Insulin, Insulin Resistance, and Platelet Signaling in Diabetes

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Insulin resistance and the metabolic syndrome are associated with a pro-thrombotic state that contributes to the pathogenesis and progression of the vascular complications of type 2 diabetes. Development of the disease is also linked to the loss of the direct antiplatelet effect of insulin and platelets obtained from patients with diabetes who are hyperreactive (i.e., demonstrate increased adhesiveness and exaggerated aggregation and thrombus generation). Conditions linked to insulin resistance and development of type 2 diabetes are generally associated with redox stress within the vasculature that, in turn, affects platelet function. There are numerous platelet signaling events that are sensitive to changes in the vascular balance of nitric oxide (NO) and oxygen-derived free radical generation; however, recent studies have highlighted the link among platelet hyperreactivity and oxidative modifications in Ca^{2+}-ATPase activity and Ca^{2+} homeostasis, altered surface expression of glycoprotein receptors and adhesive proteins on the platelet surface, and increased binding of fibrinogen (rev. in 1).

Effects of insulin on platelet function

A direct antiplatelet effect of insulin has been demonstrated by many groups; although we and others have observed an insulin-induced attenuation of the thrombin-induced Ca^{2+} response and platelet aggregation as well as the release of ADP (2,3), reports from groups assessing the same responses are inconsistent. Part of the controversy may be attributed to the fact that responses to insulin are highly heterogeneous; indeed, clear populations of "responders" and "nonresponders" have been identified in several studies

(3,4) and can be related to numerous factors including physical condition (5).

Studies in which an effect of insulin has been documented and that were aimed at addressing the molecular mechanisms that underlie the antiaggregatory effects of insulin have not yet managed to completely clarify the events involved. Thus, although insulin has been reported to stimulate the AMP-activated protein kinase and Akt in a phosphatidylinositol 3-kinase (PI3-K)-dependent manner (2) and to decrease platelet Ca^{2+} and attenuate agonist-induced platelet activation (6), these events have not been definitively linked to the activation of the insulin receptor. Indeed, researchers specifically addressing expression of the insulin receptor on washed human platelets (7) have failed to demonstrate its presence. Platelets do, however, express high levels of functional receptors for IGF-I that, when activated, lead to the tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2 and their association with the p85 subunit of PI3-K (8). At the moment, it is tempting to suggest that insulin signaling in platelets is mediated by the IGF receptor. It is equally tempting to extrapolate data obtained in cardiac muscle showing that insulin stimulates the binding of IRS-1 and IRS-2 to the COOH-terminus of sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-2 (9) to the situation in platelets and to propose that this is the mechanism by which insulin decreases platelet intracellular calcium concentration ($[Ca^{2+}]_i$). This is an attractive possibility in light of the observation that this association is significantly reduced in cardiac muscle from streptozotocin diabetic rats (9) but has yet to be addressed experimentally. However, while the activation of IGF receptor

by insulin could account for the disparate results obtained in some investigations (10), not all of the reports are currently reconcilable.

NO. It is generally accepted that platelets generate NO and that they express an NO synthase (NOS) isoform similar to that expressed in endothelial cells. Moreover, a number of stimuli, including insulin, have been reported to increase NO production (usually assayed by an increase in intracellular cyclic guanosine monophosphate [GMP] levels) in platelets from healthy subjects. Certainly, agonist-induced platelet aggregation is modified by NOS and guanylyl cyclase inhibitors and by cyclic GMP analogues. Even though platelets from endothelial NOS-deficient (NOSIII/eNOS$^{-/-}$) mice demonstrate decreased bleeding times and an attenuated insulin-induced release of ATP, eNOS deficiency is reported to only minimally affect platelet aggregation and arterial thrombosis in vivo. It has, however, proven difficult to demonstrate the expression of eNOS protein in isolated human or murine platelets (rev. in 11). Moreover, although we and others have detected a 135-kDa protein in isolated human platelets using antibodies directed against eNOS and demonstrated the generation of NO by electron-spin resonance spectroscopy (2,12), we have been unable to unequivocally confirm the presence of eNOS protein in platelet samples using modern proteomic approaches. More recent studies have failed to clear up the controversy; although eNOS is reportedly present in bovine platelets (11), other groups have concluded that human and mouse platelets do not express either eNOS or inducible NOS and that some platelet agonists directly affect the soluble guanylyl cyclase, resulting in an NO-independent activation of the cyclic GMP signaling pathway (13).

