Induction of insulin resistance by the adipokines resistin, leptin, plasminogen activator inhibitor-1 and retinol binding protein 4 in human megakaryocytes

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ABSTRACT

Background

In normal platelets, insulin inhibits agonist-induced Ca^{2+} mobilization by raising cyclic AMP. Platelet from patients with type 2 diabetes are resistant to insulin and show increased Ca^{2+} mobilization, aggregation and procoagulant activity. We searched for the cause of this insulin resistance.

Design and Methods

Platelets, the megakaryocytic cell line CHRF-288-11 and primary megakaryocytes were incubated with adipokines and with plasma from individuals with a disturbed adipokine profile. Thrombin-induced Ca^{2+} mobilization and signaling through the insulin receptor and insulin receptor substrate 1 were measured. Abnormalities induced by adipokines were compared with abnormalities found in platelets from patients with type 2 diabetes.

Results

Resistin, leptin, plasminogen activator inhibitor-1 and retinol binding protein 4 left platelets unchanged but induced insulin resistance in CHRF-288-11 cells. Interleukin-6, tumor necrosis factor-α and visfatin had no effect. These results were confirmed in primary megakaryocytes. Contact with adipokines for 2 hours disturbed insulin receptor substrate 1 Ser^{307}-phosphorylation, while contact for 72 hours caused insulin receptor substrate 1 degradation. Plasma with a disturbed adipokine profile also made CHRF-288-11 cells insulin-resistant. Platelets from patients with type 2 diabetes showed decreased insulin receptor substrate 1 expression.

Conclusions

Adipokines resistin, leptin, plasminogen activator-1 and retinol binding protein 4 disturb insulin receptor substrate 1 activity and expression in megakaryocytes. This might be a cause of the insulin resistance observed in platelets from patients with type 2 diabetes.

Key words: adipokines, insulin, insulin resistance, megakaryocytes, platelets, obesity, type 2 diabetes.

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The online version of this article has a Supplementary Appendix.

Introduction

Platelets initiate early steps in atherogenesis and adhere to prothrombotic sites of the inflamed vessel wall. The non-activated endothelial layer prevents platelet adhesion through release of the inhibitors prostaglandin I_2 and nitric oxide. Under pathological conditions, endothelial cells bind platelets through selectin- and integrin-mediated bridging. The adherent platelets recruit and activate monocytes, which start releasing chemokines, cytokines and enzymes that degrade the endothelial matrix and trigger expression of tissue factor, the main activator of the coagulation cascade. The monocytes differentiate into macrophages and contribute to atherosclerotic plaque formation.[1] Upon plaque rupture, platelets adhere to the vascular lesion providing a procoagulant surface that starts thrombin formation and systemic platelet activation. The positive correlation between intima-media thickness with number of activated platelets and platelet products released in plasma bears witness to the contribution of platelets in atherogenesis.[2]

Type 2 diabetes is a metabolic disorder characterized by insulin resistance and microvasular and macrovascular disease.[3] Prospective studies have shown that insulin resistance is a risk factor for the cardiovascular problems caused by atherosclerosis of coronary, cerebral and lower limb blood vessels.[4] Insulin resistance disturbs glucose homeostasis in muscle cells and nitric oxide production by endothelial cells and accelerates atherosclerosis.[3] In patients with type 2 diabetes, platelets circulate in an activated state and the extent of activation correlates with intima-media thickness.[2,5] Under laboratory conditions, type 2 diabetes platelets adhere better to a thrombogenic surface, form bigger aggregates at lower agonist concentration and produce more thromboxane A_2 than do control platelets.[6] The hyperactivity correlates with loss of insulin sensitivity and intensive insulin treatment partly normalizes aggregation.[7] Inhibition of platelet responsiveness with aspirin therapy reduces the relative risk of myocardial infarction and stroke by about 10%.[8]

We demonstrated earlier that insulin inhibits aggregation/secretion by platelets and tissue factor synthesis by monocytes. In healthy individuals, insulin interferes with the suppression of cAMP and accumulation of this inhibitor attenuates platelet functions and monocyte responses.[9,10] The insulin resistance observed in type 2 diabetes rescues the fall in cAMP, which promotes aggregation, secretion and procoagulant activity in platelets and tissue factor synthesis and interleukin-1β secretion in monocytes. Insulin also inhibits splicing of tissue factor pre-mRNA in platelets adhering to prothrombotic proteins and the loss of insulin responsiveness in type 2 diabetes might well contribute to the thrombogenicity of the platelet plug that forms upon plaque rupture.[6,11]

Weight gain and appearance of insulin resistance go hand in hand and are thought to be caused by abnormal adipokine release by visceral fat.[12] Adipocytes release resistin, leptin, plasminogen activator inhibitor-1 (PAI-1), retinol binding protein 4 (RBP4) and visfatin and their plasma levels are elevated in individuals with abdominal obesity. They also release adiponectin but plasma levels of this adipokine correlate inversely with body mass.[13] Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), released by macrophages in adipose tissue, might induce insulin resistance.[14] Patients with type 2 diabetes are often obese and their platelets circulate in an environment with abnormal adipokine content.[15] This might cause platelet hyperactivity but definite proof is lacking. Platelets have the leptin receptor ObRb, but they fail to respond to physiological leptin concentrations. Levels found in obese individuals (30-100 ng/mL) stimulate increases in Ca^{2+}, thromboxane A_2 formation and ADP-aggregation[16-19] and *ob/ob* mice deficient in the leptin gene show delayed occlusion in a model of arterial thrombosis.[16] These findings suggest that leptin increases platelet reactivity. In contrast to this idea are findings that prolonged leptin infusion in healthy volunteers leaves aggregation unchanged and that leptin-deficient individuals have increased rather than decreased aggregation.[18,19]

Apart from changing platelet functions through direct interference, adipokines may alter the properties of platelets during their synthesis from megakaryocytes.[20] Megakaryocytes develop from hematopoietic stem cells in the bone marrow. Their maturation is under the control of thrombopoietin, stem cell factor and other cytokines, which determine cell size, ploidy and protein expression. Variations in growth factor stimulation alter protein expression, illustrating the high responsiveness of megakaryocytes to cytokines.[20]

We speculated that these cells might be equally responsive to adipokines and addressed this question in megakaryocytic CHRF-288-11 cells[20-22] and confirmed key observations in megakaryocytes. Results show that specific adipokines induce insulin resistance in these cells. If a similar induction occurs in bone marrow, it might be one of the factors that cause insulin resistance in platelets in type 2 diabetes.

