Insulin Increases Guanosine-3′,5′-Cyclic Monophosphate in Human Platelets

A Mechanism Involved in the Insulin Anti-Aggregating Effect

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To investigate whether insulin reduces platelet aggregability through a modulation of the guanosine-3′,5′-cyclic monophosphate (cGMP) concentrations, we determined by a radioimmunoassay the cGMP values in the platelet-rich plasma (PRP) obtained from 17 healthy volunteers and incubated for 3 min with different concentrations of human recombinant insulin (0, 240, 480, 720, 960, and 1,920 pM). Insulin induced a dose-dependent cGMP increase, from 18.5 ± 3.3 to 42.0 ± 6.4 pmol/10^9 platelets (P = 0.0001). This increase was completely blunted when PRP was preincubated for 20 min with the tyrosine kinase inhibitor genistein (10 μM) or with the guanylate cyclase inhibitor methylene blue (10 μM), but the increase remained highly significant (P = 0.003 and 0.009) when PRP was preincubated for 20 min with the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX, 500 μM) or with the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA, 30 μM). Finally, the insulin-induced decrease of platelet aggregability to collagen and ADP was completely blunted when PRP was preincubated with 10 μM of the guanylate cyclase inhibitor methylene blue. This study demonstrates that the platelet anti-aggregatory effect exerted by insulin is attributable to the insulin-induced increase of cGMP that is due to a direct receptor-mediated platelet guanylate cyclase activation. *Diabetes* 43:1015–1019, 1994

It is known that insulin influences platelet function both in vitro and in vivo. In particular, it reduces the platelet responses to different agonists: ADP, collagen, thrombin, sodium arachidonate, and platelet-activating factor (1–5).

It is not surprising that platelets are targets of insulin action, because they present a well-characterized insulin receptor able to phosphorylate its β-subunit (6). The biochemical mechanisms involved in the anti-aggregating insulin effects, however, have not been completely clarified; because insulin modifies the platelet responses to agonists that interact with different receptors and activate different intracellular pathways (7), it could be supposed that the hormone influences some basic mechanisms of platelet aggregation. Previous studies demonstrated that insulin does not affect thromboxane A_2 production (3) or phospholipase C activation (6).

As is well known, the modulation of the intraplatelet concentrations of the two cyclic nucleotides, adenosine-3′,5′-cyclic monophosphate (cAMP) and guanosine-3′,5′-cyclic monophosphate (cGMP), is a relevant mechanism involved in the inhibitory control of platelet function (8). Substances that increase platelet levels of these nucleotides exert anti-aggregating effects both in vivo and in vitro (9). For instance, the anti-aggregating effects of prostacyclin and adenosine are mediated by cAMP increase (10,11); nitric oxide (NO) and organic nitrates, on the other hand, reduce platelet responses by increasing platelet cGMP content (12).

In previous studies, a putative insulin effect on platelet cAMP concentrations was considered: we were able to demonstrate a slight reduction of this nucleotide in platelets (3) similar to what has been described in adipocytes, where this phenomenon seems to account for the antilipolytic action of the hormone (13). Other authors, however, did not observe any insulin effect on platelet cAMP concentrations (6). In any case, the lack of an insulin-induced increase of platelet cAMP shows that the insulin influence on this nucleotide cannot explain the anti-aggregating insulin action.

In this study, we aimed at investigating whether insulin modifies platelet cGMP concentrations. It is known that intracellular levels of cyclic nucleotides are the result of balance between synthesis and catabolism. In human platelets, cGMP synthesis depends on a soluble guanylate cyclase, a heterodimer formed by two polypeptides containing a heme moiety as a prostetic group (14). This enzyme is activated by nitrates, and the interaction between its heme group and NO appears to be an important modulator of its activity (15).

The regulation of cGMP catabolism in platelets is not completely known. Cyclic nucleotide catabolism depends on a family of enzymes, named cyclic nucleotide phosphodiesterases (PDEs), and has been extensively reviewed (16).

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cAMP, adenosine-3′,5′-cyclic monophosphate; cGMP, guanosine-3′,5′-cyclic monophosphate; NO, nitric oxide; PDE, phosphodiesterase; L-NMMA, N^G-monomethyl-L-arginine; IBMX, 3-isobutyl-1-methyl-xanthine; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; PRP, platelet-rich plasma; PPP, platelet-poor plasma; GTN, glyceryltrinitrate; ANOVA, analysis of variance.

