

# Insulin Induces the Release of Vasodilator Compounds From Platelets by a Nitric Oxide–G Kinase–VAMP-3–dependent Pathway

Voahanginirina Randriamboavonjy,<sup>1</sup> Jürgen Schrader,<sup>2</sup> Rudi Busse,<sup>1</sup> and Ingrid Fleming<sup>1</sup>

<sup>1</sup>Institut für Kardiovaskuläre Physiologie, Klinikum der J.W. Goethe-Universität, D-60590 Frankfurt am Main, Germany

<sup>2</sup>Institut für Herz- und Kreislaufphysiologie, Heinrich-Heine-Universität Düsseldorf, D-40225 Düsseldorf, Germany

## Abstract

Insulin-induced vasodilatation is sensitive to nitric oxide (NO) synthase (NOS) inhibitors. However, insulin is unable to relax isolated arteries or to activate endothelial NOS in endothelial cells. Since insulin can enhance platelet endothelial NOS activity, we determined whether insulin-induced vasodilatation can be attributed to a NO-dependent, platelet-mediated process.

Insulin failed to relax endothelium-intact rings of porcine coronary artery. The supernatant from insulin-stimulated human platelets induced complete relaxation, which was prevented by preincubation of platelets with a NOS inhibitor, the soluble guanylyl cyclase inhibitor, NS 2028, or the G kinase inhibitor, KT 5823, and was abolished by an adenosine A<sub>2A</sub> receptor antagonist. Insulin induced the release of adenosine triphosphate (ATP), adenosine, and serotonin from platelet-dense granules in a NO-dependent manner. This response was not detected using insulin-stimulated platelets from endothelial NOS<sup>-/-</sup> mice, although a NO donor elicited ATP release. Insulin-induced ATP release from human platelets correlated with the association of syntaxin 2 with the vesicle-associated membrane protein 3 but was not associated with the activation of  $\alpha_{IIb}\beta_3$  integrin. Thus, insulin elicits the release of vasoactive concentrations of ATP and adenosine from human platelets via a NO–G kinase–dependent signaling cascade. The mechanism of dense granule secretion involves the G kinase–dependent association of syntaxin 2 with vesicle-associated membrane protein 3.

Key words:  $\alpha_{IIb}\beta_3$  integrin • adenosine • eNOS knockout mice • serotonin • syntaxin 2

## Introduction

Several mechanisms have been proposed to account for insulin-induced vasodilatation, including an interaction with the sympathetic nervous system at the vascular level (1), the activation of ion channels (2), in particular adenosine triphosphate (ATP)–dependent K<sup>+</sup> channels (3), the release of adenosine (4, 5), and an increase in the generation of nitric oxide (NO) by the vascular endothelium (6, 7). Although the latter mechanism could account for the finding that the insulin-induced vasodilatation observed in vivo is sensitive to NO synthase (NOS) inhibitors (7, 8), the acute application of insulin to endothelial cells or isolated arteries does not generally elicit an immediate increase in NO production (9, 10) or potentiate the vasodilator response to endothelial

cell agonists such as acetylcholine or a Ca<sup>2+</sup> ionophore (10). Indeed, acute effects of insulin on vascular tone have also been attributed to endothelium-independent actions related to alterations in smooth muscle calcium (11) and/or the activation of potassium channels (2). Moreover, there is also some doubt as to whether or not the effects of insulin recorded in vivo can be attributed to a direct action of the hormone at its site of application, since pronounced insulin-

*Abbreviations used in this paper:* ACD, acid citrate dextrose; ADP, adenosine diphosphate; AMPK, adenosine monophosphate-activated protein kinase; ATP, adenosine triphosphate; CPT, cyclopentyl-theophylline; CSC, 8-(3-chlorostyryl)caffeine; cyclic GMP, guanosine-3',5' cyclic monophosphate; DETA-NONOate, diethylamine nonoate; eNOS, endothelial NOS; L-NA, N<sup>o</sup>nitro-L-arginine; NO, nitric oxide; NOS, NO synthase; SNARE, N-ethylmaleimide-sensitive factor attachment protein receptor; Sp-cGMPS, guanosine-3',5' cyclic monophosphorothioate, Sp isomer; t-SNARE, target membrane SNARE; VAMP, vesicle-associated membrane protein; VASP, vasodilator-stimulated phosphoprotein.

Address correspondence to Ingrid Fleming, Institut für Kardiovaskuläre Physiologie, Klinikum der J.W.G.-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany. Phone: 49-69-6301-6972; Fax: 49-69-6301-7668; email: fleming@em.uni-frankfurt.de

induced vasodilatation has been reported to require systemic, and not local, hyperinsulinemia (12) and may be dependent on the local uptake of D-glucose (13).

Summarizing the available data, it appears that a role for NO in mediating insulin-induced changes in blood flow can only be demonstrated in vivo by comparing responses in the absence and presence of a NOS inhibitor. This means that the evidence that insulin-induced vasodilatation can be attributed to the activation of endothelial NOS (eNOS) in endothelial cells is only circumstantial. Therefore, it is possible that insulin activates eNOS in blood cells, e.g., in platelets, and that platelet-derived NO is responsible for the vasodilator effects of insulin. Alternatively, NO generated within platelets may affect the release of a more stable platelet-derived relaxing factor. Indirect evidence suggests that the latter possibility is the most likely, since platelet-derived adenosine diphosphate (ADP) (14) and other dinucleotides (15) and serotonin (16) have been reported to mediate platelet-induced vasodilatation. Therefore, the aim of the present investigation was to determine whether or not insulin affects vascular tone in a platelet- and NO-dependent manner.

## Materials and Methods

**Materials.** Citric acid and 3-([3-cholamidopropyl] dimethylammonio)-1-propanesulfonate were from Applichem; AACOCF<sub>3</sub>, Ca-Titriplex, NS2028, and KT5823 were from Merck; U46619 was from Alexis; and N<sup>ω</sup>-nitro-L-arginine (L-NA) was from Fluka. Diethylamine nonoate (DETA-NONOate), (R)-p-8-(4-chlorophenylthio)cGMP, guanosine-3',5' cyclic monophosphorothioate, Sp isomer (Sp-cGMPS), and all other drugs were from Sigma-Aldrich.

