of clopidogrel (0.5–5 mg/kg) inhibited P2Y12 receptor binding by 65 to 86% on resting platelets, it only inhibited ADP-mediated aggregation by 32 to 57%. From this observation, Haberstock-Debic and coworkers conclude that a limited number of functional receptors is sufficient to provide a sustained aggregation response, suggesting that exposure of a new clopidogrel-insensitive pool of P2Y12 receptors (25–35% increase) could, as they demonstrate, exert a significant impact on thrombosis.

The impact of an FFAs-inducible pool of P2Y12 receptors can be anticipated to be considerable given that the above findings of Haberstock-Debic and coworkers suggest that only a limited number of unblocked but ADP-activated P2Y12 receptors are required in order to maintain a sustained aggregation response. Such a phenomenon may profoundly compromise the anti-platelelet efficacy of clopidogrel in patients with raised levels of FFAs and CYP2C19 loss-of-function polymorphisms, given that low levels of the active thiol metabolite will likely result in less than maximal blockade of the P2Y12 receptor pool. Whether a mechanistic basis exists to explain the lack of sensitivity of this inducible pool of receptors to clopidogrel as defined by Haberstock-Debic (J Pharmacol Exp Ther 2011 339(1) 54-61) is something that remains to be investigated.

PART VII

THE ROLE OF FREE FATTY ACIDS AS THE FINAL ARBITER IN MODULATING CLOPIDOGREL RESISTANCE

What do we know about FFAs in different patient populations?

- *Elevated plasma concentrations of FFAs occur during different states and disorders that include:*

  Acute coronary syndrome, notably acute MI

  Diabetes mellitus

  Hypertension

  Obesity

  Following PCI

Administration of heparin

Administration of ACTH

Some things we know about FFAs and platelet function

- *Exposure of platelets to FFAs enhances responsiveness to ADP*
- *FFAs and DAG synergistically activate PKC*
- *FFAs and DAG synergistically activate human platelets*
- *FFAs induce translocation of PKC to the plasma membrane*
- *FFAs induce the accumulation of DAG within human platelets*
- *FFAs may induce DAG accumulation by inhibiting growth factor-induced DAGK* α activation
- *FFAs may regulate a DAG-independent and PKC-dependent translocation of CalDAG-GEFI to the plasma membrane*
- *FFAs mediate the Rac1-dependent and F-actin-dependent translocation of CalDAG-GEFI*
- *Effects of FFAs in mediating accumulation of DAG (by several routes) converges with a P2Y12-mediated inhibition of DAG conversion to PA*
- *As a "strong thrombogenic stimulus" FFAs above a concentration tthreshold have the potential to induce the surface expression of an inducible pool of clopidogrel-insensitive P2Y12 receptors*
- *Targeting of Rap2b to lipid rafts is promoted by palmitoylation*
- *There are 215 known proteins that constitute the palmitoylome in the human platelet of which 50% mediate signal transduction and transport processes*

The level of FFAs is a covariate (continuous variable) that determines the magnitude of clopidogrel resistance

*Higher loading doses of clopidogrel result in increased levels of active metabolite even in patients with two loss-of-function alleles (*2/*2) for the CYP2C19*2 genetic variant*

Jean-Philippe Collet and coworkers at L'Hôpital Pitié-Salpêtrière in Paris (JACC Cardiovasc Interv 2011 4(4) 392-402) provide new insights into the conversion of clopidogrel to the H4 active thiol metabolite in post-MI patients who are heterozygous (wild type/*2) or homozygous (*2/*2) for the CYP2C19*2 genetic variant. These new teachings are entirely consistent with the evidence presented throughout this document that the presence of CYP2C19 loss-of-function alleles is not a major determinant of clopidogrel resistance. Collet and coworkers showed that the use of a higher loading dose of clopidogrel in *CYP2C19*-deficient patients (i.e., *2/*2) results in an increase in the H4 thiol active metabolite plasma level measured as area under the concentration time curve (AUC) of the loading dose up to six hours (from 9.94 ± 2.88 ng · h/ml to 15.84 ± 5.26 ng · h/ml for 300 mg and 900 mg loading doses, respectively, p = 0.007), which, as these workers propose suggests a *CYP2C19*-independent metabolic pathway that could be linked to other cytochromes such as *CYP3A4* or *CYP1A2* (Drug Metab Dispos 2010 38(1) 92-9). Of interest, this increase in plasma level of the active H4 thiol metabolite with 900 mg reaches levels identical to those measured with 300 mg in wild type/*2 patients, but as Collet and coworkers add the increase with the 900 mg loading dose does not, in their hands translate into a similar degree of platelet inhibition using light transmission aggregometry or the point-of-care VerifyNow (Accumetrics) assays. Thus, in the absence of some additional variable that has the power to influence the magnitude of clopidogrel resistance then *a priori*, according to the findings of Collet and coworkers (JACC Cardiovasc Interv 2011 4(4) 392-402) an increase in loading dose in *CYP2C19*-deficient patients should overcome or attenuate clopidogrel resistance. Indeed, the findings of Laurent Bonello and coworkers at L'Hôpital Universitaire Nord in Marseille likewise suggest that the remaining CYP2C19 activity in heterozygotes and that other CYPs involved in clopidogrel metabolism, in both homozygotes and heterozygotes, enable sufficient active metabolite generation when very high doses of clopidogrel are used.

