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Review

Blood platelet abnormalities and pharmacological modulation of platelet reactivity in patients with diabetes mellitus

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Abstract:
The overall picture of platelet abnormalities in diabetes mellitus (DM), including altered adhesion and aggregation, is hypersensitivity of diabetic platelets to agonists. "Primed" diabetic platelets respond more frequently even to subthreshold stimuli, sooner become exhausted, consumed and finally hyposensitive, thus contributing to accelerated thrombopoiesis and release of 'fresh' hyperreactive platelets. In diabetes disturbed carbohydrate and lipid metabolism may lead to physicochemical changes in cell membrane dynamics, and consequently result in altered exposure of surface membrane receptors. These phenomena, together with increased fibrinogen binding, prostanoid metabolism, phosphoinositide turnover and calcium mobilization often present in diabetic patients, contribute to enhanced risk of small vessel occlusions and accelerated development of atherothrombotic disease of coronary, cerebral and other vessels in diabetes. The paper concentrates on the role of dynamic, physico-chemical properties of platelet membrane lipid bilayer, as a major determinant of platelet hypersensitivity in diabetic patients.
As a pharmacological response to platelet hypersensitivity in DM, making a major contribution to enhanced risk of thromboembolic macroangiopathy, and consequently enhanced morbidity and mortality in diabetic individuals, we have a variety of antiplatelet agents, and acetylsalicylic acid (ASA) is no doubt most commonly used world-wide. Every-day clinical practice shows that antiplatelet pharmacological approach may not always be efficient enough in people with diabetes. Although we are at the very beginning of complete understanding of so-called 'aspirin-resistance', several potential molecular mechanisms of this phenomenon in diabetes have been evidenced.

Key words:
diabetes mellitus, blood platelets, platelet hypersensitivity, platelet membrane lipid fluidity, acetylsalicylic acid, aspirin resistance

Abbreviations: ADP – adenosine diphosphate, ASA – acetylsalicylic acid, $[Ca^{2+}]_i$ – concentration of intracellular calcium, COX (COX-1, COX-2) – cyclooxygenase (1 or 2), DM – diabetes mellitus, 16-DOXYL-Ste – spin label 16-doxylstearic acid, 5-DOXYL-Ste – lipophilic spin label 5-doxylstearic acid, EDTA – ethylenediaminotetraacetate, EGTA – ethylene glycol-O,O'-bis-[2-amino-ethyl]-N,N,N',N'-tetraacetic acid, ESR – electron spin resonance, Fg – fibrinogen, GPIIb–IIIa – glycoprotein complex IIb–IIIa, integrin $\alpha_{IIb}\beta_3$ (fibrinogen receptor), h_{+1}/h_0 ratio – parameter read from ESR spectrum of a lipophilic spin label, proportional to lipid bilayer fluidity, H-12-V (HHLGGAKQAGDV) – dodecapeptide His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (corresponding to the fibrinogen γ chain C-terminus), MPV – mean platelet volume, RGDS – tetrapeptide Arg-Gly-Asp-Ser, TG – thapsigargin, t-PA – tissue-type plasminogen activator, TRAP – thrombin receptor activating peptide (SFLLRNPNDKYEPF), TSP – trombospondin, Tx (TxA_2/TxB_2) – thromboxane A_2/B_2

Introduction

The peculiar constellation of thrombotic factors and the sustained hypercoagulable state in diabetes mellitus leads to increased mortality and morbidity in this group of patients [12, 83]. Vascular inflammation, endothelial dysfunction associated with hyperglycemia and hyperinsulinemia, impaired fibrinolysis and increased coagulation factors, as well as abnormal blood platelet function, are typical for diabetes. Such a complex array of predisposing factors contributes to the overall increased thromboembolic incidence and development of arteriosclerosis in diabetic patients [13]. Typically, numerous of these risk factors may be present long before a diagnosis of diabetes mellitus (DM) is established. Commonly, the abnormalities of vascular endothelium are regarded as an important denominator of thrombosis-oriented modifications of blood platelets, coagulation and fibrinolysis, all the major factors underlying high prevalence of vascular events in diabetes [12–14, 77].

Facts on platelet abnormalities are coinciding in showing platelet hypersensitivity in diabetes

The overall picture of platelet abnormalities in DM, including altered adhesion and aggregation, is hypersensitivity of diabetic platelets to agonists. Platelets in diabetic subjects appear to be in an activated state even in the absence of vascular injury, as evidenced by greater expression of the fibrinogen-binding glycoprotein IIb/IIIa receptor, which constitutes the final common pathway of platelet activation and allows for cross-linking of individual platelets by fibrinogen molecules and formation of thrombus. "Primed" diabetic platelets respond more frequently even to subthreshold stimuli, sooner become exhausted, consumed and finally hyposensitive, thus contributing to accelerated thrombopoiesis and release of 'fresh' hyperreactive platelets [87].

Diabetic platelets are hypersensitive to agonists *in vitro*, and alterations in a number of mechanisms involved in platelet activation occur in these platelets, which could contribute to their hypersensitivity. Platelets obtained from diabetic subjects show increased adhesiveness and an exaggerated aggregation, both spontaneous and in response to stimulating agents. Also, in DM of either type, the increased populations of circulating platelets expressing activation-dependent adhesion molecules, such as activated glycoprotein complex IIb–IIIa (GPIIb–IIIa), thrombospondin, lysosomal GP53, or, perhaps most importantly, P-selectin (GMP-140), have been identified. The causes for the enhanced platelet activation in diabetes are multifold: altered exposure and/or abundance of glycoprotein receptors for agonists and adhesive proteins on the platelet surface, increased fibrinogen binding, decreased membrane fluidity, enhanced arachidonate pathway activation with increased thromboxane A_2 formation, and increased phosphoinositide turnover leading to increased protein phosphorylation, enhanced inositol trisphosphate (IP_3) production, and subsequently accelerated Ca^{2+} mobilization or a combination thereof [105]. The noteworthy implication of platelet hyperreactivity in diabetes are also their low threshold for activation with agonists and altered agonist profiles (e.g. glycated matrix ligands). Both seem of the utmost importance for the overall role of platelets in diabetic vascular complications and prothrombotic state in diabetic individuals [31, 100]. These phenomena contribute to enhanced risk of small vessel occlusions and accelerated development of atherothrombotic disease of coronary, cerebral and other vessels in diabetes [12, 13, 25].

What makes diabetic platelets hypersensitive?

In an attempt to answer the question in the title, two types of metabolic disorders, inherently associated with diabetic state, are worth considering:
(a) dyslipidemia and altered lipid profile of both blood plasma and cellular structures, and
(b) hyperglycemia, with the consequent non-enzymatic glycosylation of biomacromolecules.The important implication of the above is that in DM a disturbed carbohydrate and lipid metabolism may lead to altered physico-chemical properties of cell membranes, and physicochemical changes in cell membrane dynamics may further result in altered exposure of surface membrane receptors [87, 90, 97].

Numerous of platelet alterations in diabetes, including calcium release from intraplatelet storage pools and/or its transport across platelet membranes, are related to the alterations concerning platelet membrane components [86, 90, 92, 95, 110, 111]. The altered biophysical state of platelet membrane components in DM may thus be one of the major determinants of platelet hypersensitivity and hyperfunction and may contribute to impairments in various metabolic pathways, like intensified calcium mobilisation

and accentuated thromboxane A_2 synthesis and release [3, 90, 114].