Literature demontrates that insulin is able to increase cAMP catabolism (17), but its influence on cGMP catabolism has not been fully clarified. An abnormal platelet cGMP catabolism has been demonstrated in diabetes (18).

The aim of this study is to evaluate whether insulin exerts its anti-aggregating effect by influencing platelet cGMP concentrations. Actually, an insulin-mediated increase of this nucleotide has been described in some cell types, including adipocytes (19) and hepatocytes (19), but not in others, such as lymphocytes, which possess few insulin receptors (19). The presence of insulin receptors in platelets justifies the search for an insulin-induced cGMP increase. In particular, we aimed at investigating

1. whether insulin modifies platelet levels of cGMP
2. whether this putative insulin effect is due to the hormone action on cGMP synthesis via soluble guanylate cyclase stimulation or on cGMP breakdown via PDE inhibition
3. whether the putative insulin effect on guanylate cyclase is due to a direct hormone action or is modulated by an insulin effect on NO synthesis. Actually, in human platelets activated with collagen, an L-arginine–NO pathway was demonstrated that was able to stimulate platelet guanylate cyclase and, therefore, to increase cGMP concentrations (20)
4. whether the putative insulin effect on platelet cGMP concentrations is mediated by the insulin action on tyrosine kinase: it is well known that this enzyme is coupled with the receptors of both insulin and other growth factors (21)
5. whether the putative insulin effect on cGMP is the mechanism by which insulin exerts its anti-aggregating effect.

RESEARCH DESIGN AND METHODS

The study was conducted with 17 healthy volunteers (11 men and 6 women), 26.9 ± 1.9 years of age (mean ± SE), body mass index 23.7 ± 0.4 kg/m^2, who did not take any drugs in the previous 4 weeks. Each subject gave informed consent before investigation.

Chemicals. Human recombinant insulin was obtained from Calbiochem (La Jolla, CA); N^G-monomethyl-L-arginine (L-NMMA) was obtained from Bachem (Bubendorf, Switzerland); genistein, ADP sodium salt, bovine acid soluble collagen, 3-isobutyl-1-methyl-xantine (IBMX), and methylene blue were from Sigma (St. Louis, MO); glyceryl trinitrate was obtained from Simes S.p.A. (Milan, Italy). Human insulin was dissolved in modified Tyrode's buffer containing bovine serum albumin (BSA) (8 g/l NaCl, 0.2 g/l KCl, 1 g/l $NaHCO_3$, 0.05 g/l NaH_2PO_4, and 2.5 g/l BSA, pH 7.4). L-NMMA and methylene blue were dissolved in modified Tyrode's buffer without BSA. IBMX and genistein were dissolved in dimethylsulfoxide (DMSO). In platelet-rich plasma (PRP) samples, DMSO final concentration did not exceed 0.25% (35 mM). Based on our experiments and reports from others, it is known that DMSO final concentrations <0.5% are absolutely unable to influence human platelet function (22) or to modify platelet cGMP concentrations; in particular, our experiments performed in healthy subjects (n = 4) demonstrated that platelet cGMP with DMSO at the final concentrations of 0, 30, and 60 mM was 16.5 ± 1.4, 15.9 ± 1.3, and 15.9 ± 1.2 pmol/10^9 platelets, respectively (NS).

Protocol. Subjects were studied after overnight fasting. An 18-ml venous blood sample was withdrawn without stasis and anticoagulated with 3.8% sodium citrate, pH 7.4 (vol/vol, 1/9). PRP was obtained by a 20-min centrifugation at 180 g at room temperature; platelet-poor plasma (PPP) was prepared by a further PRP centrifugation at 2,000 g for 10 min. Platelet counts were determined on an S-Plus Coulter Counter. Different studies were carried out to take into account the different points.

