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Induction of insulin resistance by the adipokines resistin, leptin, PAI-1 and RBP4 in human megakaryocytes

Running title: Adipokine-induced insulin-resistant megakaryocytes

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The online version of this article contains a supplementary appendix.
Abstract

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

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-a and visfatin had no effect. These results were confirmed in primary megakaryocytes. A 2 hrs adipokine contact disturbed insulin receptor substrate 1 Ser^{307}-phosphorylation; a 72 hrs contact caused insulin receptor substrate 1 degradation. Plasma with disturbed adipokine profile also made CHRF-288-11 cells insulin-resistant. Type 2 diabetes platelets 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 insulin resistance observed in type 2 diabetes platelets.
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 PG\(_I\)_2 and NO. 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 illustrate the contribution of platelets in atherogenesis (2).

Type 2 diabetes is a metabolic disorder characterized by insulin resistance and microvascular and macrovascular disease (3). Prospective studies show that insulin resistance is a risk-factor of the cardiovascular problems caused by atherosclerotic coronary, cerebral and lower limb blood vessels (4). Insulin resistance disturbs glucose homeostasis in muscle cells, NO production by endothelial cells and accelerates atherosclerosis (3). In type 2 diabetes patients, 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 controls (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 IL-1b 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). Also interleukin-6 (IL-6) and tumor necrosis factor-a (TNF-a) released by macrophages in adipose tissue might induce insulin resistance (14). Type 2 diabetes patients 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 Ca^{2+} rises, thromboxane A_{2} formation and ADP-aggregation (16-19) and ob/ob mice deficient in the leptin gene show delayed occlusion in an arterial thrombosis model (16). These findings suggest, that leptin increases platelet reactivity. Opposite to this idea are findings that prolonged leptin infusion in healthy volunteers leaves aggregation unchanged and that leptin-deficient individuals have increased rather than lowered aggregation (18,19).

Apart from changing platelet functions through direct interference, adipokines may alter their properties during their synthesis by megakaryocytes (20). Megakaryocytes develop from haematopoietic stem cells in the bone marrow. Maturation is under 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), adenosin-5'-diphosphate (ADP), recombinant insulin (solubilized according to the recommendations of the manufacturer in 10 mM acetic acid, 100 mM NaCl, and 0.01% BSA to a stock concentration of 100 µM), Fura-2/AM, bovine serum albumin (BSA) and the adenyl 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 (CHX) from MP Biomedicals (Morgan Irvine, CA, USA), 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).

Antibody against phospho-Protein Kinase B (P-PKB-Ser⁴⁷³), G, a-2 and SOCS3 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), phospho-insulin receptor substrate 1 (P- insulin receptor substrate 1 Ser⁳⁰⁷), insulin receptor substrate 2 and ubiquitin from Upstate Biotechnology (Bucks, UK), PKB and human leptin antibody (MAB398) from R&D Systems (Minneapolis, MN, USA), insulin receptor, insulin receptor phospho-Tyr¹¹⁵⁰/¹¹⁵¹, F-actin from Abcam (Cambridge, UK), IGF-1 receptor from Calbiochem (Darmstadt, Germany) and horseradish peroxidase-labeled anti-rabbit antibody 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 haematopoietic stem cells isolated from umbilical cord blood, as described (20).
Subjects
The studies have been 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 metabolic syndrome subjects were obtained as described previously (24). Their characteristics are given in 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 for 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. Their further characteristics are given in 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 Na3 citrate. Platelets were isolated as described (9).