Free fatty acids represent the final arbiter in modulating clopidogrel resistance

Some concluding remarks on how FFAs modulate clopidogrel resistance

- The available evidence supports the view that within upper and lower limits, FFAs occupy a critical role in mediating platelet function. Above a certain concentration threshold it is well-documented that FFAs are associated with increased platelet reactivity and aggregation (Thrombos Haemostas (Stuttg) 1976 36(2) 325-331; Scand J Haematol 1976 17(3) 205-212; Nature 1970 228(5278) 1330-1332). It is also well-documented that FFAs non-linearly augment the effects and reactivity of mediators and messengers within platelets, that include PKC and DAG (Proc Natl Acad Sci USA 1992 89(4) 6443-6446; Proc Natl Acad Sci USA 1991 88(12) 5149-5153; Biochem Biophys Res Commun 1987 149(2) 762-768; Naunyn-Schiedeberg's Arch Pharmacol 1987 335 (Suppl 1) R36; Proc IVth Intern AICR Symposium on "Eicosanoids, Lipid Peroxidation and Cancer" (Nigam S Ed)., Springer-Verlag, Berlin, Heidelberg NY; *Biochem Biophys Res Commun 1980 95(3) 1321-1327*). Importantly, the effect of FFAs upon platelet reactivity has the potential in numerous patient subgroups, notably patients with loss-of-function CYP2C19 polymorphisms who are managed with clopidogrel to overcome total or partial blockade of the P2Y12 receptor pool. In the latter situation, the effects of FFAs have the potential to amplify signalling from fully functional agonist-stimulated P2Y12 receptors. By contrast, below the lower limit in both animals and humans, a deficiency of FFAs results in platelet aggregation that is impaired and which has been demonstrated to be causally related to FFAs deficiency (J Nutr 1987 117(9) 1520-1526; J Pediatr 1977 90(3) 439-443; Thromb Res 1978 12(5) 921-927). Moreover, platelets possess a protein palmitoylation machinery that is required for both platelet activation and platelet accumulation into thrombi and that inhibition of platelet protein palmitoylation blocks platelet aggregation and granule secretion (Blood 2011 118(13) e62-e73). Additionally, in a murine model of thrombus formation, Derek Sim and coworkers at the Beth Israel Deaconess Medical Centre in Boston (Arterioscler Thromb Vasc Biol 2007 27(6) 1478-1485) demonstrate that inhibition of protein palmitoylation markedly inhibits platelet accumulation into thrombi at sites of vascular injury.

- *A priori*, blockade of p2Y12 receptors can be surmounted by an elevated level of FFAs through an FFAs-induced increase in levels of DAG and an inhibition by FFAs of the activity of growth factor-stimulated DAG-kinase (DAGK) α. Thus, as shown by Guidetti and coworkers (J Biol Chem 2008 283(43) 2895-28805) concomitant stimulation of the P2Y12 receptor through the addition of ADP in the presence of the selective P2Y1 receptor antagonist MRS2179 (in order to observe the effects of ADP upon the P2Y12 receptor without confounding from any ADP-bound P2Y1 receptors) significantly potentiated the effect of the DAG analogue sn-1,2dioctanoylglycerol (DiC8) on platelet aggregation; and that pharmacological inhibition of DAGK restores the agonist-induced phosphorylation of PKC substrates prevented by antagonists of the P2Y12 receptor and, in parallel, allows platelets to undergo aggregation. Most importantly, even P2Y12 antagonist-promoted inhibition of Rap1 activation was overcome by pharmacologic inhibition of DAGK. Thus, above a certain threshold, these new teachings allow the suggestion that FFAs can bypass P2Y12 blockade and induce a non-receptor mediated aggregation of platelets.

- Using human platelets, Stuart Mundell and coworkers at the University of Walk in Bristol, UK (Mol Pharmacol 2006 70(3) 1132-1142) showed that novel, but not classical isoforms of PKC regulate P2Y12 function and trafficking. These findings highlight a further point of spatial convergence with FFAs signalling that may amplify signalling by functional ADP-occupied P2Y12 receptors in patients with CYP2C19 loss-of-function polymorphisms who are being managed with clopidogrel, given that in addition to activating PKC, FFAs also promote persistent and rapid translocation of PKC to the cell membrane (Am J Physiol Endocrinol Metab 2001 280(2) E229-37; J Cell Biol 1998 143(2) 511-521; Jpn J Pharmacol 1998 78(4) 411-417; J Cell Sci 1997 110(14) 1625-1634; J Biol Chem 1993 268(7) 5063–5068).

