Impaired Insulin-Induced Platelet Antiaggregating Effect in Obesity and in Obese NIDDM Patients

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To investigate the effects of insulin on platelets in obesity and in non-insulin-dependent diabetes mellitus (NIDDM) —classic insulin-resistant states—we determined ADP-induced platelet aggregation and platelet cGMP (guanosine 3′,5′-cyclic monophosphate) content in platelet-rich plasma obtained from nine obese subjects and nine age-matched healthy volunteers and from eight NIDDM obese patients and nine age-matched healthy volunteers after a 3-min incubation with human recombinant insulin (0, 240, 480, 960, and 1,920 pmol/l). Platelet aggregation was evaluated using different ADP doses to measure the ADP concentration determined on the basis of a dose-response curve necessary to elicit a maximal aggregation of 50% (ED_{50}). Insulin induced a dose-dependent decrease of platelet aggregation to ADP ($P = 0.0001$) in healthy subjects. A significant effect was evident starting from an insulin concentration of 240 pmol/l. On the contrary, in insulin-resistant subjects, insulin reduced platelet sensitivity to ADP only at a concentration of 1,920 pmol/l. When ADP ED_{50} values obtained in platelet-rich plasma incubated with insulin were expressed in percentage of the ADP ED_{50} values obtained in platelet-rich plasma without insulin, considered as 100%, we observed that ADP ED_{50} with 1,920 pmol/l insulin was 153.6 ± 13.2% in the younger healthy subject group ($P = 0.004$), 150.0 ± 3.8% in the older healthy subject group ($P = 0.0001$), 116.1 ± 6.1% in obese subjects ($P = 0.031$), and 120.0 ± 8.6% in NIDDM patients ($P = 0.05$). In healthy subjects, insulin induced a dose-dependent increase of platelet cGMP ($P = 0.0001$). A significant effect was evident starting from an insulin concentration of 240 pmol/l; cGMP values rose from 7.9 ± 1.4 to 14.6 ± 2.5 pmol/10^9 platelets with 1,920 pmol/l insulin in the younger healthy subject group ($P = 0.0001$) and from 7.5 ± 0.1 to 13.4 ± 1.4 pmol/10^9 platelets in the older healthy subject group ($P = 0.003$). In obese subjects and in NIDDM patients, insulin induced an increase of cGMP only at 1,920 pmol/l: from 7.6 ± 0.7 to 9.8 ± 1.4 pmol/10^9 platelets in obese subjects ($P = 0.07$) and from 7.5 ± 0.5 to 9.5 ± 0.9 pmol/10^9 platelets in NIDDM patients ($P = 0.07$). The platelet antiaggregating effect exerted by insulin, attributable to the increase of platelet cGMP content, is impaired in obesity and in obese NIDDM patients. These data indicate that human platelets are a site of insulin resistance. *Diabetes* 44:1318–1322, 1995

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ANOVA, analysis of variance; BMI, body mass index; HDL, high-density lipoprotein; NIDDM, non-insulin-dependent diabetes mellitus; OD, optical density.

Insulin resistance is a complex syndrome defined by a reduced insulin action (1) that has been well characterized in relation to the metabolic effects of the hormone but not in relation to its vascular effects.

Platelets have an insulin receptor able to phosphorylate its β-subunit (2), and insulin can reduce platelet function both in vitro and in vivo at physiological concentrations (3–7). We have demonstrated that the antiaggregating effect of insulin is attributable to the insulin-induced increase of platelet cGMP (guanosine 3′,5′-cyclic monophosphate) caused by guanylate cyclase activation (8).

Our data concerning platelets agreed with the results obtained in studies of other cell types, which demonstrate the ability of insulin to increase cGMP in adipocytes (9), hepatocytes (9), and vascular smooth muscle cells (10) and to activate soluble guanylate cyclase in the liver (11).