Measurement of Ca2+ mobilization
CHRF-288-11 cells (2×10^5 cells per sample) and platelet-rich plasma were incubated in the dark with Fura 2-AM (3 M, 1 hr, 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 Ca2+-free HEPES–Tyrode (HT) buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na2HPO4, 1 mM MgSO4, 10 mM HEPES, pH 7.25) supplemented with 5 mM D-glucose. The final platelet concentration was adjusted to 2.0×10^{11} cells L^{-1}. Five minutes before the start of analyses, suspensions were prewarmed at 37°C. Measurements and calibrations were performed as described (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 NaVO₃ and 1 mM cantharidin. For equal loading of total lysates protein concentrations were determined by a BCA (bicinchoninic acid) protein assay from Pierce (Thermo Scientific, Rockford, IL, USA). Samples were incubated overnight (4°C) with protein G-sepharose and anti-insulin receptor substrate 1 phospho-Ser³⁰⁷ or anti-insulin receptor substrate 1 antibodies (1 mg/mL). Precipitates were washed 3 times with lysis buffer and taken up in reducing Laemmli sample buffer. The phosphorylation of insulin receptor, PKBa, upregulation of SOCS3 and total Gi α-2 protein, was measured in lysates in reducing Laemmli sample buffer. Samples were separated by SDS-PAGE and proteins transferred to polyvinylidine difluoride membranes. After blocking with 4% BSA in TBS or Odyssey blockbuffer (1 h, 22 °C), membranes were incubated with appropriate primary antibodies (16 hrs, 4°C). Immunoblots for PKBa phospho-Ser⁴⁷³, 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. Detection of insulin receptor phospho-Tyr¹¹⁵⁰¹¹⁵¹, insulin receptor substrate 1 phospho-Ser³⁰⁷, ubiquitinated insulin receptor substrate 1 and insulin receptor-, insulin receptor substrate 1-, PKBa- and SOCS3 protein, was performed with HRP-labelled secondary antibodies and enhanced chemiluminescence. Quantification of the bands was performed using ImageJ.

**Leptin and resistin measurements**

Leptin and resistin levels in plasma from metabolic syndrome men and controls were determined by ELISA (R&D systems, Minneapolis, MN, USA).

**Statistics**

Data is expressed as mean ± S.E.M. with n observations and was analyzed with Student’s test for unpaired observations or Mann Whitney U test, as indicated. Differences were considered significant at p<0.05, as indicated.*
Results

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

We showed previously 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 fall in Ca^{2+} mobilization induced by insulin (Figure 1A,B). Platelets respond weakly to growth factors in contrast to their progenitors 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 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 in 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-a 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.

Incubations with leptin concentrations in the physiological range (10-40 ng/mL, 25) and above showed a dose-dependent decrease in ISI inducing 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 concentration of >30 ng/mL rarely have been measured, a 4 to 5 fold increase in resistin levels was observed in humans after injection with LPS (28), suggesting that these high levels can be reached in vivo. Combinations of physiological levels leptin and resistin induced more inhibition than the adipokines alone, suggesting synergistic interactions in induction of insulin resistance (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 and inactivates the inhibitory G-protein of adenylyl cyclase, Gi\(_{a2}\). This action interferes with the fall 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 ADP-P2Y12 association. This treatment induced a dose-dependent fall in Ca\(^{2+}\) rises, confirming the presence of P2Y12 signaling in CHRF-288-11 cells (Figure S2A). Insulin inhibition was absent in the presence of the adenylyl cyclase inhibitor SQ22536, showing that insulin acts by interfering with cAMP regulation (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 (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\(^{2+}\) (Figure S2D).

**Short adipokine contact induces reversible insulin resistance**

To separate rapid and delayed effects of adipokines, CHRF-288-11 cells were incubated with adipokines for 2 and 72 hrs. A 2 hrs incubation 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 mg/mL cycloheximide, 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 (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/protein kinase B (PKB), the GTP-ase Ras and mitogen-activated protein kinases pathways, in addition to Gα₂ (9, 29). Tyr phosphorylation of insulin receptor substrate 1 is under control of insulin receptor substrate 1 Ser307 but whether phospho-Ser307 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-Ser307 and cantharidin neutralized this inhibition (Figure 2C). These findings accord with activation of a Ser-phosphatase by resistin, which is blocked by cantharidin. The effect of Ser307 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 PKBa Ser473, an indicator of PKBa activation (30) (Figure 2D,E). These findings suggest that in CHRF-288-11 cells, phospho-Ser307 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 address this possibility, CHRF-299-11 cells were stimulated with insulin and a thrombin/ADP combination and PKB was measured. Thrombin/ADP combination and the separate activators alone failed to activate PKB. Furthermore, P2Y12 blockade did not change PKB activation by insulin (Figure S3B and not shown). Together, these findings indicate in CHRF-288-11 cells PKB is an exclusive indicator of insulin receptor substrate-1 activation and not affected by P2Y12 signaling. A second candidate for interference by adipokines is the insulin receptor. Leptin failed to change insulin-induced β-subunit Tyr1150-1151 phosphorylation, which determines receptor kinase activity suggesting that it leaves insulin signaling by the receptor unchanged (data not shown).