Design and Methods

Materials

We obtained human α-thrombin from Enzyme Research Laboratories (South Bend, IN, USA), adenosine-5'-diphosphate (ADP), recombinant insulin (solubilized according to the recommendations of the manufacturer in 10 mM acetic acid, 100 mM NaCl, and 0.01% bovine serum albumin (BSA) to a stock concentration of 100 μM), Fura-2/AM, BSA and the adenylyl cyclase inhibitor SQ22536 from Sigma-Aldrich (St. Louis, MO, USA), resistin, PAI-1, cantharidin, JAK2 inhibitor AG490 and proteasome inhibitor MG132 from Calbiochem (Darmstadt, Germany), leptin and IL-6 from R&D Systems (Minneapolis, MN, USA), RBP4 from Cayman Chemical (Ann Arbor, MI, USA), TNF-α from Reprotech Inc (Rocky Hill. NJ, USA), visfatin from Biovision (Mountain View, CA, USA), cycloheximide from MP Biomedicals (Morgan Irvine, CA, USA) and Arg-Gly-Asp-Ser (RGDS) from Bachem (Bubendorf, Switzerland). The P2Y12 antagonist, N6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX) was a kind gift from Astra Zeneca (Loughborough, UK).

Antibodies against phospho-protein kinase B (P-PKB-Se^{r473}), G_i α–2 and SOCS3 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), phospho-insulin receptor substrate 1 (P-insulin receptor substrate 1 Ser^{307}), insulin receptor substrate 2 and ubiquitin were from Upstate Biotechnology (Bucks, UK), PKB and human leptin antibody (MAB398) were from R&D Systems (Minneapolis, MN, USA), insulin receptor, insulin receptor phospho-$Tyr^{1150/1151}$ and F-actin were from Abcam (Cambridge, UK), insulin growth factor-1 receptor was from Calbiochem (Darmstadt, Germany) and horseradish peroxidase-labeled anti-

rabbit antibody was from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were of analytical grade.

Cell culture

Megakaryocytic CHRF-288-11 cells were cultured as described earlier.[23] Human megakaryocytes were cultured from hematopoietic stem cells isolated from umbilical cord blood, as described elsewhere.[20]

Subjects

The studies were approved by the Medical Ethical Review Board of the University Medical Center Utrecht, Utrecht (UMCU), The Netherlands; the department is certified for ISO-9001:2008. Plasma samples from subjects with metabolic syndrome were obtained as described previously.[24] Their characteristics are given in *Online Supplementary Table S1*.

The patients were recruited from the out-patient clinic of the UMCU. All patients were anti-GAD negative and had plasma C-peptide levels higher than 0.30 nM. We recruited insulin-using patients in order to circumvent the possibility that oral hypoglycemic agents interfered with the study; only metformin use was acceptable (it was stopped the evening before the study). One patient used one injection, two patients used two injections and six patients used four injections with insulin per day, one used CSII. Further details of the patients' characteristics and their treatment are given in *Online Supplementary Table S2*. Patients and matched control subjects gave their informed consent prior to participation in the study.

Platelet isolation

Freshly drawn venous blood from healthy, medication-free volunteers and type 2 diabetes patients was collected into 0.1 volume of 130 mM Na_3 citrate. Platelets were isolated as described previously.[9]

Measurement of Ca^{2+} mobilization

CHRF-288-11 cells ($2x10^5$ cells per sample) and platelet-rich plasma were incubated in the dark with Fura 2-AM (3 μM, 1 h, 37°C). CHRF-288-11 cells were centrifuged (150xg, 5 min, 20°C) and platelet-rich plasma was acidified with ACD to pH 6.5 and thereafter centrifuged (330xg, 15 min, 20°C). Cells were resuspended in Ca^{2+}-free HEPES–Tyrode (HT) buffer (145 mM NaCl, 5 mM KCL, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM HEPES, pH 7.25) supplemented with 5 mM D-glucose. The final platelet concentration was adjusted to $2.0x10^{11}$ cells/L. Five minutes before the start of analyses, suspensions were pre-warmed to 37°C. Measurements and calibrations were performed as described elsewhere.[9]

Immunoprecipitation and blotting

To study phosphorylation and ubiquitination of insulin receptor substrate 1, CHRF-288-11 cells were incubated with insulin for 15 min and adipokines at indicated concentrations at 37°C and collected in lysis buffer (20 mM Tris, 5 mM EGTA, 1% TX-100, pH 7.2) supplemented with 10% protease inhibitor cocktail, 1 mM $NaVO_3$ and 1 μM cantharidin. For equal loading of total lysates, protein concentrations were determined by a bicinchoninic acid protein assay from Pierce (Thermo Scientific, Rockford, IL, USA). Samples were incubated overnight (at 4°C) with protein G-sepharose and anti-insulin receptor substrate 1 phospho-Ser^{307} or anti-insulin receptor substrate 1 antibodies (1 μg/mL). Precipitates were washed three times with lysis buffer and taken up in reducing Laemmli sample buffer. The phosphorylation of insulin receptor, PKBα, upregulation of SOCS3 and total Gi α-2 protein, was measured in lysates in reducing Laemmli sample buffer. Samples were separated by sodium dodecylsulfate polyacrylamide electrophoresis and the proteins were transferred to polyvinyldine difluoride membranes. After blocking with 4% BSA in TBS or Odyssey block-buffer (1 h, 22°C), membranes were incubated with appropriate primary antibodies (16 h, 4°C). Immunoblots for PKBα phospho-Ser^{473}, F-actin and Gi α-2 were visualized by Odyssey infrared imaging (LI-COR Biosciences) using Alexa-labeled antibodies according to the manufacturer's instructions. Insulin receptor phospho-$Tyr^{1150/1151}$, insulin receptor substrate 1 phospho-Ser^{307}, ubiquitinated insulin receptor substrate 1 and insulin receptor, insulin receptor substrate 1, PKBα and SOCS3 protein were detected with horseradish protein-labeled secondary antibodies and enhanced chemiluminescence. The bands were quantified using ImageJ.

