Diabetologia (1998) 41: 609–622

Diabetologia

Review

Insulin, insulin resistance and platelet function: similarities with insulin effects on cultured vascular smooth muscle cells

M. Trovati, G. Anfossi

Diabetes Unit, Department of Clinical and Biological Sciences of the University of Turin, San Luigi Gonzaga Hospital, Orbassano (Turin), Italy.

Keywords Insulin, Insulin resistance, Platelets, Vascular Smooth Muscle, Calcium, cGMP, cAMP, Nitric oxide, Prostacyclin.

1. Introduction

The relationships between insulin, insulin resistance and atherosclerosis are still a matter of intense debate [1–4] even though it was suggested as long as 30 years ago that insulin is involved in the pathogenesis of atherosclerosis [5]. It is not clear whether insulin plays the role of a culprit, an innocent bystander, a factor involved in the attenuation of some steps in the atherogenic process or an agent with multifaceted actions having different and possibly contrasting effects. The question is: is the prevalence of atherosclerosis in the insulin-resistant states due to hyperinsulinaemia or to the presence of cellular resistance to some anti-atherogenic insulin actions? To answer we need to know the mechanisms by which insulin influences the cells involved in the pathogenesis of atherosclerosis.

In this review we examine the way in which insulin affects platelet function. Platelets are essential elements in the thrombotic and atherosclerotic processes because of their own functional properties and their ability to interact with endothelial and vascular smooth muscle cells (VSMC) [6]. As VSMC share common features in their contractile structure and its regulation with platelets [7] we also compare the effect of insulin and insulin resistance on these two types of cells. In particular we lay emphasis on the modulation of calcium fluxes, cyclic nucleotide concentrations, nitric oxide and prostacyclin actions.

2. Basic information on platelet physiology

Some information on platelet physiology is necessary to understand the insulin effects on platelets [8–12].

Main events in platelet activation

According to the "response to injury hypothesis" [6] atherosclerosis develops as a response to endothelial damage due to: hypercholesterolaemia, oxidized LDL, arterial hypertension, diabetes, cigarette smoking, hyperhomocysteinaemia, etc. When endothelial cells are damaged or lost, they are unable to produce vasodilating, anti-aggregating and antithrombotic substances such as prostacyclin (PGI_2), nitric oxide (NO), adenosine and heparin. In these conditions, platelets:

i) adhere to subendothelium by interacting with exposed collagen and other cytoadhesive proteins of basal membranes through specific surface glycoproteins;

ii) lose their resting dish-like shape, becoming sphere-like cells with pseudopods (shape change);

iii) metabolize the phospholipid-derived arachidonic acid into different eicosanoids, in particular, into thromboxane A_2 (TXA_2) which causes fibrino-

Corresponding author: M. Trovati, MD, Diabetes Unit, Department of Clinical and Biological Sciences of the University of Turin, San Luigi Gonzaga Hospital, 10043 Orbassano (Turin), Italy

Abbreviations: VSMC, Vascular smooth muscle cells; PGI_2, prostacyclin; NO, nitric oxide; NOS, nitric oxide synthase; TXA_2, Thromboxane A_2; IP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, phosphatidylinositol 1,4,5-trisphosphate; DAG, diacylglycerol; Ca^{2+}, calcium; PKC, protein kinase C; ADP, adenosine diphosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; ATPase, adenosine triphosphatase; AVP, arginine vasopressin; AT II, angiotensin II; PAF, platelet activating factor; PGE_1, Prostaglandin E_1; : L-NMMA, L-N^G-monomethyl arginine; ET-1, endothelin 1.

gen binding to platelets, aggregation, granule secretion and vasoconstriction;

iv) show an activation of phospholipase C, acting on the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (IP_2) to produce the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases cytosolic free calcium (Ca^{2+}) via a direct action on the Ca^{2+} stores contained in the dense tubular system and thereby activates Ca^{2+}/calmodulin-dependent protein kinases, whereas DAG directly activates protein kinase C (PKC) and, via PKC, phosphorylates proteins involved in the release reaction and increases cytosolic pH;

v) interact with other platelets by forming aggregates;

vi) release their granule content in the extracellular space through the canalicular system and microtubular contractile proteins (release reaction);

vii) contribute to the activation of the coagulation cascade, since some intrinsic coagulation reactions occur preferentially on their surface;

viii) modulate fibrinolysis, since they contain both plasminogen activators and inhibitors;

ix) induce chemotaxis and proliferation of VSMC, since proteins contained in platelet granules exert chemotactic and proliferating effects on these cells;

x) modulate vasomotion, since they release both vasodilating – such as adenosine-diphosphate (ADP) and adenosine-triphosphate (ATP) – and vasoconstricting substances (TXA_2 and serotonin).

Platelets are involved both in the early stages of atherosclerosis (VSMC chemotaxis, migration to the intima and proliferation) and in the advanced lesions: after plaque formation, they contribute to vessel occlusion by promoting vasoconstriction, activation of the coagulative system and intravascular thrombus formation. Platelets can be activated not only by components of the subendothelial layer but also by other physiologic mediators able to bind to specific receptors, such as ADP, thrombin, catecholamines, 5-hydroxytryptamine, arginine vasopressin (AVP), angiotensin II (AT II), platelet activating factor (PAF) and TXA_2.