Prolonged adipokine contact triggers persistent insulin resistance
CHRF-288-11 cells incubated with leptin and resistin for 72 hrs were resistant against cantharidin, indicating that interference with insulin receptor substrate 1 phospho-Ser307 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, Gα2 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 hrs stimulation with leptin, CHRF-288-11 cells showed a strong increase in SOCS3 expression, which decreased to the pre-stimulation range 32 hrs 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, upon prolonged contact with resistin and leptin, CHRF-288-11 cells lose insulin sensitivity by the ubiquitin-mediated degradation of insulin receptor substrate 1.

Metabolic syndrome plasma induces insulin resistance and type 2 diabetes platelets 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) (Supplement 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 beyond the normal range (Table S1), a single factor causing increased ISI reduction can not be established. MetS plasma contained a 3-fold higher leptin level than control plasma whereas resistin levels were not elevated. This makes leptin a potential contributor to ISI suppression in CHRF-288-11 cells (Figure S4A,B). Interestingly, an antibody that was capable to neutralize
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 (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 a decreased insulin receptor substrate 1 content compared with matched controls (Figure 4C,D and Supplement Table 2 for patient 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 fall in cAMP induced by P2Y12 ligation making insulin an inhibitor of Ca^{2+} rises and aggregation, secretion and pro-coagulant 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_{iα2} (23). The present data show that CHRF-288-11 cells and primary megakaryocytes also respond to insulin with a fall in Ca^{2+} mobilization. This property makes the thrombin-induced Ca^{2+} increase a sensitive marker for insulin responsiveness in platelets (9) and megakaryocytes (this study).

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 development of type 2 diabetes. At levels found in vivo (26, 28), these adipokines interfere with insulin receptor substrate 1 regulation (short contact) and induce its degradation (prolonged contact). The rapid interference by resistin and leptin is accompanied with a fall in phospho-insulin receptor substrate 1-Ser^{307} and prevented by cantha-ridin, suggesting involvement of a phosphatase that targets phospho-Ser^{307}. A similar interference 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, a pattern of phosphorylations and dephosphorylations control insulin receptor substrate 1 function and this pattern might change in time (44). At first, activating phospho-sites may protect insulin receptor substrate 1 function against inhibitory phospho-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 control of glucose uptake by muscle cells and adipocytes and the interference by adipokines shown here may well have implications for these and other cell types.

Prolonged contact between resistin/leptin and CHRF-288-11 cells reveals interference of insulin-inhibition independent of the state of phospho-Ser residues and is accompanied with 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, Gα2 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 type 2 diabetes patients shows a lowered insulin receptor substrate 1/F-actin ratio seems to support this hypothesis but it is clear that larger patient groups 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 discrepancy 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 increased risk of type 2 diabetes and a similar correlation was found in Japanese men (36,37). Also resistin, PAI-1 and RBP4 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.

**Authorship and Disclosures**

AJG wrote the manuscript and performed the studies with patient material, controls and primary megakaryocytes; EG performed measurements of Ca\(^{2+}\) mobilization and blotting; CAK performed western blotting experiments and studied patient material; FLV helped with data analysis and interpretation; TWH recruited the patients, helped with data analysis and interpretation; JWA coordinated the project and wrote the manuscript. The authors report no potential conflicts of interest.

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References


Figure legends

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 hrs, 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-a 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 the 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-a (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 reduce 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 significant difference, p<0.05).

Figure 2. Short adipokine contact induces insulin resistance by interfering with insulin receptor substrate 1 regulation.