The ability of insulin to increase cGMP in platelets has important physiological consequences, since it is the mechanism by which insulin attenuates platelet responses to agonists (8).

It is not known, however, whether human platelets are a site of insulin resistance. This unanswered question is of clinical importance, since platelet hyperactivation plays a role in the pathogenesis of atherosclerosis both in the general population and in diabetic patients (12,13). Insulin-resistant states, such as obesity and non-insulin-dependent diabetes mellitus (NIDDM), show increased levels of circulating insulin (1). If insulin-resistant, hyperinsulinemic subjects had a normal platelet sensitivity to insulin, they should show an increased insulin-induced attenuation of platelet function resulting in a protective effect against thrombosis and atherogenesis. On the contrary, if insulin resistance occurred also at the platelet level, the lack of the physiological insulin-induced attenuation of platelet function should play a role in the pathogenesis of atherosclerosis, contributing to the complex interplay between insulin resistance and vasculopathy. In particular, diabetes is characterized by an increased prevalence of large vessel disease (14), which is not satisfactorily explained by the presence of the major vascular risk factors such as arterial hypertension, hypercholesterolemia, and cigarette smoking (15). We can suppose that the reduction of the insulin-induced attenuating effect on platelet function would play an additional atherogenic role in these patients.

The aim of our study is to evaluate the effects of insulin on platelet aggregation and platelet content of cGMP in insulin-resistant states such as obesity and obese NIDDM.

TABLE 1
Clinical characteristics of age-matched healthy and obese subjects

	Healthy subjects	Obese subjects
n	9	9
Sex (M/F)	5/4	6/3
Age (years)	32.7 ± 1.4	33.4 ± 4.0
BMI (kg/m^2)	21.9 ± 0.6	29.6 ± 1.0*
Glucose (mmol/l)	4.9 ± 0.03	5.1 ± 0.1
Insulin (pmol/l)	47.4 ± 7.8	91.2 ± 8.4†
HbA_{1c} (%)	4.9 ± 0.1	5.2 ± 0.2
Systolic blood pressure (mmHg)	120.5 ± 2.7	125.0 ± 5.0
Diastolic blood pressure (mmHg)	75.5 ± 2.6	78.3 ± 2.8
Triglycerides (mmol/l)	0.8 ± 0.1	1.5 ± 0.3‡
Cholesterol (mmol/l)	4.8 ± 0.3	5.2 ± 0.4
HDL cholesterol (mmol/l)	1.6 ± 0.1	1.4 ± 0.1

Data are means ± SE. *P = 0.0001. †P = 0.002. ‡P = 0.038.

RESEARCH DESIGN AND METHODS

Subjects. We studied nine obese subjects matched by age with nine healthy subjects and eight diet-treated obese NIDDM patients matched by age with nine healthy subjects. NIDDM was defined in accordance with the National Diabetes Data Group criteria (16). Obesity was defined as a body mass index (BMI) >25 kg/m^2. Obese subjects were otherwise healthy and did not present a family history of diabetes. Diabetic patients were free from late complications of diabetes, evaluated by means of history, clinical examination, funduscopy, fluoroangiography, electrocardiography, large vessel Doppler ultrasonography, renal clearances, proteinuria and microalbuminuria, electromyography, and tests for the detection of autonomic neuropathy.

Some clinical characteristics detected the same day of the study after overnight fasting and rest are shown in Tables 1 and 2.

Fasting venous plasma glucose was measured by a Beckman glucose analyzer (Beckman, Fullerton, CA); fasting plasma insulin was measured by radioimmunoassay (Kit Ares Serono, Milan, Italy; specificity 100% for human insulin, 15% for human proinsulin, <0.01% for C-peptide and glucagon); HbA_{1c} was measured by high-performance liquid chromatography (Bio-Rad, Richmond, VA); fasting serum cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured by automated chemistry.