**Platelet Isolation.** Blood was donated by healthy individuals who had not taken any medication known to interfere with platelet aggregation for at least 10 d. 50 ml blood was collected into acid citrate dextrose (ACD: 85 mmol/L sodium citrate, 65 mmol/L citric acid, and 125 mmol/L dextrose; 5 ml ACD:50 ml of blood). Platelet-rich plasma was obtained by centrifugation at 130 g for 20 min. Plasma-free platelet suspensions were obtained by centrifugation (900 g, 7 min) of the platelet-rich plasma, and the resulting pellet was suspended in either Tyrode's solution (132 mmol/L NaCl, 4 mmol/L KCl, 1.6 mmol/L CaCl<sub>2</sub>, 0.98 mmol/L MgCl<sub>2</sub>, 23.8 mmol/L NaHCO<sub>3</sub>, 0.36 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 10 mmol/L glucose, 0.05 mmol/L Ca-Titriplex, and gassed with 20% O<sub>2</sub>, 5% CO<sub>2</sub>, and 75% N<sub>2</sub> to give a pO<sub>2</sub> of ~140 mmHg and pH 7.4 at 37°C) or a Hepes-modified Tyrode solution (132 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L CaCl<sub>2</sub>, 0.5 mmol/L MgCl<sub>2</sub>, 9.5 mmol/L Hepes, 0.36 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, and 5 mmol/L glucose). After a further centrifugation step (900 g, 4 min), washed platelets were resuspended in Hepes-Tyrode or Tyrode's solution to achieve a final platelet density of 3 × 10<sup>8</sup>/ml. Platelet suspensions were allowed to equilibrate (37°C, 30 min) in the absence or presence of different inhibitors before stimulating with insulin (1 nmol/L–1 μmol/L) for 10 min. After stimulation, the platelet suspension was centrifuged for 2 min at 900 g, and 2 ml of the supernatant was added to U46619-precontracted arteries mounted in an organ chamber (final volume, 10 ml). The final dilution of the platelet-derived relaxing factor (starting from 50 ml whole blood) was approximately eightfold. In the platelet preparations used throughout (from 53 different donors), the av-

erage number of platelets was 393/nL versus 0.03 leukocytes/nL (of which 10–20% were monocytes). There was no difference in the purity of preparations which responded to insulin versus those which did not.

**Cyclic Guanosine-3',5' Cyclic Monophosphate Assay.** Cyclic guanosine-3',5' cyclic monophosphate (GMP) levels were determined in isobutyl methylxanthine (3 μmol/L IBMX)-treated platelets using a specific radioimmunoassay (PerkinElmer).

**Vascular Reactivity Studies.** Second branches of coronary arteries (internal diameter 300–500 μm) were dissected from the hearts of freshly slaughtered pigs, cleaned of adventitial adipose and connective tissue, and cut into 4-mm-long segments. Coronary artery rings were mounted on stainless steel triangles connected to a force transducer (Hugo Sachs Elektronik-Harvard Apparatus) and a rigid support for measurement of isometric force in organ baths containing Tyrode's solution. Passive tension was gradually adjusted over a 60-min period to 1 g; thereafter, arterial rings were repeatedly exposed to a modified Tyrode's solution rich in KCl (80 mmol/L) until stable contractions were obtained as described (17). The presence of functional endothelium was assessed in all preparations by the ability of bradykinin (1 μmol/L) to induce the relaxation of vessels precontracted with U46619 (0.1 μmol/L), and vessels that exhibited <80% relaxation were discarded. After additional washing and reestablishment of baseline tension, vessels were contracted to 80% of the maximal KCl-induced contraction, and the relaxant response to the cumulative application of either adenosine (0.1–30 μmol/L) or ATP (0.1–30 μmol/L) or to the bolus application of platelet supernatant (2 ml) was determined. In some experiments, the endothelium was removed by intraluminal perfusion with 3-([3-cholamidopropyl] dimethylammonio)-1-propanesulfonate (0.5% in Tyrode's solution) for 30 s. In this case, all rings which responded to the application of bradykinin were discarded.

**ATP, Adenosine, and Serotonin Assays.** The release of ATP into the platelet supernatant was determined using a luciferin/luciferase ATP kit (Enliten<sup>®</sup> ATP assay system; Promega). For this purpose, 500 μl aliquots of platelet supernatant containing the ectonucleotidase inhibitor ARL 67156 (10 μmol/L) were mixed with 100 μl of ATP assay buffer, and light emission was measured using a luminometer. Raw data were collected as relative light units integrated over 10 s and calibrated with the aid of a standard ATP calibration curve.

The concentration of adenosine in the supernatant from insulin-stimulated platelets was determined by HPLC as described (18). Serotonin in the platelet supernatant was quantified using a commercially available ELISA (IBL).

**Preparation of Murine Platelets.** Wild-type (c57 black b6) and age- and gender-matched eNOS<sup>-/-</sup> mice (obtained from the Heinrich-Heine-Universität) were anesthetized with isoflurane, and blood was immediately collected in 10% ACD by punctuating the right cardiac ventricle. Approximately 500 μl of murine blood was centrifuged at 130 g for 20 min and thereafter at 900 g for 7 min. Platelets were suspended in Hepes-Tyrode solution, and after a further centrifugation step (900 g, 4 min), the volume of Hepes-Tyrode solution was adjusted to give a final platelet count of 2 × 10<sup>8</sup>/ml. Platelet suspensions were allowed to equilibrate (37°C, 30 min) in the absence or presence of different inhibitors before stimulating with insulin (1 μmol/L) or DETA-NONOate (1 μmol/L) for 10 min. After stimulation, the platelet suspension was centrifuged for 2 min at 900 g, and the supernatant was used for ATP measurement.

**Immunoblotting and Immunoprecipitation of Syntaxin 2.** Platelets were solubilized in buffer of the following composition: 20