- Exciting and novel findings suggest that in patients with CYP2C19 loss-of-function polymorphisms who are treated with clopidogrel, signalling from clopidogrel-unoccupied, but functional ADP-occupied P2Y12 receptors (because of low levels of the active metabolite of clopidogrel) will converge with signalling by FFAs upon the activity of DAG-kinase α and the PKC-DAG-DAGK axis and potentiate the effects of DAG upon platelet activation. In

2008, Gianni Guidetti and coworkers at the University of Pavia in Italy (J Biol Chem 2008 283(43) 2895-28805) showed that stimulation of the P2Y12 receptor or direct inhibition of DAG-kinase (DAGK) potentiated the effect of the cell-permeable DAG analogue sn-1,2dioctanoylglycerol (DiC8) on platelet aggregation and pleckstrin phosphorylation, in association with inhibition of its phosphorylation to phosphatidic acid (PA). These results reveal a novel and unexpected role of the G_i-coupled P2Y12 receptor in the regulation of diacylglycerol-mediated events in activated platelets. That is, in these patients it is likely that even a normal physiological level of FFAs will amplify the output from unblocked, but ADP-occupied functional P2Y12 receptors and diminish the effects of an increase in loading dose of clopidogrel (that may be administered following CYP2C19 genetic testing) that fails to achieve 100% blockade of the P2Y12 receptor pool (but see comments on work by Helena Haberstock-Debic and coworkers below).

- At a high concentration, FFAs have the capacity to induce thrombosis that can be massive and extensive and result in death. Importantly, this document shows that the effects of FFAs converge upon numerous mediators within signalling pathways induced by activated G protein-coupled receptors (GPCRs) in platelets. Consequently, it is not unreasonable to propose that FFAs in common with the diverse agonists studied by Haberstock-Debic and coworkers (J Pharmacol Exp Ther 2011 339(1) 54-61) will induce the surface expression of an intracellular inducible pool of P2Y12 receptors. That is, internalized P2Y12 receptors will recycle to the platelet surface with subsequent resensitization (Mol Pharmacol 2006 70(3) 1132-1142). Superimposed upon this background of FFAs-induced tonic mobilization there will be phasic increases in recycling of P2Y12 receptors associated with context-dependent spike elevations of FFAs levels (associated with administration of heparin for example).

- The outcomes of the study by Helena Haberstock-Debic and coworkers at Portola Pharmaceuticals in California (J Pharmacol Exp Ther 2011 339(1) 54-61) show that a limited number of functional receptors is sufficient to provide a sustained aggregation response. That is, in patients with CYP2C19 loss-of-function polymorphisms who are treated with clopidogrel and in whom FFAs concentrations are elevated, ideally, 100% blockade by the active metabolite of clopidogrel must be achieved in order to

abrogate the convergence of P2Y12 signalling with that of FFAs upon DAG metabolism and growth factor-stimulated DAGK α activity. However, and most importantly, an FFAs-induced recycling of internalized P2Y12 receptors to the platelet surface may be predicted to overwhelm the capacity of clopidogrel to achieve total blockade of the platelet surface p2Y12 receptor population, notably in patients with CYP2C19 loss-of-function polymorphisms. Whether a mechanistic basis exists to explain the lack of sensitivity of this inducible pool of receptors to clopidogrel as defined by Haberstock-Debic (J Pharmacol Exp Ther 2011 339(1) 54-61) is something that remains to be investigated.

- CalDAG-GEFI activates Rap1 in response to calcium ionophores (J Biol Chem 275(41) 32260-32267) and the direct association of its N-terminal domain with F-actin regulates the subcellular localization of CalDAG-GEFI (J Biol Chem 2004 279(19) 20435-20446). The F-actin-dependent translocation of CalDAG-GEFI is regulated by FFAs by both non-covalent and covalent interactions. Importantly, regulation of the translocation of CalDAG-GEFI by FFAs is concentration-dependent and consequently levels of FFAs above a critical threshold can be expected to result in platelet hyper-responsiveness with the potential to both augment the response of platelets to ADP-occupied functional P2Y12 receptors and surmount blockade by P2Y12 antagonists in a non-receptor mediated fashion.