To match both obese subjects and NIDDM patients for age, we studied two groups of healthy volunteers. Actually, NIDDM patients were older than obese subjects (P = 0.0001). The BMIs of obese and NIDDM subjects did not differ. Obese and NIDDM subjects had higher values of circulating insulin than did healthy subjects (P = 0.002 and P = 0.01, respectively). Plasma glucose and HbA_{1c} values of obese subjects were similar to those of healthy subjects, whereas NIDDM patients showed higher values of plasma glucose (P = 0.003) and HbA_{1c} (P = 0.0001). Obese and NIDDM subjects had higher values of triglycerides than healthy control subjects (P = 0.038 and P = 0.005, respectively). NIDDM patients also had lower values of HDL cholesterol (P = 0.0001).

TABLE 2
Clinical characteristics of age-matched healthy and NIDDM subjects

	Healthy subjects	NIDDM subjects
n	9	8
Sex (M/F)	6/3	6/2
Age (years)	52.1 ± 1.6	52.1 ± 3.3
BMI (kg/m^2)	23.4 ± 0.5	32.1 ± 1.5*
Diabetes duration (years)		4.6 ± 1.4
Glucose (mmol/l)	5.2 ± 0.2	8.3 ± 0.9†
Insulin (pmol/l)	50.8 ± 8.2	116.4 ± 21.6§
HbA_{1c} (%)	4.8 ± 0.2	7.6 ± 0.4*
Systolic blood pressure (mmHg)	131.7 ± 3.2	141.2 ± 5.8
Diastolic blood pressure (mmHg)	81.7 ± 1.2	85.6 ± 2.4
Triglycerides (mmol/l)	1.1 ± 0.1	1.8 ± 0.2‡
Cholesterol (mmol/l)	4.8 ± 0.3	5.0 ± 0.3
HDL cholesterol (mmol/l)	1.6 ± 0.1	1.0 ± 0.03*

Data are means ± SE. *P = 0.0001. †P = 0.003. ‡P = 0.005. §P = 0.01.

The subjects had not taken any drug influencing platelet function in the previous 4 weeks and gave an informed consent before investigation.

Materials and methods. Human recombinant insulin was obtained from Calbiochem (La Jolla, CA); ADP sodium salt was obtained from Sigma (St. Louis, MO). Human insulin was dissolved in modified Tyrode's buffer containing bovine serum albumin (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 bovine serum albumin, pH 7.4).

The subjects were studied after an overnight fast. Venous blood samples were withdrawn without stasis and anticoagulated with 3.8% sodium citrate (1:9, vol/vol), pH 7.4. Platelet-rich plasma was obtained by a 20-min centrifugation at 100*g* at room temperature; platelet-poor plasma was prepared by a further platelet-rich plasma centrifugation at 2,000*g* for 10 min. Platelet counts were determined on a S-Plus Coulter Counter (Coulter, Hertfordshire, U.K.).

Platelet aggregation responses to ADP were determined after a 3-min platelet-rich plasma preincubation with insulin (0, 240, 480, 960, and 1,920 pmol/l).

Platelet aggregation was carried out according to Born's method (17) using a model 500 Chrono Log aggregometer (Chrono Log, Havertown, PA) with constant stirring at 900 rpm. In aggregometric studies, the parameter considered is light absorption, which is evaluated on a scale of 0–100%. Because platelets are randomly dispersed in platelet-rich plasma and virtually absent in platelet-poor plasma, light absorption is very high with platelet-rich plasma and very low with platelet-poor plasma. At the beginning of each aggregometric experiment, light absorption of the platelet-rich plasma examined is conventionally set at 90% and light absorption of platelet-poor plasma at 10%. When platelets aggregate, light absorption is reduced, and the reduction correlates with the extent of platelet aggregation. The aggregating response to each agonist concentration is therefore evaluated as reduction of light absorption and quantified as maximal aggregation. Maximal aggregation is calculated using the formula of Weiss and Rogers (18):