Role of cyclic nucleotides in the modulation of platelet function

Intraplatelet Ca^{2+} is the final mediator of platelet functional changes [8–15]. Platelet agonists induce depletion-refilling of Ca^{2+} stores: i) by generating IP_3, which promotes Ca^{2+} release from intracellular stores in the cytosol, followed by Ca^{2+} re-accumulation into the stores via a Ca^{2+}-Adenosine triphosphatase (Ca^{2+}-ATPase); ii) by inducing an influx of Ca^{2+} from the extracellular space, directly by interaction with agonist receptors and indirectly by depletion of intracellular stores [13–15]. Similarly, Ca^{2+} fluxes are the main regulators of VSMC contraction [16–17].

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), the main inhibitory second messengers for platelet activation acting predominantly via specific protein kinases [18], reduce cytosolic Ca^{2+} by different mechanisms. In particular, cAMP: i) decreases binding of thrombin to its receptors [19]; ii) inhibits activation of phospholipase C, with a consequent reduction of IP_3 and DAG [20–21]; iii) interferes with processes distal to phospholipase C [21]; iv) by stimulating the Ca^{2+}-ATPase, enhances Ca^{2+} extrusion from platelets [22] and Ca^{2+} uptake by the dense tubular system [23]. cAMP, therefore, decreases intraplatelet Ca^{2+} by promoting its uptake into the internal stores and its extrusion from the cells.

Similarly, cGMP: i) inhibits agonist-induced Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx determined by store depletion [24]; ii) reduces the activation of phospholipase C also at the level of G-proteins [25], iii) modifies processes following protein phosphorylation close to the effector system [26].

Furthermore, both cAMP and cGMP: i) prevent Ca^{2+}-releasing activity of IP_3 from stores by inducing phosphorylation of IP_3 receptors [27]; ii) inhibit thrombin-dependent Ca^{2+} influx [28], and, via this mechanism, modulate the Na^+/H^+ antiporter and reverse the thrombin-evoked alkalinization [29]. Owing to the importance of Ca^{2+} fluxes in platelet function, it is not surprising that the increase of either cAMP or cGMP should prevent the main platelet responses: phosphoinositide metabolism, Ca^{2+} elevation, protein phosphorylation, platelet aggregation and release reaction [20, 30–31]. Similar effects of cyclic nucleotides on Ca^{2+} fluxes in VSMC mediate their vasodilating properties [32–35].

Modulation of cyclic nucleotide synthesis and catabolism

It is useful to summarise the mechanisms of synthesis and catabolism because of the relevance of cyclic nucleotides in platelet function.

cAMP and cGMP are synthesized by adenylate cyclase and guanylate cyclase from ATP and guanosine triphosphate (GTP), respectively.

Adenylate cyclase is activated by substances coupled to specific G_s-proteins: prostaglandins, such as prostaglandin E_1 (PGE_1) and PGI_2, through specific receptors [36–38]; adenosine through the purinergic receptor A_2 [39]; catecholamines through β_2 adrenergic receptors [40] and D_1 dopaminergic receptors [41]. The catalytic subunit of adenylate cyclase can be activated directly by forskolin [42]. Adenylate cy-

clase is inhibited by substances activating receptors coupled to G_i-proteins; in particular, α_2-adrenergic receptors [43] and D_2 dopaminergic receptors [41]. Physiological substances able to stimulate adenylate cyclase activity, such as PGE_1 and PGI_2, exert anti-aggregating and vasodilating properties by increasing cAMP both in platelets and in VSMC [44] and play a key role in the prevention of atherosclerosis and thrombosis [45].

cGMP is synthesized in platelets through a soluble guanylate cyclase, inhibited by methylene blue and activated by nitro-vasodilators and NO [46]. NO, a diffusible substance with a half-life of a few seconds formed from L-arginine by oxidation of its guanidine-nitrogen terminal [47], is the main endothelial-derived relaxing factor [48] and a powerful platelet inhibitor [49, 50]. It is able to reduce platelet adhesion to the vessel wall [51], platelet aggregation [52], and platelet surface glycoprotein expression, including P-selectin and activated glycoprotein IIb-IIIa complex [53]. As in endothelium, NO is constitutively produced by a cytosolic NO-synthase (NOS) requiring calmodulin, Ca^{2+} and NADPH, in platelets and megakaryocytes [54–58]. The aminoacid sequence of human platelet NOS has been identified [59]. Through their NOS activity, platelets produce detectable amounts of NO [60–61] able to exert physiological effects such as inhibition of additional platelet recruitment after platelet activation [62]. Analogues of L-arginine, such as L-N^G-monomethyl arginine (L-NMMA), inhibit NO synthesis in a stereospecific manner [63]. NOS is also inhibited by methylene blue [64], a guanylate cyclase inhibitor [46]. With the help of NOS inhibitors it was possible to determine that platelet-derived NO regulates platelet function, the activation of which is enhanced in vivo by infusion of NOS inhibitors [65] and reduced by L-arginine, the NOS-substrate [66].

In human platelets, PGI_2 and NO, which modulate cAMP and cGMP respectively, act synergistically to inhibit aggregation [67–69]. This synergism is further enhanced in vivo, since NO activates cyclo-oxygenase via a cGMP-independent mechanism leading to the endothelial release of PGI_2 and therefore promoting the synthesis of cAMP [70].