- Covalent modification of proteins by FFAs includes N-myristoylation, where myristate is co-translationally attached to proteins at N-terminal glycine residues (J Biol Chem 1991 266(14); Annu Rev Biochem 1997 63 869-914; Annu Rev Biochem 1988 57 69-99) and S-acylation where palmitate and, less frequently, other FFAs are added to cysteine residues (Subcell Biochem 2004 37 217-232; Annu Rev Biochem 2004 73 559-587). The post-translational covalent linking of the 16-carbon FFA palmitate, usually by formation of a thioester bond (thioacylation/thioalkanoylation) to cysteine and less frequently to serine and threonine residues of proteins is a process known as “palmitoylation”. Palmitoylation is reversible, and cycles of palmitoylation–depalmitoylation enable proteins to transiently associate with membranes, thereby regulating their sorting, localization and function (Cell 2010 141(3) 458-471; Nat Rev Mol Cell Biol 2007 8(1) 74-84; J Cell Biol 2007 176(3) 249-254; Curr Opin Neurobiol 2005 15(5) 527-35). Since protein palmitoylation

influences protein trafficking, protein stability, and protein aggregation, then aberrant or uncontrolled protein palmitoylation could disrupt many of these events, including anterograde and endocytic trafficking of membrane proteins, inappropriate lateral segregation of proteins into membrane lipid microdomains, or interference with palmitate anchors in heterotrimeric G protein subunit recycling (Am J Physiol Endocrinol Metab 2012 302(11) E1390-1398). Platelets possess a protein palmitoylation machinery that is required for both platelet activation and platelet accumulation into thrombi and that inhibition of platelet protein palmitoylation blocks platelet aggregation and granule secretion (Blood 2011 118(13) e62-e73). Additionally, in a murine model of thrombus formation, Derek Sim and coworkers at the Beth Israel Deaconess Medical Centre in Boston (Arterioscler Thromb Vasc Biol 2007 27(6) 1478-1485) demonstrate that inhibition of protein palmitoylation markedly inhibits platelet accumulation into thrombi at sites of vascular injury. Conversely, as discussed within the main text of this document, excessive incorporation of palmitate can be expected to result in dysfunctional platelet activity that can include hyper-responsiveness to ADP for example.

- In patients with CYP2C19 loss-of-function polymorphisms who are being managed with clopidogrel, given that low levels of the active thiol metabolite will likely result in less than maximal blockade of the P2Y12 receptor pool, then Rap2b palmitoylation will proceed unattenuated given that functional ADP-stimulated P2Y12 receptors are conditional for the activation of Rap2B. Conversely, with total blockade of the P2Y12 receptor pool then the rate and magnitude of Rap2b activation and subsequently its contribution to platelet activation, mediated in some part by the simultaneous translocation of integrin αIIbβ3, would be markedly attenuated.

APPENDIX I/II

Some Notes

The potential role of HGF in modulating clopidogrel resistance

Abstract

In addition to the effects of FFAs in surmounting clopidogrel blockade of the P2Y12 receptor the effects of HGF and possibly that of FFAs upon expression and activity of CYP 450 enzymes will additionally impact upon clopidogrel responsiveness through attenuation of the formation of the active thiol metabolite.

Systemic HGF levels in patients with atherosclerosis display considerable within and between study variation in levels of systemic HGF

The normal range for serum/plasma concentrations is reported as 0.06-0.4 ng/ml (J Clin Oncol 2009 27(33) 5519–28; Biochem Pharmacol 2011 Aug 5th Epub ahead of print), (small differences exist between serum and plasma levels of HGF).

The values documented within Table 1 below show considerable variation in systemic levels of HGF both within and between studies of patients with atherosclerosis. The wide confidence intervals frequently reported indicate a substantial variation within a single study, and the range of values between studies for patients such as those with acute myocardial infarction (0.27-20.7 ng/ml) show values within the normal range, or greater than two orders of magnitude above the lower limit of normal (A level of 37 ng/ml is cited in J Biol Chem 2003 278(7) 4705-4712).

TABLE 1

REPORTED PLASMA OR SERUM LEVELS OF HGF IN PATIENTS WITH ATHEROSCLEROSIS AND RELATED DISORDERS

All values in ng/ml

Source	Population	Min value (median/mean if no min)	Max value	Control (normal or reference)
Leukemia 2001 15 1165	Healthy n=11	0.164	0.522	0.36
J Clin Endocrinol Metab 1999 84 2425	Healthy n=40			0.19+/- 0.01
Ibid	Healthy (Smokers)			0.19+/ 0.01
Ibid	Healthy (Non-smokers)			0.18+/- 0.01
Ibid	PAOD n=37		0.04+/- 0.02	
Ibid	PAOD (Smokers)		0.45+/- 0.03	
Ibid	PAOD (Non-smokers)		0.35+/- 0.02	
Biochem Biophys Res Commun 1996 221 391	Acute MI n=11	9.4+/- 3.2	13.1+/- 5.7	<0.39
Heart Vessels 1997 12 241	Acute MI n=12	12.5+/- 4.6	15.7+/- 9.1	0.3+/- 0.1 (angina)
Growth Factors 2010 28 75	ACS n=104	0.7	1.9	
Clin Res Cardiol 2009 98 477	STEMI n=23		4.5+/- 0.46	
Zhonghua Yi Xue Za Zhi	Chronic unstable angina (angiography with PCI) n=49	12.32+/- 3.72	13.57+/- 3.77	1.74+/- 0.60 (pre-angiography)
Ibid	Chronic unstable angina (angiography without PCI) n=21	10.93+/- 2.20	11.46+/- 2.30	0.97+/- 0.35 (pre-angiography)