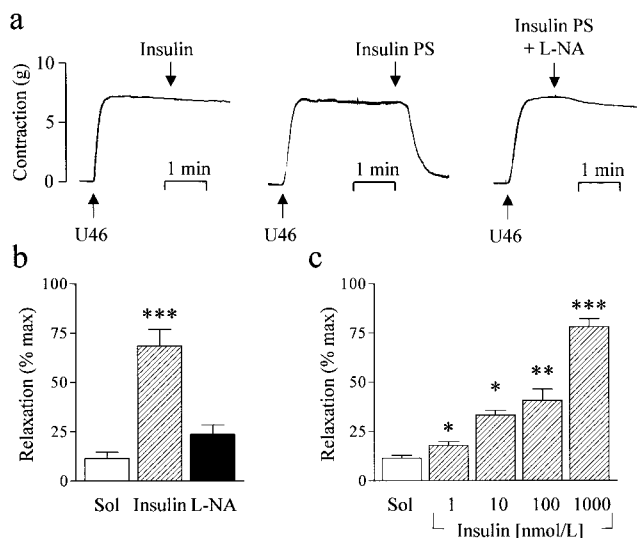
mmol/L Tris-HCl, 150 mmol/L NaCl, NaPPi 10 mmol/L, 20 mmol/L NaF, 25 mmol/L  $\beta$ -glycerophosphate, 2 mmol/L sodium orthovanadate, 1% Nonidet, 10 nmol/L okadaic acid, 100  $\mu$ g/ml leupeptin, 100  $\mu$ g/ml aprotinin, and 100  $\mu$ mol/L phenylmethylsulfonyl fluoride. Then, platelets were left on ice for 30 min and centrifuged at 13,000 rpm for 10 min, and the supernatants were used for immunoprecipitation. In some experiments, syntaxin 2 was immunoprecipitated with a monoclonal anti-syntaxin 2 antibody (Santa Cruz Biotechnology, Inc.) after preclearing of the cell lysate with a mixture of protein A-G sepharose. Platelet lysates or immunoprecipitates were boiled in SDS sample buffer, separated by SDS-PAGE, transferred to a nitrocellulose membrane as described (19), and visualized using a commercially available enhanced chemiluminescence kit (Amersham Biosciences). Samples were blotted twice, and the blots probed with the respective antibodies. Phosphorylated vasodilator-stimulated phosphoprotein (VASP) was detected using a specific monoclonal antibody (Nanotools), and the equal loading of each lane was assessed using an antibody recognizing total VASP protein (Transduction Laboratories). Syntaxin 2 immunoprecipitates were also blotted twice, one membrane being used to detect vesicle-associated membrane protein (VAMP)-3/cellubrevin, using a specific antibody (Abcam), and the other to detect syntaxin 2 and monitor the efficiency of the immunoprecipitation.

**Flow Cytometry.** Platelet suspensions (100  $\mu$ L, containing  $2 \times 10^6$  platelets) were prepared as described and incubated for 10 min at room temperature in the dark with either a FITC-conjugated anti-human PAC-1 antibody (BD Biosciences), a FITC-conjugated anti-human P-selectin antibody (BD Biosciences) or matched mouse IgM and IgK isotype controls. Platelets were then stimulated for 10 min with either insulin (1  $\mu$ mol/L), DETA-NO (1  $\mu$ mol/L), or thrombin (0.1 U/ml) in the presence of H-Gly-Pro-Arg-pro-NH<sub>2</sub> (5 mmol/L) to prevent fibrin polymerization and platelet aggregation. After stimulation, the samples were fixed by the addition of 1 ml of paraformaldehyde (2%, vol/vol) in PBS and analyzed by flow cytometry (FACSCalibur flow cytometer; BD Biosciences) within 2 h of stimulation. The FITC channel baseline value for resting and agonist-stimulated platelets was set using unlabeled platelets. An average of 30,000 platelet events per sample was collected and analyzed for PAC-1 and P-selectin fluorescence intensity.

**Statistical Analysis.**  $R_{\max}$  represents the maximal relaxation recorded in response to the cumulative addition of a given agonist. Data are expressed as mean  $\pm$  SEM, and statistical evaluation was performed using Student's *t* test for unpaired data, one-way analysis of variance (ANOVA) followed by a Bonferroni *t* test, or ANOVA for repeated measures, where appropriate. Values of  $P < 0.05$  were considered statistically significant.

## Results

**Relaxing Factor Derived from Insulin-stimulated Washed Human Platelets.** The direct application of insulin (1  $\mu$ mol/L) to precontracted, endothelium-intact, small porcine coronary artery rings failed to elicit any change in tone (Fig. 1 a). However, the addition of washed human platelets to the organ chamber resulted in platelet aggregation, as a consequence of the activation by the thromboxane analogue used to precontract the coronary arteries (not depicted). However, the supernatant from insulin (1 mmol/L)-stimulated human platelets elicited the rapid relaxation of arterial rings;  $R_{\max}$  values were  $11.4 \pm 3.1$  in response to the su-

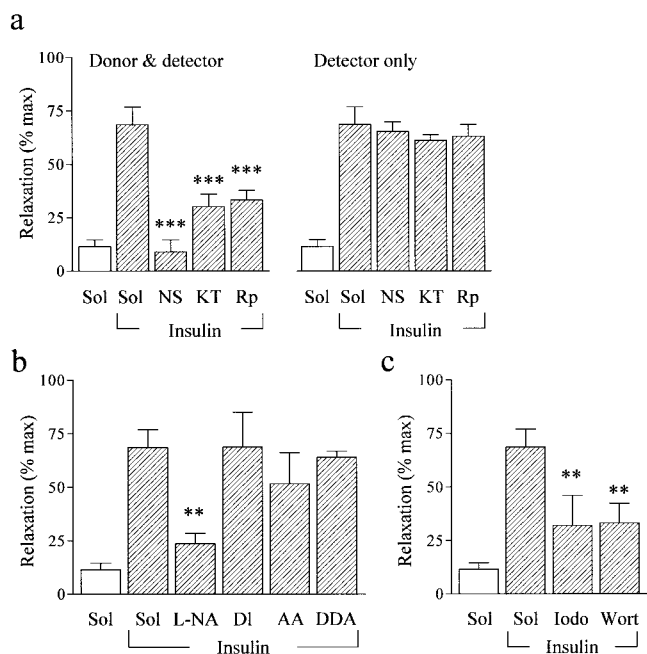


**Figure 1.** Relaxing factor derived from insulin-stimulated washed human platelets. (a) Original tracings and (b) statistical summary showing the effect of the direct application of human insulin (1  $\mu$ mol/L), the supernatant from unstimulated platelets (Sol), the supernatant from insulin-stimulated (1  $\mu$ mol/L, 10 min) platelets, and from platelets stimulated with insulin in the presence of L-NA (300  $\mu$ mol/L) on the tone of endothelium-intact small porcine coronary arteries precontracted with the thromboxane analogue U46619. (c) Statistical summary showing the concentration-dependent effect of insulin (1 nmol/L–1  $\mu$ mol/L) on the release of a relaxing factor from platelets. The results shown represent the mean  $\pm$  SEM of data obtained in six independent experiments; \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus the response obtained using the supernatant from unstimulated platelets (Sol).

pernatant of unstimulated platelets versus  $68.5 \pm 8.3\%$  in response to the supernatant from insulin-stimulated platelets. The supernatant from insulin-stimulated platelets, which were pretreated with the NOS inhibitor L-NA (300  $\mu$ mol/L, 30 min), failed to relax coronary artery rings. The effect of insulin on the release of the platelet-derived relaxing factor was concentration dependent, and a small but significant relaxation was observed in response to the application of supernatant removed from platelets stimulated with 1 nmol/L insulin (Fig. 1 c).