Both cAMP and cGMP are catalysed by phosphodiesterases, enzymes subdivided into at least seven families, with family-specific agonists and inhibitors, able to hydrolyse selectively cAMP and cGMP or both [71–72]. Platelets contain different phosphodiesterase families, among them: i) the cGMP-stimulated cAMP phosphodiesterase which hydrolyses both cAMP and cGMP; ii) the low Km cGMP-inhibited cAMP phosphodiesterase which is the most abundant in platelets and hydrolyses cAMP more effectively than cGMP; iii) the cGMP-specific phosphodiesterase [71]. Substances that increase cAMP by means of adenylate cyclase activation, such as PGE_1, PGI_2, Iloprost (a PGI_2 analogue) and forskolin, stimulate cAMP phosphodiesterase activity in platelets with a negative feed-back mechanism which regulates cAMP concentrations: this is particularly so in the case of the cGMP-inhibited cAMP phosphodiesterase [71]. Furthermore, nitro-vasodilators, which stimulate the production of cGMP by activating the soluble guanylate cyclase, cause a dose-dependent increase of cAMP also, since cGMP reduces cAMP catabolism by inhibiting the cGMP-inhibited cAMP phosphodiesterase [73].

In summary, platelet aggregation is deeply involved in atherothrombosis and requires important changes in platelet Ca^{2+} fluxes, with a final increase in intracellular Ca^{2+} content.

The cyclic nucleotides cAMP and cGMP, the platelet content of which is determined by a balance between the activity of anabolic and catabolic enzymes, are physiological inhibitory modulators of Ca^{+} fluxes and, therefore, also of platelet aggregation. For this reason, the main mechanism by which platelet antagonists reduce platelet function is to increase cyclic nucleotide concentrations. A similar modulation of cyclic nucleotides regulates Ca^{2+} fluxes in VSMC and mediate vasodilation.

3. Insulin influence on platelet function

Insulin receptors in platelets

The insulin receptors in human platelets have a concentration per surface area similar to that described in other cells [74] and have a beta-subunit phosphorylated by the hormone itself [75–76]. In patients with Type II diabetes mellitus, the platelet insulin receptor number and affinity are reduced [77]. Platelets, therefore, are a site of the insulin action and can be subject to variation of insulin sensitivity.

Insulin influence on platelet aggregation

We observed that, if human platelets are incubated with physiological concentrations of insulin for short periods of time (3–20 min), they have a reduced aggregating response to agonists: ADP, thrombin, adrenaline, PAF, collagen and sodium arachidonate [78–83]. Similar results have been obtained by other authors who observed that insulin reduces AT II- and thrombin-induced platelet aggregation and attenuates AT II ability to increase thrombin-stimulated platelet aggregation [84]. The same insulin-induced reduction of platelet responses to agonists can be reproduced in vivo by a euglycaemic hyperinsulinaemic clamp or similar techniques [79, 81, 85, 86].

Some authors, however, did not observe a direct insulin action on platelet aggregation in vitro

Table 1. Effects of insulin in platelets

– Activation of insulin receptors ([a])
– Increase of cGMP (NO-mediated) ([a]), as in VSMC
– Increase of cAMP (NO-mediated, possibly via cGMP), as in VSMC
– Increased effects on cAMP of adenylate cyclase activators (NO-mediated), as in VSMC
– Reduced Ca^{2+} fluxes elicited by Ca^{2+}-mobilizing agents ([a]), as in VSMC
– Reduced agonist-elicited platelet aggregation ([a])
– Increased antagonist-induced platelet anti-aggregation
– Increased binding of anti-aggregating prostanoids to platelets via receptor up-regulation, with an increase of their effects
– Decreased binding of catecholamines to platelets via α_2-adrenergic receptor down-regulation, with reduction of adrenaline-induced aggregation
– Increased platelet uptake of magnesium
– Increased platelet release of plasminogen activator

([a]) it has been demonstrated that this insulin effect is reduced in the insulin-resistant states

[87–89]. Others, using huge, supra-physiological amounts of insulin (100 nmol/l), even noted an insulin-induced pro-aggregating effect [90] which was confirmed in our laboratory [91].

The physiological role of insulin in inhibiting platelet function could play a protective role in the prevention of thrombus formation and in the release of vasoactive mediators and chemotactic/mitogenic substances, thus contributing to reduce the pathological events of thrombosis, hypertension and atherosclerosis. Furthermore, if platelets were a site of insulin resistance, the associated loss of insulin's beneficial effects could play a role in the increase in cardiovascular risk [92, 93]. This intriguing hypothesis has been confirmed experimentally. In effect, in the insulin-resistant states of obesity, obese Type II diabetes mellitus and arterial hypertension, the anti-aggregating effect exerted by insulin is attenuated or lost [83, 84].

Insulin and Ca^{2+} fluxes in platelets: similarities with VSMC

Insulin attenuates the action of different agonists able to activate platelets with different receptor and post-receptor events [78, 79, 84]. This means that the hormone interplays with very basic mechanisms of platelet function.