Measurements of leptin and resistin

Leptin and resistin levels in plasma from men with metabolic syndrome and controls were determined by enzyme-linked immunosorbent assay (R&D systems, Minneapolis, MN, USA).

Statistics

Data are expressed as mean ± SEM with number of observations and were analyzed with Student's test for unpaired observations or the Mann-Whitney U test, as indicated. Differences were considered statistically significant when the *P* value was less than 0.05, indicated by an asterisk in the figures.

Results

Resistin, leptin, plasminogen activator inhibitor-1 and retinol binding protein 4 induce insulin resistance in megakaryocytes

We previously showed that platelets respond to thrombin stimulation with a rise in cytosolic Ca^{2+}, and that this response is about 25% lower following pre-incubation with insulin.[9] To investigate whether adipokines could make platelets resistant to insulin, rises in Ca^{2+} were measured in the presence of resistin, leptin and IL-6 at concentrations 10-fold those found under physiological conditions. These treatments did not change the decrease in Ca^{2+} mobilization induced by insulin (Figure 1A,B). In contrast to their progenitors, platelets respond weakly to growth factors and megakaryocytes may, therefore, be more sensitive to adipokines. This possibility was addressed in CHRF-288-11 cells, which have a Ca^{2+} homeostasis closely similar to that of primary megakaryocytes. As in platelets, insulin dose-dependently inhibited the thrombin-induced Ca^{2+} increase (Figure 1C,D). The inhibition induced by 100 nM insulin was set at 100% and converted into an insulin-sensitivity index, ISI (Figure 1E). CHRF-288-11 cells were incubated with different adipokines at concentrations 10-fold the mean normal range for 1 day to study rapid interference by adipokines and for 7 days to investigate possible interference with protein synthesis. In both conditions, resistin, leptin, PAI-1 and RBP4 induced an 80-100% fall in insulin sensitivity but visfatin, IL-6 and TNF-α had no effect (Figure 1F). Adipokines alone failed to change Ca^{2+} (*data not shown*). Thus, CHRF-288-11 cells become insulin resistant upon contact with resistin, leptin, PAI-1 and RBP4.

Incubation with leptin at concentrations in the physiological range (10-40 ng/mL)[25] and above produced a dose-dependent decrease in the ISI, leading to complete insulin

resistance at 150 ng/mL, which is in the upper range of concentrations found in obese individuals (50 -150 ng/mL).[26] Normal levels of resistin (5-20 ng/mL),[27] induced a smaller fall in ISI, but higher concentrations (>75 ng/mL) also induced insulin resistance. Although resistin concentrations higher than 30 ng/mL have rarely been measured, a 4- to 5-fold increase in resistin levels was observed in humans after lipopolysaccharide injection,[28] suggesting that these high levels can be reached *in vivo*. Combinations of physiological levels of leptin and resistin induced more inhibition than the adipokines alone, suggesting synergistic interactions in induction of insulin resistance (*Online Supplementary Figure S1A-C*). To confirm these results in primary cells, megakaryocytes cultured from CD34-positive cord blood cells were incubated with resistin, leptin and IL-6 for 1 day. Consistent with observations in CHRF-288-11 cells, resistin and leptin, but not IL-6, completely suppressed the ISI (Figure 1G,H).

In platelets, insulin signals through the insulin receptor and insulin receptor substrate 1, which associates with and inactivates the inhibitory G-protein of adenylyl cyclase, $G_i\alpha_2$. This action interferes with the drop in cAMP induced by P2Y12 ligation, making insulin an inhibitor of Ca^{2+} rises and platelet functions.[9] To clarify whether a similar mechanism was operational in CHRF-288-11 cells, thrombin-induced Ca^{2+} mobilization was measured in the presence of AR-C69931MX, an inhibitor of the ADP-P2Y12 association. This treatment induced a dose-dependent decrease in Ca^{2+} rises, confirming the presence of P2Y12 signaling in CHRF-288-11 cells (*Online Supplementary Figure S2A*). Insulin inhibition was absent in the presence of the adenylyl cyclase inhibitor SQ22536, showing that insulin acts by interfering with cAMP regulation (*Online Supplementary Figure S2B*). It was also undisturbed by an antibody against the insulin-like growth factor-1 receptor, confirming that insulin signaled through the insulin receptor, as seen in platelets (*Online Supplementary Figure S2C*). Together, these findings suggest that resistin, leptin, PAI-1 and RPB4 make CHRF-288-11 cells insulin resistant by neutralizing its interference with P2Y12 signaling to cAMP/Ca^{2+} (*Online Supplementary Figure S2D*).