Study A. To verify whether insulin increases platelet concentrations of cGMP, the PRP samples (500 μl) from the 17 subjects were incubated at 37°C for 3 min, without stirring, in the presence of different human recombinant insulin concentrations: 0, 240, 480, 720, 960, and 1,920 pM. Platelet reactions were then stopped with 30% trichloroacetic acid. Precipitated proteins were removed by means of a centrifugation at 2,000 g for 20 min at 4°C. After the addition of 100 μl of 1 M HCl, supernatant was submitted to 10 extractions with ethylic ether to remove trichloroacetic acid. The samples were then lyophilized and kept at −70°C until the radioimmunoassay of cGMP (Kit Advanced Magnetics, Cambridge, MA; with the following characteristics: specificity: 100% for cGMP, 0.027% for cAMP, and <0.001% for GMP, GDP, ATP, and GTP; sensitivity, <0.01 pmol/0.1 ml). For control, experiments with 40 μM glyceryltrinitrate (GTN) were carried out, because nitrates sharply increase platelet cGMP concentrations.

Study B. To verify whether the insulin-induced increase of cGMP is due to an insulin-induced guanylate cyclase activation, we repeated the experiments described in study A in five subjects, with a 20-min PRP preincubation with the guanylate cyclase inhibitor methylene blue at the final concentration of 10 μM or with the same volume of buffer.

Study C. To verify whether a putative insulin influence on cGMP PDE would play some role in the insulin-induced increase of cGMP, we repeated the experiment described in study A in four subjects after a 20-min PRP preincubation with the unselective PDE inhibitor IBMX at the final concentration of 500 μM or DMSO in the same volume.

Study D. To verify whether insulin affects platelet cGMP concentrations through an effect on the platelet synthesis of NO, we repeated in seven subjects the experiments described in study A with a 20-min PRP preincubation with the NO synthase inhibitor L-NMMA at the final concentration of 30 μM or with the same volume of buffer.

Study E. To verify whether the insulin effects on platelet cGMP concentrations are mediated by an insulin-induced activation of tyrosine kinase, we studied in four subjects the effects of 1,920 pM insulin with a 20-min PRP preincubation with the tyrosine kinase inhibitor genistein at the final concentration of 10 μM or DMSO in the same volume.

Study F. To verify whether the insulin effects on platelet cGMP account for the insulin-induced reduction of platelet aggregability, we determined, in five subjects, platelet responses to ADP and collagen, with a 3-min PRP preincubation with insulin (960 and 1,920 pM) or with the same volume of buffer and with a 10-min preincubation with the guanylate cyclase inhibitor methylene blue at a final concentration of 10 μM or with the same volume of buffer. Platelet aggregation was carried out as described previously (1,3), according to Born's method (23), quantifying the maximal aggregation according to the Weiss formula (24) and using different concentrations of ADP (1–4 μM) and collagen (2.5–8 mg/l) to measure the agonist ED_{50}. In Fig. 5, ED_{50} values are expressed as percent of values without insulin addition.

Statistical analysis. Data, in the text and in the figures, are expressed as means ± SE. Statistical analysis has been carried out by means of analysis of variance (ANOVA) for repeated measures and, when appropriate, by Student's t test for paired data.

RESULTS

Study A. In our conditions, the intraplatelet cGMP levels of unstimulated platelets were 18.5 ± 3.3 pmol/10^9 platelets. As shown in Fig. 1, the exposure to different insulin concentrations induced a dose-dependent increase of intracellular cGMP, which reached 42.0 ± 6.4 pmol/10^9 platelets in the presence of 1,920 pM insulin. The results are highly significant (ANOVA for repeated measures: P = 0.0001). The values are significantly more than the control levels starting from the physiological insulin concentration of 240 pM (Student's t test for paired data: P = 0.05). The control study carried out with GTN showed a platelet cGMP increase from 18.5 ± 3.3 to 82.05 ± 14.9 pmol/10^9 platelets (P = 0.0001). The response to GTN showed a significant correlation with the response to 1,920 pM insulin (r = 0.628, P = 0.007).

Study B. The preincubation with methylene blue completely blunted the insulin-induced increase of platelet cGMP (Fig. 2).