(A,B) CHRF-288-11 cells lose insulin sensitivity upon short contact with resistin. Cells were pre-incubated with resistin, leptin and IL-6 (2 hrs, 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 mmol/L, 15 min, 37°C). Resistin and leptin but not IL-6 induce insulin resistance; cantharidin rescues insulin sensitivity. (C) Loss of insulin sensitivity is accompanied with loss of insulin receptor substrate 1 Ser^{307} phosphorylation. CHRF-288-11 cells were incubated with resistin
(2 hrs, 37°C) and stimulated with insulin (100 nM). Resistin reduced phospho-insulin receptor substrate 1 Ser³⁰⁷ and cantharidin (1 mmol/L, 15 min, 37°C) prevented the decrease. (D,E) Loss of insulin sensitivity is accompanied with loss of PKBa activity. CHRF-288-11 cells were incubated with resistin, leptin and IL-6 (2 hrs, 37°C) and stimulated with 1 and 100 nM insulin (15 min, 37°C). Analysis of phospho-PKBa Ser⁴⁷³ shows interference by resistin and leptin and not by IL-6. Further details as in Fig.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 hrs, 37°C) and insulin inhibition of thrombin-induced Ca²⁺ mobilization was measured in the absence and presence of the Ser phosphatase inhibitor cantharidin (CA, 1 mmol/L, 15 min, 37°C). Resistin and leptin but not IL-6 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 hrs, 37°C), expression of insulin receptor substrate 1, insulin receptor, Gα₂ 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 hrs 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 mM) or both (1 or 2 hrs, 37°C) and expression of ubiquitinated insulin receptor substrate 1 was measured.
Figure 4. Plasma from men with metabolic syndrome induces insulin resistance and lower insulin receptor substrate 1/F-actin ratio’s in type 2 diabetes platelets. (A) CHRF-288-11 cells were incubated with plasma from men with metabolic syndrome (MetS) for 2 hours (37°C). Thrombin (1 U/mL)-induced Ca²⁺ mobilization was measured in 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 hrs and insulin interference with thrombin-induced Ca²⁺ mobilization was expressed as ISI. Both after 2 and 72 hrs, incubation in MetS plasma induced increased insulin resistance compared to control plasma. (C,D) Type 2 diabetes platelets 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 was given as 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.
A

B

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Fig. 3 A-G

C

D

E

F

G
SUPPLEMENTARY APPENDIX

Figure S1. Dose-dependent interference with insulin resistance by leptin and resistin. CHRF-288-11 cells were incubated with different concentrations of leptin (A), resistin (B) and a combination of leptin and resistin (C) for 2 hrs (37°C). Insulin inhibition of Ca^{2+} mobilization was measured and expressed as the ISI, as defined in Figure 1E.

Figure S2. Adipokines interfere with insulin suppression of thrombin-induced Ca^{2+} mobilization in CHRF-288-11 cells. (A) Functional P2Y12 signaling in CHRF-288-11 cells. Cells were pre-incubated with different concentrations AR-C69931MX (5 minutes, 37°C) and thrombin-induced Ca^{2+} mobilization was measured. (B) Insulin inhibition is mediated by adenylyl cyclase. CHRF-288-11 cells were pre-incubated with the adenylyl cyclase inhibitor SQ22536 (50 µM, 15 min, 37°C) and insulin inhibition of thrombin-induced Ca^{2+} mobilization was measured. (C) No role for the IGF-1 receptor in the inhibition by insulin. CHRF-288-11 cells were pre-incubated with a blocking antibody against the insulin-like growth factor-1 receptor (IGF-1 R; 1 mg/mL, 20 min, 37°C) and insulin inhibition of thrombin-induced Ca^{2+} mobilization was measured. (D) Schematic representation of signaling pathways initiated by thrombin and ADP and the interference by insulin in platelets and CHRF-288-11 cells. (For A-C: means ± SEM, n=3, data was analyzed with Student’s test).

Figure S3. Short adipokine contact. (A) Cantharidin did not change thrombin induced Ca^{2+} mobilization. CHRF-288-11 cells were left untreated or incubated with cantharidin (1 mmol/L, 15 min, 37°C) stimulated with thrombin (1 U/mL) and Ca^{2+} mobilization was measured. means ± SEM, n=5, data was analyzed with Student’s test). (B) P2Y12 stimulation does not result in PKB activation. CHRF-288-11 cells were left untreated or preincubated with AR-C69931MX (100 nM, 10 min, 37°C) and subsequently stimulated with different agonists (100 nM insulin or a combination of 20 mM ADP and 1 U/mL thrombin (15 min, 37°C). Total lysates were analyzed for phospho-PKBa Ser^{473} and total PKB levels. The figure shows a representative example of 4 experiments with similar results.
**Figure S4.** Leptin and resistin concentrations in normal and MetS plasma and effect of a leptin neutralizing antibody. *(A,B)* MetS plasma samples contain elevated concentrations of leptin but resistin levels are normal (*n*=9). *(C)* Cells were incubated in control and MetS plasma in the absence and presence of a leptin neutralizing antibody (40 mM, 37°C). The ISI was determined after 2 hrs (means ± SEM, *n*=3; Data was analyzed with Student’s test).
Fig. S3A,B