Transitions in membrane lipid bilayer fluidity are physiological phenomena

Membrane lipid bilayer fluidity plays an important role in cellular functions, and blood platelets are not unique in that respect [58]. Overwhelming evidence has accumulated confirming that membrane fluidity is involved in the control of an increasing number of physiological processes and that derangements of normal fluidity may occur in some pathological states [40, 63, 88]. The major effect of lipid fluidity is on the dynamics of functional units which are embedded in the lipid matrix. Membrane proteins are mobile in the lipid environment and their lateral diffusion is affected by both the effects of protein crowding and to the constraints from an aqueous matrix [7, 63]. While variations in the fluid nature of the hydrophobic core of the membrane may directly affect various transmembrane events, other changes within membrane, such as protein-protein spacing or receptor position within the membrane, may also be important [7, 28, 37, 63]. When the lipid fluidity is decreased, the new equilibrium position of an overall weaker lipid-protein interaction but with correspondingly greater protein-water association may become more optimal, in which membrane proteins may be displaced towards the aqueous phase on either side of the membrane [28, 64]. Although the correlation between lipid fluidity and the rate of lateral diffusion can be only partially accounted for, the lipid microviscosity becomes the major retarding force for rotational diffusion of proteins in lipid matrix [63]. Numerous reported changes in protein projection could be accounted for by such displacement mechanisms. Moreover, ligand binding to a membrane protein (i.e. ligand-receptor interaction) has also been suggested to induce a modulation of charge distribution [102]. Furthermore, the immediate lipid fluidity around the occupied receptor may alter, thus affecting unoccupied receptors and the resultant changes in the exposure of both occupied and unoccupied receptors may lead to local membrane aggregations [57, 63]. Overall, the modulation of membrane lipid fluidity may determine the apparent degree of accessibility of receptors and their response to fluidity changes. In long-term or sustained changes of membrane lipid microviscosity (as in atherosclerosis, diabetes or during aging), the total number of receptors may also change by various metabolic processes [45, 49, 63, 107].

Mobility of membrane phospholipids determines projection of membrane proteins

Specific association between a membrane receptor and its ligand is generally of a high affinity and mediates significant conformational changes in the occupied receptors, which can even alter the packing density of the neighbouring lipid bilayer. The resulting fluidity changes are expected to fade away from the occupied receptor, but opposing fluidity gradients from other occupied receptors may direct the system to a new steady-state level of lipid fluidity. The modulation of lipid fluidity in the vicinity of an occupied receptor raises the possibility that the accessibility and turnover of adjacent receptors may be affected by the occupied receptors [57, 63] (Fig. 1). Based on what was discussed above, there is no doubt at present that alterations in membrane lipid fluidity, and the consequent new "equilibrium" states of functional membrane receptors/proteins may have a direct impact on platelet intracellular signalling. The precise

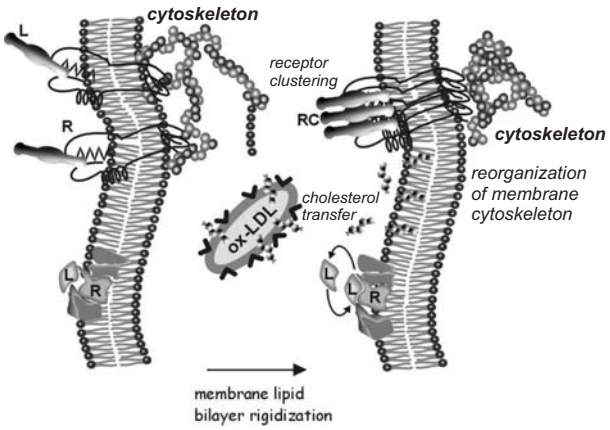

Fig. 1. Mechanisms underlying hypercholesterolaemia-mediated platelet hypersensitivity. Reduced platelet membrane lipid fluidity may be a consequence of altered lipid composition in platelet membranes, e.g. increased cholesterol content. Cholesterol accumulation in membranes is dependent on plasma lipid profile and may be mediated by the interaction of platelets with some low-density lipoprotein (LDL) fractions, mainly oxidised LDL (ox-LDL). The latter facilitate cholesterol transfer and activate platelets. Increased membrane cholesterol to phospholipid ratio makes membrane receptors (R) less mobile in lipid environment, affects their positioning and induces their displacement toward aqueous environment. Enhanced receptor projection promotes ligand (L) binding. Changes in membrane lipid fluidity in the vicinity of occupied receptors affects also unoccupied receptors that leads to receptor clustering. Formation of receptors clusters ('aggregates') (RC), that may be further stabilized by cytoskeleton proteins (cytoskeleton), leads to increased platelet sensitivity to agonists and facilitates platelet aggregation and/or adhesion

relation between platelet membrane fluidity and signal transduction in human platelets remains indefinable, however, we have evidence that the fluidity of platelet plasma membranes affect the responsiveness of blood platelets [58, 67, 90, 92, 95, 110–112]. The belief that impaired macromolecular interactions in platelet membranes, associated with the reduced diffusion of various membrane components, modulate cell receptor-mediated signal transduction [63], is supported by the observations suggesting that the lack of dynamic lipid changes following receptor-ligand interactions results in defective transmembrane signalling [7].

The evidence supporting the crucial role of membrane lipid dynamics in triggering the initial steps of signal transduction in activated platelets comes essentially from two types of research: (*a*) studies on the modulation of membrane-associated phenomena by dietary lipid composition, f.i. in terms of cholesterol content in lipid bilayer, and (*b*) effects of anesthetics and organic solvents on membrane fluidity.

Basic implications of altered platelet membrane fluidity in diabetes

The lipid fluidity of platelet membranes may be influenced by either the altered composition of membrane lipids, the altered structure and conformation of membrane proteins embedded into lipid matrix, or the altered lipid-protein interactions. While the former is likely to be modulated by dietary lipids, the latter may be associated with the binding of ligands to membrane receptors. In fact, the evidence has been presented which demonstrates that enrichment of cellular membranes with cholesterol, that reduces membrane lipid bilayer fluidity, causes an increase in the exposure of certain membrane proteins to the aqueous environment [6]. Associated with such changes in membrane lipid composition, blood platelets have been demonstrated to become more sensitive to aggregation induced by adrenalin or adenosine diphosphate (ADP) [35, 67, 68, 73]. On the other hand, there are reports showing that the interaction of protein and peptide ligands with platelet membrane receptors leads to the changes in the organization of membrane components, and such displacements in membrane protein projection could be mediated by the alterations of membrane lipid fluidity [45, 58, 67, 96–98].

In DM, the disease with a peculiar metabolic background, the following implications of altered cell membrane fluidity seem crucial:

(a) as the fluctuations in lipid bilayer fluidity are able to redirect membrane protein components to thermodynamically new equilibrium state of protein projection and accessibility, some protein domains, previously buried in hydrocarbon core may become newly exposed,
(b) thus, new potential targets for non-enzymatic protein modifications (glycosylation, carbonylation, acetylation and so on) become exposed,
(c) as far as numerous platelet proteins function as receptors, it means more exposed membrane receptors with potentially extended overall accessibility to ligands,
(d) more exposed platelet membrane receptors make an important contribution to platelet hypersensitivity.

