Leptin-mediated activation of human platelets: involvement of a leptin receptor and phosphodiesterase 3A-containing cellular signaling complex

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Submitted 18 March 2005; accepted in final form 5 May 2005

Elbatarny, Hisham S., and Donald H. Maurice. Leptin-mediated activation of human platelets: involvement of a leptin receptor and phosphodiesterase 3A-containing cellular signaling complex. Am J Physiol Endocrinol Metab 289: E695–E702, 2005. First published May 10, 2005; doi:10.1152/ajpendo.00125.2005.—An elevated circulating level of the adipocyte-derived satiety hormone leptin is an independent risk factor for cardiovascular disease. Because thrombus formation is a major cause of acute coronary events and leptin was shown previously to facilitate ADP-induced platelet aggregation, we chose to define the signaling events involved in leptin-mediated platelet activation. Using pharmacological, biochemical, and cell biological approaches, we show that leptin-induced platelet activation required activation of a signaling cascade that included the long form of the leptin receptor, three kinases [Janus kinase 2 (JAK2), phosphatidylinositol 3-kinase (PI3K), and protein kinase B (PKB/Akt)], the insulin receptor substrate-1 (IRS-1), and the major human platelet cAMP phosphodiesterase phosphodiesterase 3A (PDE3A). Moreover, we identify a role for an intraplatelet LEPR/JAK2/IRS-1/PI3K/PKB/PDE3A molecular complex that allows for the selective leptin-mediated activation of platelets. Our data demonstrate that leptin promotes platelet activation, provides a mechanistic basis for the prothrombotic effect of this hormone, and identifies a potentially novel therapeutic avenue to limit obesity-associated cardiovascular disease.

Obesity contributes significantly to the risk of atherothrombosis and impacts cardiovascular morbidity and mortality through its influence on several associated conditions, including insulin resistance, hypertension, and dyslipidemia (8, 14). Although numerous factors contribute to a link between obesity and cardiovascular disease, leptin has recently been shown to represent an important potential candidate to link these disorders (31). Consistent with this, epidemiological studies have reported that hyperleptinemia and its associated leptin resistance represent an independent risk factor for cardiovascular disease in humans (29). In this context, studies have begun to elaborate a potentially important role for this hormone in the regulated functioning of several important systems involved in cardiovascular and inflammatory diseases. Indeed, recent reports have established that leptin directly inhibits insulin secretion from pancreatic islet cells (9, 33), stimulates angiogenesis (3), induces cardiac hypertrophy (25), stimulates hematopoiesis (11), promotes platelet aggregation (5, 16, 17, 21), and regulates inflammation (10, 26).

Leptin actions in cells are coordinated through the leptin receptor (LEPR), a member of the large family of class I cytokine receptors encoded by the diabetes (db) gene (28, 32). LEPRs are composed of common extracellular and transmembrane-spanning domains and can encode variable intracellular domains (28). One “long form” of LEPR (LEPRL), encoding a 303-amino acid intracellular domain, and several “short forms” (LEPRs) have been described. Many of leptin’s effects are coordinated through effects in the central nervous system, and LEPRs are abundantly expressed in the hypothalamus. Peripherally, LEPR is expressed in several tissues including kidney, lung, adipose, and pancreas, as well as in vascular endothelial cells, immune cells, and blood platelets (2, 27). Although most known effects of leptin result from activation of LEPRL, LEPRs can also transduce leptin-dependent signals in cell types devoid of LEPRL (22, 35). Leptin-induced signals utilize at least two signaling systems, the conventional cytokine receptor-linked JAK2-STAT3 system (6, 23) and one involving a phosphatidylinositol 3-kinase (PI3K)-dependent, protein kinase B (PKB/Akt)-mediated activation of a cAMP phosphodiesterase, namely phosphodiesterase 3B (PDE3B) (34). In this context, acute leptin-mediated effects in hypothalamus, pancreatic islet cells, and hepatocytes are dependent, at least in part, on PKB-mediated activation of PDE3B and a resultant blunting of cAMP signaling (33, 34, 35). In contrast, the mechanistic basis of leptin-mediated effects on glucose transport and glycogen synthesis in skeletal muscle (1, 15), migration of human vascular endothelial cells (12), inflammatory potential of mononuclear cells (26), and, of relevance to this report, stimulated platelet activation (5, 16, 17, 21) have yet to be fully elucidated.

