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Phosphatase Activity in Rat Adipocytes: Effects of Insulin and Insulin Resistance

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Abstract Insulin regulates the activity of both protein kinases and phosphatases. Little is known concerning the subcellular effects of insulin on phosphatase activity and how it is affected by insulin resistance. The purpose of this study was to determine insulin-stimulated subcellular changes in phosphatase activity and how they are affected by insulin resistance. We used an in vitro fatty acid (palmitate) induced insulin resistance model, differential centrifugation to fractionate rat adipocytes, and a malachite green phosphatase assay using peptide substrates to measure enzyme activity. Overall, insulin alone had no effect on adipocyte tyrosine phosphatase activity; however, subcellularly, insulin increased plasma membrane adipocyte tyrosine phosphatase activity 78 ± 26% (n = 4, $P < 0.007$), and decreased high-density microsome adipocyte tyrosine phosphatase activity 42 ± 13% (n = 4, $P < 0.005$). Although insulin resistance induced specific changes in basal tyrosine phosphatase activity, insulin-stimulated changes were not significantly altered by insulin resistance. Insulin-stimulated overall serine/threonine phosphatase activity by 16 ± 5% (n = 4, $P < 0.005$), which was blocked in insulin resistance. Subcellularly, insulin increased plasma membrane and crude nuclear fraction serine/threonine phosphatase activities by 59 ± 19% (n = 4, $P < 0.005$) and 21 ± 7% (n = 4, $P < 0.007$), respectively. This increase in plasma membrane fractions was inhibited 23 ± 7% (n = 4, $P < 0.05$) by palmitate. Furthermore, insulin increased cytosolic protein phosphatase-1 (PP-1) activity 160 ± 50% (n = 3, $P < 0.015$), and palmitate did not significantly reduce this activity. However, palmitate did reduce insulin-treated low-density microsome protein phosphatase-1 activity by 28 ± 6% (n = 3, $P < 0.04$). Insulin completely inhibited protein phosphatase-2A activity in the cytosol and increased crude nuclear fraction protein phosphatase-2A activity 70 ± 29% (n = 3, $P < 0.038$). Thus, the major effects of insulin on phosphatase activity in adipocytes are to increase plasma membrane tyrosine and serine/threonine phosphatase, crude nuclear fraction protein phosphatase-2A, and cytosolic protein phosphatase-1 activities, while inhibiting cytosolic protein phosphatase-2A. Insulin resistance was characterized by reduced insulin-stimulated serine/threonine phosphatase activity in the plasma membrane and low-density microsomes. Specific changes in phosphatase activity may be related to the development of insulin resistance. J. Cell. Biochem. 77:445–454, 2000.

Key words: fatty acids; subcellular fractions; plasma membrane; fat cells

Phosphatases are likely to play an important part in regulating insulin-signaling pathways due to the importance of reversible phosphorylation in the propagation of downstream signals [Chan et al., 1988; Ding et al., 1994; Wood et al., 1993].

Previous studies investigating the effects of insulin on phosphatase activity have usually determined phosphatase activities in soluble and particulate subcellular fractions. These studies are important, as they link alterations in phosphatase activity to the development of insulin resistance and type 2 diabetes [Zierath et al., 1997; Begum et al., 1991; Ahmad et al., 1995; Corvera et al., 1991; Meyerovitch et al., 1991; Begum and Ragolia, 1998]. For example, in genetically obese and diabetic animals, there is an approximately 50% decrease in liver cytosolic and particulate PTPase activity compared with nonobese heterozygotes [Meyero-

Abbreviations used: PTPase, protein tyrosine phosphatase; S/T, serine/threonine; OA, okadaic acid; PP-1, protein phosphatase-1; PP-2A, protein phosphatase-2A, LCSFA, long chain saturated fatty acid; LAR, leukocyte antigen-related phosphatase; LRP, leukocyte common antigen-related phosphatase; HDM, high-density microsomes; LDM, low-density microsomes; PM, plasma membranes; CNF, crude nuclear fraction; Cyt, cytoplasm, FAF, fatty acid free.