Advanced platelet protein glycation in diabetes is associated with reduced platelet membrane fluidity

Much of the observed hyperaggregability of diabetic platelets results from the interactions of platelets with diabetic plasma proteins and this effect appears to be proportional to the extent of glycosylation of membrane and plasma proteins [19, 27, 32, 36, 110, 112]. A chronic hyperglycemia, and the resulting increased nonenzymatic glycosylation of platelet membrane proteins, have been suggested as major determinants underlying platelet hypersensitivity [33, 87, 105]. Also, the relationship between platelet protein modification by glucose and the reduced membrane lipid fluidity in diabetes has been evidenced in some studies [92, 95, 110–112]. Several reports have evidenced that an explanation for the reduction in membrane lipid fluidity in platelets from diabetic patients may relate to the increased nonenzymatic glycosylation of platelet membrane proteins [110–112]. The mechanism(s) of such a relation seem more even clear considering that the nonenzymatic attachment of glucose moieties to platelet membrane proteins most likely induces a steric hindrance in the hydrocarbon core of the membrane lipid bilayer. The rigidisation of platelet membranes leads to the more viscous hydrophobic core of the lipid matrix, in which membrane proteins become displaced towards the aqueous phase on either side of the membrane [28, 63, 104]. Hence, hypersensitivity of diabetic platelets to aggregating agents could potentially result in the increased accessibility of platelet membrane receptors in diabetic platelets [87, 90, 92, 95, 97], as it was revealed in the case of platelet fibrinogen receptors [48, 95].

Numerous reports concern devastating role of hyperglycemia as well as the resulting protein glycation in altering platelet functions. These studies point to positive associations between either hyperglycemia or induced protein glycation and the impaired platelet function, mainly increased platelet aggregability and release of intraplatelet granule contents [33, 38]. Also, the reports on preventive effects of dietary or pharmacological glucose-lowering strategies in the improvement of platelet functions are worth mentioning [72]. Whereas various platelet abnormalities, such as hyperaggregability, hypersensitivity to agonists or increased circulating platelet activation have been correlated with hyperglycemia [32, 92, 95, 97, 110, 112], the normalization of platelet hyperfunction in diabetes has been observed as a consequence of near-normoglycemia [24].

Release of Ca^{2+} and P-selectin in blood platelets is hampered by procaine

The modulators of membrane lipid fluidity have several features in common, which seem to be of a crucial importance in affecting platelet membrane dynamics:
(a) they considerably affect the compactness of phospholipid molecules in platelet membrane lipid bilayer, and thus lead to more or less restrained lipid mobility,
(b) accordingly, they may directly modulate various transmembrane events, such as protein-protein spacing or receptor position within a membrane,
(c) they influence lipid-protein interactions within membrane bilayer, and this concerns particularly boundary lipid molecules,
(d) they remain efficient regardless of whether they act as 'fluidizers' or 'rigidizers' of membrane lipid bilayer [22].
Effects of local anesthetics on the impairing membrane-associated signalling has been well documented, and their protective effects result from the constellation of physico-chemical changes in membrane lipid bilayer, including destroyed lateral phase separation in the region of protein-lipid interface, weakened association forces between water and macromolecules, a release of bound water molecules or/and direct interactions with membrane components [22]. Apart from above described non-specific biophysical effects [93], the modulators of platelet membrane fluidity might specifically influence some membrane-associated platelet components, crucial for platelet signal transduction, like protein kinase C or adenylate cyclase [66, 114].

The local anesthetic, procaine, not only rigidized platelet membranes [reduced electron spin resonance (ESR) h_{+1}/h_0 ratio of lipophilic spin label 5-doxyl-stearic acid (5-DOXYL-Ste)] and hampered the release of intraplatelet granules [reduced expression of P-selectin in platelets stimulated with either ADP, thrombin receptor activating peptide (TRAP) or thrombin], but also reduced Ca^{2+} mobilization from intraplatelet storage pools. The latter was demonstrated in both the platelets agonized with 15 μmol/l ADP, where procaine hampered Ca^{2+} release from intraplatelet storage pools, and under conditions of poor extracellular calcium, where procaine intensified calcium removal in the presence of Ca^{2+} chelator, ethylenediaminotetraacetate (EDTA)K_2. The preventive effects of procaine on platelet degranulation and Ca^{2+} mobilization were related to procaine-mediated dislocations of some membrane components, crucial in triggering of platelet release and activation. Procaine-induced distortions of lipid-protein interactions are believed to generate a steric hindrance, interfering with signal transduction, and thus leading to to impaired mobilization of Ca^{2+} and other components from platelet storage pools [91].

Reduced procaine's ability to hamper platelet activation in diabetes results from distorsions in platelet membrane components

Conformational changes leading to enhanced calcium mobilization may be considered a peculiar self-perpetuating mechanism in a number of intracellular events. Membrane-associated triggering of signal transduction initiates calcium release from intraplatelet storage pools. The rise in Ca^{2+} underlies further changes in membrane lipid fluidity and membrane bilayer 'order parameter', because calcium itself acts as a rigidizer in membrane lipid bilayer. Thereby the fluctuations in Ca^{2+} concentration and/or flux of Ca^{2+} across platelet membrane may possibly facilitate further Ca^{2+} mobilisation and platelet activation [90, 91], and these effects could have consequences for the general receptor functioning [26].

Majority of published data concerning calcium homeostasis and mobilization in diabetic individuals are rather consistent in showing: (i) increased platelet $[Ca^{2+}]_i$ in the resting state, (ii) higher Ca^{2+} mobilization after stimulation with thrombin and ADP, entirely

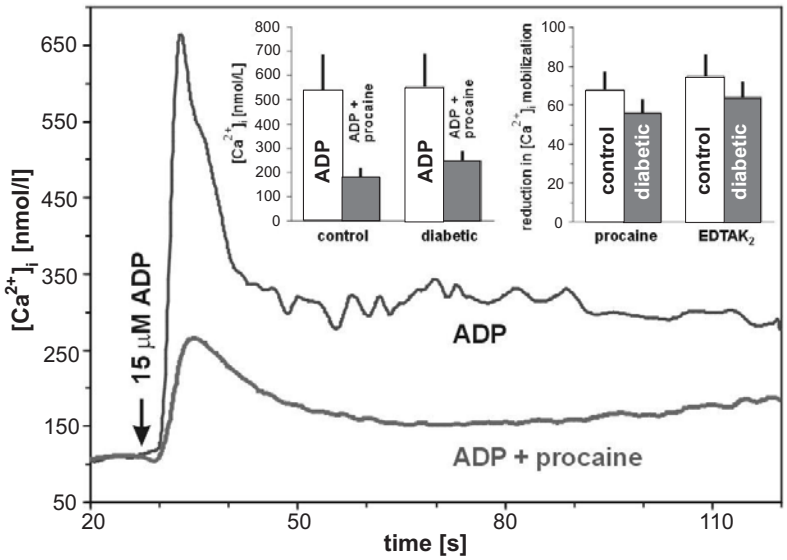

Fig. 2. Changes in $[Ca^{2+}]_i$ in Fura-2-loaded platelets isolated from type 2 diabetic patients and control healthy individuals. Procaine (10 mg/ml) reduced release of calcium from ADP-activated isolated platelets loaded with Fura-2 (4 M, λ_{exc} = 340/380 nm [free/bound], λ_{em} = 500 nm). *Insert:* procaine was less efficient to hamper ADP-stimulated Ca^{2+} mobilization in diabetic platelets ($p < 0.001$) (*left*), and similar reduction in preventive effects on Ca^{2+} mobilization was also observed for Ca^{2+} chelator, EDTA ($p < 0.05$) (*right*) [90]

due to increased resting concentrations, (iii) increased activity of Ca^{2+}-ATPase, and (iv) reverse-mode acting Na^+/Ca^{2+} exchanger mediating Ca^{2+} influx [43, 55, 68, 90].