In the present study, we used a combination of pharmacological, biochemical, and cell biological approaches to test the hypothesis that leptin activates the major platelet cAMP PDE PDE3A, and that this event allows leptin to activate these cells. PDE3A, a member of the PDE3 gene family of enzymes, is highly homologous to PDE3B and represents the dominant PDE3 enzyme expressed in several cardiovascular tissues, including blood platelets (20). We present our data in the context that PDE3A could represent a common molecular target for leptin in cardiovascular tissues and might represent a novel therapeutic target in continuing efforts to limit obesity-related activation of platelets and reduce cardiovascular diseases associated with obesity.

MATERIALS AND METHODS

Preparation of platelet-rich plasma and washed platelets. After formal consent was obtained from drug-free, healthy, nonobese volunteers, samples (30–50 ml) of their blood were collected in 1
hemin (15 U/ml), when used for aggregation studies; 2) 3.8% trisodium citrate (1:9) for PDE assay and immunoblotting; or 3) 1 M phosphate dextrose [ACD, 2.5% trisodium citrate, 1.5% citric acid monohydrate, 2% dextrose (pH 4.5), 1:6], when used for platelet adhesion/cAMP measurements. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by centrifugation of anticoagulated blood at 284 g for 15 min at room temperature (PRP) or 2750 g for 5 min at room temperature, respectively. Platelets were isolated from PRP by centrifugation at 900 g for 10 min at room temperature. Washed platelets were obtained when isolated platelets were washed in a Ca2+-free Tyrode’s buffer containing 0.35% BSA, 137 mM NaCl, 2.7 mM potassium chloride (KCl), 11.9 mM NaHCO3, 1 mM magnesium chloride (MgCl2·6H2O), 0.26 mM EGTA, 3 mg/ml aprotinin, and 5 mM 1,4-piperazinediethanesulfonic acid (PIPES, pH 6.5) and then subsequently, in this buffer, supplemented with 2 mM Ca2+ and buffered with 5 mM HEPEs (pH 7.4) rather than PIPES. Platelet cAMP measurements. Platelet cAMP levels were determined as described (19). Briefly, washed platelets (2.5 × 10^8/ml) were incubated with 4 μM [3H]adenine (4 Ci/mmol) for 1 h, centrifuged, and resuspended at 4.5 × 10^8/ml in Ca2+-containing Tyrode’s solution at 37°C. Aliquots of prelabeled platelets were transferred to tubes containing forskolin, cilostamide, leptin, or ADP, alone or in combination, for predetermined periods (see individual figure legends) at 37°C. Incubations were terminated by addition of an equal volume of a 10% (wt/vol) ice-cold trichloroacetic acid (TCA) to a final concentration of 5% (wt/vol). After addition of the recovery marker [14C]cAMP (1,000 dpm), platelet cAMP was purified by column chromatography and quantitated by liquid scintillation. [3H]cAMP was expressed as a percentage of total [3H]. Platelet aggregation studies. Platelet aggregation in PRP was monitored optically using an optical aggregometer, with PPP used as a reference. Aliquots (500 μL, 4 × 10^8 platelets/ml of platelet suspensions) of platelets were incubated with pharmacological agents of interest at 37°C for prescribed periods and transferred to siliconized glass cuvettes containing 2 μM ADP and allowed to aggregate for 3 min. Platelet adhesion studies. Platelets were labeled with [3H]adenine as described above. Labeled platelets (1 × 10^8/ml) were incubated with additions (100 μl/well, 30 min at room temperature) in individual wells of microtiter plates that had been previously coated with either fibronectin (10 μg/ml) or collagen (20 μg/ml). Unbound platelets