There were marked differences in the responses observed to insulin treatment between platelets from different donors, so that responders (65% of all preparations), where the supernatant from insulin-stimulated platelets elicited a relaxation of  $>60\%$ , and nonresponders (35% of all preparations), where the relaxation was generally  $<20\%$ , could be identified. The donor-dependent ability of the supernatant from these platelets to release a relaxing factor was also correlated with the ability of insulin to inhibit thrombin-induced platelet aggregation (not depicted).

**Pharmacological Characterization of the Pathway Involved in the Insulin-induced Release of a Platelet-derived Relaxing Factor.** The experiments using L-NA suggested that the activation of NOS was involved in regulating the release of a relaxing factor from insulin-stimulated platelets. To clarify this point, we assessed cyclic GMP levels in insulin-stimulated platelets. Cyclic GMP levels were  $20.9 \pm 1.4$ ,  $40.6 \pm$



**Figure 2.** Pharmacological characterization of the pathway involved in the insulin-induced release of a platelet-derived relaxing factor. (a) Effect of the guanylyl cyclase inhibitor, NS 2028 (NS, 10  $\mu\text{mol/L}$ , 30 min), and the G kinase inhibitors, KT 5823 (KT, 1  $\mu\text{mol/L}$ , 30 min) and Rp-8CPT-cGMPs (Rp, 10  $\mu\text{mol/L}$ , 30 min), on the insulin (1  $\mu\text{mol/L}$ , 10 min)-induced release of a relaxing factor from washed human platelets, i.e., inhibitors applied to the platelet donor and the detector artery ring compared with the effects observed when the inhibitors were applied only to the detector ring. (b) Statistical summary showing the effect of L-NA (300  $\mu\text{mol/L}$ , 30 min), diclofenac (DI, 10  $\mu\text{mol/L}$ , 30 min), the phospholipase  $A_2$  inhibitor AACOCF<sub>3</sub> (AA, 3  $\mu\text{mol/L}$ , 30 min), and the adenylyl cyclase inhibitor 2',3'-dideoxyadenosine (DDA, 100  $\mu\text{mol/L}$ , 30 min) on the insulin (1  $\mu\text{mol/L}$ , 10 min)-induced release of a relaxing factor from washed human platelets. (c) Summary showing the effect of the AMPK inhibitor iodotubercidin (Iodo, 1  $\mu\text{mol/L}$ , 30 min) and the phosphatidylinositol 3-kinase inhibitor wortmannin (Wort, 40 nmol/L, 30 min) on the insulin (1  $\mu\text{mol/L}$ , 10 min)-induced release of a relaxing factor from washed human platelets. The results shown represent the mean  $\pm$  SEM of data obtained in five to seven independent experiments. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus the response obtained using the supernatant from unstimulated platelets (Sol).

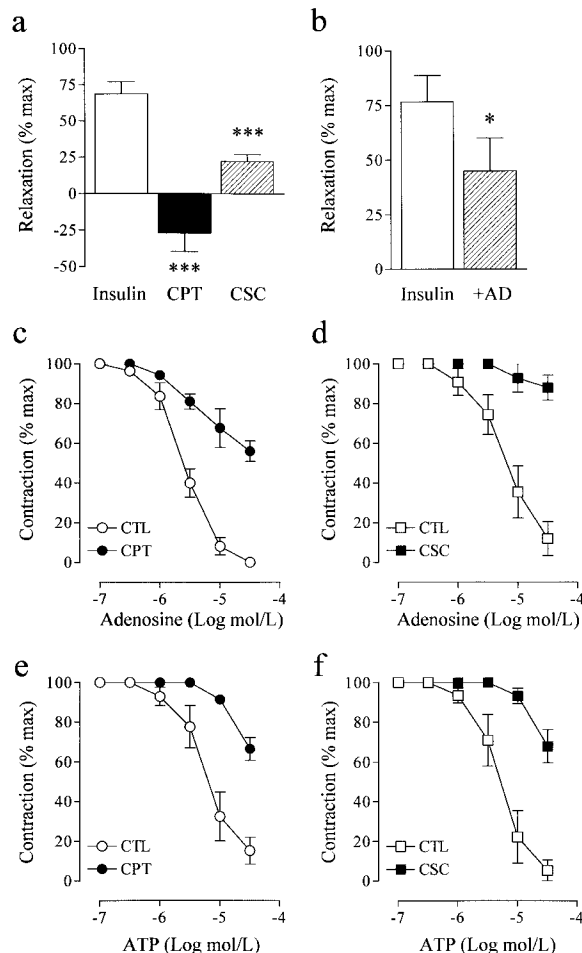
2.7,  $20.1 \pm 1.1$ , and  $19.5 \pm 0.9$  fmol/mg protein in the presence of solvent, insulin, L-NA, and insulin plus L-NA, respectively ( $P < 0.01$ ,  $n = 5$ ).

When the soluble guanylyl cyclase inhibitor, NS2028 (10  $\mu\text{mol/L}$ ), or the G kinase inhibitors, KT 5823 (1  $\mu\text{mol/L}$ ) or Rp-8CPT-cGMPs (10  $\mu\text{mol/L}$ ), were present throughout the platelet stimulation and the organ chamber experiments, the relaxation of porcine coronary artery rings was significantly attenuated. However, when these compounds were present in the organ chamber but not present throughout the preparation of the platelet supernatant, no effect on the insulin-induced release of a platelet-derived relaxing factor was observed (Fig. 2 a).

Pretreatment of washed human platelets with either the cyclooxygenase inhibitor diclofenac (10  $\mu\text{mol/L}$ , 30 min), the phospholipase  $A_2$  inhibitor AACOCF<sub>3</sub> (3  $\mu\text{mol/L}$ , 30 min), or the adenylyl cyclase inhibitor 2',3'-dideoxyaden-

osine (100  $\mu\text{mol/L}$ , 30 min) did not affect the insulin (1  $\mu\text{mol/L}$ )-induced release of a relaxing factor from platelets (Fig. 2 b). However, the adenosine monophosphate-activated protein kinase (AMPK) inhibitor iodotubercidin (1  $\mu\text{mol/L}$ ) and the phosphatidylinositol 3-kinase inhibitor wortmannin (40 nmol/L), which attenuate the insulin-induced phosphorylation and activation of eNOS in platelets (20), attenuated the insulin-induced release of a platelet-derived relaxing factor (Fig. 2 c).