We have already mentioned that a rise in intracellular Ca^{2+} is a crucial aspect of platelet activation [8–15]. In these cells, the AT II-stimulated increases in intracellular Ca^{2+} are attenuated by a 5-min in vitro incubation with physiological insulin concentrations (70 μU/ml); furthermore, insulin reduces the increase in cytosolic pH induced by AT II and by endothelin 1 (ET-1); finally, the insulin effects on Ca^{2+} fluxes are accompanied by a concomitant reduction of platelet aggregation in response to thrombin [84]. Interestingly, the same study shows that insulin, when added alone to platelets, induces a rapid and transient rise in free Ca^{2+} [84].

In cultured VSMC and mesangial cells, that are structurally similar to platelets as far as the Ca^{2+}-regulated contractile processes are concerned [7, 16], insulin, even at physiological concentrations, attenuates Ca^{2+} fluxes and contractile responses to different vasoconstrictors (AT II, AVP, noradrenaline, serotonin, and ET-1) [94–107]. This direct insulin action, together with VSMC-endothelium interactions [106], could take part in the insulin-induced vasodilation documented by many studies in vivo, consideration of this is beyond the scope of this review.

Sites at which insulin acts in modulating intracellular Ca^{2+} content: evidence from studies in VSMC

To understand the mechanisms by which insulin reduces Ca^{2+} fluxes in platelets, we should consider similar effects in VSMC.

Since insulin inhibits Ca^{2+} responses elicited by different agonists [95, 98, 101, 104, 107], it is possible that its effects occur at a common point in the transmembrane signalling cascade: therefore, insulin probably does not interplay primarily with receptor binding of the different agonists. The vasoconstrictors tested generate IP_3, which interacts with specific receptors in the sarcoplasmic reticulum membrane of VSMC, opening Ca^{2+} channels and permitting Ca^{2+} contained in the intracellular stores to diffuse from the sarcoplasmic reticulum to the cytosol [17, 108, 109]. Insulin, therefore, could reduce the increase of intracellular Ca^{2+} induced by agonists by attenuating Ca^{2+} release from the IP_3-sensitive intracellular stores. This hypothesis has been confirmed experimentally in cultured rat VSMC, when a 20-min insulin incubation did not alter basal intracellular Ca^{2+} concentrations, but reduced AT II-induced IP_3-releasable Ca^{2+} flux, through a mechanism mimicked by treatment with the cGMP analogue 8-bromo-cGMP or with sodium nitroprusside, and blocked by the NOS inhibitor L-NMMA [102]. Interestingly, insulin did not modify AT II receptor affinity and density, AT II-stimulated phospholipase C activity or IP_3 production [102] which demonstrates that it simply reduces the sensitivity of Ca^{2+} stores for IP_3. Similar effects have been documented in porcine coronary artery VSMC [107]. The ability of insulin to reduce cytosolic Ca^{2+} by interplaying with intracellular Ca^{2+} fluxes stimulated by agonists is further confirmed by the persistence of insulin effects in the absence of extracellular Ca^{2+} [102, 107]. In our labora-

tory, it has been observed that the insulin-induced platelet anti-aggregating effect is also maintained in a Ca^{2+}-free medium [80].

Insulin is also able to decrease Ca^{2+} influx in VSMC by stimulating the Na^+-K^+ ATPase pump [110–112], a transport system that is inhibited by ouabain. Based on the exchange of intracellular K^+ with extracellular Na^+, stimulation of Na^+-K^+-ATPase results in hyperpolarization of the cell membrane and subsequent closure of voltage-dependent Ca^{2+} channels, resulting in a decrease of intracellular Ca^{2+} [113]. An insulin effect on this ion pump has been reported in different cell types in vitro [114, 115] and has been described in in vivo experiments [116]. In human platelets, ouabain increases both cytosolic Ca^{2+} and Na^+ by blocking Na^+-K^+ countertransport [117]. By analogy it could also be hypothesized that insulin affects this cellular pump also in platelets. In the rare condition of thyrotoxic periodic paralysis, hyperinsulinaemia is accompanied by an elevated Na^+-K^+ ATPase activity in platelets [118].

The insulin role in the regulation of the different Ca^{2+} transport systems in VSMC has been reviewed recently [119]. In any case, insulin reduces intracellular Ca^{2+} both in platelets and in VSMC, and, by this basic mechanism, attenuates both platelet activation and VSMC constriction.

Attentuation of the insulin effects on platelet Ca^{2+} fluxes in insulin-resistant states: similarities with VSMC

First, platelets from insulin-resistant animals and man (e.g. affected by arterial hypertension, obesity or Type II diabetes mellitus) exhibit increased cytosolic free Ca^{2+} concentrations in resting and stimulated conditions [84, 120–128]. A positive correlation between resting intracellular Ca^{2+} and serum insulin concentrations, an indirect marker of insulin resistance, has been observed [128]. The abnormalities in Ca^{2+} handling in platelets from insulin-resistant subjects could be due to a defective insulin action on Ca^{2+} fluxes. In particular: i) platelets from hypertensive subjects show higher basal and AT II-stimulated intracellular Ca^{2+} concentrations; ii) in the presence of arterial hypertension, the insulin-induced attenuation of Ca^{2+} responses to AT II is impaired; iii) serum insulin concentrations, a marker of insulin resistance, are correlated positively with the increases in intracellular free Ca^{2+} elicited by AT II in the presence of insulin, meaning that the greater insulin resistance is, the less effective insulin is in the attenuation of AT II-stimulated Ca^{2+} fluxes [84]. These results suggest that an attenuated insulin-induced inhibition of platelet responses stimulated by agonists is a manifestation of insulin resistance, potentially involved in platelet hyperactivity occurring in arterial hypertension [84].