$$\text{Maximal aggregation (\%)} = 100 \times (OD_0 - OD_m)/OD_0$$

where OD_0 is the optical density (OD) before agonist addition and OD_m is the minimum OD reached after agonist addition. Maximal aggregation is calculated for each of the agonist doses used. A very reliable parameter to quantify platelet aggregation is the agonist concentration able to induce a value of maximal aggregation of 50% (50% effective dose, ED_{50}). For this purpose, at least four doses of each agonist are used. With the maximal aggregation values corresponding to different agonist concentrations, it is possible to obtain a dose-response curve; subsequently, the curve is transferred to a semilogarithmic scale by plotting the logarithms of agonist concentrations on the x axis and the corresponding maximal aggregation values on the y axis. The agonist ED_{50} is calculated by interpolation. In the case of ADP, the doses are usually chosen in a range from 1 to 8 μmol/l, according to the sensitivity of each subject's platelets to ADP, and the ADP ED_{50} is measured in micromoles per liter. In our previous studies evaluating the insulin effects on platelets (3,5,8), as in our present study, we transformed the absolute ADP ED_{50} values obtained in platelet-rich plasma incubated with insulin to a percentage of the ADP ED_{50} value obtained in platelet-rich plasma without insulin, considered as 100%. The rationale for this transformation is the relatively wide range of agonist ED_{50} values that can be observed in healthy subjects and patients.

The platelet content of cGMP was measured in platelet-rich plasma samples (500 μl) incubated at 37°C for 3 min without stirring in the presence of 0, 240, 480, 960, and 1,920 pmol/l human recombinant insulin. The platelet reactions were stopped with 30% trichloroacetic acid (100 μl). The precipitated proteins were removed by centrifugation at 2,000*g* for 20 min at 4°C. After the addition of 1 mol/l (100 μl) HCl, the supernatant was submitted to 10 extractions with ethyl 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; specificity 100% for cGMP, 0.027% for cAMP, <0.001% for GMP, GDP, ATP, GTP; sensitivity, 0.01 pmol/0.1 ml). Because nitrates sharply increase platelet cGMP concentrations, positive control experiments were carried out with 40 μmol/l glyceryltrinitrate.