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vitch et al., 1991]. Also, streptozotocin-induced diabetic rat adipocytes have demonstrated increases in particulate protein tyrosine phosphatase (PTPase) activity in vitro, with simultaneous decreases in cytosolic fractions [Begum et al., 1991]. Recent work by Begum and Ragolia, 1998 has demonstrated basal and insulin-stimulated reductions in protein phosphatase-1 (PP-1) activity by 32% and 65%, respectively, in nonobese, insulin-resistant, type 2 diabetic rats compared with control Wistar rats.

Direct involvement of phosphatase activity in insulin action has been demonstrated using the serine/threonine (S/T) phosphatase inhibitor okadaic acid (OA). In adipocytes, okadaic acid, per se has been shown to increase basal GLUT4-mediated glucose transport while reducing insulin-stimulated glucose transport [Corvera et al., 1991; Lawrence et al., 1990]. The precise mechanism by which OA inhibits insulin-stimulated glucose transport is unknown; however, it is thought to affect a S/T phosphatase critical to the translocation of GLUT4 to the cell surface [Corvera et al., 1990]. Recently it has been demonstrated that mice lacking the PTPase-1B gene exhibit increased insulin sensitivity [Elchebly et al., 1999]. In adipocytes, insulin greatly influences glucose transport, lipolysis, and glycogen synthesis. Insulin-regulated phosphatase activity plays a role in controlling each of these functions, which are localized to particular subcellular fractions. Relatively little is known concerning the subcellular effects of insulin and insulin resistance on phosphatase activity; therefore, the purpose of this study was to determine insulin-stimulated subcellular changes in phosphatase activity and how they are influenced by insulin resistance.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA) fraction V, 2-deoxy-D-glucose, and palmitate were obtained from Sigma Chemical Co. (St. Louis, MO). Collagenase was obtained from Worthington (Freehold, NJ). Tritiated 2-deoxy-D-glucose was obtained from Du Pont-NEN (Boston, MA). The WAKO nonesterified fatty acid (NEFA) C kit for the quantitative determination of the total concentration of fatty acids added to cells was obtained from WAKO Pure Chemicals (Osaka, Japan). Immobilon-P membrane was obtained from Millipore (Bedford, MA). The tyrosine, S/T phosphatase assay kits, anti-PP-2A, and anti-phosphotyrosine antibody (4G10) were obtained from Upstate Biotechnologies (Lake Placid, NY). Enhanced chemiluminescent Western blotting detection reagents were purchased from Amersham Life Science (Buckinghamshire, UK). All other reagents were from standard suppliers.

Preparation of Fatty Acid-Free BSA

Fatty acid-free (FAF) BSA was prepared via the acid charcoal treatment method of Chen, 1967. Palmitate was added to the FAF BSA by the method of Spector and Hoak [1969]. Albumin is a physiologic carrier of fatty acids avoiding the introduction of organic solvents. These methods have been used by our laboratory previously, and are described in greater detail elsewhere [Hardy et al., 1991; Thode et al., 1989].

Preparation of Isolated Rat Adipocytes

Epididymal adipocytes were isolated from male Sprague-Dawley rats (100–160 g), fed ad libitum using the collagenase digestion method of Rodbell [1984].