Similar results have been obtained in obese, hypertensive Type II diabetic patients. In these subjects, the reduced ability of insulin to attenuate AT II-stimulated free Ca^{2+} concentrations is corrected by 12 weeks of antihypertensive treatment with Ca^{2+}-channel blockers (isradipine or diltiazem). These results demonstrate that the removal of abnormalities in Ca^{2+} fluxes restores insulin sensitivity in platelets and suggest that increased cytosolic Ca^{2+} can be both a cause and consequence of insulin resistance [129].

Furthermore, in contrast to insulin sensitive subjects, providing insulin-resistant, hypertensive and hyperinsulinaemic subjects with insulin increases their platelet Ca^{2+} concentrations, suggesting that insulin resistance extends to the regulation of platelet Ca^{2+} metabolism [130].

A resistance to the insulin effects on Ca^{2+} fluxes has also been observed in VSMC of insulin-resistant animals, such as spontaneously hypertensive rats (SHR) and Zucker obese rats [97, 98, 131]. These data indicate that insulin resistance at the VSMC level contributes to the increased intracellular Ca^{2+} response to vasoconstrictors and to the enhanced vascular reactivity of the insulin-resistant states [132–134]. An altered VSMC intracellular Ca^{2+} metabolism, therefore, may be a fundamental abnormality linking hypertension and insulin resistance [135].

These findings, both in platelets and in VSMC, support the hypothesis that a defect in the regulation of intracellular Ca^{2+} accompanies the reduction of insulin action and leads to an increase in vascular resistance, to arterial hypertension and to platelet hyperaggregability, features commonly observed in insulin-resistant states [127, 136–138].

Insulin, cyclic nucleotides and NO in platelets: similarities with VSMC and alterations in the insulin-resistant states

Some studies investigated in platelets the insulin effects on cAMP and cGMP, the main inhibitory systems of platelet activation. In other cell types, insulin reduces platelet levels of cAMP by inhibiting adenylate cyclase [139] and by stimulating cAMP phosphodiesterases [140–141]. In adipose cells, this mechanism accounts for the anti-lipolytic effect of the hormone [142]. On the other hand, both in adipose and in liver cells, insulin induces a prompt increase of cGMP (with a maximum effect at 2 and at 6 min, respectively) [143]. Finally, in rats with streptozotocin-induced diabetes mellitus, there is a decreased hepatic guanylate cyclase activity [144].

The first effect noted of insulin on cyclic nucleotides in platelets is an increase in the activity of phosphodiesterases [76, 145]: insulin activates, via a serine phosphorylation, the cGMP-inhibited cAMP

phosphodiesterase, that represents more than 75 % of cAMP phosphodiesterase activity in a platelet extract [145]. The biological meaning of this insulin action could be a reduction of cyclic nucleotides, and in particular of cAMP. This has never been observed in platelets, indicating that other biochemical events also modulate the insulin action on cyclic nucleotides in these cells.

Studies from our laboratory on platelet-rich plasma show that insulin increases platelet concentrations of cGMP [82]. This effect is mediated by the phosphorylation of the insulin receptor, being blunted by genistein, a tyrosine kinase inhibitor [82], and is completely blocked by methylene blue [82] and by L-NMMA [146]. These last observations induced us to speculate that insulin increases cGMP in platelets via NO [146]. Recent studies of our group demonstrated that insulin increases the synthesis of NO, measured by the ^{3}H-arginine/^{3}H-citrulline method, in human platelets [147]. This is one of the very few studies demonstrating a direct insulin action on NO synthesis in human cells in vitro, and the first one in platelets. An insulin-induced increase of NO production has been demonstrated in endothelial cells [148].

The insulin action on cGMP in platelets shows a plateau between 2 and 20 min, declines after 20 min but is still significant at 120 min [147]. The decline with time of the cGMP response to insulin is not surprising, since insulin increases cGMP via NO. Also pretreatment of tissues with nitro-vasodilators results in a molecular desensitization of soluble guanylate cyclase [149, 150], and the NO-induced activation of guanylate cyclase is reversible over time [151, 152].

We also demonstrated that insulin increases platelet concentrations of cAMP in a very short time [147]. These results do not conflict with those of other authors who were unable to demonstrate an insulin effect on platelet cAMP after at least 120 min of incubation [87] because the insulin action on cAMP reaches a plateau between 2 and 20 min, declines after 20 min and is no more significant at 60 min [147]. Interestingly, the insulin effect on cAMP is inhibited by methylene blue and by L-NMMA, demonstrating that it is a NO-mediated event [147]. This phenomenon is not surprising, since nitro-vasodilators, that are NO-donors, also enhance both cGMP and cAMP in platelets [69, 73, 153]. Actually, the increase of cGMP they induce blunts the cGMP-inhibited cAMP phosphodiesterase, thus decreasing cAMP catabolism and increasing cAMP concentrations [73]. Since insulin increases cGMP via NO, a similar phenomenon could take place after insulin incubation. In this case, insulin should be able to indirectly inhibit, through cGMP, the same cAMP phosphodiesterase that it activates per se [145].