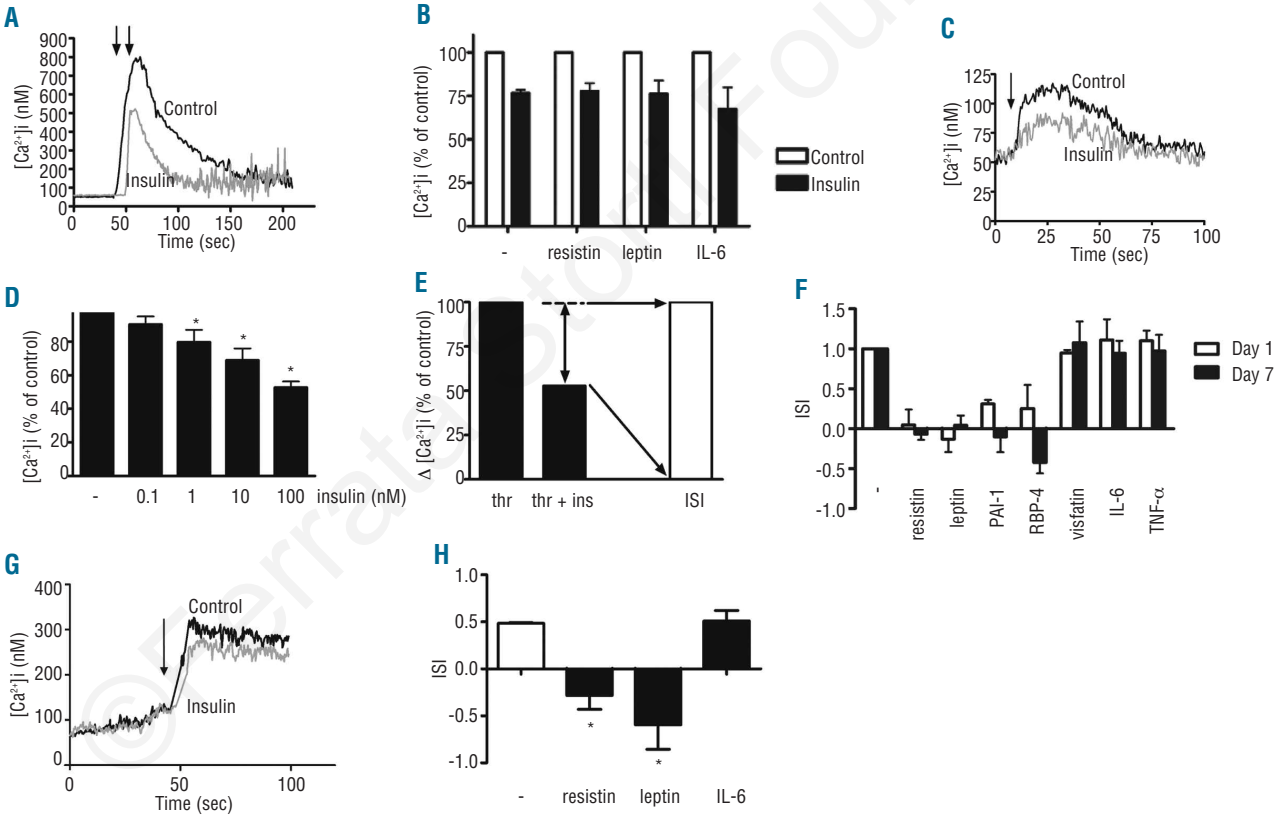

Figure 1. Resistin, leptin, PAI-1 and RBP4 induce insulin resistance in CHRF-288-11 cells. (A,B) Inhibition by insulin of thrombin-induced Ca^{2+} mobilization ($[Ca^{2+}]_i$) in platelets is unaffected by adipokines. Thrombin (1 U/mL)-induced Ca^{2+} mobilization in platelets pre-incubated without and with insulin (100 nM, 5 min, 37°C) in the presence (2 h, 37°C) of resistin (150 ng/mL), leptin (300 ng/mL) and IL-6 (12 ng/mL). (C,D) Inhibition by insulin of thrombin induced Ca^{2+} mobilization in CHRF-288-11 cells. Thrombin (1 U/mL)–induced Ca^{2+} mobilization in CHRF-288-11 cells pre-incubated without and with indicated insulin concentrations (f.c.; 5 min, 37°C). (E) Insulin inhibition was expressed as an insulin sensitivity index, ISI. (F) Resistin, leptin, PAI-1 and RBP4 induce insulin-resistance, but visfatin, IL-6 and TNF-α do not. Inhibition of Ca^{2+} mobilization by insulin (100 nM) in CHRF-288-11 cells after 1 and 7 days culture in the presence of resistin (150 ng/mL), leptin (300 ng/mL), PAI-1 (260 ng/mL), RBP4 (500 ng/mL), visfatin (20 ng/mL), IL-6 (12 ng/mL) and TNF-α (50 pg/mL). These concentrations were used in all experiments, unless indicated otherwise. (G) Thrombin (1 U/mL)-induced Ca^{2+} mobilization in primary megakaryocytes pre-incubated without and with insulin (100 nM, 5 min, 37°C) in the absence of adipokines. (H) Resistin and leptin, but not IL-6 completely reduced insulin sensitivity in primary megakaryocytes. Cells were incubated with vehicle, resistin, leptin and IL-6 (1 day, 37°C) and insulin inhibition of Ca^{2+} mobilization was measured. An ISI=1.0 refers to insulin inhibition in the absence of adipokines. (data are means ± SEM, n=3 and were analyzed with Student's test, *denotes a statistically significant difference, $P<0.05$).