In spite of an existing controversy as to whether the rigidification of plasma membranes during cell activation caused by an increase in $[Ca^{2+}]_i$ is a primary or late event, it is believed that the modulation of membrane dynamics by calcium displacements might essentially contribute to signal transduction [59, 63, 70, 90]. In the light of these findings, also the altered biophysical state of platelet membrane components in DM that is regarded a crucial determinant of platelet hyperfunction [97, 110–112], has been interpreted as a factor contributing to the intensified calcium mobilization in diabetic platelets [90]. We found that blood platelets from diabetic humans remained less susceptible to preventive effects of procaine, the agent hampering calcium release from intraplatelet storage pools (by up to 40%) and platelet degranulation. Consequertly, diabetic platelets were more vulnerable to stimuli facilitating cytosolic calcium mobilization (ADP) in the presence of procaine or $EDTAK_2$ (by more than 40%) (Fig. 2). Both the increased intracellular Ca^{2+} mobilization and higher levels of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in the presence of procaine corresponded to reduced platelet membrane fluidity in platelets from diabetic patients [90]. This rather complex array of experimental data clearly points that the biophysical state of platelet membrane components in DM is likely the crucial determinant of platelet hyperfunction and probably contributes to more intensified Ca^{2+} mobilization in diabetic platelets. Also, the diminished preventive effects of procaine on platelet release reaction and Ca^{2+} mobilization in platelets from diabetic patients may result from primary distortions and/or dislocations of membrane components caused by a diabetic state [89, 90].

Platelets in diabetes mellitus are more vulnerable to releasers and more resistant to blockers of cytosolic Ca^{2+} mobilisation

Thrombospondin (TSP) is a soluble, adhesive glycoprotein of blood platelet α-granules. Upon release, in the presence of high levels of Ca^{2+} this molecule binds to platelet surface membranes and to components of nascent and mature clot [44]. Thus, it promotes formation of large platelet aggregates and stable clot formation, and influences clot resistance to fibrinolysis [5]. Due to enhanced Ca^{2+} mobilisation and generally augmented release of intraplatelet granules in platelets of diabetic patients, TSP release and binding may be of particular relevance to prothrombotic status in DM [54, 75, 77, 103].

In the course of platelet activation TSP becomes released from intraplatelet granules and the ongoing Ca^{2+} mobilisation and its traffic outside platelets facilitates TSP binding to platelet surface membranes. Thus, Ca^{2+} mobilisation and release in diabetic platelets support TSP binding in a double way: indirectly, *via* enhancing Ca^{2+}-depended cellular signalling, and directly – *via* promoting the interaction of TSP with its receptor(s) [103].

In healthy non-diabetic individuals thapsigargin (TG, 1 μM), the inhibitor of Ca^{2+} sequestration by dense tubular systems, elevated the binding of TSP to surface membranes of TRAP-activated platelets (by up to 50%, $p < 0.001$), whereas ethylene glycol-O,O'-bis-[2-amino-ethyl]-N,N,N',N'-tetraacetic acid (EGTA, 5 mM), the extracellular Ca^{2+} chelator, reversed this effect (by up to 85%, $p < 0.001$). These effects were less profoundly expressed in type 2 diabetic patients. In both control and diabetic subjects, TG increased the presentation of platelet membrane receptor for TSP, glycoprotein IV (CD36), in platelets stimulated with TRAP ($p < 0.05$), whereas EGTA lowered the TRAP-stimulated expression of CD36. While the EGTA-mediated inhibition of CD36 expression was significantly stronger in healthy volunteers (41% *vs*. 32% resp., $p < 0.05$), TG-mediated activating effect was higher in diabetic individuals (11% *vs*. 27%, $p < 0.05$). When acting together, the suppresive effects of EGTA on TG-dependent Ca^{2+} mobilization were much attenuated in diabetic patients ($p < 0.05$). These observations clearly point out that platelets from diabetic subjects seem more vulnerable to the releasers of cytosolic Ca^{2+} and more resistant to the blockers of $[Ca^{2+}]_i$ mobilization.

Biophysical state of platelet membrane is crucial determinant of platelet dysfunction in diabetes mellitus

In less fluid diabetic platelet membranes, protein molecules embedded into the rigidised lipid bilayer would acquire much less motional freedom to accomplish the possible rearrangements due to the ligand-induced alterations in lipid-protein interactions [97]. Such a reduced lipid fluidity response of diabetic platelet membranes implies hypersensitivity of diabetic platelets [87, 95, 97, 108, 110]. In the line of the latter finding are the observations pointing that the fluctuations in membrane lipid fluidity associated with the interactions of natural ligands with platelet surface membrane receptors are restrained in patients with diabetes [97]. It has been suggested that much hampered effects of membrane receptor ligands on a lipid bilayer dynamics in diabetic platelets might have resulted from the altered exposure of platelet membrane proteins and the considerable rearrangements in the lipid-protein interactions in more 'rigid' membranes of diabetic platelets [90, 97].

We monitored microenvironmental lipid bilayer changes associated with the interaction of fibrinogen and fibrinogen-derived peptides with blood platelet membranes, using fluorescence quenching and ESR technique. A dodecapeptide HHLGGAKQAGDV (H-12-V), corresponding to the fibrinogen γ chain C-terminus and the tetrapeptide Arg-Gly-Asp-Ser (RGDS), corresponding to fibrinogen Aα chain sequence 572–575, induced the opposed changes in platelet membrane lipid dynamics: whereas the first had a fluidising effect and increased h_{+1}/h_0 parameter of 5-doxylstearic acid and 16-doxyl- stearic acid (increased mobilities of 5-DOXYL-Ste and 16-DOXYL-Ste), the second acted as a rigidizer (lowered mobilities of both spin labels) in platelet membrane lipid bilayer. These effects remained consistent with RGDS-mediated decrease and H-12-V-mediated increase in membrane protein tryptophan exposure towards the external membrane environment, as well as the conformational changes due to mobilization/immobilization of some membrane protein domains [increased relative rotational correlation time (τ_c) of 4-(ethoxyfluorophosphinyloxy)-2,2,6,6-tetramethylpiperidine-1-oxyl (ethoxyfluorophosphinyloxy-TEMPO) and increased h_w/h_s ratio (parameter proportional to mobility of labeled protein domains) in the ESR spectrum of 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (maleimide-TEMPO)] (Fig. 3A) [96].

When monitoring the effects of selected platelet ligands on the dynamics of membrane lipid bilayer, we observed in patients with type 2 diabetes much smaller reductions in the 5-DOXYL-Ste h_{+1}/h_0 ratio upon the interaction of either RGDS peptide or tissue-type plasminogen activator (t-PA), and much less profound increase in this parameter in the presence of fibrinogen (Fg) or Fg-derived peptides containing γ-chain carboxy-terminal sequence H-12-V. It means that the effects of various interacting ligands on platelet membrane fluidity were much lower in diabetic platelets: lower rigidizing (RGDS, t-PA) and lower fluidising effects (Fg, H-12-V) were particularly distinct at the lower depths of a lipid bilayer of