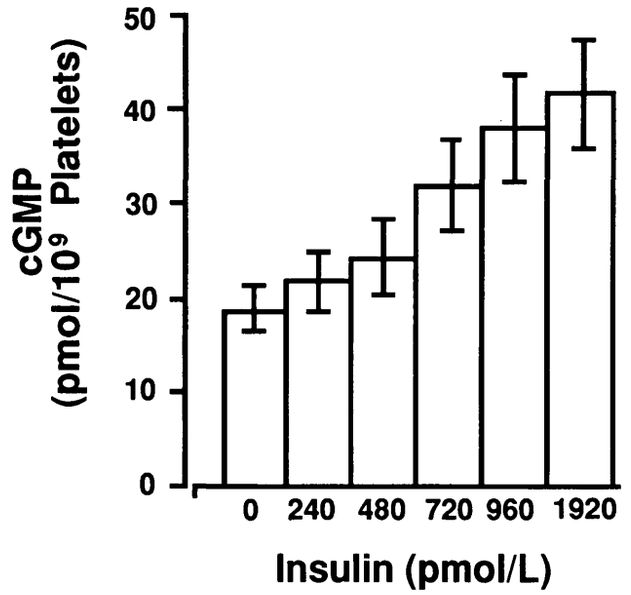

FIG. 1. Influence of 3-min PRP incubation with increasing insulin concentrations on the platelet content of cGMP (n = 17, P = 0.0001).

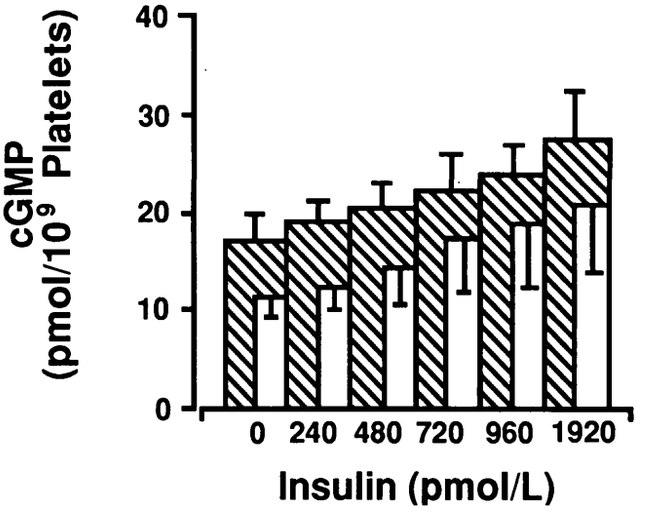

FIG. 3. Influence of 3-min PRP incubation with increasing insulin concentrations on the platelet content of cGMP in the absence (▭) and presence (▨) of a 20-min PRP preincubation with the PDE inhibitor IBMX at the final concentration of 500 μM (n = 4; P = 0.016 for insulin alone, P = 0.003 for insulin plus IBMX). IBMX caused a significant increase of basal cGMP concentrations (P = 0.022).

Study C. The PDE inhibitor IBMX induced a significant increase of the basal cGMP platelet concentrations (P = 0.022) (Fig. 3). Insulin, also in the presence of IBMX, caused a dose-dependent increase of platelet cGMP concentrations (ANOVA: P = 0.016 for insulin alone, P = 0.003 for insulin plus IBMX). As shown clearly in Fig. 3, the slope of the dose-dependent curves with insulin alone and with insulin plus IBMX was similar; in particular, if cGMP values with 1,920 pM insulin are expressed as percent of basal values, the results are 178.7 ± 25.7% with insulin alone and 167.7 ± 25.3% with insulin plus IBMX (NS).

Study D. The NO synthase inhibitor L-NMMA caused a reduction in basal cGMP concentrations, but this reduction was not statistically significant (Fig. 4). Insulin, also in the presence of L-NMMA, induced a significant, dose-dependent increase of platelet cGMP (ANOVA: P = 0.0001 for insulin alone, P = 0.009 for insulin plus L-NMMA) (Fig. 4).

Study E. Genistein did not modify the basal intraplatelet cGMP concentrations (18.0 ± 7.9 pmol/10^9 platelets without and 21.0 ± 9 pmol/10^9 platelets with genistein) and completely blunted the insulin-induced increase of the intraplatelet cGMP concentrations (17.9 ± 8.9 pmol/10^9 platelets with 1,920 pM insulin plus 10 μM genistein).