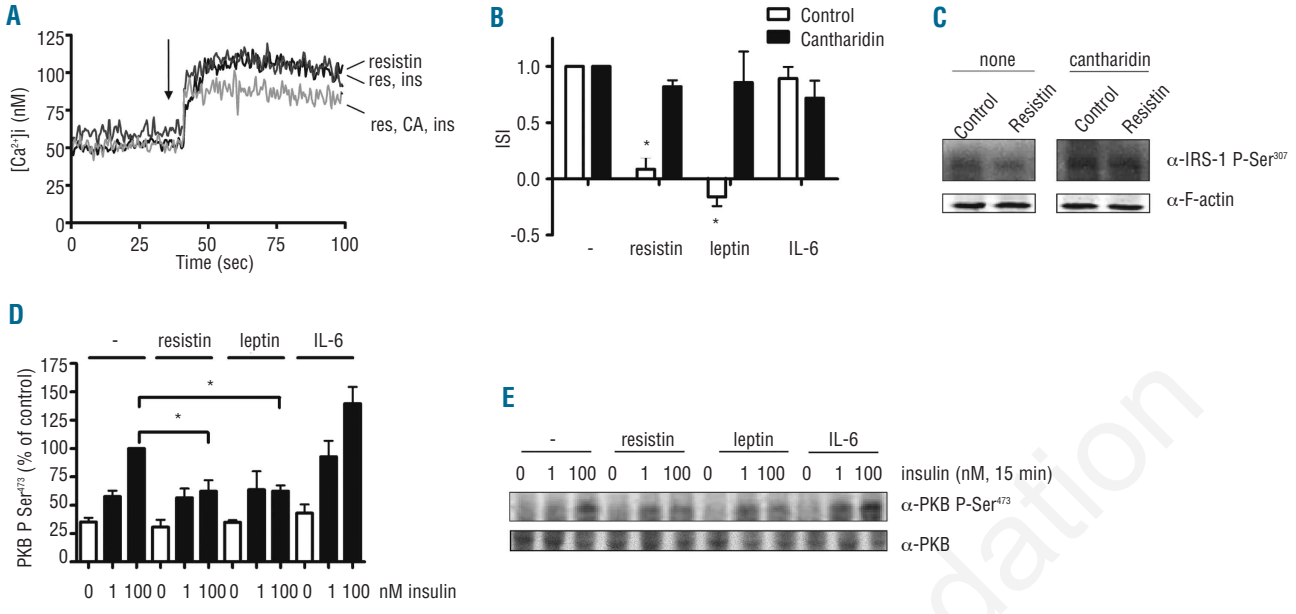

Figure 2. Brief adipokine contact induces insulin resistance by interfering with insulin receptor substrate 1 regulation. (A,B) CHRF-288-11 cells lose insulin sensitivity upon brief contact with resistin. Cells were pre-incubated with resistin, leptin and IL-6 (2 h, 37°C) and insulin-inhibition of thrombin-induced Ca^{2+} mobilization was measured in the absence and presence of the Ser phosphatase inhibitor cantharidin (1 μmol/L, 15 min, 37°C). Resistin and leptin but not IL-6 induce induce insulin resistance; cantharidin rescues insulin sensitivity. (C) Loss of insulin sensitivity is accompanied by loss of insulin receptor substrate 1 Ser^{307} phosphorylation. CHRF-288-11 cells were incubated with resistin (2 h, 37°C) and stimulated with insulin (100 nM). Resistin reduced phospho-insulin receptor substrate 1 Ser^{307} and cantharidin (1 μmol/mol/L, 15 min, 37°C) prevented the decrease. (D,E) Loss of insulin sensitivity is accompanied by loss of PKBα activity. CHRF-288-11 cells were incubated with resistin, leptin and IL-6 (2 h, 37°C) and stimulated with 1 and 100 nM insulin (15 min, 37°C). Analysis of phospho-PKBα Se^{r473} shows interference by resistin and leptin and not by IL-6. Further details as in Figure 1.

Brief contact with adipokines induces reversible insulin resistance

To separate the rapid and delayed effects of adipokines, CHRF-288-11 cells were incubated with adipokines for 2 and 72 h. Incubation for 2 h was sufficient to induce insulin resistance by resistin and leptin, whereas IL-6 was again inactive (Figure 2A,B). The rapid response remained unchanged in the presence of a protein synthesis inhibitor (100 μg/mL cycloheximide, *data not shown*) suggesting direct interference with insulin signaling. We tested different pharmacological inhibitors and found that the Ser-phosphatase inhibitor cantharidin preserved insulin sensitivity in the presence of leptin and resistin. Without these adipokines (and with the negative control IL-6), cantharidin left the inhibition by insulin intact. Cantharidin alone did not change Ca^{2+} mobilization (*Online Supplementary Figure S3A*). These findings suggest that leptin and resistin induce insulin resistance in CHRF-288-11 cells by dephosphorylating a Ser-phosphorylation site in the insulin signaling pathway.

A key element in insulin signaling is insulin receptor substrate 1, which is phosphorylated at multiple Tyr residues by the activated insulin receptor, forming docking sites for proteins of the phosphoinositide-3 kinase/PKB, the GTP-ase Ras and the mitogen-activated protein kinases pathways, in addition to $G_i\alpha_2$.[9,29] Tyr phosphorylation of insulin receptor substrate 1 is under the control of insulin receptor substrate 1 Ser^{307} but whether phospho-Ser^{307} stimulates or inhibits insulin receptor substrate 1 function differs among cell types. In insulin-treated CHRF-288-11 cells, resistin induced a fall in phospho-Ser^{307}, while cantharidin neutralized this inhibition (Figure 2C). These findings are concordant with activation of a Ser-phosphatase by resistin, which is blocked by cantharidin. The effect of Ser^{307} phosphorylation on insulin receptor substrate 1 function was deduced from the activity of PKB. Resistin and leptin (but not IL-6) reduced insulin-induced phosphorylation of PKBα Ser^{473}, an indicator of PKBα activation[30] (Figure 2D,E). These findings suggest that in CHRF-288-11 cells, phospho-Ser^{307} keeps insulin receptor substrate 1 in a functional state for signaling to PKB. In platelets, P2Y12 receptor ligation suppresses cAMP increases and stimulates PKB.[31-33] The lower PKB activation by insulin in the presence of adipokines might, therefore, reflect interference with P2Y12 signaling. To investigate this possibility, CHRF-288-11 cells were stimulated with insulin and a thrombin/ADP combination and PKB was measured. The thrombin/ADP combination and the separate activators alone failed to activate PKB. Furthermore, P2Y12 blockade did not change PKB activation by insulin (*Online Supplementary Figure S3B* and *data not shown*). Together, these findings indicate that, in CHRF-288-11 cells, PKB is an exclusive indicator of insulin receptor substrate-1 activation and is not affected by P2Y12 signaling. A second candidate for interference by adipokines is the insulin receptor. Leptin failed to change insulin-induced β-subunit $Tyr^{1150\text{-}1151}$ phosphorylation, which determines receptor kinase activity, suggesting that it leaves insulin signaling by the receptor unchanged (*data not shown*).

Prolonged contact with adipokines triggers persistent insulin resistance

CHRF-288-11 cells incubated with leptin and resistin for 72 h were resistant to cantharidin, indicating that interference with insulin receptor substrate 1 phospho-Ser[307] was no longer involved (Figure 3A,B). To investigate whether these adipokines interfered with the expression of mediators of insulin signaling, CHRF-288-11 cells were incubated with resistin, leptin (and with IL-6 as a negative control) and insulin receptor substrate 1 was detected on immunoblots (Figure 3C,D). Both adipokines induced a strong reduction in insulin receptor substrate 1 expression whereas IL-6 had no effect. Under the same conditions, expression of insulin receptor, $G_i\alpha_2$ and insulin receptor substrate 2, another member of the insulin receptor substrate family present in megakaryocytes,[34] was not disturbed.