Another possible interpretation of the insulin-induced increase of platelet cAMP derives from the observation that, when guanylate cyclase is stimulated by NO donors, it shows striking alterations in its properties, becoming able to synthesize not only cGMP but also cAMP [154]. A similar mechanism could explain the insulin-induced increase of cAMP, since insulin enhances NO in platelets [147] and stimulates platelet guanylate cyclase via NO.

Finally, we observed that a 8 min-insulin incubation enhances dose-dependently the effects on cAMP of substances able to activate adenylate cyclase with a receptor-dependent mechanism, such as Iloprost, a PGI_2 analogue [37], or with a receptor-independent mechanism, such as forskolin [42] and that these effects are also NO-mediated, being blunted by L-NMMA [147]. As a consequence, insulin dose-dependently enhances the anti-aggregating effects of these substances [147]. Thus, insulin increases the influence on cAMP of adenylate-cyclase stimulating agents because it increases cAMP per se via a NO-mediated mechanism [147]. The ability of insulin to increase the anti-aggreating effects of PGI_2 explains the very effective platelet modulating action exerted by the hormone in vivo [79, 81, 85], where PGI_2 is released into the circulation by endothelial cells. PGI_2 and NO, in fact, act synergistically to reduce platelet aggregation [67–69], and insulin interplays with both of these physiological anti-aggregating substances by enhancing NO synthesis and, through NO, by increasing PGI_2 effects [147].

The relevance of cyclic nucleotides in the anti-aggregating effect exerted by insulin is further supported by the fact that insulin does not reduce platelet sensitivity to agonists in the presence of methylene blue, a substance able to inhibit both guanylate cyclase and NOS [82].

Data from our laboratory demonstrate that insulin increases both cGMP and cAMP through NO also in human VSMC, as in platelets, since its effects are blunted by L-NMMA [155–156]. Furthermore, in VSMC insulin enhances the effects on cAMP of adenylate cyclase activators, such as Iloprost [155], forskolin [155] and β-adrenergic agonists [156] with a mechanism mediated by NO, being inhibited by L-NMMA [156]. Very recently, we observed that human VSMC show a NOS activity able to be stimulated within a few minutes by the Ca^{2+} ionophore ionomycin and by insulin and to induce, via NO, rapid increases of cyclic nucleotides [157]. Together with other recent evidence, these data support the contention that VSMC have not only an inducible, Ca^{2+}-independent NOS, responsible for a delayed release of NO following activation by cytokines, but also a Ca^{2+}-dependent NOS, that could be a constitutive isoform, able to be activated within a few minutes by insulin [119, 157]. Thus, insulin-induced vasodilation could be attributed not only to the effects on VSMC of endothelium-derived NO but also to that exerted by insulin directly on VSMC.

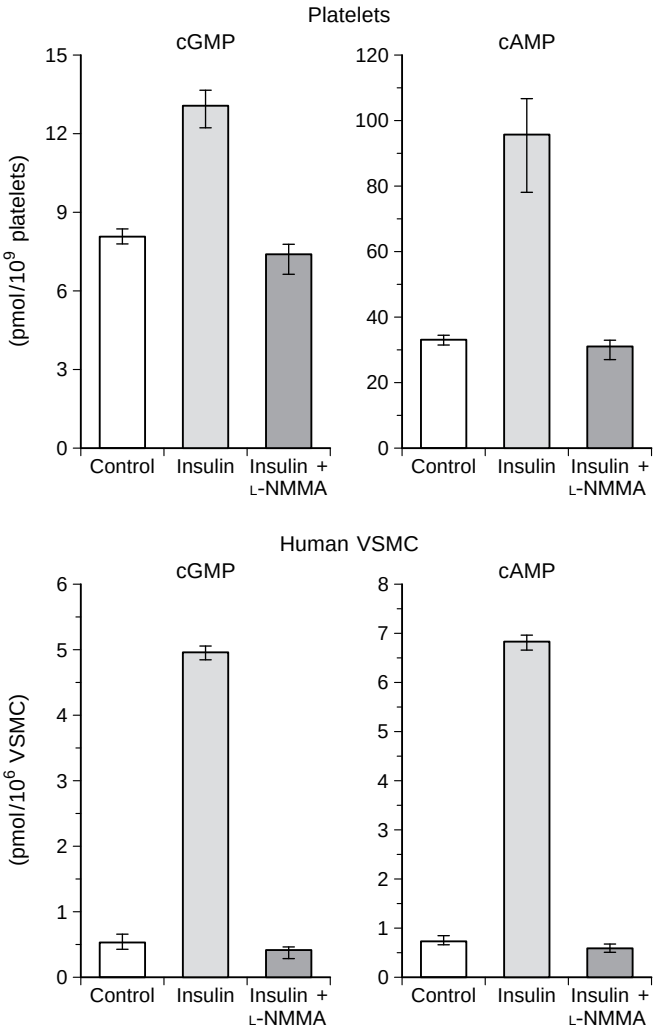

Fig. 1. Insulin increases both cGMP and cAMP in platelets and in human VSMC via NO, its effects being completely blunted by the NO synthase inhibitor L-NMMA. cGMP and cAMP are the main inhibitory mediators of platelet activation and of VSMC constriction, being able to attenuate calcium fluxes. The ability of NO to increase not only cGMP but also cAMP is discussed in the text. In these experiments ($n = 6$), representative of many others carried out in our laboratory, the cells were incubated for 15 min with 2 nmol/l insulin, with and without a 20 min preincubation with L-NMMA (100 μmol/l in platelets, 2 mmol/l in hVSMC), and the insulin-induced increases of cGMP and cAMP were significant ($p = 0.03$–0.0001)

Figure 1 shows the similarities in the NO-mediated insulin actions on cyclic nucleotides in platelets and in VSMC.