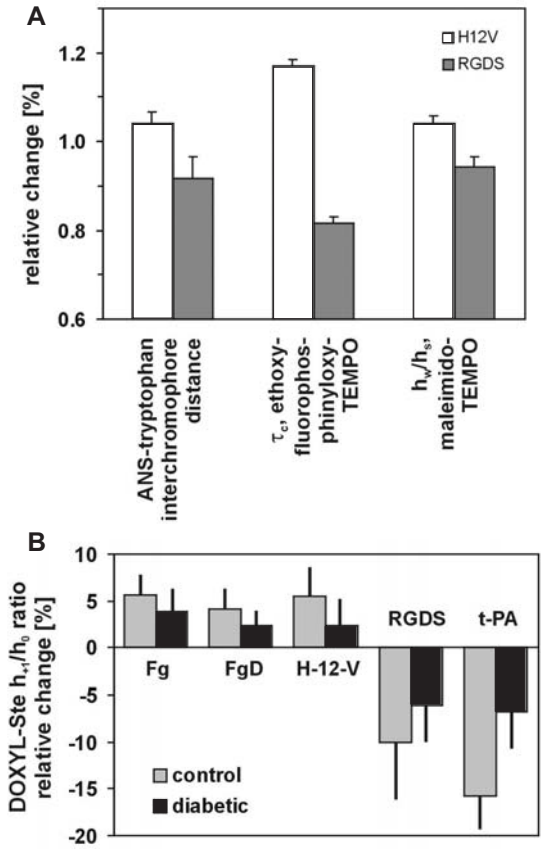

Fig. 3. Changes in dynamics of platelet membrane lipid bilayer induced by the interaction with fibrinogen-derived peptide ligands. (**A**) RGDS decreased relative apparent interchromophore separation (lowered ANS-tryptophan distance in platelet membrane proteins) ($p < 0.001$), and immobilized platelet membrane protein domains labeled with ethoxyfluorophosphinyloxy-TEMPO ($p < 0.001$) or maleimide-TEMPO ($p < 0.025$), for H-12-V the changes were opposite [96]. (**B**) Fluidizing effects of fibrinogen (0.01 μM), FgD (0.01 μM) or H-12-V (10 μM) and rigidizing effects of RGDS (10 μM) or t-PA (0.01 μM) were less pronounced in platelets from diabetic patients ($p < 0.04$ or less) [97]

platelet membranes (Fig. 3B). Further, these changes in dynamics of diabetic platelet membranes paralleled increased glycation of platelet membrane proteins in diabetes, which encourages to conclude that these much hampered effects on dynamics of platelet membrane lipid bilayer likely result from rearrangements in the lipid-protein interactions in rigidized membranes of diabetic platelets [97].

Platelet hypersensitivity contributes to accelerated platelet aging in diabetes mellitus

As the more viscous lipid matrix implies the greater projection of membrane proteins toward the external environment, it may instantaneously result in more exhaustive glycation of newly unshielded protein residues, which become accessible for glucose moieties. Such a self-supporting mechanism may render some membrane proteins becoming more displaced toward the external site of a membrane and considerably enhance the probability of the accelerated shedding the displaced proteins off membrane [63, 87]. Thus, we might expect that circulating diabetic platelets that often encounter episodes of activation may loose their functional membrane glycoproteins. The hypothesis has been raised that subsequent replacements of membrane glycoproteins shed off activated platelets may contribute to faster exhaustion of platelet intrinsic storage pools, and finally to accelerated platelet 'consumption' in diabetes [95]. In the course of such a process platelets from diabetic individuals would gradually attenuate their natural reactivity much faster than control platelets. Indeed, the accelerated 'consumption' of the functional platelet membrane proteins has been occasionally reported in type 1 and type 2 diabetic patients, where platelets presented diminished total amount of β_3 subunit of fibrinogen receptor complex GPIIb-IIIa, and the response of platelets challenged *in vitro* with strong agonists, like thrombin, was much reduced [92].

Platelet volume distribution comprises subpopulations of smaller and larger platelets

Each platelet volume distribution may be decomposed mathematically to partial volume distributions (the so-called exponentially-modified Gaussian distributions), representing subpopulations of cells differing in their dimensions (Fig. 4A). Such an approach revealed that platelet volume distributions in diabetic individuals are characteized by an apparent size bimodality, resulting from higher numbers of platelets of the extreme dimensions: very small platelets and very large platelets (Fig. 4B) [92]. These two platelet subpopulations of extreme dimensions are believed to differ considerably in their function, e.g. their reactivity and susceptibility to release granule contents. Small platelets are less dense cells and are often regarded as older platelets, which have undergone more episodes of release reaction and hence they are more exhausted off their granule contents. In turn, the increased fraction of platelets with larger size appears to optimise platelet aggregation [113]. Large platelets are considered younger cells, which have been reported as more sensitive and more rapidly recruited

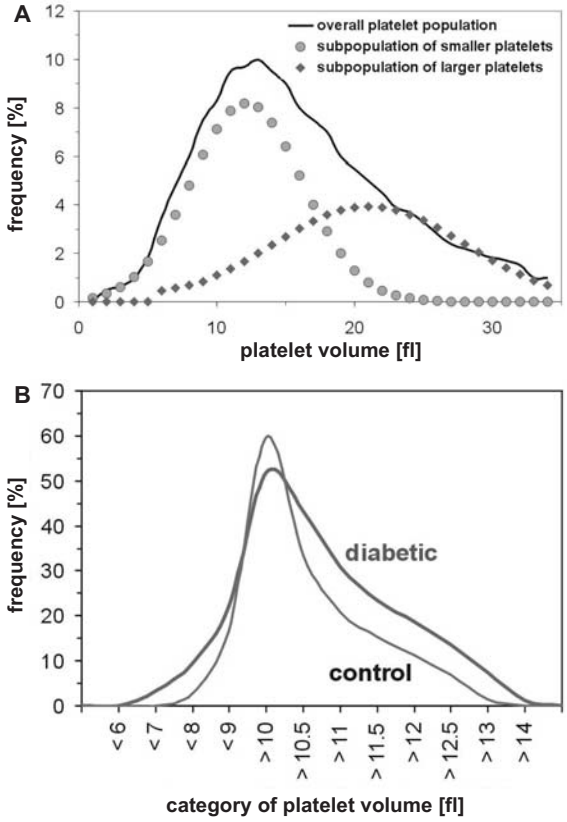

Fig. 4. Analysis of platelet volume heterogeneity. (**A**) Platelet volume distributions, acquired by the automated cell counters, may be decomposed to the partial exponentially-modified Gaussian (EMG) distributions, describing the componential platelet subpopulations. These resultant componential distributions are characterised by four individual measures of platelet volume dispersion: the distribution mode (volume of the most frequently represented platelets), deviation corresponding to platelet distribution width (PDW), area below the curve circumscribing a given platelet subpopulation and the so-called "dispersion" parameter, characterizing the right-side exponentially vanishing 'tail' of the distribution. (**B**) Frequency distributions show that in diabetic individuals there are higher number of platelets of extreme dimensions: very small platelets and very large platelets are more frequent in diabetic compared to control individuals [92]

into both micro- and macroaggregates in response to stimulating agents [78, 81, 113]. It may be deduced that with the increased frequency of the episodes of platelet release reaction, like in diabetes, there is the increased fraction of smaller and exhausted platelets, which become gradually replaced by large and hyperactive platelets [92].

Large platelets are more frequent in diabetic patients and platelet turnover is faster in diabetes mellitus

Some reports have claimed that larger platelets are more frequent or the mean platelet volume (MPV) is higher in diabetic patients, and these alterations have been often attributed to the quality of diabetes control [29, 61]. Besides the recognized clinical indicators of metabolic quality control of diabetes (fasting plasma glucose, fructosamine, hemoglobin A_1), several other parameters have been related to MPV, including membrane receptor expression or release of intraplatelet granule contents from activated platelets [61, 78, 80]. Since platelet size is generally perceived as a determinant of platelet function, with larger platelets being more reactive per unit volume [81, 92], an increased platelet size is thought to be a potential precursory factor in micro- and macrovascular complications of DM [61]. The enlargement of the mean platelet volume induced by increased severity of the diabetic state might reflect decreased mean age of the circulating platelets, which in turn implies shorter survival time and an increased turnover of the platelet population in DM [80, 81, 92, 109]. The increased fraction of larger, hyperaggregable platelets and/or the increased averaged platelet volume (MPV) may reflect the increased frequency of the episodes of platelet activation, and subsequently exaggerated platelet consumption and increased platelet renewal [79, 80, 92]. Thus, the increased MPV, related to increased numbers of large platelets in diabetic patients, is often regarded as a hallmark of impaired thrombopoiesis in DM [78, 81]. It has been reported that the accelerated thrombopoiesis, reflected by the increased fractions of reticulated (rich in residual RNA) platelets, directly contribute to platelet volume bimodality in diabetic individuals [92].