Study F. As far as the aggregation studies are concerned, we confirmed previous results from our laboratory (3), evidencing that insulin reduces the platelet responses to ADP and collagen and consequently enhances the ED_{50} values for each agonist (ANOVA: P = 0.0001). Furthermore, we observed that preincubation with 10 μM methylene blue prevents the anti-aggregating effect of insulin, as shown in Fig. 5, where ADP ED_{50} values are expressed as percent of values without insulin addition. The same phenomenon is observed

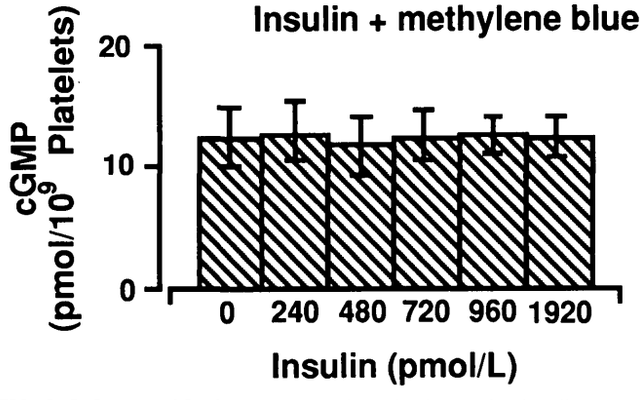

FIG. 2. Influence of 3-min PRP incubation with increasing insulin concentrations on the platelet content of cGMP in the presence of a 20-min PRP preincubation with the guanylate cyclase inhibitor methylene blue at the final concentration of 10 μM (n = 5, NS).

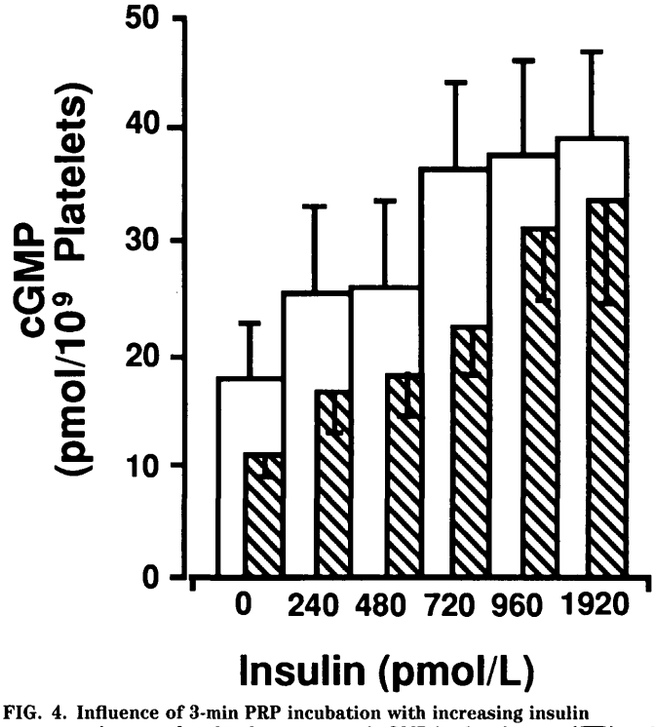

FIG. 4. Influence of 3-min PRP incubation with increasing insulin concentrations on the platelet content of cGMP in the absence (▭) and presence (▨) of a 20-min PRP preincubation with the NO synthase inhibitor L-NMMA at the final concentration of 30 μM (n = 7; P = 0.0001 for insulin alone, P = 0.009 for insulin plus L-NMMA).