Leptin and resistin receptors induce signaling through the JAK/STAT pathway to expression regulation of suppressor of cytokine signaling (SOCS) proteins, which attenuate cytokine signaling by interfering with receptor function, JAK activity or by targeting activated signaling proteins for degradation by the proteasome.[35] CHRF-288-11 cells incubated with the JAK inhibitor AG-490 preserved insulin sensitivity in the presence of resistin and leptin, confirming that these adipokines signal through JAK (Figure 3E). After 2 h of stimulation with leptin, CHRF-288-11 cells showed a strong increase in SOCS3 expression, which decreased to the pre-stimulation range 32 h later (Figure 3F). These findings suggest that prolonged contact with resistin and leptin impair insulin signaling by inducing SOC3-mediated degradation of insulin receptor substrate 1. Immunoprecipitation of insulin receptor substrate 1 followed by blotting with an antibody against ubiquitin showed that leptin alone and especially leptin in combination with the proteosome inhibitor MG-132 triggered a shift in molecular mass from 180 to 250 kD (Figure 3G). Thus, following prolonged contact with resistin and leptin, CHRF-288-11 cells lose insulin sensitivity by the ubiquitin-mediated degradation of insulin receptor substrate 1.

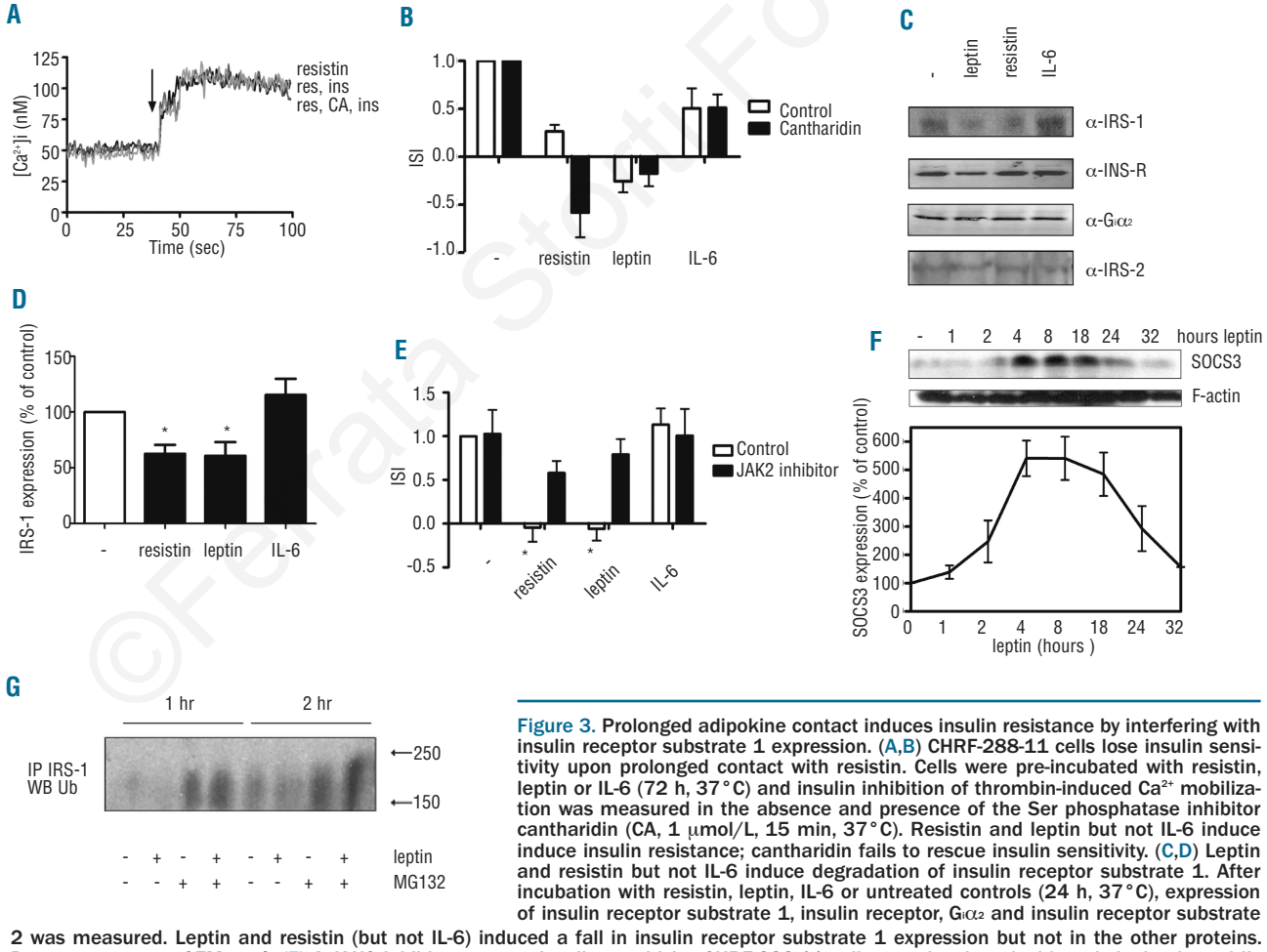

Figure 3. Prolonged adipokine contact induces insulin resistance by interfering with insulin receptor substrate 1 expression. (A,B) CHRF-288-11 cells lose insulin sensitivity upon prolonged contact with resistin. Cells were pre-incubated with resistin, leptin or IL-6 (72 h, 37°C) and insulin inhibition of thrombin-induced Ca^{2+} mobilization was measured in the absence and presence of the Ser phosphatase inhibitor cantharidin (CA, 1 μmol/L, 15 min, 37°C). Resistin and leptin but not IL-6 induce induce insulin resistance; cantharidin fails to rescue insulin sensitivity. (C,D) Leptin and resistin but not IL-6 induce degradation of insulin receptor substrate 1. After incubation with resistin, leptin, IL-6 or untreated controls (24 h, 37°C), expression of insulin receptor substrate 1, insulin receptor, $G_i\alpha_2$ and insulin receptor substrate 2 was measured. Leptin and resistin (but not IL-6) induced a fall in insulin receptor substrate 1 expression but not in the other proteins. Data are means ± SEM, n=4. (E) A JAK2 inhibitor rescues insulin sensitivity. CHRF-288-11 cells were incubated with resistin, leptin and IL-6 without and with the JAK2 inhibitor AG490 (100 nM, 15 min, 37°C). (F) Leptin initiates upregulation of SOCS3. CHRF-288-11 cells were incubated with leptin for 0 – 32 h and expression of SOCS3 was measured. (G). Leptin induces ubiquitination of insulin receptor substrate 1. CHRF-288-11 cells were incubated with leptin, the proteasome inhibitor MG132 (10 μM) or both (1 or 2 h, 37°C) and expression of ubiquitinated insulin receptor substrate 1 was measured.

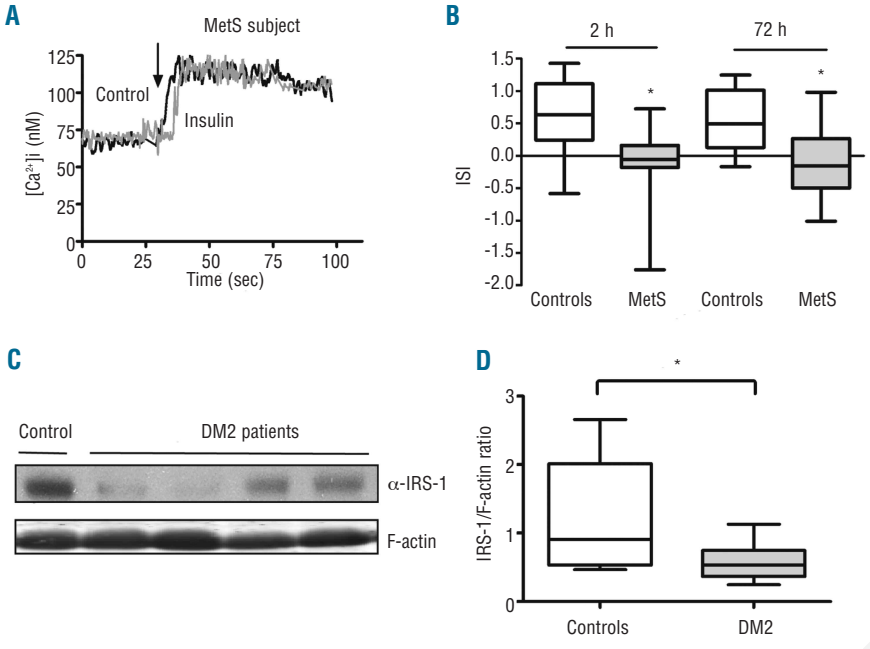