Fatty Acid-Induced Insulin Resistance

The method for fatty acid-induced insulin resistance in rat adipocytes was performed as described previously [Hunnicutt et al., 1994]. Adipocytes ($1–2 \times 10^5$ cell/ml) were suspended in HBS buffer (21 mM Hepes, 22 mM dextrose, 140 mM NaCl, 5.0 mM KCl, 1.4 mM $CaCl_2$, pH 7.6) containing 0.45 mM (3% wt/vol) FAF BSA (control), or FAF BSA with 1.0 mM palmitate. Cells were incubated for 4 h at 37°C. The incubation buffer was removed, the cells were resuspended in HBS buffer [HBS with 0.15 mM (1%) FAF BSA (pH 7.0)], and incubated for an additional hour under identical conditions. This buffer was removed, and the cells were washed with HBS buffer with 1% FAF BSA (pH 7.4) and briefly centrifuged. The remaining HBS buffer was removed, and a portion of cells was allocated for glucose uptake assays. The remaining cells were incubated in 1% FAF BSA (pH 7.4) with 1 nM (final concentration) insulin or without (control) for 15 min at 37°C. After the HBS buffer was removed, cells were washed with 1% FAF BSA buffer (pH 7.4) and fractionated as described below.

2-Deoxyglucose Uptake

Glucose uptake was performed in triplicate as previously described [Hunnicutt et al., 1994]. Briefly, adipocytes (1–2 $\times$ 10^5 cells/ml) were resuspended in Krebs-Ringer phosphate (KRP) buffer, with 0.45 mM (3% wt/vol) BSA, and 1.5 mM pyruvate and incubated without or including insulin (1 nM final concentration) at 37°C for 15 min. 6.0 μCi/ml 1-[^{3}H]-2-deoxy-D-glucose (2-deoxyglucose final concentration of 34 μM) was then added to the mixture and transport was measured after 3 min. Cells were separated by centrifugation through dinonyl phthalate oil, and 1-[^{3}H]-2-deoxyglucose uptake was quantified by scintillation counting. Nonspecific 2-deoxyglucose uptake was measured in the presence of 50 μM cytochalasin B.

Subcellular Fractionation of Rat Adipocytes

Adipocytes were fractionated according to the differential centrifugation method of McKeel and Jarett [1970] and Jarett [1974]. Respective subcellular fractions were solubilized in ice-cold HBS wash buffer (21mM HEPES, 140 mM NaCl, 5.0 mM KCI, 1.4 mM CaCl2, 1 mM PMSF, 1 mM benzamidine, 1 mM EDTA, 1% NP-40, pH7.4), vortexed for 30 sec, and rotated at 4°C for 1.5–3 h. Each sample was then centrifuged for 20 min at 4°C and 8,200 x g, and the supernatant was aliquoted and stored at –70°C. In parallel fractionations, the purity of PM preparations was verified by 5′-nucleotidase assays as previously described [Avruch and Wallach, 1971]. Results obtained indicated that LDM fractions contained only low levels of 5′-nucleotidase activity (6–10%) in all treatments compared with the corresponding PM fractions. The differences observed with palmitate show no pattern that would indicate a generalized adverse effect on cellular enzyme activity.

Immunoblot

Briefly, subcellular fractions were prepared as described above. Protein concentrations were determined using the Bio-Rad DC protein concentration determination kit (Bio-Rad, Hercules, CA); 15 μg of PM protein was boiled in an equal volume of 2× Laemmli buffer (with β-mercaptoethanol) and separated by 7.5% SDS-PAGE. Proteins were then transferred to Immobilon P membrane (Millipore, Bedford MA) and probed with anti-phosphotyrosine (mAb 4G10, UBI, Lake Placid NY). Proteins were visualized by chemiluminescent detection (Amersham, Piscataway NJ).