In general, the overall platelet reactivity, as measured by aggregation and total release of granular content, correlates with platelet size [61, 78, 82]. The shifts in platelet volume heterogeneity in type 2 diabetic patients, reflected by higher numbers of platelets of extreme dimensions (Fig. 4B), were associated with increased platelet response to agonists, and elevated consumption of circulating platelets, as deduced from increased fractions of platelet microparticles [92]. Tschoepe et al. who found the elevated average number of $\alpha_{IIb}\beta_3$ complexes in the platelets originating from type 1 and type 2 adult diabetic patients [79], have raised the suggestion that platelet hyperactivity reported in the diabetic state may thus be due to primarily altered production of platelets with an increased number of functional glycoproteins [78, 79]. Thus, the finding that peripheral platelets circulate in an activated state in newly diagnosed or even in pre-

diabetic individuals suggests a chronic condition of ongoing platelet consumption [106]. These observation suggests that it may be a primary alteration of platelet precursors, megakaryocytes, already at the level of bone marrow [76].

The concept that the most dense platelets are young platelets that become less dense as they age in the circulation implies that this process may be also modulated by some pathogenic factors. Of these, lipid alterations and nonenzymatic glycosylation, as well as the resulting reduced platelet membrane fluidity relevant to diabetic state, have been pointed out as the modulators of the utmost importance [87]. The association between reduced platelet membrane lipid fluidity occurring in diabetes and the enhanced activation of circulating blood platelets implies that platelet membrane receptors are more exposed to the external environment [87, 92, 95, 110], and thus the altered membrane dynamics might contribute to platelet hypersensitivity in diabetic state [95, 107, 111, 110], the formation of platelet volume heterogeneity [92], and consequently to an increased platelet turnover and reduced survival of platelets from the diabetic individuals [80, 81, 87, 92, 109].

Pharmacological modulation of platelet function in people with diabetes

Platelets of diabetic patients are found in a permanent prethrombotic state. Platelet 'priming' to more spontaneous activation and aggregation with resultant arterial thrombus formation, are considered central mechanisms in the pathophysiology of acute arteriothrombotic events in people with DM [80]. It is largely the consequence of the outcomes originating from large-scale randomized trials showing that, besides the requirements of tight glucose control improving the vascular status of diabetic patients, antiplatelet agents have also been shown to be effective in primary and secondary prevention of cardiovascular and stroke events in diabetes [17, 18]. Of them, aspirin and thienopyridine derivatives are the most commonly used antiplatelet agents, effective in reducing the associated risk of myocardial infarction and stroke [1, 2, 16]. Although clinical efficacy and safety of aspirin (acetylsalicylic acid, ASA) has been well recognised for decades, it has always been regarded as a relatively weak antiplatelet agent and several clinical trials have also exposed the limitations of ASA in some particular groups of patients, including individuals with diabetes [1, 2].

Platelets from diabetic patients show reduced sensitivity to ASA

Recent research indicates that platelet response to aspirin treatment shows a huge individual variability and the same dose of aspirin which is effective in some patients may be characterized by low therapeutic index in others [50]. The direct consequence of the aforementioned variability in response to ASA is that the optimum dose of aspirin as an antithrombotic drug can differ in different approaches and different groups of patients [4]. Along with such a variability there is also a considerable heterogeneity in the yield of a protection by ASA against thromboembolic complications, reported particularly in some clinical states [11]. This phenomenon is known as reduced blood platelet sensitivity to aspirin and often referred to as the so called "aspirin-resistance". Nowadays, there is no unambiguous evidence pointing to the cause of reduced blood platelets response to ASA, nevertheless multiple mechanisms for resistance have been proposed, including both genetic and environmental factors [21, 34, 50, 51, 60, 101].

Occasional data in the literature indicate that aspirin-induced suppression of platelet thromboxane synthesis may be lower in diabetic than non-diabetic subjects [46]. Our own observations point that lowered sensitivity to aspirin may concern not only the arachidonic acid-mediated platelet aggregation but also platelet response to collagen. The maximal inhibition of platelet aggregation by ASA was found to be lower and IC_{50} higher in diabetic compared to control subjects, both in the presence of arachidonic acid and collagen. Thus, the diminished platelet sensitivity to aspirin in diabetic individuals in the presence of various agonists might suggest that aspirin-induced reduction in platelet function is a more generalized phenomenon, not merely attributed to the inhibition of platelet cyclooxygenase. Moreover, the observed reduced response of platelets from diabetic subjects to aspirin was associated with a higher level of fraction of glycated hemoglobin (HbA_{1c}) lower concentration of high-density lipoprotein cholesterol and a higher total cholesterol concentration [94]. The latter observation points to possible metabolic factors underlying the reduced platelet sensitivity to the action of ASA in patients with diabetes.

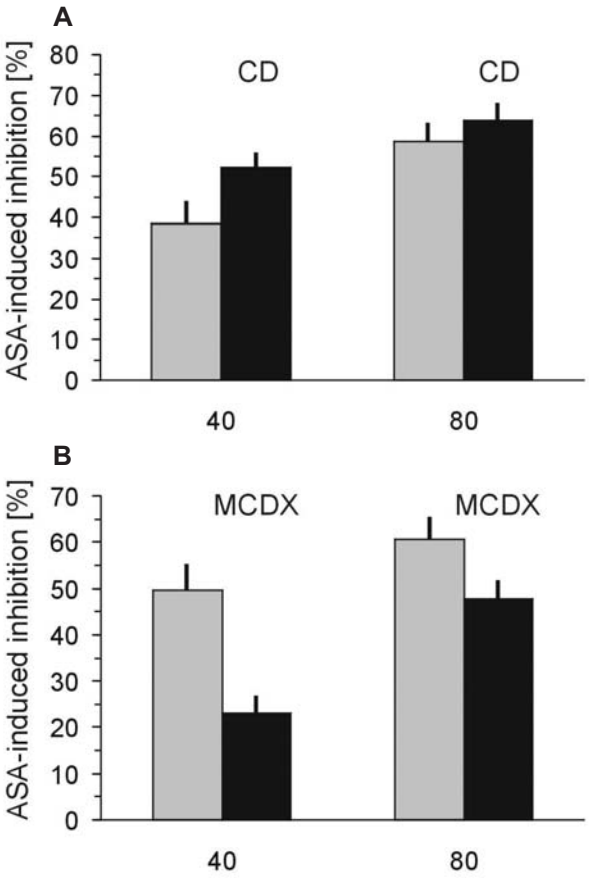

Fig. 5. Aspirin-induced changes in platelet aggregation in cholesterol-depleted (**A**) and cholesterol-enriched platelets (**B**); (**A**) CD (5 mM, 30 min, 37°C) (dark bars) improves ASA ability to inhibit platelet (optical) aggregation induced by collagen (2 μg/ml) ($p = 0.021$ at 40 μM ASA, *NS* at 80 μM ASA), (**B**) MCDX (3 mM, 60 min, 37°C) [52] attenuates ASA effect on collagen-induced platelet aggregation ($p = 0.002$ at 40 μM ASA, $p = 0.0075$ at 80 μM ASA), mean ± SE (11–14) [8, 9]. CD – β-cyclodextrin; MCDX – methyl-β-cyclodextrin

High glucose lowers ASA effectiveness to inhibit blood platelet reactivity

Given that the relative insensitivity of platelets from diabetic patients to ASA relates to some markers of metabolic control in diabetic patients (like glycemia control and plasma lipid profile), it appears clear that an inadequate metabolic control of diabetes may constitute an important determining factors of the reduced platelet sensitivity to ASA [46, 56, 65, 94]. In this respect chronic hyperglycemia, and the resultant protein glycation, are unique, since they are observed in no other clinical state but DM.