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

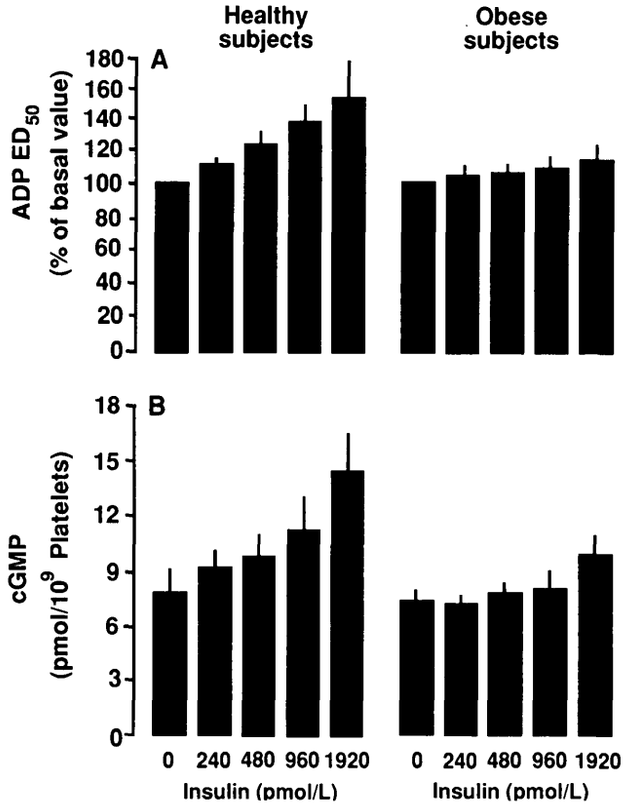

FIG. 1. Influence of a 3-min platelet-rich plasma incubation with increasing insulin concentrations on platelet sensitivity to ADP (*A*) and on cGMP content (*B*) in nine obese subjects and in nine age-matched healthy control subjects. Platelet aggregation is evaluated by measuring ADP ED_{50}. ADP ED_{50} values are expressed as a percentage, considering ADP ED_{50} values in samples without insulin addition as 100%. In healthy subjects, insulin decreased platelet aggregation to ADP, as shown by the dose-dependent increase of ADP ED_{50} (ANOVA for repeated measures, $P = 0.0001$) and increased platelet content of cGMP (ANOVA for repeated measures, $P = 0.0001$). A significant effect was evident at all insulin concentrations tested for platelet aggregation to ADP (at 240 and 480 pmol/l, $P = 0.013$; at 960 pmol/l, $P = 0.007$; at 1,920 pmol/l, $P = 0.004$) and for platelet content of cGMP (at 240 pmol/l, $P = 0.05$; at 480 pmol/l, $P = 0.017$; at 960 pmol/l, $P = 0.002$; at 1,920 pmol/l, $P = 0.0001$). In obese subjects, insulin caused a decrease of platelet aggregation to ADP and an increase of platelet cGMP content only at 1,920 pmol/l ($P = 0.03$ and $P = 0.07$, respectively).

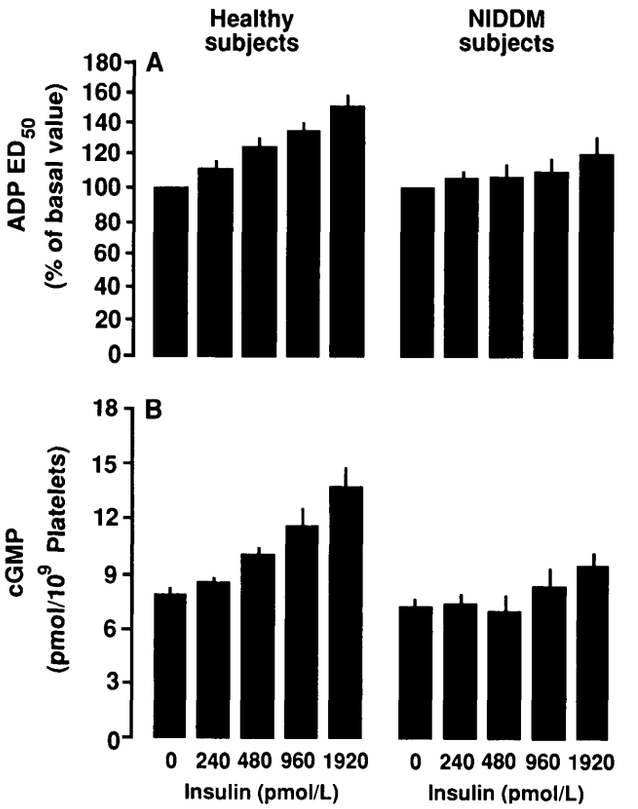

FIG. 2. Influence of a 3-min platelet-rich plasma incubation with increasing insulin concentrations on the platelet sensitivity to ADP (*A*) and cGMP content (*B*) in eight obese NIDDM patients and in nine age-matched healthy control subjects. Platelet aggregation is evaluated by measuring ADP ED_{50}. ADP ED_{50} values are expressed as a percentage, considering ADP ED_{50} values in samples without insulin addition as 100%. In healthy subjects, insulin decreased platelet aggregation to ADP, as shown by the dose-dependent increase of ADP ED_{50} (ANOVA for repeated measures, $P = 0.0001$) and increased platelet content of cGMP (ANOVA for repeated measures, $P = 0.0001$). A significant effect was evident at all the insulin concentrations tested for platelet aggregation to ADP (at 240 pmol/l, $P = 0.006$; at 480, 960, and 1,920 pmol/l, $P = 0.0001$) and for platelet content of cGMP (at 240 pmol/l, $P = 0.027$; at 480, 960, and 1,920 pmol/l, $P = 0.003$). In obese NIDDM patients, insulin caused a decrease of platelet aggregation to ADP and an increase of platelet cGMP content only at 1,920 pmol/l ($P = 0.05$ and 0.07, respectively).