Although the application of insulin to platelets stimulates platelet eNOS, the relatively long preparation time of the



**Figure 3.** Pharmacological characterization of the platelet-derived relaxing factor. (a) Effect of the nonselective adenosine receptor antagonist CPT (10  $\mu\text{mol/L}$ , 30 min) and the selective  $A_2$  receptor antagonist CSC (1  $\mu\text{mol/L}$ , 30 min) on the relaxation elicited by a factor derived from insulin (1  $\mu\text{mol/L}$ , 10 min)-stimulated washed human platelets. (b) Effect of inclusion of adenosine deaminase (50 U/ml) on the relaxing effect of the supernatant from insulin-stimulated platelets. (c-f) Effect of adenosine receptor antagonists on the adenosine- and ATP-induced relaxation of the porcine coronary artery. Endothelium-intact rings of porcine coronary artery were precontracted with U46619 (0.01–0.5  $\mu\text{mol/L}$ ), and concentration-relaxation curves to adenosine (c and d) and ATP (e and f) were obtained. Experiments were performed in the absence (CTL) and the presence of CPT (10  $\mu\text{mol/L}$ ) or CSC (1  $\mu\text{mol/L}$ ). The results shown represent the mean  $\pm$  SEM of data obtained in six to eight independent experiments. \* $P < 0.05$  and \*\*\* $P < 0.001$  versus the response obtained in the absence of inhibitor.



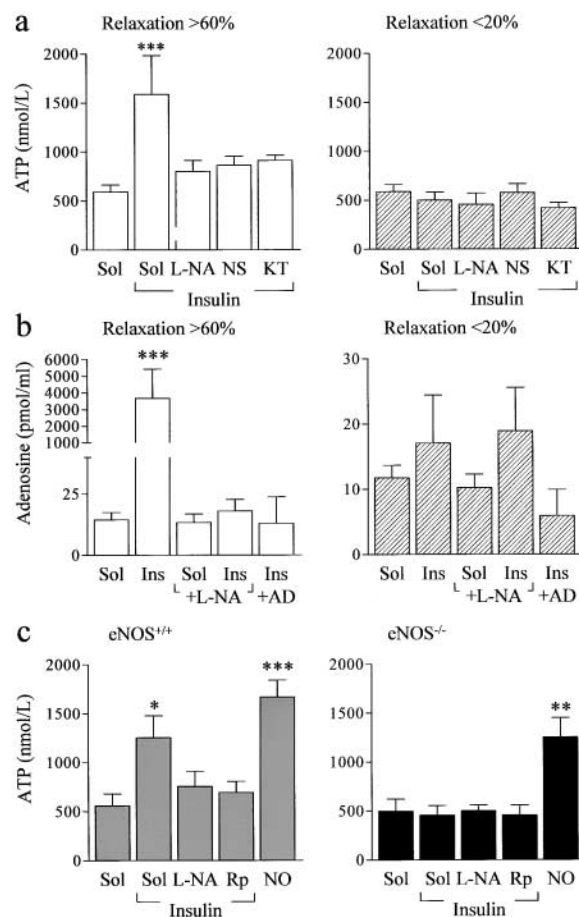
platelet supernatant together with the insensitivity of the platelet supernatant-induced relaxation of coronary arteries to guanylyl cyclase and G kinase inhibition, imply that NO itself is not the platelet-derived relaxing factor.

**Characterization of the Platelet-derived Relaxing Factor.** Both the nonselective adenosine receptor antagonist cyclopentyl-theophylline (CPT, 10  $\mu\text{mol/L}$ ) and the selective  $A_2$  adenosine receptor antagonist 8-(3-chlorostyryl)caffeine (CSC, 1  $\mu\text{mol/L}$ ) significantly inhibited the relaxation elicited by the supernatant from insulin-stimulated platelets (Fig. 3 a). Moreover, inclusion of adenosine deaminase in the supernatant to convert adenosine to inosine significantly inhibited the relaxing effect of the supernatant from insulin-stimulated platelets (Fig. 3 b). Neither the  $P_2$  purinoceptor antagonist, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt (50  $\mu\text{mol/L}$ ), nor the  $P_{2Y}$  receptor antagonist 3'-phosphoadenosine 5'-phosphosulfate (10  $\mu\text{mol/L}$ ) affected the relaxation elicited by insulin-stimulated platelets (not depicted).

**Effect of Adenosine and Purine Receptor Antagonists on the Adenosine- and ATP-induced Relaxation of the Porcine Coronary Artery.** Since ATP would be expected to be released in larger amounts than adenosine from insulin-stimulated platelets, concentration-relaxation curves to adenosine (0.01–0.5  $\mu\text{mol/L}$ ) and ATP (0.1–30  $\mu\text{mol/L}$ ) were assessed using endothelium-intact rings of porcine coronary artery precontracted with U46619.

The adenosine receptor antagonist CPT (10  $\mu\text{mol/L}$ ) significantly inhibited relaxations elicited by adenosine and ATP (Fig. 3, c and e);  $R_{\text{max}}$  values were  $100 \pm 0.1$  versus  $44 \pm 5.2\%$  in solvent- and CPT-treated artery rings stimulated with adenosine ( $P < 0.01$ ,  $n = 5$ ) and  $85 \pm 7$  versus  $33 \pm 6\%$  in solvent- and CPT-treated artery rings stimulated with ATP ( $P < 0.01$ ,  $n = 5$ ). A similar effect was observed in adenosine- and ATP-stimulated coronary artery rings treated with CSC (Fig. 3, d and f);  $R_{\text{max}}$  values were  $95 \pm 3$  versus  $12 \pm 6\%$  in solvent- and CSC-treated artery rings stimulated with adenosine ( $P < 0.01$ ,  $n = 5$ ) and  $95 \pm 5$  versus  $32 \pm 8\%$  in solvent- and CSC-treated artery rings stimulated with ATP ( $P < 0.01$ ,  $n = 6$ ). Neither of the purinoceptor antagonists, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt (50  $\mu\text{mol/L}$ ) or 3'-phosphoadenosine 5'-phosphosulfate (10  $\mu\text{mol/L}$ ) affected the relaxation elicited by adenosine or ATP (not depicted). Similar results were obtained using endothelium-denuded porcine coronary arteries (not depicted).

**Insulin-stimulated and NO-dependent Release of ATP and Adenosine from Washed Human Platelets.** To determine whether the release of ATP and/or adenosine in response to insulin stimulation could account for the platelet-derived relaxing factor, we assessed ATP and adenosine levels in the supernatant of platelets incubated with either solvent or insulin in the absence and presence of inhibitors of the NO-cyclic GMP signaling pathway and compared the results obtained with the ability of the same supernatant to elicit the relaxation of precontracted rings of porcine coronary artery.