were removed by three washes with PBS (pH 7.4), and bound platelets were solubilized with 1% Triton X-100 (200 μl/well). Solubilized contents of each well were transferred to scintillation vials, and [3H] was counted by liquid scintillation. Platelet adhesion, measured as [3H] present in each well, was expressed as a percentage of the total [3H] that had been added to each well. Because platelet adhesion was measured in a static system without stirring, platelets did not aggregate during adhesion experiments. Determination of cAMP PDE activity. After incubation with agents of interest, platelets were lysed in a buffer consisting of 1 mM EDTA (pH 7.4), 100 mM NaCl, 5 mM MgCl2, 50 mM Tris·HCl (pH 7.4), 1% Triton X-100, 5 mM benzamidine, 1 μM/ml aprotinin, 5 μg/ml bestatin, 2 μg/ml leupeptin, 100 μM DTT, 10 mM phenylmethylsulfonyl fluoride (PMSF), and supplemented with phosphatase inhibitors [10 mM Na β-glycerophosphate, 10 mM Na pyrophosphate, 10 mM sodium fluoride (NaF), and 10 mM Na vanadate]. Reactions were initiated by addition of lysates (2–5 μg) to a PDE assay buffer (3 mM EGTA, 5.26 mM Tris, and 0.526 mM MgCl2) containing 1 μM [3H]cAMP (100,000 dpm). The 30-min reactions were terminated by the addition of 1 ml of a solution containing 20 μM HEPES (pH 8.5), 20 μM NaCl, 17.5 μM NaOH, and 25 μM EDTA (pH 7.4). The product of the PDE reaction, 5′-[3H]cAMP, was isolated and quantified as described previously (29). Immunoprecipitations. Platelet lysates generated as described in the previous section were preclarified with 1 μg of rabbit IgG and 50 μl of a solution of protein A/G-agarose beads for 2 h at 4°C. Preclarified platelet lysates were subsequently incubated with 1 μg of an antibody raised to the common extracellular domain of all known LEPR (anti-LEPR), or 1 μg of an antisera raised against human PDE3A and 50 μl of a solution of protein A/G-agarose beads for 16 h at 4°C. After this incubation, agarose beads were isolated by centrifugation (10,000 g) and washed three times with 1 ml of ice-cold lysis buffer (as in previous section). Isolated immune complexes were resolved by SDS-PAGE and immunoblotted for LEPR, PDE3A, JAK2, PKB, Src homology 2-B (SH2-B), IRS-1, or phosphorylated tyrosine residues (see Immunoblot analysis). Immunoblot analysis. Platelet lysates or isolated immune complexes were resuspended in Laemmli buffer, processed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. After treatment with 5% nonfat dried milk in Tween-Tris-buffered saline (T-TBS) [5 mM Tris·HCl (pH 7.4), 0.9% NaCl, 0.1% Tween-20] for 1.5 h at room temperature, membranes were incubated with antibodies raised against either PDE3A (1:500), phospho-PKA substrate (1:10,000), phospho-PKB substrate (1:5,000), JAK2 (1:1,000), or phospho-JAK2 (1:1,000) in 5% milk T-TBS for 1.5 h at room temperature. After incubation with primary antibodies, membranes were washed five times with T-TBS and then further incubated for 1.5 h with horseradish peroxidase-conjugated anti-goat (1:4,000, PDE3A) or anti-rabbit antisera (1:7,000, all others) in 5% milk T-TBS. Immunoreactive proteins were visualized by chemiluminescence, as suggested by Bio-Rad. General reagents. Recombinant human leptin was purchased from Leinco Technologies (St. Louis, MO). Radioactive materials [3H]adenine, [3H]AMP, [3H]cAMP, and [3H]5′-AMP were purchased from PerkinElmer Life Sciences (Boston, MA). Cilostamide, forskolin, LY 294002, BSA, and bestatin were purchased from Calbiochem (La Jolla, CA). BAY 31–9472, 