Further studies are needed to clarify the mechanisms by which insulin stimulates NOS activity in platelets and in VSMC. In platelets, insulin activates the cGMP-inhibited cAMP phosphodiesterase via a serine phosphorylation [145]. A putative insulin effect on NOS, which is to be proven experimentally, could be a serine phosphorylation, which would result in its activation [158].

In the insulin-resistant states of obesity and obese Type II diabetes mellitus, we observed that the ability of both insulin and the NO donor glyceryl-trinitrate (GTN) to increase cGMP and to reduce platelet activation is blunted [83, 159], whereas lean Type II diabetic patients show a normal cGMP response to both insulin and GTN [159]. Similarly, in platelets from patients affected by essential hypertension which is an insulin-resistant condition [93], the inhibition of platelet Ca^{2+} fluxes induced by another NO donor, sodium nitroprusside, is impaired [160]. Thus, insulin/NO and NO/cGMP pathways are altered in the insulin-resistant states.

In summary, insulin activates NOS activity, both in platelets and VSMC; via NO, it rapidly increases the intracellular content of both cGMP and cAMP and enhances the effects of cAMP-increasing substances; through cyclic nucleotides, it decreases intracellular Ca^{2+}, and therefore reduces platelet activation and vasoconstriction. These insulin effects in platelets are blunted in the insulin-resistant subjects.

We have mentioned that a putative mechanism by which insulin reduces Ca^{2+} fluxes is the activation of Na^{+}-K^{+} ATPase [110–112]. This insulin effect could also be mediated by NO, since it stimulates Na^{+}-K^{+} ATPase activity [161, 162], a phenomenon accounting for the NO-induced depolarization of vascular smooth muscle [163]. Furthermore, NO directly activates Ca^{2+}-dependent K^{+} channels, which reduce Ca^{2+} influx through voltage-operated Ca^{2+} channels [164].

Insulin and platelet binding of prostanoids and catecholamines

Insulin up-regulates prostacyclin binding and down-regulates α_2-adrenergic receptors in human platelets, thus attenuating platelet responses. In particular, studies carried out by incubating platelet preparations with insulin for at least 2 h at 23 °C demonstrated that the hormone increases PGE_1 binding to platelets, and thereby enhances platelet sensitivity to the inhibitory effect of the prostanoid through the increased formation of cAMP [87]. The time course of the insulin effect on PGE_1/PGI_2 receptor number follows that of the insulin binding to platelets [87]. Further studies of the same research group confirmed that insulin interacts with the binding of PGE_1/PGI_2 with prostanoid receptors [165], by a mechanism involving G-proteins [166]. Insulin also amplifies the antiaggregatory effect of PGE_1 in rat platelets [167].

Experiments carried out in vivo by an iv bolus injection of insulin followed by a 2.5 h insulin infusion, with plasma glucose kept constant by a simultaneous glucose infusion, demonstrated that insulin increases PGE_1 and PGI_2 binding to platelets by two- to three-fold over control. This is due to an increase of both high and low affinity receptor number, with small

changes in receptor affinity. This increased binding was associated with more than a twofold decrease in the minimum prostanoid concentrations needed to inhibit platelet aggregation through cAMP formation [86]. Other authors demonstrated that a 2-h insulin infusion during a euglycaemic insulin clamp results in a 65% increase of PGE_1-stimulated cAMP concentrations [168]. Infusion of insulin in vivo without changing blood glucose concentrations not only increases prostanoid binding to platelets, but even enhances PGI_2 in plasma, compatible with an increased synthesis by endothelial cells [86].

During acute spontaneous angina and acute ischaemic heart disease, platelet hyperactivation plays a pivotal role in vessel occlusion [169, 170]. This phenomenon is in part due to a reduced platelet sensitivity to vasodilating prostanoids [171], owing to a reduction of their specific receptors [172, 173]. These alterations can be corrected by treating platelets with insulin: a 3-h incubation of platelets with the hormone, in effect, resulted in increased specific binding of PGI_2 in 75% of patients with acute myocardial infarction or unstable angina [174]. This effect was due to an increase of both high- and low-affinity receptor number, without changes in affinity [174]. An increase in cAMP corresponded to the increase in binding [174]. These data suggest that giving insulin after an acute ischaemic event inhibits, at least in part, thrombus formation by the restoration of the prostanoid receptor activity. The same authors verified this intriguing hypothesis and found that giving insulin to patients with unstable angina pectoris or acute myocardial infarction returned to normal the impaired response of platelets to PGE_1/PGI_2 and the synthesis of PGI_2 [175].

During acute ischaemic heart disease, there is also a transient decrease of insulin binding to platelets, which is related to changes in the sensitivity to PGE_1 [176]. Thus, acute alterations in the insulin-platelet inter-relationships are involved in the prothrombotic state accompanying acute vascular events. Similarly, in subjects with chronic spinal cord injury, in whom coronary artery disease is a leading cause of death, PGI_2 fails to inhibit the platelet-stimulated thrombin generation owing to the loss of high-affinity receptor sites for the prostanoid [177]. These platelet defects are corrected with platelet treatment with insulin [177].