The mechanism of the inhibitory effect of acetylsalicylic acid involves irreversible acetylation of one particular platelet protein – cyclooxygenase (COX), which leads to the permanent platelet defect [39]. However, there is a substantial evidence in a literature pointing that ASA is capable of acetylating in a non-selective manner all susceptible amino and hydroxyl groups in a variety of platelet and plasma proteins In diabetic subjects such a non-specific acetylation is believed to compete with other non-enzymatic modifications of proteins, mainly non-enzymatic glycosylation. Evidence originating from *in vitro* and *in vivo* studies has furnished to support the idea on the possible role of chemical competing between these two non-enzymatic modifications affecting a diversity of proteins, such as collagens, albumins, hemoglobin, lens crystallins or cellular proteins [15, 20, 41, 69, 112]. In patients with type 2 DM the observed differences in the susceptibility of platelet proteins for the *in vitro* acetylation corresponded to the significantly increased glycation of platelet proteins ($r = –0.652$ $p << 0.0001$) [99].

The *in vitro* studies showed that not only the extent of protein glycation was attenuated by increasing ASA concentrations, but also high glucose interfered with ASA reactivity towards protein amino groups [99]. Hence, the effects of high glucose and high ASA on the overall occupancy of protein free amino groups are not additive: while at higher concentrations ASA is able to overcome the effects of hyperglycemia and retards glycation, high glucose makes acetylation less efficient, so the resultant chemical modification becomes greatly reduced. Importantly, the ASA-mediated protein acetylation precludes the attachment of 'spacious' glycosyl (ketoamine) residues by former modification with much smaller acetyl residues. Consequently, the spheric hindrance originating from smaller acetyl residues may be reasonably expected to remain negligible compared to possible conformational changes induced by the attachment of glucose. Moreover, chemical competing between ASA and glucose should be considered more in the aspect of chemical kinetics and the formation of a peculiar equilibrium between glucose, ASA and free reactive protein amino groups, that the ultimate *a posteriori* effect of chemical modification.

Hence, in chronic hyperglycemia the occupancy of amino groups by glucose moieties, and thus diminished susceptibility and/or vulnerability of various platelet proteins and receptors on blood platelet membranes to acetylation, might contribute to the apparently differentiated sensitivity of blood platelets to ASA and determine "aspirin-insensitivity" in diabetic patients.

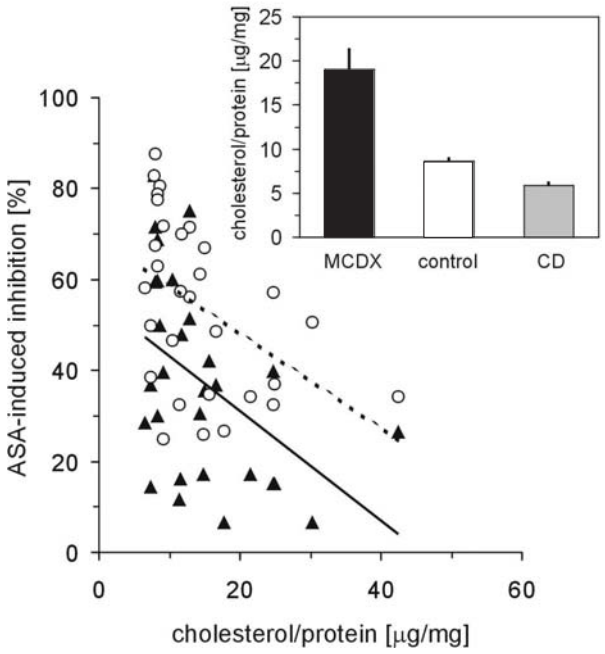

Fig. 6. Associations between platelet cholesterol content and ASA-mediated inhibition of platelet aggregation. Significant non-linear correlations were observed for ASA used at concentrations of either 40 μM (▲) ($\square_{Kendall}$ = –0.377, p < 0.025) or 80 μM (○) ($\square_{Kendall}$ = –0.493, p < 0.004). *Insert:* Effects of platelet incubation with CD or MCDX on cholesterol content, p < 0.0001 (CD) and p < 0.0002 (MCDX) [8, 9]

Increased platelet cholesterol retards ASA penetration through platelet membranes

Dyslipidemia is considered one of the reasons of impaired antiplatelet action of aspirin *in vivo*. Based on the studies of prostanoid compounds formation in hypercholestrolemic patients Davi et al. [23] described an aspirin-insensitive mechanism possibly linking lipid peroxidation to amplified platelet activation. In turn, Szczeklik et al. [71] has explained a blunted aspirin action in hypercholesterolemia by the lesser accessibility of platelet membrane proteins for acetylation by ASA in previously altered lipid-protein mosaic of platelet membrane. Recently, we demonstrated an association between reduced platelet sensitivity to aspirin and a higher total cholesterol concentration in diabetic patients [94]. Here, it should be emphasized that hypercholesterolemia causes alterations in platelet membrane lipid profile and significantly affects platelet function by changing the dynamic properties of cell membranes. Increased platelet membrane cholesterol content, as intimately associated with lipid membrane fluidity and thus also with signal transduction, is believed to correspond to elevated platelet response to agonists and augmented thromboxane metabolism [62, 74, 85]. As such it may potentially modulate also a therapeutic action of ASA.

In cholesterol-depleted platelets in the presence of β-cyclodextrin (CD) the effect of ASA-mediated inhibition of collagen-induced platelet aggregation appeared greater, which was particularly distinct at lower, subthreshold ASA concentrations (Fig. 5A). Otherwise, the enrichment of platelets in cholesterol by using methyl-β-cyclodextrin (MCDX) significantly attenuated the inhibitory effect of ASA on collagen-induced platelet aggregation, and again, such a modulation in lipid profile remained particularly profoundly marked at lower concentrations of ASA (Fig. 5B). There was a significant, though non-linear, correlation between platelet cholesterol content and the extent of ASA-mediated inhibition of platelet function (Fig. 6) [8, 9]. Furthermore, the reduction in platelet membrane cholesterol lead to significant suppression of platelet thromboxane (Tx)A_2 production and such a depletion influenced the extent of the inhibition of TxA_2 release by ASA (by up to 23%, 38% and 72% for 0, 1 and 5 μM ASA) [10]. In general, platelet cholesterol content significantly affects platelet sensitivity to ASA, with higher cellular cholesterol impairing ASA ability to inhibit TxA_2 generation and platelet reactivity. The possible molecular mechanisms of this association remain elusive. One may speculate that overall cellular content of cholesterol certainly affects the lipid profile of platelet surface membranes, thereby influencing a plethora of membrane-associated phenomena, including membrane fluidity, transmembrane transport, membrane signalling etc. Hence, the altered lipid status of platelet membranes seems likely to retard ASA penetration across platelet membranes and to lower ASA potency to acetylate its intraplatelet target, COX-1. At present, we have no firm evidence whether it is the case. However, the role of platelet membrane cholesterol in platelet response to ASA seems to be pivotal with respect to the phenomenon of platelet resistance to aspirin. Hypercholesterolemia might be the important metabolic factor predisposing diabetic patients to increased platelet refractoriness to ASA.