5-(4-[1-(hydroxyethyl)-4-phenylbutyl]-6-oxo-6,9-di-hydro-1H-purin-2-yl)methyl)-2-methoxy-N-methylbenzenesulfonamide was a gift from Dr. Erwin Bischoff, Bayer, Leverkusen, Germany. Adenosine diphosphate (ADP) and NaF were purchased from Sigma-Aldrich (Oakville, ON, Canada). Hepalene hemin sodium was purchased from the Kingston General Hospital Pharmacy (Kingston, ON, Canada). Benzamidine, EDTA, EGTA, PMSF, and citric acid were purchased from ICN Biomedicals (Aurora, OH). Leupeptin was purchased from Roche Applied Science (Mississauga, ON, Canada). KCl, potassium dihydrogen orthophosphate, calcium chloride dihydrate, MgCl2, magnesium chloride hexahydrate, and di-sodium orthophosphate were purchased from BDH Incorporated (Toronto, ON, Canada). Aquasil silicizing fluid was purchased from Pierce (Rockford, IL). Each of the PDE3A polyclonal antibodies, M14, C18, and G20, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and a human PDE3A-specific monoclonal antisera was a gift from ICOS. The anti-LEPR and SH2-B antisera were generous gifts from Drs. R. V. Considine, Indiana University School of Medicine (Indianapolis, IN) and C. Carter-Su (University of Michigan, Ann Arbor, MI), respectively. Phospho-(Ser/Thr) PKA and PKB/Akt substrate, anti-phospho-JAK1 or JAK2, and anti-IRS-1 antisera were purchased from Cell Signaling Technology (Beverly, MA). The anti-JAK2 antibody was purchased from Upstate Cell Signaling Solution (Lake Placid, NY). Horseradish peroxidase-conjugated secondary antisera, kaleidoscope prestained protein markers, chemiluminesence (ECL) reagents, and nitrocellulose membranes were from Bio-Rad (Hercules, CA). A dual-chamber optical aggregometer (490–2D), glass cuvettes, and disposable stir bars were from CHRONO-LOG (Haverton, PA). Statistical analysis. Data presented are means ± SE from at least three independent experiments, with each mean derived from either three or four separate determinations in separate experiments. Where representative immunoblots or representative platelet aggregation tracings are shown, similar data were obtained in at least three separate experiments utilizing separate platelet populations. Results obtained from immunoblot analysis were quantified by densitometric analysis, and values are reported as means ± SE. Statistical differ-
Leptin-induced platelet activation. Consistent with recent reports (5, 16, 17, 21), we report that leptin significantly potentiated ADP-induced aggregation of human platelets (Fig. 1A). Indeed, in the 25 separate experiments in which this was assayed, leptin (500 ng/ml) potentiated 2 μM ADP-induced aggregation of human platelets by 16 ± 3% (n = 25). To extend these studies to include other aspects of platelet activation, we next investigated the effects of leptin on platelet adhesion. Consistent with the idea that leptin stimulates platelet adhesion, leptin concentration dependently (50–500 ng/ml) increased this response of platelets in our experiments. Indeed, in the six independent experiments in which this effect was studied, leptin concentration dependently stimulated platelet adhesion with a maximal effect of 52 ± 11% at the highest concentration of leptin studied (500 ng/ml, Fig. 1B). In addition to its ability to stimulate platelet adhesion when used alone, leptin markedly potentiated platelet adhesion stimulated by 2 μM ADP. Thus, although 2 μM ADP increased basal platelet adhesion by 85 ± 8% when used alone, when measured in the presence of 50 or 500 ng/ml of leptin ADP-induced platelet adhesion was 125 ± 10 and 155 ± 11%, respectively (means ± SE from 6 experiments).