RESULTS

The results concerning the insulin effect on platelet aggregation to ADP are shown in Figs. 1 (obese subjects) and 2 (NIDDM patients).

ADP ED_{50} (μmol/l) was 4.4 ± 0.3 in the younger healthy subjects, 4.5 ± 0.3 in the older healthy subjects, 4.1 ± 0.5 in obese subjects, and 4.3 ± 0.3 in NIDDM patients. The differences between healthy subjects and insulin-resistant patients were not statistically significant.

Insulin dose-dependently decreased the platelet aggregation to ADP in the two groups of healthy subjects (ANOVA for repeated measures, $P = 0.0001$). A significant effect was evident at all the insulin concentrations tested (in the younger healthy subjects at 240 and 480 pmol/l, $P = 0.013$; at 960 pmol/l, $P = 0.007$; at 1,920 pmol/l, $P = 0.004$; in the older healthy subjects at 240 pmol/l, $P = 0.006$; at 480, 960, and 1,920 pmol/l, $P = 0.0001$). In particular, when ADP ED_{50} values obtained in platelet-rich plasma incubated with insulin were expressed as a percentage of the ADP ED_{50} values obtained in platelet-rich plasma without insulin, considered as 100%, we observed that ADP ED_{50} with 1,920 pmol/l insulin was 153.6 ± 13.2% in the younger healthy subject group ($P = 0.004$) and 150.0 ± 3.8% in the older healthy subject group ($P = 0.0001$).

In obese subjects and NIDDM patients, a significant decrease of platelet aggregation to ADP occurred only at the supraphysiological insulin concentration of 1,920 pmol/l; in particular, the ADP ED_{50} with 1,920 pmol/l insulin was 116.1 ± 6.1% of the ADP ED_{50} without insulin in obese subjects ($P = 0.031$) and 120 ± 8.6% in NIDDM patients ($P = 0.05$). The platelet responses to insulin in obese subjects and NIDDM patients did not differ.

The results concerning the influence of insulin on platelet content of cGMP are given in Figs. 1 (obese subjects) and 2 (NIDDM patients).

Insulin dose-dependently increased the platelet content of cGMP in both groups of healthy subjects (ANOVA for repeated measures, $P = 0.0001$). A significant effect of insulin was evident at all concentrations tested (in the younger healthy subjects at 240 pmol/l, $P = 0.05$; at 480 pmol/l, $P = 0.017$; at 960 pmol/l, $P = 0.002$; at 1,920 pmol/l, $P = 0.0001$; in the older healthy subjects at 240 pmol/l, $P = 0.027$; at 480, 960, and 1,920 pmol/l, $P = 0.003$). In particular, with 1,920 pmol/l insulin, cGMP rose from 7.9 ± 1.4 to 14.6 ± 2.5

pmol/10^9 platelets in the younger healthy subjects (P = 0.0001) and from 7.5 ± 0.1 to 13.4 ± 1.4 pmol/10^9 platelets in the older healthy subjects (P = 0.003).

In obese subjects and in NIDDM patients, insulin caused an increase of platelet cGMP content approaching the statistical significance only at 1,920 pmol/l: in particular, cGMP values with 1,920 pmol/l insulin were 9.8 ± 1.4 pmol/10^9 platelets in obese subjects (P = 0.07 vs. the basal value of 7.6 ± 0.7 pmol/10^9 platelets) and 9.5 ± 0.9 pmol/10^9 platelets in NIDDM patients (P = 0.07 vs. the basal value of 7.5 ± 0.5 pmol/10^9 platelets). The influence of insulin on platelets was similar in obese subjects and obese NIDDM patients.