Role of calpain in platelet activation

Calpains are Ca^{2+}-regulated cysteine proteases that have been implicated in cytoskeletal organization, cell proliferation, apoptosis, cell motility, and hemostasis. Platelets have been reported to express both μ-calpain (calpain 1) and m-calpain (calpain 2), named for the Ca^{2+} concentration required to activate them in vitro

(14). As Ca^{2+} is the main regulator of protease activity, it follows that calpain activation occurs rapidly following an increase in platelet $[Ca^{2+}]_i$ and induces limited proteolysis of a number of proteins implicated in cytoskeletal rearrangement, degranulation, and aggregation. Proteins identified to date that are targeted by calpain include spectrin, adducin, and talin as well as platelet endothelial cell adhesion molecule (PECAM)-1, the myosin light-chain kinase, and N-ethylmaleimide–sensitive factor attachment receptor (SNARE) proteins such as N-ethylmaleimide–sensitive factor attachment protein (SNAP)-23 and vesicle-associated membrane protein (VAMP)-3. Furthermore, μ-calpain modulates $\alpha_{IIb}\beta_3$ integrin–mediated outside-in signaling and platelet spreading by cleaving the β_3 subunit of the $\alpha_{IIb}\beta_3$ integrin (rev. in 15). The importance of μ-calpain activation in platelet function has been highlighted by the disruption of the mouse μ-calpain gene: μ-calpain$^{-/-}$ mice demonstrate pronounced defects in platelet aggregation and clot retraction. Mechanistically, the latter effects have been attributed to the dephosphorylation of platelet proteins inasmuch as μ-calpain$^{-/-}$ platelets exhibit high levels of protein tyrosine phosphatase 1B (PTP1B) and activity. Moreover, either an inhibitor of the phosphatase or the generation of μ-calpain and PTP1B double knockout mice was able to rescue the platelet defect (16). Although one consequence of calpain activation is reported to be a change in platelet tyrosine phosphorylation, it seems that this modification also determines the susceptibility of a protein to proteolytic cleavage by calpains (17). It has not been studied in detail whether insulin acts as a physiological modulator of calpain activation, but the reported Ca^{2+}-lowering effects of the hormone would suggest that this may well be the case.

Insulin resistance and platelet signaling in diabetes

Insulin resistance has been linked with a prothrombotic risk and suppressed fibrinolysis as a consequence of elevated levels of plasminogen activator inhibitor-1 (18). To date, it has not been investigated in humans in detail to what extent chronic hyperglycemia and the posttranslational modification of proteins by O-GlcNAcylation affect platelet function. However, a recent report failed to detect this modification in platelets isolated from either a hyperglycemic murine model of type 1 diabetes or a genetically-based model (*ob/ob*) of type 2 diabetes (19).

Reactive nitrogen and oxygen species. A large body of evidence from animal models and patient studies indicates that redox stress plays a major role in the pathogenesis of vascular complications of diabetes. There is convincing evidence linking decreased vascular NO production coupled with the overproduction of reactive oxygen species (ROS) and the generation of potent oxidants such as peroxynitrite ($ONOO^-$) to altered platelet function. Although the regulation of a platelet NOS is currently controversial, insulin-induced cyclic GMP production in platelets from subjects with diabetes is reported to be attenuated, and agonist-induced platelet aggregation becomes insensitive to NOS inhibitors (20). The generation of ROS in diabetes may either lead to or result from platelet activation, suggesting that oxidative stress and platelet activation may be closely interrelated. Indeed, superoxide anions and hydrogen peroxide, which are both reported to play an important role in platelet activation, are continuously produced in these cells, and diabetes is associated with reduced platelet antioxidant levels (21). High concentration of ROS can modify platelet function in different ways; e.g., hydrogen peroxide elevates protein tyrosine phosphorylation by activating Bruton's tyrosine kinase, the Janus kinases, and the Src family tyrosine kinases (22,23). $ONOO^-$ generation is also increased in diabetes, presumably as a consequence of eNOS uncoupling (i.e., under conditions in which tetrahydrobiopterin levels are low, the enzyme generates superoxide anions in addition to NO, which then can react to generate $ONOO^-$), and diabetes is associated with the elevated tyrosine nitration of serum proteins.