Leptin-induced platelet activation involves Jak2, IRS-1, P13K, and PKB. Incubation of human platelets with leptin caused activation-dependent phosphorylation of several proteins previously shown to participate in leptin-mediated signaling in other cell types (1, 2). Thus incubation of platelets with leptin caused the activation-dependent phosphorylation of Jak2, but not Jak1 (Fig. 2A), IRS-1 (Fig. 2B), and PKB (Fig. 2C) in these cells. In agreement with the idea that leptin-induced phosphorylation/activation of Jak2 and P13K were involved in allowing leptin to activate human platelets, the addition of either the P13K selective inhibitor LY-294002 (10–50 μM) or the Jak2 inhibitor AG-490 (10 μM) each virtually abolished leptin-induced platelet adhesion in our experiments (Fig. 2D and Fig. 2E). In addition, AG-490-mediated Jak2 inhibition also markedly attenuated leptin-induced potentiation of ADP-induced platelet aggregation, but not that stimulated by ADP in the absence of leptin (Fig. 2F).

Role of PDE3A in leptin-induced platelet activation. In addition to stimulating platelet aggregations and adhesion, leptin (500 ng/ml) also significantly blunted forskolin-induced inhibition of platelet aggregation (Fig. 3) and adhesion (not shown). Because forskolin inhibits platelet aggregation by activating adenylyl cyclase and increasing cAMP, we investigated whether leptin altered cAMP signaling in platelets. Both our pharmacological and biochemical data are consistent with the idea that leptin attenuated forskolin-induced inhibition of platelet aggregation by activating the major platelet cAMP PDE, namely PDE3A. Thus addition of the PDE3-selective inhibitor cilostamide (0.1–1.0 μM) significantly blunted J the ability of leptin to potentiate ADP-induced aggregation (Fig. 3), 2) the antagonism of forskolin-mediated inhibition of ADP-induced aggregation caused by leptin (Fig. 3), and 3) the proadhesive effect of leptin (Fig. 4). In contrast, addition of a selective inhibitor of the other cAMP-PDE expressed by human platelets (PDE2, Bay 31–9472) (20) did not impact leptin-mediated platelet adhesion (Fig. 4).

Consistent with the idea that leptin activated platelet PDE3A, incubation of these cells with leptin (500 ng/ml) increased total platelet PDE3A activity by 19 ± 2% (Table 1). Because this effect of leptin on PDE3A activity was modest and recent reports had shown that intracellular targeting of PDEs played a central role in allowing these enzymes to effectively regulate cellular functions, we investigated the possibility that PDE3A and LEPR interacted in platelets. Our data are consistent with this idea. Thus platelet LEPR and PDE3A were coimmunoprecipitated from resting platelets by
Fig. 2. Identification of signaling elements involved in leptin-mediated activation of human platelets. Aliquots of human washed platelets (5 ml at 1 x 10^8 platelets/ml) were incubated with either vehicle or leptin, isolated by centrifugation (2500 g), and lysed (see MATERIALS AND METHODS). Platelet lysates were either immediately boiled in 2× SDS-loading buffer or incubated with a polyclonal antiserum raised against human LEPR overnight at 4°C. LEPR immune complexes were isolated after incubation with protein A/G-agarose and centrifugation (10,000 g; see MATERIALS AND METHODS). Platelet lysates or immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted (IB) with anti-activated JAK1 or anti-activated JAK2 antisera (A), an anti-insulin receptor substrate (IRS-1) or an anti-phosphotyrosine [anti-P(Y), clone 4G10] (B), or an anti-activated PKB antisera (C). D: impact of selective phosphatidylinositol 3-kinase inhibition with LY-294002 (10, 25, 50 μM) on leptin-induced adhesion of [3H]adenine-prelabeled human platelets to fibronectin was assessed as described in the legend to Fig. 1B. Impact of JAK2-selective inhibitor AG-490 (50 μM) on leptin-induced adhesion of platelets (E) or aggregation (F) was determined as described in the legend to Fig. 1.
use of either an LEPR antiserum (Fig. 5A) or a PDE3A antiserum (not shown). Obviating a role for leptin-mediated PDE3A recruitment to this complex in cells, leptin treatments did not alter the amount of PDE3A found in complex with LEPR (Fig. 5A). In contrast, leptin incubation did increase the activity of the PDE3A associated with LEPR (Table 1). Indeed, PDE3A activity associated with LEPR was increased by 85% after leptin treatment of platelets. Interestingly, PDE3A activity not associated with LEPR was not increased by leptin treatment of platelets (not shown). Immunoblot analysis using an antiserum directed against PKB substrates showed that LEPR-associated PDE3A was not phosphorylated by PKA in either resting or leptin-treated platelets (Fig. 5B). In contrast, immunoblot analysis utilizing a PKA substrate-specific antiserum showed that LEPR-associated PDE3A was not phosphorylated by PKA in either resting or leptin-treated platelets (not shown). Although leptin treatment did not alter the amount of PDE3A that associated with LEPR in platelets (Fig. 5B), leptin treatment did cause a significant increase in the amount of IRS-1 that could be detected in this complex (Fig. 5C). Indeed, in the four separate experiments in which this was investigated, leptin increased PDE3A-associated IRS-1 by 380 ± 70%.