TABLE 1

REPORTED PLASMA OR SERUM LEVELS OF HGF IN PATIENTS WITH ATHEROSCLEROSIS AND RELATED DISORDERS

All values in ng/ml

Source	Population	Min value (median/mean if no min)	Max value	Control (normal or reference)
Eur Heart J 2005 26 2387	CAD with stable angina n=338	1.23	1.93	0.4 (suspected angina but normal angiogram)
Ibid	CAD with stable angina (pre-treated with heparin) n=18	1.71	8.70	1.54 (CAD with no heparin)
Pol Merker Lekarski 2004 17 88-91	ACS	Plasma concentration of HGF increased "dramatically" after heparin injection, particularly unfractionated heparin; the authors comment on the role of drugs, especially ACE inhibitors in modulating HGF levels		
Circ J 2004 68 645	Acute MI n=61		0.34+/- 0.20	0.17+/- 0.56 (stable angina)
Ibid	Unstable angina n=18		0.27+/- 0.15	0.17+/- 0.56 (stable angina)
Ibid	Aortic dissection n not reported (n=?)		0.32+/- 0.12	0.17+/- 0.56 (stable angina)
Ibid	Pulmonary Thromboembolism n=?		0.29+/- 0.10	0.17+/- 0.56 (stable angina)
Clin Sci (Lond)	Acute MI with early reperfusion n=17	3.68+/- 0.13 (pre-reperfusion)	15.15+/- 0.22 (post-reperfusion)	0.06+/- 0.13
Coron Artery Dis 2003 14 301	Angina n=60	The authors report that HGF levels in the angina and acute MI groups were significantly higher than the control group, and the level in the acute MI group was significantly higher than the angina group		
	Acute MI n=62			

TABLE 1

REPORTED PLASMA OR SERUM LEVELS OF HGF IN PATIENTS WITH ATHEROSCLEROSIS AND RELATED DISORDERS

All values in ng/ml

Source	Population	Min value (median/mean if no min)	Max value	Control (normal or reference)
Circ J 2002 66 1003	Acute MI n=40		0.33+/- 0.09	0.24+/- 0.08
Circ J 2002 66 253	Unstable angina n=22		0.30+/- 0.03	0.19+/- 0.01
Ibid	Acute MI n=60		0.27+/- 0.02	0.19+/- 0.01
		The authors report a "dramatic" increase in serum HGF level after heparin		
Ibid	Acute MI (with PCI) n=51		0.34+/0.04	0.19+/- 0.01
Am Heart J 2002 143 272	Patients with suspected aorta-iliac atherosclerosis		0.35+/- 0.11 (demonstrable disease)	0.27+/- 0.09 (not demonstrable)
J Hypertens 2001 19 1975	Cardiovascular disease	Plasma HGF levels were raised in patients with atherosclerosis. The authors add that HGF levels were significantly associated with age, current smoking, and diabetes mellitus		

PCI and heparin appear to represent significant covariates

Interestingly, the variation in levels of HGF was significantly modulated by interventions such as PCI, and notably heparin which resulted in marked increases (heparin also increases levels of FFAs).

HGF and the platelet

To my knowledge only one group has studied the interaction between HGF and platelet function. Sinigaglia and Pietrapiana were the first workers to report that human platelets express the HGF receptor, the tyrosine kinase encoded by c-*MET* gene (J Thrombosis Haemostasis 2005 3(Suppl 1) Abstr P0296; FEBS Lett 2005 579(20) 4550-4554). Unfortunately, these early studies were not progressed due to a loss of

key personnel and a lack of funding (Fabiola Sinigalia, Universita del Piemonte Orientale, Via Solaroli 17, Italy, Personal Communication Sept 2011).

HGF stimulates PLC and PLD-catalyzed formation of DAG

Phospholipase D (PLD) catalyzes formation of phosphatidic acid from the major membrane phospholipid, phosphatidylcholine (PtdCho), and this reaction is implicated in the regulation of diverse cellular processes, such as vesicular trafficking and cell growth and differentiation. A large variety of receptor tyrosine kinases (RTKs), including the HGF receptor, c-Met and receptors coupled to heterotrimeric G proteins in a wide range of cell types has been reported to mediate PLD stimulation in response to their specific agonists. Thus, this represents an additional mechanism for the formation of DAG. The growth factor (eg HGF)-mediated elaboration of DAG via receptor tyrosine kinases (eg c-Met) in platelets remains to be demonstrated.