DISCUSSION

Our present study shows for the first time that human platelets have a reduced response to the antiaggregating effects of insulin in the classic insulin-resistant states of obesity and NIDDM. As in our previous studies (3,5,8), we quantified platelet aggregation measuring the concentration of the agonist necessary to elicit a maximal aggregation of 50% (agonist ED_{50}). This parameter is obviously much more reliable than the simple measurement of the platelet response to a fixed agonist concentration, since it takes into account the platelet responses to different agonist doses.

Because our study was carried out in obese NIDDM patients, its results cannot be extended to lean NIDDM subjects. However, obesity is associated with diabetes in the large majority of NIDDM patients (16). In our study, subjects with obesity alone and those with obesity and NIDDM showed a similar impairment of the platelet response to insulin. Actually, at physiological insulin concentrations (240–960 pmol/l), neither obese subjects nor obese NIDDM patients showed the inhibitory modulation of platelet function exerted by insulin in healthy subjects through platelet cGMP increase. In insulin-resistant subjects, insulin reduced platelet aggregability and increased platelet cGMP concentrations only at the supraphysiological concentration of 1,920 pmol/l. Since both healthy and obese subjects had similar values of plasma glucose, HbA_{1c}, and serum HDL cholesterol, we can exclude a role for these factors in the reduced platelet response to insulin occurring in the insulin-resistant subjects. As far as triglycerides are concerned, both obese subjects and NIDDM patients had values higher than values of healthy subjects, as frequently occurs in the presence of the insulin resistance syndrome (1). The role that this phenomenon may play in the reduced platelet response to insulin requires further investigation. Finally, we can exclude the influence of age on the platelet responses to insulin, since younger and older healthy volunteers presented a similar platelet behavior.

The parallelism observed in the insulin-induced changes of platelet aggregation and cGMP content in healthy and insulin-resistant subjects confirms that the increase of platelet cGMP is the main mechanism by which insulin modulates platelet function (8). In obesity and in obese NIDDM patients, a supraphysiological insulin concentration of 1,920 pmol/l is required to obtain the same effects on platelet aggregation and cGMP content that insulin exerts in healthy subjects at concentrations in the range of 240–480 pmol/l. It is clear, therefore, that the insulinemic concentrations occurring in vivo in insulin-resistant subjects, although elevated, are not able to physiologically modulate platelet function.

It has been observed recently that the vasodilating effect of insulin is impaired in obesity and NIDDM (19,20), inducing one to suppose that human vessels are a site of insulin resistance and providing a possible explanation for the high prevalence of arterial hypertension in the insulin-resistant states. Similarly, our present study shows that human platelets are insulin resistant in obesity and in obese NIDDM patients, suggesting that a reduced insulin action could play a role in the pathophysiology of atherothrombosis in these insulin-resistant states.

It has been proposed that NIDDM, obesity, arterial hypertension, dyslipidemia, and atherosclerotic cardiovascular disease are different aspects of a multifaceted syndrome characterized by the presence of insulin resistance (1). In addition, because insulin-resistant conditions present elevated insulin concentrations, it has been supposed that insulin could be the pathogenetic link between insulin resistance and atherosclerosis, in view of its potential atherogenic activities. Among those are its influence on vascular smooth muscle cell proliferation (21), the sodium-hydrogen antiporter activation (22), and the release of angiotensin II (23) and endothelin 1 (24,25). For platelet function, however, our data demonstrate that insulin reduces platelet activation, an important pathogenetic factor in atherosclerosis (12,13), and that this beneficial action is impaired in obesity and in obese NIDDM. We therefore suggest that in this case, platelet resistance to a beneficial effect of insulin could play a role in the pathogenesis of atherothrombosis in insulin-resistant states.

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