To sum up the last remarks concerning the possible impact of metabolic disorders encountered in diabetic patients on development of reduced blood platelet sensitivity to the antiplatelet action of ASA, we may conclude that:

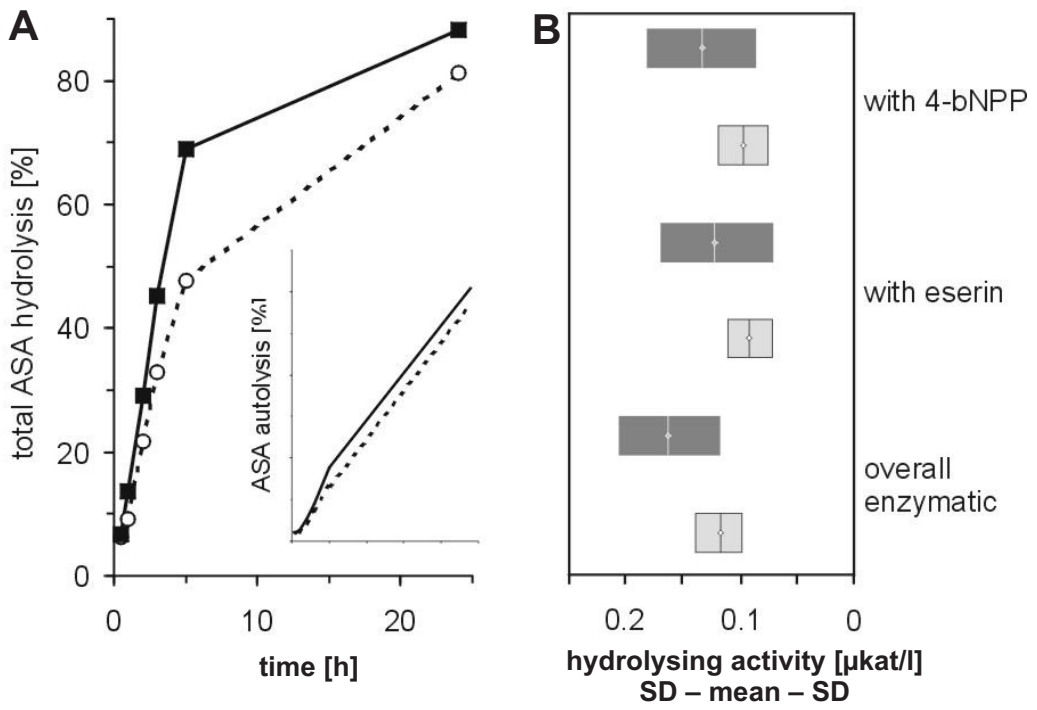

Fig. 7. Hydrolysis rate of ASA in blood plasma from non-diabetic and diabetic individuals. (**A**) Rate constants (*k*) of total (enzymatic and spontaneous) hydrolysis of ASA in blood plasma from diabetic patients (■) and healthy donors (○) were: 0.13 ± 0.05 h^{-1} and 0.08 ± 0.02 h^{-1}, $p < 0.006$. *Insert:* Spontaneous ASA hydrolysis (autolysis) was not different in blood plasma from non-diabetic and diabetic subjects. (**B**) ASA-hydrolyzing activities in blood plasma from non-diabetic and diabetic individuals. Diabetic patients (dark bars) showed elevated overall enzymatic ASA hydrolysing activity compared to non-diabetic individuals (light bars) (0.16 ± 0.05 µkat/l *vs.* 0.12 ± 0.02 µkat/l, $p < 0.003$), also in the presence of specific esterase inhibitors, eserin (0.12 ± 0.05 µkat/l *vs.* 0.09 ± 0.02 µkat/l, $p < 0.02$) and 4-bis-nitrophenyl phosphate (0.14 ± 0.05 µkat/l *vs.* 0.10 ± 0.02 µkat/l, $p < 0.04$) [30]

(a) reduced platelet response to ASA in diabetes melitus is transient and may be directly derived of some metabolic impairments more or less typical for diabetic state,
(b) as far as the nature of such an impaired platelet response to ASA is concerned, we may think that an apparent resistance to ASA in people with diabetes might be 'amended' *via* the compounding pharmacological intervention (like f.i. the agents normalizing hyperglycemia and/or hypercholesterolemia),
(c) the issue of whether and to which extent such a combined therapy in diabetes (f.i. antiplatelet agents + statins) would be effective in overcoming the impaired action of some antiplatelet drugs (like ASA) remains open for further investigations.

ASA "lives" shorter in blood of diabetic patients

Still another possible mechanism explaining the defected clinical effectiveness of ASA in patients with diabetes concerns its bioavailability.

After the introduction of ASA into the body, the drug undergoes decomposition into the salicylate (SA) and acetate. Both ASA and SA comply with each other as analgesic agents, however, their anti-inflammatory and antiplatelet effects differ significantly [12, 80]. The faster ASA is degraded into SA and acetate, the less likely it is for ASA to reach platelet COX-1 and suppress platelets' reactivity. Therefore the rate of aspirin hydrolysis underlying the bioavailability of drug in the circulation seems to be crucial for the effectiveness of the aspirin-mediated antiplatelet therapy. First, the initial contact of platelets with ASA occurs in the presystemic circulation, where the majority of platelets' COX-1 is inhibited [4]. As much as 30% of ASA introduced to the body is further hydrolyzed *via* the *first pass* mechanism in liver by the specific enzymes called aspirin esterases (aspirinases, EC 3.1.1.55) [80]. Subsequently, ASA reaches the blood circulation, where it is further inactivated by two different pathways of ASA hydrolysis: pH-dependent autolysis (autolysis or spontaneous hydrolysis, with the highest rate at basic pH values) and enzymatic hydrolysis [80]. The latter has been shown

to be much more dynamic and catalyzed largely by specific aspirinases [13, 14].

It has been found that that the rate of total (i.e. spontaneous and enzymatic) ASA hydrolysis was elevated in plasma from type 2 diabetic patients compared to non-diabetic individuals, while no differences in the rate of spontaneous ASA hydrolysis were noted (Fig. 7) [30]. Of two tested plasma aspirin esterase activities, only the activity at pH 7.4 was revealed to be higher in diabetic patients. This increased aspirin esterase activity at pH 7.4 was due to butyrylcholinesterase (EC 3.1.1.8, also called pseudocholinesterase or serum cholinesterase), since the activity was inhibited by eserine. The difference remained discriminative also in the presence of other esterase inhibitor, 4-bis-nitrophenyl phosphate (4-bNP). Otherwise, the aspirin esterase activity monitored at pH 5.5 was similar in healthy and diabetic subjects, and this activity was probably due to albumin [42, 47, 53, 84].

Of the interest are the associations between the increased rates of blood plasma ASA decomposition and the refractoriness of blood platelets to ASA [30], which indicate that the altered blood plasma ASA hydrolysing status may imply lowered ASA bioavailability, and thus may contribute to the reduced effectiveness of ASA-mediated antiplatelet therapy in diabetic patients.

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