Figure 4. Plasma from men with metabolic syndrome induces insulin resistance and lowers the insulin receptor substrate 1/F-actin ratio in platelets from patients with type 2 diabetes. (A) CHRF-288-11 cells were incubated with plasma from men with metabolic syndrome (MetS) for 2 h (37°C). Thrombin (1 U/mL)-induced Ca^{2+} mobilization was measured in the absence (control) or presence of insulin (100 nM, 5 min, 37°C). (B) CHRF-288-11 cells were incubated in control or MetS plasma (n=9 for both groups) for 2 and 72 h and insulin interference with thrombin-induced Ca^{2+} mobilization was expressed as the ISI. Both after 2 and 72 h, incubation in MetS plasma induced increased insulin resistance compared to control plasma. (C,D) Platelets from the patients with type 2 diabetes have reduced insulin receptor substrate 1 expression. Platelets were isolated from matched controls and type 2 diabetes patients (n=9 for both groups) and expression of insulin receptor substrate 1 and F-actin is given as the insulin receptor substrate 1/F-actin ratio. Data are based on duplicate measurements with a maximal variation of 11.1% and 3.4% in controls and type 2 diabetes patients, respectively, and were analyzed with the Mann-Whitney U test.

Plasma from subjects with metabolic syndrome induces insulin resistance and platelets from patients with type 2 diabetes have reduced insulin receptor substrate 1 expression

Having established that resistin and leptin induce insulin resistance in cell cultures, we searched for individuals whose plasma would make CHRF-288-11 cells insulin-resistant. Blood was collected from matched controls and diabetes-free subjects with metabolic syndrome (MetS), who are known to have an abnormal plasma adipokine profile[12] (*Online Supplementary Table S1*[24]). Both control and MetS plasma samples reduced the ISI established in buffer, but the reduction by MetS plasma was significantly stronger (Figure 4A,B). Since MetS plasma contains many adipokines and other blood constituents in levels outside the normal range (*Online Supplementary Table S1*), a single factor causing a greater reduction in the ISI could not be established. MetS plasma contained a 3-fold higher level of leptin than control plasma whereas resistin levels were not elevated. This makes leptin a potential contributor to the suppression of ISI in CHRF-288-11 cells (*Online Supplementary Figure S4A,B*). Interestingly, an antibody that was capable of neutralizing the function of leptin restored insulin sensitivity to the range found in incubations with buffer, suggesting that leptin reduces insulin sensitivity in plasma from healthy individuals and MetS subjects (*Online Supplementary Figure S4C*).

The abnormalities in plasma adipokine content seen in MetS are also found in type 2 diabetes.[36-39] This implies that in type 2 diabetes patients, megakaryocytes mature in an environment that might compromise normal insulin receptor substrate 1 expression, resulting in release of deficient platelets. Indeed, platelets from obese type 2 diabetes patients showed lower insulin receptor substrate 1 content compared with the content of matched controls (Figure 4C,D and *Online Supplementary Table S2* for the patients' characteristics).