Consistent with our data demonstrating that leptin selectively activated the small fraction of platelet PDE3A that associated with LEPR in these cells, leptin did not alter global platelet cAMP levels. Thus although leptin treatment of resting or activated platelets tended to reduce global cAMP levels (Table 2), these effects did not reach statistical significance. Similarly, leptin did not significantly affect forskolin (1 μM) or cilostamide (1 μM)-induced increases in total platelet cAMP (Table 1). Although further studies will be required to fully

<table>
<thead>
<tr>
<th>Sample</th>
<th>Platelet Treatment</th>
<th>PDE3 Activity pmol·min⁻¹·mg protein⁻¹</th>
<th>%Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet lysate</td>
<td>None</td>
<td>544±46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leptin, 500 ng/ml</td>
<td>650±50*</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>156±14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leptin, 500 ng/ml</td>
<td>289±15*</td>
<td>85</td>
</tr>
</tbody>
</table>

Values are means ± SE from 6 independent experiments. Phosphodiesterase (PDE3) activity was determined in platelet lysates or leptin receptor (LEPR) immunoprecipitate from resting or leptin (500 ng/ml)-treated platelets. *P < 0.05 signifies a significant difference when compared with PDE3 activity in cells not treated with leptin.
assess the movement of the various proteins involved in regulating leptin effects into this LEPR complex, our data are consistent with a role for selective leptin-mediated activation of LEPR-associated PDE3A and with the idea that leptin treatment could locally increase cAMP hydrolysis. Regrettably, cellular transfection approaches are the only experimental avenue to investigate localized changes in cellular cAMP levels, and the anucleated platelets are not suitable for these types of analyses.

DISCUSSION

Obesity is a noncontroversial risk factor for cardiovascular disease, and abnormal platelet activation is a direct pathognomonic factor for thrombotic disorders. In this context, recent attention has focused on a potential role for physiological and pathological processes influenced by the adipocyte-derived satiety hormone leptin in this association, including its potential role as a platelet activator. With one exception (24), published reports are consistent with the hypothesis that leptin potentiates the platelet-aggregating effects of ADP (5, 16, 17, 21). On the basis of these earlier reports and our laboratory’s continuing interest in the role of leptin in mediating diabetes-associated cardiovascular diseases (22), we chose to study leptin-mediated effects on platelet activation and to begin to determine the molecular basis by which this hormone was acting in these cells. To achieve these objectives, we first confirmed the earlier findings indicating that leptin potentiated with ADP to aggregate human platelets. Our findings are unequivocal and indicate that leptin significantly promotes ADP-induced human platelet aggregation. Consistent with this effect of leptin being pharmacologically and potentially physiologically relevant, we further demonstrated that leptin could blunt the effects of forskolin, an inhibitor of platelet aggregation that operates by increasing the synthesis of cAMP. Because forskolin, by increasing platelet cAMP, inhibits platelet functions by mechanisms analogous to those utilized by the physiological inhibitor of platelet function prostacyclin, our data are consistent with the broader hypothesis that circulating leptin could also act to predispose platelets to aggregating to subthreshold aggre-

### Table 2. Impact of leptin on basal and forskolin-induced increases in cAMP in resting and adhering human platelets

<table>
<thead>
<tr>
<th>Platelet Status</th>
<th>Additions</th>
<th>Basal</th>
<th>Fsk, 1 μM</th>
<th>Cil, 1 μM</th>
<th>Fsk, 1 μM + Cil (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>None</td>
<td>0.04±0.00</td>
<td>0.59±0.02*</td>
<td>0.11±0.01*</td>
<td>0.68±0.01**</td>
</tr>
<tr>
<td></td>
<td>Leptin, 500 ng/ml</td>
<td>0.03±0.01</td>
<td>0.50±0.06*</td>
<td>0.10±0.01*</td>
<td>0.55±0.04*</td>
</tr>
<tr>
<td></td>
<td>ADP, 2 μM</td>
<td>0.18±0.04</td>
<td>0.20±0.02</td>
<td>0.22±0.03</td>
<td>0.28±0.01*</td>
</tr>
<tr>
<td>Adhesion</td>
<td>ADP (2 μM) + leptin (500 ng/ml)</td>
<td>0.16±0.01</td>
<td>0.18±0.03</td>
<td>0.20±0.01</td>
<td>0.26±0.01*</td>
</tr>
</tbody>
</table>

Fsk, forskolin; Cil, cilostamide. [3H]cAMP levels are expressed as means ± SE of 3 separate experiments and were determined as described in MATERIALS AND METHODS. *P < 0.05 signifies a significant difference when compared to cAMP level without addition; †P < 0.05 signifies a significant difference when compared to effect of forskolin (1 μM).
gating stimuli. A more complex paradigm to test directly, this latter hypothesis will require further ex vivo and in vivo experimentation to be fully tested.