HGF downregulates the expression of cytochrome P450 isoenzymes in human hepatocytes

Using concentrations of HGF above the normal physiological range, but within the range seen in patients with ACS, Donato et al (J Pharmacol Exp Ther 1998 284(2) 760-7) studied the effects of recombinant human HGF, on the cytochrome P450 (CYP) system and conjugating reactions in cultured human hepatocytes. HGF produced a general decrease in the activity of all the CYP isozymes studied, that included CYP1A1/2, CYP2B6, CYP2A6, CYP2E, and CYP3A4. Given the role of CYP1A2, CYP2B6 and especially that of CYP3A4 in the metabolism of clopidogrel, then raised levels of HGF in patients with ACS can be predicted to impact upon the formation of the active metabolite. From their observations that the changes in the activity and protein levels of CYP1A2 and CYP3A4 correlated with a reduction in the specific messenger RNA levels, these workers infer that HGF could down-regulate CYP expression at a pre-translational level.

Some FFAs inhibit cytochrome P450 enzymes

It would be interesting to determine whether the downregulation by HGF of CYP 450 enzymes, reported above in some way interact with the apparently direct inhibitory effects of some poyunsaturated FFAs that include linoleate, linolenate and arachidonate, that were demonstrated by Yao et al (Life Sci 2006 79(26) 2432-40). Although in this study by

Yao et al the IC50 and K_i values tended to be above the physiological limit, nevertheless a synergy between HGF and FFAs cannot be discounted, given the different sites of action.

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TABLE 2

FFAs and platelet activation	Reference
Platelet aggregation occurred when saturated FFAs were added to platelet-rich plasma or to washed platelets.	Nature 1963 200 1331-2; Nature 1964 202 765-8
Extensive thrombosis and death occurred after the intravenous infusion of long-chain, saturated fatty acids into dogs. These effects did not occur when unsaturated fatty acids or short-chain, saturated fatty acids were administered.	J Clin Invest 1963 42(June) 860-6
Elevated concentrations within the physiological range of albumin-bound saturated FFAs enhance platelet responsiveness to ADP. The long chain FFAs stearate (C18) and palmitate (C16) produced the greatest effect with a moderate effect using myristate (C14) and minimal effect with laurate (C12). By contrast with the outcomes using saturated FFAS, little or no response was observed using the unsaturated FFAs linoleate (C18) and oleate (C18) even at the highest molar ratio of FFA to albumin.	Nature 1970 228(5278) 1330-1332)
Conscious subjects undergoing cardiac catheterization and other diagnostic procedures showed an increase in platelet-aggregation response to ADP one hour before and during the procedure. The responses returned towards normal one hour afterwards. Plasma FFA levels were increased during the procedure, and one hour afterwards, but had returned to normal by the following day.	J Clin Pathol 1973 26(12) 958-962

FFAs and platelet activation	Reference
In ten healthy male volunteers, fasting for seventy-two hours resulted in an increase of mean plasma concentration of FFAs of more than two fold, and this was associated with an increase in assayed platelet aggregation scores ($p<0.02$). This correlation increased significantly when plasma levels of the saturated FFA, oleate were used alone ($p<0.01$). Fasting was also associated with an increase in plasma levels of the small CXC family cytokine platelet factor 4 (PF4) (this cytokine is released from platelet alpha granules) suggesting that platelet reactivity had been enhanced.	Thrombos Haemostas (Stuttg) 1976 36(2) 325-331
In a group of patients with acute MI group the concentration of plasma FFAs was significantly higher at admission (1.058+/- 0.334 at 3 hours) by comparison with values obtained at 16 hours (0.752+/- 0.297 $p<0.05$) and at 24 hours (0.798+/-0.283 $p<0.01$). The percentage of venous reversible platelet aggregates was significantly elevated during the first hours of acute MI, and a similar pattern was also found within a subgroup of patients with severe angina pectoris without ECG changes but with an established history of CAD. In reviewing these findings in patients with MI and severe angina, the investigators propose that in some part, based upon the earlier findings of Hoak et al (Nature 1970 228(5278) 1330-1332) (above) FFAs may reduce the threshold for ADP-induced aggregation or induce aggregation directly.	Scand J Haematol 1976 17(3) 205-212

FFAs and platelet activation	Reference
Platelet aggregation was studied following intravenous injection of heparin to nine healthy adults. Heparin is known to produce an increase in the concentration of free fatty acids (FFA) within 10 minutes of intravenous injection. A significant correlation was found between the intensity of primary platelet aggregation and changes in plasma FFA levels.	Am J Hematol 1978 4(1) 17-22
Homa et al provide one of the earliest reports that exogenous FFAs dose-dependently induce the accumulation of DAG within intact human platelets. They observed that when FFAs, notably unsaturated FFAs, were added to whole human platelets pre-labelled with ^{14}C arachidonate, radioactive DAG accumulated within the platelets. Polyunsaturated and mono-unsaturated FFAs were equally effective and the saturated FFA, palmitate produced the lowest effect.	Biochem Biophys Res Commun 1980 95(3) 1321-1327
Zentner et al studied the aggregation response of washed porcine platelets to the sodium salt of stearic, oleic, palmitic, and myristic acids. The FFAs were prepared as aqueous suspensions and as taurocholate- or albumin-solubilized systems. The final concentration of FFA in the platelet preparation varied between 70 μM and 600 μM. Platelet aggregation was observed with both the suspended and taurocholate-solubilized FFA. The extent of platelet aggregate formation increased with the FFA concentration and chain length. With the exception of stearate, the taurocholate-solubilized fatty acids were more active than the suspensions. Albumin-solubilized FFAs were devoid of platelet aggregating activity. Platelet aggregation induced by FFAs was not inhibited by epoprostenol, alprostadil, or indomethacin. These investigators conclude that the FFA-induced platelet aggregation was independent of cyclic AMP-related calcium shift, cyclooxygenase-arachidonate, or granular nucleotide release mechanisms.	J Pharm Sci 1981 70(9) 975-981