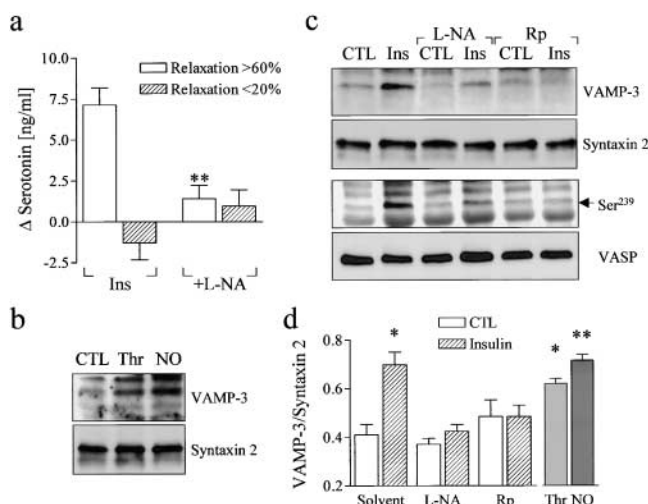


**Figure 4.** Insulin-stimulated and NO-dependent release of ATP and adenosine from washed human and murine platelets. (a) Effect of L-NA (300  $\mu\text{mol/L}$ , 30 min), the guanylyl cyclase inhibitor, NS 2028 (10  $\mu\text{mol/L}$ , 30 min), and the G kinase inhibitor, KT 5823 (1  $\mu\text{mol/L}$ , 30 min), on the insulin (1  $\mu\text{mol/L}$ , 10 min)-induced release of ATP from washed human platelets. (b) Effect of L-NA (300  $\mu\text{mol/L}$ , 30 min) on the release of adenosine from washed human platelets incubated with either solvent (Sol) or insulin (1  $\mu\text{mol/L}$ , 10 min). In some experiments, adenosine deaminase (50 U/ml) was added to the supernatant before stimulation. In each case, the ability of the supernatant to relax precontracted rings of porcine coronary artery was assessed, and the results were divided into two categories, i.e., the relaxation induced by the supernatant of insulin-stimulated platelets was  $>60\%$  (responders) or relaxation was  $<20\%$  (non responders). (c) Effect of insulin (1  $\mu\text{mol/L}$ , 10 min) and NO (DETA NONOate, 1  $\mu\text{mol/L}$ , 10 min) on the release of ATP from platelets derived from either wild-type (eNOS<sup>+/+</sup>) or eNOS<sup>-/-</sup> mice. Experiments were performed in the presence of solvent (Sol), L-NA, or Rp-8CPT-cGMPs (10  $\mu\text{mol/L}$ ). The results shown represent the mean  $\pm$  SEM of data obtained in 6–10 independent experiments; \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus the response obtained using the supernatant from insulin-stimulated platelets.

In experiments in which insulin elicited the generation of a platelet-derived relaxing factor, there was a significant, insulin-induced release of ATP from platelets (Fig. 4 a). However, ATP levels were not increased by insulin in samples (non responders) which failed to relax the arterial rings. The insulin-induced release of ATP was attenuated by L-NA and by NS2028 (10  $\mu\text{mol/L}$ ) and the KT 5823 (1  $\mu\text{mol/L}$ ). On the other hand, both the NO donor DETA-NONOate

(1  $\mu\text{mol/L}$ ) and the cyclic GMP analogue—G kinase activator Sp-cGMPS (10  $\mu\text{mol/L}$ ) stimulated the release of ATP from platelets. ATP concentrations were  $574.9 \pm 21.25$  versus  $2,006.3 \pm 540.7$  and  $1,394.0 \pm 207.7$  nmol/L ATP in the supernatant of solvent-stimulated platelets versus DETA-NONOate- and Sp-cGMPS-stimulated platelets ( $P < 0.01$ ,  $n = 5-6$ ). The levels of ATP released from these platelets did not elicit aggregation and were markedly lower than the levels detected in the supernatant from thrombin-stimulated platelets. The ATP concentration was  $574.0 \pm 21.3$  nmol/L ATP in the supernatant of solvent-stimulated platelets versus  $4,567.0 \pm 794.0$  nmol/L ATP in the supernatant from thrombin-stimulated platelets ( $P < 0.01$ ,  $n = 6$ ). Similar results were obtained when adenosine levels in the supernatant were assayed (Fig. 4 b). Moreover, adenosine levels in the supernatant were attenuated in the presence of adenosine deaminase (Fig. 4 b).

To clarify the role of eNOS in the regulation of ATP release, experiments were performed using platelets from either wild-type or eNOS<sup>-/-</sup> mice. Insulin induced the release of ATP from platelets from wild-type mice, a re-



**Figure 5.** Effect of insulin on serotonin release from  $\alpha$ -granules, the phosphorylation of VASP, and the association of VAMP-3 with syntaxin 2. (a) Effect of L-NA (300  $\mu\text{mol/L}$ ) on the insulin-induced release of serotonin from washed human platelets. Values are presented relative to the serotonin levels detected in the supernatant from platelets treated with solvent. The supernatant-induced relaxation of porcine coronary artery rings was assessed, and the results were divided into two categories, i.e., responders and nonresponders. (b) Effect of solvent (CTL), thrombin (Thr, 0.1 U/ml), and the NO donor DETA-NONOate (NO, 1  $\mu\text{mol/L}$ ) on the association of VAMP-3 with syntaxin 2, immunoprecipitated from washed human platelets. (c) Effect of solvent (CTL) and insulin (1  $\mu\text{mol/L}$ ) on the association of VAMP-3 with syntaxin 2, immunoprecipitated from washed human platelets. Experiments were performed in the absence and presence of L-NA and the G kinase inhibitor Rp-8CPT-cGMPs (Rp, 10  $\mu\text{mol/L}$ ). The phosphorylation of VASP (Ser<sup>239</sup> VASP), as a marker of NOS activation, was also determined in the Triton X-100-soluble fraction of the same platelets preparations. To demonstrate the equal loading of each lane, the blots were reprobed with antibodies recognizing either syntaxin 2 or total VASP protein. The bar graph (d) summarizes the results of VAMP-3 association with syntaxin 2 obtained in seven independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$  versus unstimulated platelets (CTL and solvent).

sponse that was sensitive to L-NA and Rp-8CPT-cGMPs and mimicked by DETA NONOate (Fig. 4 c). Insulin failed to elicit the release of ATP from platelets from eNOS<sup>-/-</sup> mice, whereas ATP was secreted in response to the NO donor (Fig. 4 c).