In addition to confirming that leptin promotes ADP-induced aggregation of human platelets, we further reported the novel finding that leptin could, through its own actions on platelets, stimulate platelet adhesion to extracellular matrix proteins. Leptin-induced platelet adhesion was studied because we reasoned that this event, representing an early step in the process of platelet activation, might be more sensitive to the effects of leptin. Our findings clearly identified leptin as a platelet adhesion factor and were consistent with reports of cytokine-induced adhesion of several distinct cell types to extracellular matrix components (13). For the mechanistic reasons listed above, in addition to further broadening the scope of leptin actions on platelets, the finding that leptin, without the assistance of other factors, could stimulate platelet adhesion may also be physiologically and pathologically important. Thus, taken together, we propose that our findings that leptin promotes platelet adhesion when used alone and potentiates ADP-induced adhesion and aggregation support the hypothesis that elevated circulating leptin may amplify local thrombotic episodes by simultaneously potentiating actions of platelet activators and reducing the impacts of inhibitory factors operating by increasing cAMP.

Mechanistic insight into the actions of leptin in human platelets was derived from two of our research approaches. First, the observation that leptin was capable of preventing the antiplatelet effect of the adenyl cyclase-activating agent forskolin was consistent with the involvement of a cAMP-based mechanism. Second, our data showed that cilostamide, a selective inhibitor of PDE3A, the dominant cAMP-PDE present in human platelets, blunted the effects of leptin in platelets. Together, these data identified PDE3A as a likely molecular target for the actions of leptin in platelets. Although a novel hypothesis for the actions of leptin in human platelets, our proposed link between leptin and PDE3A is consistent with findings from other systems, in which activation of PDE3B was proposed to modulate some of the effects of leptin. Thus, recent data have identified PDE3B as playing an important role in several actions of leptin. Indeed, Zhao et al. (34) have presented compelling data that leptin regulates cellular functions by stimulating a P13K- and PKB-mediated phosphorylation and activation of PDE3B. Because PDE3A is highly homologous to PDE3B (20, 30) and is hypothesized to contain the conserved residues required for regulation by PKB, we proposed that a similar mechanism was involved in platelets, and much of our biochemical data are consistent with this. Thus, although leptin addition to platelets increased PDE3 activity and the phosphorylation of PDE3A by PKB, addition of a P13K inhibitor abolished all of these effects. Consistent with a role for LEPR and JAK2 in coordinating the upstream events of activation of P13K and PKB in platelets, leptin significantly increased activation of Jak2 in platelets. Taken together, our biochemical data are entirely consistent with the proposal that leptin addition to human platelets activates a signaling cascade that includes an LEPR-Jak2-P13K-PKB axis and that activation of this signaling axis by leptin promotes the PKB-mediated phosphorylation and activation of PDE3A in these cells. Whether and how our findings are linked to the very recently reported (4) observation that leptin increases free calcium concentrations in platelets through phospholipase C-protein kinase C-dependent events will require further study.

Recent studies by several laboratories, including ours, have demonstrated that specific PDEs regulate selective cellular functions by virtue of being specifically targeted to distinct intracellular domains and, as such, regulating distinct intracellular “pools” of cellular cAMP reviewed in Ref. 20). In this context, our studies suggest that leptin acts to regulate a distinct pool of cAMP in human platelets. Moreover, our biochemical data identify a LEPR/PDE3A molecular complex that may represent a molecular basis for this effect. Thus, although leptin did not alter the amount of PDE3A that associated with LEPR, this hormone did selectively activate LEPR-associated PDE3A compared with its effect on total platelet PDE3A. Regrettably, since the only method available to date to study regioselective changes in cellular cAMP requires transfection of cells, and this is not possible in the anucleated platelet, a direct analysis of the impact of leptin on regioselective changes in platelet cAMP will necessarily have to await development of more directly applicable techniques.

Possible Therapeutic Implications Of Our Study

As confirmed by our study, leptin-induced platelet activation via activation of PDE3A may represent a molecular basis for the association between hyperleptinemia and cardiovascular diseases. Because our data define a role for an LEPR/Jak2/IRS-1/P13K/PKB/PDE3A cascade, we postulate that interruption of signaling through this axis in platelets may represent a pharmacological avenue to limit the risk of cardiovascular disease associated with hyperleptinemia.

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