Discussion

Novel findings in the present study are: (i) the rise in Ca^{2+} in stimulated megakaryocytic CHRF-288-11 cells is inhibited by insulin; (ii) resistin, leptin, PAI-1 and RBP4 induce insulin resistance; (iii) a similar insulin resistance is induced by plasma from MetS subjects; (iv) some patients with type 2 diabetes have platelets with reduced insulin receptor substrate-1 content, possibly caused by adipokine interference during platelet formation.

In platelets, insulin signals interferes with the decrease in cAMP induced by P2Y12 ligation making insulin an inhibitor of Ca^{2+} rises and aggregation, secretion and procoagulant activity.[9] The present data show that a similar mechanism is operative in CHRF-288-11 cells. Megakaryocytic CHRF-288-11 cells have many properties in common with mature primary megakaryocytes, including receptors for thrombin, the presence of α- and δ-granules and phospholipase C, which is an important step in receptor signaling to Ca^{2+}.[21,40] They also respond to thrombin with Ca^{2+} mobilization,[22] which is suppressed by high cAMP,[20] and express $G_i\alpha_2$.[23] The present data show that CHRF-288-11 cells and primary megakaryocytes also respond to insulin with a decrease in Ca^{2+} mobilization. This property makes the thrombin-induced Ca^{2+} increase a sensitive marker for insulin responsiveness in platelets[9] and, as shown in this study, megakaryocytes.

The observation that resistin and leptin (and possibly PAI-1 and RBP4) induce insulin resistance by interfering with insulin receptor substrate 1, adds to the list of plasma abnormalities that link obesity with the development of type 2 diabetes. At levels found *in vivo*,[26,28] these adipokines interfere with insulin receptor substrate 1 regulation (brief contact) and induce its degradation (prolonged contact). The rapid interference by resistin and leptin is accompanied by a fall in phospho-insulin receptor substrate 1-Ser[307] and prevented by cantharidin, suggesting involvement of a phosphatase that targets phospho-Ser[307]. A similar inter-

ference is seen in normal adipocytes incubated with RBP4 and in type 2 diabetes adipocytes.[41,42] These findings suggest that phospho-Ser[307] preserves insulin receptor substrate 1 function.

Phosphorylation of Ser residues in insulin receptor substrate 1 has positive and negative effects on insulin signaling, depending on cell types and incubation conditions.[43] In mice, Ser phosphorylation mainly negatively regulates insulin receptor substrate 1.[44] Positive regulation has been demonstrated in CHO-cells,[45] hepatoma Fao cells[46] and in human adipocytes.[46] At present, 13 Ser residues undergoing stimulus-induced phosphorylation have been identified. It is clear that rather than a change in a single phospho-Ser residue, there is a pattern of phosphorylation and dephosphorylation control of insulin receptor substrate 1 function and this pattern might change over time.[44] At first, activating phospho-sites may protect insulin receptor substrate 1 function against inhibitory phosphor-sites but later the balance may shift, shutting insulin signaling down.[43] The regulation of insulin receptor substrate 1 function is an important step in the control of glucose uptake by muscle cells and adipocytes and the interference by adipokines shown here may well have implications for these and other types of cells.

Prolonged contact between resistin/leptin and CHRF-288-11 cells reveals an interference of insulin inhibition that is independent of the state of phospho-Ser residues and is accompanied by insulin receptor substrate-1 degradation. Cytokine receptors induce signaling through the JAK/STAT pathway.[47] At least three classes of negative regulators contribute to cytokine inhibition during prolonged incubation: the -SH2 containing protein tyrosine phosphatases, the protein inhibitors of activated STAT and SOCS proteins.[35] SOCS proteins attenuate cytokine signaling by interfering with receptor function, JAK activity or by targeting activated signaling proteins for degradation by the proteasome. Apparently, signaling by leptin/resistin follows this pathway since it is inhibited by a JAK blocker and accompanied by upregulation of SOCS3. Expression of SOCS3 was transient and preceded the specific degradation of insulin receptor substrate 1, whereas levels of insulin receptor, $G_i\alpha_2$ and insulin receptor substrate 2 remain unchanged. These data agree with the SOCS-induced degradation of insulin receptor substrate proteins in hepatic cells in mice.[48] Reduced expression was also observed in adipocytes of type 2 diabetes patients[49] and in conditions resembling those in MetS subjects.[50] Apart from enhanced degradation, low levels of insulin receptor substrate 1 may result from disturbed transcription in obesity. Both IL-6 and TNF-α reduce transcription of the insulin receptor substrate 1 gene in 3T3-L1 cells.[51,52] In severely obese humans, a low fat cell insulin receptor substrate 1 content is a predictor of insulin resistance and type 2 diabetes.[53]

The reduced insulin receptor substrate-1 expression CHRF-288-11 treated with leptin/resistin raises the possibility that a similar defect occurs when bone marrow megakaryocytes make contact with plasma with elevated leptin/resistin concentrations. The result would be shedding of platelets with reduced insulin receptor substrate 1 content and impaired insulin inhibition of Ca^{2+} increases. The observation that a small group of patients with type 2 diabetes has a lowered insulin receptor substrate 1/F-actin ratio seems to support this hypothesis but it is clear that larger groups of patients are required to separate effects of adipokines from platelet abnormalities by other acquired and congenital causes.

The observation that MetS plasma induces similar alterations in CHRF-288-11 cells as the separate addition of leptin or resistin suggests that the abnormal plasma profile of MetS individuals might gradually lead to impaired insulin receptor substrate 1 regulation and insulin resistance. The elevated leptin levels in MetS plasma and the ISI correction by a leptin antibody suggest that this adipokine plays a major role. No leptin effect is seen with platelets, a difference possibly due to the contribution of insulin-like growth factor receptor-1 in these cells.[54] In diabetes-free Aboriginal Canadians high leptin levels at baseline were associated with an increased risk of type 2 diabetes and a similar correlation was found in Japanese men.[36,37] Restistin, PAI-1 and RBP4 also make CHRF-288-11 cells insulin-resistant. In a follow-up study, baseline PAI-1 levels predicted risk of type 2 diabetes and RBP4 levels correlated with the magnitude of insulin resistance.[38,39] At present about 50 different adipokines have been identified and their effects alone and in combination should be clarified before their impact on platelet sensitivity for insulin can be understood.

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