**Insulin-stimulated Dense Granule Secretion.** To determine whether or not the ATP/adenosine was derived from dense granules, we determined whether serotonin, a classical marker of dense granules, was also released from insulin-stimulated platelets. Again, insulin elicited the release of serotonin from those platelets that also generated a relaxing factor but not from platelets which failed to generate a relaxing factor (Fig. 5 a).

**Effect of Insulin on the Association of Syntaxin 2 and VAMP-3.** Since dense granule release is inhibited by antibodies against the target membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptor (t-SNARE) protein syntaxin 2 (21) and by soluble recombinant VAMP-3 (22), we determined whether or not insulin stimulated the NO-dependent association of VAMP-3 with syntaxin 2.

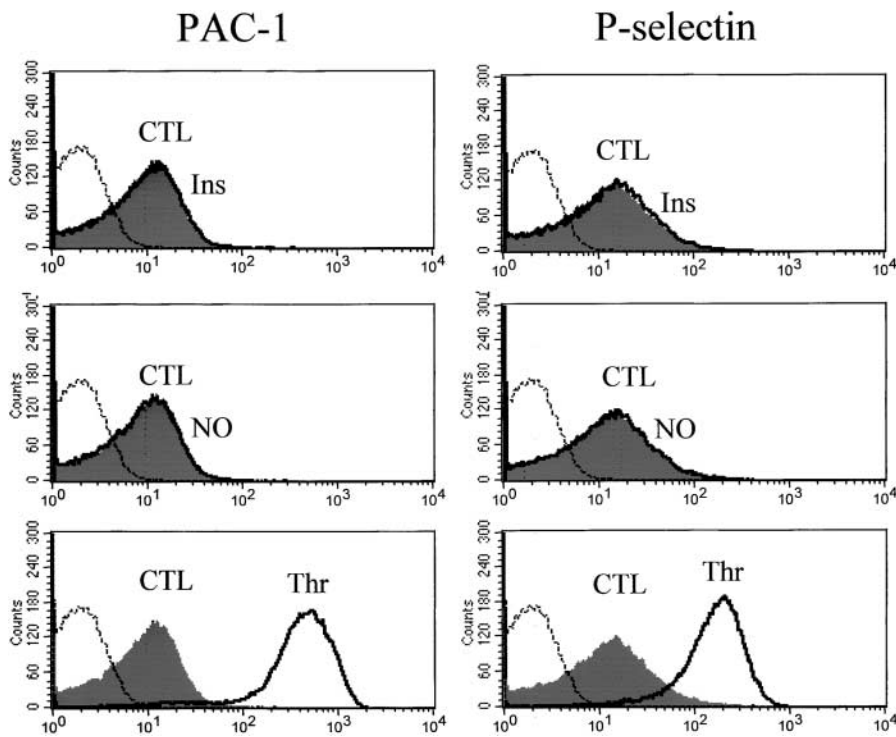
In unstimulated platelets, a small amount of VAMP-3 coprecipitated with syntaxin 2. Stimulation with either the potent platelet activator thrombin (0.1 U/ml) or the NO donor diethylamine nonoate (DETA-NO, 1  $\mu\text{M}$ , 10 min) increased the association of VAMP-3 with syntaxin 2 (Fig. 5, b and d).

In platelets in which insulin elicited the L-NA-sensitive and G kinase-dependent phosphorylation of VASP (an index of NO production), there was also an increased association of VAMP-3 with syntaxin 2 (Fig. 5, c and d). The association of these proteins was also sensitive to L-NA and Rp-8CPT-cGMPs (10  $\mu\text{mol/L}$ ). No change in the association of VAMP-3 with syntaxin was observed in platelets in which insulin did not elicit the phosphorylation of VASP (not depicted).

**Effect of Insulin on the Activation of  $\alpha_{\text{IIb}}\beta_3$  Integrin and the Secretion of  $\alpha$ -Granules.** Neither insulin nor DETA NONOate activated  $\alpha_{\text{IIb}}\beta_3$  integrin, as assessed using a FITC-conjugated PAC1 antibody that recognizes only the activated conformation of the  $\alpha_{\text{IIb}}\beta_3$  complex (Fig. 6). Moreover, neither stimulus elicited the cell surface expression of P-selectin. These observations contrasted markedly with the effects of thrombin, which both activated  $\alpha_{\text{IIb}}\beta_3$  integrin and elicited the cell surface expression of P-selectin (Fig. 6). Insulin also failed to effect either the release of the  $\alpha$ -granule marker  $\beta$ -thromboglobulin or the generation of thromboxane A<sub>2</sub> (not depicted).

## Discussion

The results of the present investigation demonstrate that insulin-stimulated platelets release sufficient amounts of ATP and/or adenosine to relax precontracted porcine coronary arteries. The signaling pathway initiated by insulin involves the activation of platelet eNOS, the soluble guanylyl cyclase and G kinase, and the association of VAMP-3



**Figure 6.** Comparison of the effects of insulin, NO, and thrombin on the activation of  $\alpha_{IIb}\beta_3$  integrin and platelet  $\alpha$ -granule secretion. Representative FACS<sup>®</sup> analysis showing the effects of stimulation (10 min) with either solvent (CTL), insulin (Ins, 1  $\mu$ mol/L), DETA NONOate (NO, 1  $\mu$ mol/L), or thrombin (Thr, 0.1 U/ml) on the surface expression of activated  $\alpha_{IIb}\beta_3$  integrin (PAC1) or P-selectin. Identical results were obtained in four additional experiments. The dashed line represents the population of unlabeled platelets.

with the t-SNARE protein, syntaxin 2, and the release of adenine nucleotides and serotonin from dense granules. Since insulin-induced vasodilatation *in vivo* is sensitive to NOS inhibitors (8) but insulin does not acutely enhance NO production by endothelial cells (20), the NO-dependent release of platelet-derived vasodilator compounds may well account for the phenomenon of insulin-induced vasodilatation *in vivo*.

The effects of insulin on platelets is controversial since some studies have demonstrated that insulin attenuates thrombin-induced platelet aggregation (23–25), whereas others report that insulin may enhance platelet activation and aggregation (26). The results of the present study suggest that insulin activates platelets to a certain extent but that the effects initiated stop short of the threshold for a classical activation response. Indeed, although insulin elicited the release of ATP and serotonin from platelets, the amount of ATP and serotonin released was much smaller than that released from thrombin-stimulated platelets. Moreover, insulin and NO failed to either activate  $\alpha_{IIb}\beta_3$  integrin (the GPIIb–IIIa complex) or to induce  $\alpha$ -granule secretion, as determined by assaying the release of  $\beta$ -thromboglobulin and the surface expression of P-selectin.