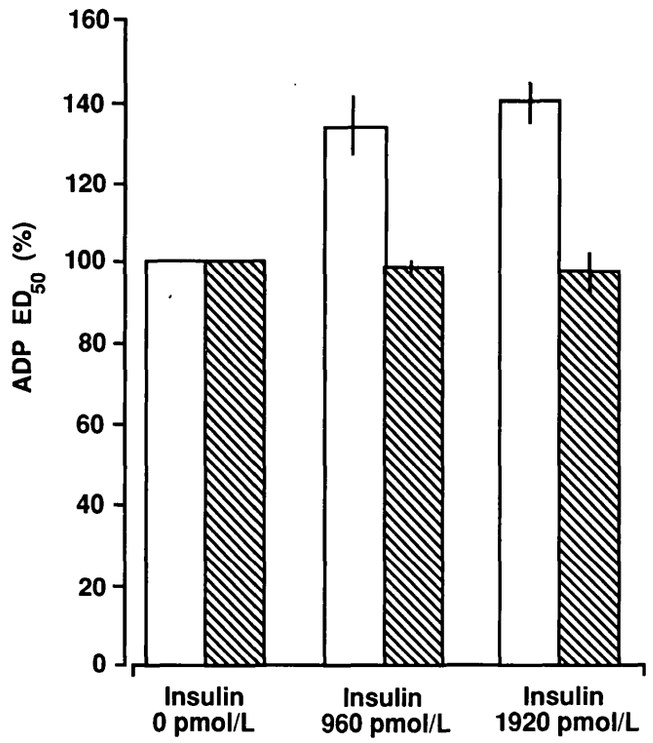

FIG. 5. Influence of 3-min PRP incubation with increasing insulin concentrations on the platelet sensitivity to ADP in the absence (□) and presence (▨) of a 10-min PRP preincubation with the guanylate cyclase inhibitor methylene blue at the final concentration of 10 μM (n = 5; P = 0.0001 in experiments with insulin alone, NS in experiments with insulin plus methylene blue).

with collagen: collagen ED_{50} values with 1,920 pM insulin are 139 ± 4% of basal values (P = 0.02), and with 1,920 pM insulin plus 10 μM methylene blue, collagen ED_{50} values are 99.0 ± 2% of basal values (NS).

DISCUSSION

This study shows for the first time that insulin induces a dose-dependent increase of the intracellular cGMP content in human platelets. The effect can be demonstrated in the presence of insulin concentrations as low as 240 pM. This observation indicates that, as far as cGMP is concerned, the responses of human platelets to insulin are similar to those observed in adipocytes and hepatocytes (19).

To clarify the mechanisms involved in this insulin action, we investigated the hormone effects on cGMP breakdown and on cGMP synthesis.

For cGMP catabolism, the experiments conducted in this study using high concentrations of the unselective PDE inhibitor IBMX, which is able to completely abolish any PDE activity, clearly indicate that PDE inhibition does not modify the insulin-induced increase of platelet cGMP concentrations. This fact, per se, indicates that this phenomenon is not due to an insulin-induced reduction of cGMP catabolism and, therefore, suggests a possible role for an insulin-induced increase of cGMP synthesis. In our study, the lack of an insulin effect on cGMP concentrations in the presence of a guanylate cyclase inhibitor shows for the first time that insulin directly stimulates this enzyme activity in human platelets. This effect is independent of a putative insulin-induced enhancement of NO production in platelets because it is not influenced by the platelet exposure to a selective NO synthase inhibitor (L-NMMA).

We hypothesized that the insulin-induced activation of the platelet soluble guanylate cyclase could be mediated by insulin receptors that are present on the platelet surface (6). The experiments performed with platelet exposure to genistein, an inhibitor of various insulin effects through a selective antagonism of tyrosine kinase activity, provided evidence that the insulin effect on platelet cGMP is mediated by a receptor-induced tyrosine kinase activation. Obviously, because tyrosine kinase is coupled with the receptors of many growth factors (21), among them insulin-like growth factor I, our study is not able to exclude a possible insulin role through these receptors, even if we are not aware of studies evaluating their presence on human platelets.

Our experiments on the platelet aggregating responses to ADP and collagen in the presence of the guanylate cyclase inhibitor methylene blue demonstrate that the insulin-induced cGMP increase is the mechanism by which insulin directly reduces platelet aggregation. From the physiopathological point of view, this is an important effect mediated by insulin receptors in platelets. It is known that insulin seems to be involved in the pathogenesis of diabetic complications. It has been supposed that the hormone influences water and sodium reabsorption (25); catecholamine, renin, angiotensin II, and endothelin secretion (26–29); and vascular smooth muscle cell proliferation and lipid accumulation (30). These different actions can explain why insulin is considered a potential factor in the pathogenesis of arterial hypertension and atherogenesis.

On the other hand, we and others previously demonstrated that insulin is also able to decrease platelet sensitivity to aggregating agents, both in vitro and in vivo (1–5). This study provides the first demonstration that insulin inhibits platelet functions by increasing the platelet concentrations of cGMP, an action similar to that of endogenous and exogenous nitrates. Insulin effects on platelets, therefore, seem to exert a protective influence against thrombus formation, which suggests that the role of insulin on the pathogenesis of diabetes vascular complications is very complex and particularly intriguing.

Finally, because "in diabetes a vicious cycle may be set up in which vascular disease may lead to platelet damage and altered platelet function may contribute to vascular disease" (31), this study demonstrates how insulin directly contributes to the reduction of the platelet hyperaggregability, even independently of the well-known effects mediated through its action on glucose and lipid metabolism (31).

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