Calcium signaling. One prominent change in the "diabetic platelet" is an altered Ca^{2+} homeostasis (24). Dysregulation of the platelet Na^+/Ca^{2+} exchanger has been reported to be one of the mechanisms underlying the increased platelet $[Ca^{2+}]_i$ and hyperactivity. However, the modified Ca^{2+} homeostasis is also partially attributable to changes in the activity of Ca^{2+}ATPases, which are highly sensitive to oxidative damage (25). In platelets from healthy individuals, the plasma membrane Ca^{2+} ATPase is regulated by tyrosine phosphorylation, and platelet-activating agonists such as thrombin stimulate its phosphorylation by Src, thus inhibiting the pump and in-

creasing intracellular Ca^{2+} (26). It follows that modifications that interfere with such a regulatory process would affect several platelet signaling pathways and lead to platelet activation. Indeed, although the regulation of SERCA-2 activity by tyrosine phosphorylation has yet to be specifically documented in platelets, the tyrosine nitration of the protein has been detected in platelets from patients with type 2 diabetes, could be mimicked by incubating healthy platelets with authentic $ONOO^-$, and was associated with the loss of Ca^{2+}ATPase activity (27). Such findings indicate that the Ca^{2+} ATPases are a potential link between oxidative stress, dysregulation of Ca^{2+} handling, protease activity, and platelet activation.

Is there any way to improve platelet function in diabetes?

Vitamin E deficiency is associated with increased platelet aggregation, and α-tocopherol supplementation was initially reported to decrease platelet thromboxane A_2 production in response to ADP (28). Although several such studies concluded that vitamin E treatment could be beneficial with respect to platelet function and platelet-vessel-wall interactions, larger-scale trials failed to demonstrate a decreased risk of myocardial infarction, stroke, or cardiovascular death in diabetic patients receiving vitamin E (29). Insulin therapy, on the other hand, may improve at least platelet sensitivity/responsiveness to NO. The results of a recent clinical study revealed that platelet responsiveness to the nitrovasodilator sodium nitroprusside was increased by insulin administration to patients with acute coronary syndrome, which is an insulin-resistant state (30).

Despite the proven benefits of insulin, optimal protection against the cardiovascular complications of diabetes has been obtained when insulin was used in combination with oral antidiabetes drugs, such as the thiazolidinediones and biguanides. Thiazolidinediones and glitazones are agonists of the peroxisome proliferator–activated receptor γ, and the treatment of diabetic subjects with rosiglitazone decreased oxidative stress, inasmuch as plasma nitrotyrosine levels were reduced. Moreover, therapy prevented tyrosine nitration of platelet SERCA while simultaneously decreasing μ-calpain activation and improving platelet function (27). Although the beneficial effect of glitazones on platelet function has been demonstrated in vivo, the effect on isolated

platelets is more controversial. Troglitazone, not pioglitazone, has been reported to inhibit thrombin-induced aggregation—an effect mimicked by vitamin E, suggesting that the in vitro actions of troglitazone can be attributed to its structural similarity to the former (31). Taking the available data together, it seems that the beneficial effects of the different antidiabetes drugs on platelet function can be mostly attributed to a reduction in oxidative stress.

Outlook

The effects of insulin on isolated platelets have become controversial over the last few years as researchers use more and more refined techniques to isolate pure populations of nonactivated platelets, which can effect enormous differences in the results obtained. However, it seems safe to state that the responses to insulin that have been detected in samples from healthy individuals are generally compromised in diabetic patients. Clearly, responses to many stimuli are affected by the disease, and the consequences of diabetes on platelet function cannot be solely attributed to altered insulin signaling. Indeed, tyrosine phosphorylation and limited proteolysis of platelet proteins is altered, and the activation of calpain by a mechanism apparently linked to redox stress plays a major role in the diabetes-associated changes in platelet function.

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