FFAs and platelet activation	Reference
Srivastava and Awasthi studied the effects of *cis* and *trans* FFAs as sodium salts and as free acids upon ADP- and collagen-induced platelet aggregation. Their results indicate that unsaturated FFAs inhibit platelet aggregation induced by ADP and collagen.	Prostaglandins Leukot Med 1983 10(4) 465-472.
The unsaturated octadecadienoic FFA (C18 with two double bonds) *cis, cis*- linoleic acid and *trans, trans*- linolelaidic acid and the diacylglycerol, 1-oleoyl-2-acetyl-rac-glycerol (OAG) concentration-dependently induced activation of gel-filtered human platelets (aggregation and phosphorylation of 20 kDa and 47 kDa peptides). By contrast, the unsaturated octadecenoic FFAs oleic and elaidic acid, and the saturated octadecanoic FFA stearic acid were inactive. Octadecadienoic acid-induced platelet activation was suppressed by the protein kinase C inhibitor, polymyxin B, but not by the cyclooxygenase inhibitor, indomethacin. OAG-induced activation was potentiated by octadecadienoic acids present at non-stimulatory concentrations. From these observations, these investigators propose that octadecadienoic acids and diacylglycerol synergistically induce platelet activation via protein kinase C and that these findings suggest a critical role for DAG in mediating FFA-induced platelet activation. They conclude that their data indicate that unsaturated octadecadienoic acids and DAG synergistically induce platelet activation via PKC. They add that these new findings using intact human platelets support their earlier observations and conclusions derived using purified PKC (Naunyn-Schiedeberg's Arch Pharmacol 1987 335 (Suppl 1) R36; Proc IVth Intern AICR Symposium on "Eicosanoids, Lipid Peroxidation and Cancer" (Nigam S Ed)., Springer-Verlag, Berlin, Heidelberg NY).	Biochem Biophys Res Commun 1987 149(2) 762-768

FFAs and platelet activation	Reference
In studies of gel-filtered human platelets, MacIntyre et al found that *cis*-unsaturated fatty acids at very concentrations (1-35 μM) inhibited platelet shape change, aggregation, and secretion of 5-hydroxytryptamine induced by thrombin, ADP, collagen, U46619 (a thromboxane A2 analog), or plant lectins, but not that induced by A23187, a calcium ionophore. *Trans*-unsaturated and saturated fatty acids had little or no inhibitory effect. The inhibitory effects of cis-unsaturated fatty acids were not affected by inhibition of adenylate cyclase or cyclooxygenase. The maximum platelet-inhibitory effect of *cis*-unsaturated fatty acids was seen when over 90% of the platelet label was still in the form of free fatty acids, and platelet inhibition could be reversed by washing the platelets by gel filtration. Binding of platelet agonists to the platelet was not inhibited by the fatty acids.	Blood 1984 63(4) 848-857
The inhibitory effects of three *cis*-unsaturated C18 fatty acids (oleic, linoleic, and linolenic acids, sodium salts) on ADP- and sodium-arachidonate-induced aggregation of washed rabbit platelets were investigated by Dratewka-Kos et al. When the platelets were suspended in protein-free medium containing dextran, consistent with the earlier observations of MacIntyre et al (Blood 1984 63(4) 848-857), Dratewka-Kos et al also observed that at very low concentrations (2-45 μM) the above *cis*-unsaturated fatty acids were potent inhibitors of platelet responsiveness; the inhibitory effect was rapid and occurred within seconds. The inhibition of ADP-induced aggregation was not affected by abolishing the activity of platelet cyclooxygenase using aspirin. Human serum albumin relieved the inhibition caused by fatty acids for both ADP- and arachidonate-induced aggregation.	Biochem Cell Biol 1986 64(9) 906-913