The effect of insulin on the release of ATP, adenosine, and serotonin from platelets was dependent on the generation of NO, since the responses were prevented by a NOS inhibitor and by inhibitors of the guanylyl cyclase and G kinase. Moreover, the NO donor, DETA-NO, mimicked the effect of insulin and elicited the degranulation of human and murine platelets, whereas insulin-induced ATP release was not detectable in platelets from eNOS<sup>-/-</sup> mice. The pathway leading to the activation of eNOS in insulin-stimulated

platelets has not been completely elucidated but is thought to involve the activation of the metabolic stress-sensing kinase, AMPK (20). Given that changes in AMPK expression and activity have been associated with insulin resistance and diabetes (27, 28), alterations in AMPK activity may be linked to aggregatory disturbances in diabetic patients. The involvement of the AMPK in the insulin-induced release of a platelet-derived relaxing factor was also indicated in the present study, since relaxation was attenuated in samples from platelets treated with the AMPK inhibitor iodotubercidin. Although it has been reported that one of the inhibitors used, KT 5823, does not inhibit G kinase in intact human platelets (29), we observed similar effects using KT 5823 and Rp-8CPT-cGMPs, and in our hands, KT 5823 attenuated the insulin-induced phosphorylation of VASP in washed human platelets (unpublished data).

The existence of platelet-derived relaxing factors is widely accepted (30), but the physiological relevance of the release of these compounds is not generally appreciated. The results of the present investigation indicate that the release of ATP/ADP and adenosine from insulin-stimulated platelets can account for insulin-induced platelet-dependent relaxation of porcine coronary arteries. Although platelet-derived serotonin does elicit vasodilatation in some vascular beds (16), serotonin failed to affect porcine coronary artery tone (unpublished data). Although platelet-derived adenine nucleotides and serotonin were proposed to elicit vasodilatation by initiating the generation of an endothelium-derived relaxing factor (15), the responses observed to platelet-derived ATP/ADP and adenosine in the present study were endothelium independent, and the relaxant response was mediated entirely via the activation of

adenosine receptors, especially the A<sub>2A</sub>-adenosine receptor. The fact that the ATP-induced relaxation of coronary arteries was insensitive to purinoceptor antagonists but was markedly attenuated by adenosine receptor antagonists implies that ATP is rapidly metabolized by the ecto-5'-nucleotidase within the vascular wall, as described previously (31–33). The concept that adenosine mediates insulin-induced vasodilatation is supported by forearm blood flow data obtained in healthy subjects in response to a hyperinsulinemic euglycemic clamp, since both drafazine (an adenosine uptake blocker) and theophylline (an adenosine receptor antagonist) significantly prevented insulin-induced changes in blood flow (5).

ATP, adenosine, and serotonin are stored within platelet-dense granules, and the molecular mechanisms of platelet exocytosis are thought to be homologous to those in neurons and other cells in that SNARE complexes are formed between VAMPs and proteins in the target membranes (t-SNAREs) (34). Platelet membranes contain the t-SNARE proteins syntaxins 2 and 4, which play distinctive roles in granule exocytosis, with syntaxin 2 being involved in dense granule release (35). Platelets also contain abundant amounts of SNAP-23 (36) and VAMP-3 and -8 (22;37), which interact and form SNARE complexes (for review see reference 34). Since dense granule release is reported to be inhibited by antibodies against the syntaxin 2 (21) and by soluble recombinant VAMP-3 (22), we determined the effects of insulin on the association of syntaxin 2 and VAMP-3. We observed that insulin initiated the association of the two proteins and that this effect is also dependent on the activation of eNOS and the G kinase. Moreover, a NO donor also stimulated the association of the two proteins and the association of syntaxin 2 with VAMP-3 was only detected in samples in which insulin induced the L-NA-sensitive phosphorylation of VASP as a marker for eNOS activation.

Although all of the platelets derived from wild-type mice responded to insulin with an increase in ATP release, ~35% of the human platelet preparations used did not respond to insulin. This phenomenon was not donor specific since a responder could be nonresponder at a different bleeding time and vice versa. Heterogeneity in responsiveness to insulin is well documented (38) and can be related to numerous factors including physical condition (39). Although it was outside the scope of the present study to determine the mechanisms affecting insulin sensitivity, we found an excellent correlation between the insulin-induced activation of platelet eNOS (assayed via cyclic GMP), the release of a platelet-derived relaxing factor, the association of VAMP-3 with syntaxin 2 and dense granule secretion. In all cases in which insulin activated platelet eNOS, the entire signaling cascade leading to dense granule secretion was also activated. This implies that the phenomenon determining insulin resistance is located upstream of the activation of eNOS.

There appear to be at least two actions of NO in platelets. The first is to limit platelet activation, adhesion, and aggregation by activating the soluble guanylyl cyclase, in-

itiating the G kinase-mediated phosphorylation of the thromboxane receptor (40), inhibiting kinase activity (41, 42) and decreasing capacitative Ca<sup>2+</sup> entry (43, 44). A second effect of NO in platelets appears to be to facilitate the release of ATP/ADP and serotonin from dense granules via a G kinase-dependent pathway involving the association of VAMP-3 with syntaxin 2. This implies that low concentrations of NO actually promote platelet activation. However, rather than being a proaggregatory signal, the limited degranulation that occurs in response to insulin-induced NO production results in the release of potent vasodilator compounds. These data are consistent with a recent report demonstrating that cyclic GMP enhances thrombin-induced platelet activation, whereas G kinase inhibitors attenuate the response (45). The role of the G kinase in mediating platelet responses is reported to be biphasic, consisting of an initial transient stimulatory response that promotes platelet aggregation and a subsequent inhibitory response that limited thrombus size (45).

How can the apparently contradictory effects of the NO-cyclic GMP-G kinase pathway on platelet activation be reconciled? Although it is impossible to answer this question at this stage, the key is probably related to further factors, i.e., the platelet microenvironment and the concomitant activation of additional signaling pathways. Indeed, although the von Willebrand factor- and thrombin-induced activation of platelets is impaired in G kinase-deficient mice (45), the response to collagen is normal (46).

Together, the results of the present investigation, somewhat heretically, suggest that the acute vasodilator action of insulin, which has until now been attributed to the generation of endothelium-derived NO, can actually be attributed to the effect of the hormone on platelet eNOS and the subsequent release of adenine nucleotides, which are rapidly metabolized by the ectonucleotidase present on the endothelial cell surface to the potent vasodilator adenosine.

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