A

B

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[Graph showing Ca<sup>2+</sup> levels with and without cantharidin treatment and an ns annotation]

[Western blots showing α-PKB and P-Ser<sup>473</sup> levels under different conditions: control, insulin, thrombus-ADP, and AR-C69931MX]
**Fig. S4A-C**

A. Leptin levels in controls and MetS (controls vs. MetS: *p* < 0.05).

B. Resistin levels in controls and MetS (controls vs. MetS: ns).

C. ISI values with and without leptin antibody in controls and MetS.
Table S1. Characteristics of male control subjects and male subjects with metabolic syndrome as defined by the Adult Treatment Panel III of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults¹.

<table>
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<th>Control group</th>
<th>Metabolic syndrome patients</th>
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<tr>
<td></td>
<td>(n=9)</td>
<td>(n=9)</td>
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<tr>
<td>Age (years)</td>
<td>52±8</td>
<td>54±7</td>
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<tr>
<td>Height (m)</td>
<td>1.80±0.08</td>
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<td>Weight (kg)</td>
<td>80.2±11.3</td>
<td>101.5±10.2</td>
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<td>Body mass index [kg (m²)⁻¹]</td>
<td>24.6±2.6</td>
<td>30.1±2.7</td>
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<tr>
<td>Body fat (%)</td>
<td>23±2</td>
<td>30.3±4.3</td>
<td>&lt;0.001</td>
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*Laboratory parameters*

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<td>(n=9)</td>
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<tr>
<td>Creatinine (µM)</td>
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<td>95±7</td>
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<td>HbA1c (%)</td>
<td>5.5±0.2</td>
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<td>Hs-CRP (mg L⁻¹)</td>
<td>0.96±1.2</td>
<td>3.39±2.3</td>
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<td>Total cholesterol (mM)</td>
<td>6.05±1.2</td>
<td>6.46±1.2</td>
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<td>LDL-cholesterol (mM)</td>
<td>2.98±0.63</td>
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<tr>
<td>Plasma Insulin (mU L⁻¹)</td>
<td>6±4</td>
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<td>Homocysteine (µM)</td>
<td>12.6±1.6</td>
<td>9.3±1.7</td>
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<tr>
<td>HOMA-IR</td>
<td>1.26±0.27</td>
<td>5.5±2.2</td>
<td>&lt;0.0001</td>
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</table>

*Components of the metabolic syndrome*

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<tbody>
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<td>(n=9)</td>
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<tr>
<td>Glucose (mM)</td>
<td>5.2±0.6</td>
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<td>Waist (cm)</td>
<td>90±7</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>139±19</td>
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<td>Diastolic blood pressure (mmHg)</td>
<td>86±10</td>
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<td>Triglycerides (mM)</td>
<td>1.65±0.83</td>
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<tr>
<td>HDL-c (mM)</td>
<td>1.22±0.20</td>
<td>1.11±0.12</td>
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Table S2. Characteristics of male control subjects and male subjects with DM2.

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<td>Systolic blood pressure (mmHg)</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
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<td>80±4</td>
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<tr>
<td>HbA1c (%)</td>
<td>5.4±0.08</td>
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<td>&lt;0.0006</td>
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<tr>
<td>Cholesterol (mM)</td>
<td>6.3±0.3</td>
<td>4±0.3</td>
<td>&lt;0.0003</td>
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<tr>
<td>Triglycerides (mM)</td>
<td>1.5±0.3</td>
<td>1.9±0.3</td>
<td>0.4</td>
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<tr>
<td>HDL-cholesterol (mM)</td>
<td>1.3±0.07</td>
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<tr>
<td>Creatinine (µM)</td>
<td>100±5.2</td>
<td>100±6</td>
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<td>Duration of diabetes (years)</td>
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