Thus, these studies support the conclusion that insulin interplays with prostanoid effects on platelets not only by enhancing within a few minutes the increase of cAMP they induce via its own, NO-mediated, effect on cAMP [147] but also by enhancing prostanoid binding to platelets after 2 h.

In vitro studies also demonstrated that incubation of human platelets with insulin for 2.5 h at 23 °C increases their resistance to adrenaline aggregating effects and inhibits the potentiating effect of adrenaline on ADP-induced aggregation [88]. These phenomena have been attributed to a decrease by insulin of the number of α_2-adrenergic receptors that account for the aggregating response to adrenaline [88]. A possible explanation for the insulin-induced up-regulation of prostanoid receptors and down-regulation of α_2-adrenergic receptors is an insulin influence on cell membrane properties [178].

In summary, when platelets are exposed to insulin for a period of 120–150 min, both in vivo and in vitro, they show an up-regulation of prostanoid receptors and a down-regulation of α_2-adrenergic receptors resulting in anti-aggregation. Furthermore, platelet binding to anti-aggregating prostanoids is impaired during acute cardiovascular events and restored by insulin.

Insulin and magnesium fluxes in platelets

Insulin dose- and time-dependently increases platelet uptake of Mg^{2+} from the extracellular space with a maximal effect achieved by incubation with 200 μU/ml insulin for 30 min [179]. This effect is mediated by insulin receptors being blunted in the presence of an anti-insulin receptor monoclonal antibody [179]. The insulin effects on Ca^{2+} and Mg^{2+} could be inter-related since decreases in Mg^{2+} concentrations lead to an increase in intracellular Ca^{2+} and to enhancement of platelet aggregation [180]. Insulin resistance states are characterized by decreased intracellular Mg^{2+} and increased intracellular Ca^{2+} [127, 181]. This fact could be attributed to lack of physiological insulin action on Mg^{2+} and Ca^{2+} handling.

Insulin and platelet-mediated modulation of fibrinolysis

Platelets modulate fibrinolysis since they show both anti-fibrinolytic and pro-fibrinolytic activities, containing both plasminogen activator inhibitor I (PAI-I) [182] and plasminogen activators [183, 184].

It has been demonstrated that: i) incubation of washed platelets with insulin (200 μU/ml) at 37 °C for 3 h results in a 3-fold increase in plasminogen activator activity in the supernatant; ii) treatment of plasma membranes with insulin also enhances the release of platelet plasminogen activator, a phenomenon completely blunted by agents able to increase platelet cAMP [185]. Since platelets do not synthesize proteins, this insulin effect could be ascribed to enzyme activation or its release from platelet membrane or both. The insulin action on platelets, therefore, could account for an increase of the fibrinolytic activity suggesting that both insulin deficiency and insulin resistance can increase the incidence of thrombosis in diabetic patients.

Interestingly, in Type II diabetic patients, fasting plasma insulin is correlated with platelet PAI-1, demonstrating an influence of insulin per se, or of insulin resistance, in platelet anti-fibrinolytic activity [186]. In VSMC, insulin stimulates PAI-1 secretion and gene expression [187].

4. Conclusion

Platelets, as VSMC, are targets of insulin action and subject to changes in insulin sensitivity. The main insulin effect in these cells is to inhibit Ca^{2+} fluxes, especially those from internal stores to cytosol, with a consequent reduction of agonist-stimulated platelet aggregation and vessel contraction.

The insulin effects on Ca^{2+} fluxes are regulated in both cell types by the cyclic nucleotides cGMP and cAMP, that insulin increases via NO, which thus becomes the key to understanding insulin action on platelet and VSMC function. Even if the insulin-induced vasodilation occurring in vivo is mainly attributable to the influence on VSMC of endothelium-derived NO, insulin also exerts direct effects on these cells. Furthermore, both in platelets and in VSMC, insulin increases via NO the influence on cAMP of other anti-aggregating and vasodilating substances (i.e. prostacyclin and β-adrenergic agonists), thereby enhancing their biological effect.

Finally, insulin increases platelet binding of anti-aggregating prostanoids, and decreases platelet binding of α_2-adrenergic agonists, with a final anti-aggregating effect.

In the insulin-resistant states, insulin effects on platelet cyclic nucleotides are attenuated, and the insulin-dependent reduction of Ca^{2+} fluxes is impaired both in platelets and in VSMC. These changes might explain why the insulin resistance syndrome is characterized by an altered intracellular ionic milieu, by an enhanced platelet activation and by a great prevalence of arterial hypertension, which can account for the increased vascular risk [93].

However, the described vasoprotective effects exerted by insulin on platelets and VSMC are only some of the many aspects of the influence of insulin on factors involved in atherogenesis and arterial hypertension: we should also remember the stimulatory role exerted by insulin on VSMC proliferation [188, 189], and on the sympathetic tone and sodium reabsorption [93].

Finally, this review offers some elements to better understand the role of insulin deficiency and of insulin resistance in determining the platelet alterations described in diabetes mellitus, which play a major role in the pathogenesis of diabetic angiopathy [190].

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