FFAs and platelet activation	Reference
ADP-induced platelet aggregation in diluted whole blood from rats fed a fat-free diet supplemented with 10% (by weight) hydrogenated coconut oil [essential fatty acid(EFA)-deficient rats] were significantly lower than that in animals fed 10% safflower oil [rich in linoleic acid] or 10% marine oil [rich in eicosapentaenoic acid and docosahexaenoic acid]. Platelet responsiveness to ADP was restored when plasma from the EFA-deficient rats was replaced by plasma obtained from rats fed a normal diet. ADP responsiveness in diluted whole blood was also restored after injections of 100 mg ethyl linoleate every 48 hours for two weeks, and partially restored by injections of 100 mg ethyl alpha-linolenate. These workers suggest that impaired platelet aggregation in EFA deficiency is related more to plasma factors than to inherent platelet properties.	J Nutr 1987 117(9) 1520-1526
In seven healthy non-smoker male subjects, a bolus injection of HMWH (5000 iu) was associated with significantly enhanced platelet aggregation. These experiments were repeated following the oral administration of acetylsalicylic acid (ASA). HMWH again caused enhancement of platelet aggregation despite the ASA-mediated inhibition of platelet aggregation. The administration of HMWH was associated with a rapid increase (minutes) in mean plasma levels of FFAs of approximately three fold.	J Nutr 1987 117(9) 1520-1526
Jastrzebska et al studied the effect of unfractionated heparin on platelet aggregation in normal subjects and in patients with primary hypertriglyceridemia and following myocardial infarction. Adrenaline and ADP-induced platelet aggregation was examined in plasma-rich platelets. Following the intravenous injection of 50 units of heparin per kg body weight they observed that heparin was associated with an increase in platelet aggregation and that the magnitude of the increase correlated with the plasma level of FFAs.	Kardiol Pol 1992 37(9) 136-141

FFAs and platelet activation	Reference
Yoshida et al undertook a systematic investigation in order to establish whether in activated human platelets, activation of PKC can be sustained by the synergistic interaction between *cis*-unsaturated FFAs and DAG, particularly when calcium ion concentration returns to basal levels. Initially, they observed that the transient phosphorylation of a PKC-specific 47-kDa protein by the short-lived membrane-permeant DAG analogue, 1,2-dioctanoylglycerol (1,2 DG) was significantly enhanced by the addition of the *cis, cis*-unsaturated FFA linoleic acid, which by itself was completely inactive. The potentiation of the phosphorylation of the 47-kDa protein by linoleic acid was also observed for many other naturally-occurring cis-unsaturated FFAs that include oleic, linolenic, eicosapentanoic and docosahexanoic acids. By contrast, neither saturated FFAs such as palmitic and stearic acids nor trans-unsaturated FFAs such as elaidic and linolelaidic acids were active in enhancing protein phosphorylation induced by 1,2-DG. These investigators propose that consistent with their earlier *in vitro* observations (Proc Natl Acad Sci USA 1991 88(12) 5149-5153), in the presence of DAG, *cis*-unsaturated fatty acids appear to increase the sensitivity of PKC activation to calcium ion, rendering the enzyme more active at lower calcium ion concentrations. In that study, kinetic analysis shows that these *cis*-unsaturated fatty acids almost fully activate the enzyme at the basal level of calcium ion concentration.	Proc Natl Acad Sci USA 1992 89(4) 6443-6446

FFAs and platelet activation	Reference
Oleic acid inhibited platelet activating factor-induced aggregation and serotonin release of rabbit platelets. However, at concentrations above 20 μM, oleic acid induced platelet aggregation and calcium ion mobilization.	Arch Biochem Biophys 1992 298(2) 471-479
The enhancement of ADP-induced aggregation by steric acid was shown to be prevented by using albumin to bind the stearic acid at a molar ratio of FFA to albumin of < 2. From these observations, Hoak proposes that clinical conditions in which the FFA:albumin ratio exceeds 2 could represent a thrombogenic threat in some patient groups. They suggest that a high FFA:albumin ratio could result from an aberration of fatty acid transport in which the FFA-albumin molar ratio exceeds 2 either because of very high plasma FFA concentrations as a result of excessive lipid mobilization or a low concentration of albumin in the blood.	Am J Clin Nutr 1994 60(6 Suppl) 1050S-1053S)
Tesserommatis et al show that the short term administration of standard heparin and low molecular weight fractions provoke an increase in the serum concentration of FFAs and that these concentrations remain significantly elevated seven days after stopping the administration. These investigators reason that because long chain FFAs are known to enhance the susceptibility to experimental thrombosis by a mechanism that remains to be characterized, then the effects on haemostasis of an elevation of FFA concentrations merits further attention.	Eur J Drug Metab Pharmacokinet 1996 21(3) 213-215

FFAs and platelet activation	Reference
Thirteen free-living healthy males consumed two experimental diets for four weeks with a seven week washout between the two dietary periods. The diets consisted of approximately 30% of energy as fat (66% of which was the treatment fat) providing approximately 6.6% of energy as stearic acid or approximately 7.8% of energy as palmitic acid. The investigators report that under these conditions, whereas the stearic acid diet was associated with beneficial effects upon thrombogenic and atherogenic risk factors, by contrast platelet aggregation was significantly increased by the palmitic acid diet.	Eur J Clin Nutr 2001 55(2) 88-96