Phosphatase Activity Assays

Tyrosine and S/T phosphatase activities were measured using malachite green detection assays, initially described by Harder et al. [1994] and obtained from UBI (Lake Placid, NY). The synthetic PTPase-specific phosphopeptide used in these assays was R-R-L-I-E-D-A-E-pY-A-A-R-G and the S/T phosphatase-specific peptide was composed of a synthetic K-R-pT-I-R-R peptide. Assays were performed with 5–15 μg of protein, initiated by the addition of phosphopeptide, and incubated at 23°C for 15 min. Reactions were stopped by addition of malachite green. Resulting data from these assays are expressed in pmol/μg of protein over the 15-min incubation period. Particulate fractions were diluted in an extra 20 μl of HBS wash buffer, without NP-40. Experiments with OA included a 15-min preincubation with the phosphatase inhibitor before the addition of the phosphopeptide substrate. PP-1 and PP-2A activities were determined based on the differential susceptibility of these phosphatases to OA. PP-2A activity was defined as the difference in S/T phosphatase activity between the 3 nM treated fraction and untreated (0 nM OA) fraction, while PP-1 activity represents the activity between 300 nM and 3 nM OA-treated fraction [Cohen et al., 1990].

Statistical Analysis

The results are expressed as mean ±SEM unless otherwise stated. Comparisons between experiments were assessed using Student's *t*-test (paired where appropriate). *t*-tests were performed by setting each control value to 100% and the treatment values were set to percentage of control. $P < 0.05$ was considered statistically significant. All significant differences are indicated.

RESULTS

Insulin-Resistant Adipocytes

Palmitate-induced insulin resistance was verified by glucose uptake assays, as described in the Materials and Methods. Insulin-stimulated glucose transport 5.6 ± 0.3-fold, while palmitate treatment inhibited insulin-

TABLE I Effects of Insulin and Palmitate Treatment on 5′ Nucleotidase Activity in Adipocyte Plasma Membranes and Low-Density Microsomes*

		5′ Nucleotidase activity (nmoles AMP/mg protein/ 30 min)	
Insulin	Palmitate	Plasma membrane (PM)	Low-density microsomes (LDM)
−	−	2,630 ± 51	251 ± 64
+	−	2,926 ± 60	176 ± 30
−	+	3,220 ± 377	193 ± 14
+	+	2,471 ± 87	193

*Insulin and palmitate treatments are for conditions of insulin resistance, as described under Materials and Methods. Results shown are from a single experiment performed in duplicate. Errors shown indicate a range except for LDM palmitate plus insulin, which is a single sample.

stimulated glucose uptake 59 ± 7% (n = 9) compared with FAF-treated cells. Importantly, basal glucose uptake was unaltered by palmitate treatment indicating that palmitate is not having a general adverse effect on cells. Consistent 5′ nucleotidase activities between treatments (Table I) further supports the specificity of the palmitate effect. Palmitate treatment under identical conditions has been demonstrated to have no effect on insulin-stimulated glycogen synthesis [Van Epps-Fung et al., 1997], also indicating palmitate does not have a nonspecific inhibitory effect on insulin signaling.

Adipocyte Protein Phosphatase Activity

Overall, insulin alone had no effect on adipocyte PTPase activity but increased S/T phosphatase activity by 16 ± 5% (n = 4, $P < 0.005$). Palmitate inhibited basal PTPase activity in control cells by 24 ± 3% (n = 5, $P < 0.003$); however, it did not alter basal S/T phosphatase activity. Palmitate significantly inhibited both insulin-stimulated PTPase, 25 ± 4% (n = 4, $P < 0.008$), and S/T phosphatase activity 11 ± 2% (n = 4, $P < 0.01$). Generally, the effects of both insulin and palmitate on cellular phosphatase activity are relatively minor and demand closer scrutiny to determine significant alterations.

Subcellular Tyrosine Phosphatase Activity

Among subcellular fractions, PTPase activity in the PM fraction was the most sensitive to insulin and palmitate treatment. Insulin alone significantly decreased high-density microsome (HDM) PTPase activity by 42 ± 13% (n = 4, $P < 0.005$) and increased PM PTPase activity by 78 ± 26% (n = 4, $P < 0.007$) (Fig. 1A). These results indicate that insulin specifically alters subcellular tyrosine phosphatase activity possibly by changing distribution and/or activity in specific compartments.

Palmitate alone significantly inhibited PTPase activity in PM fractions by 33 ± 5%

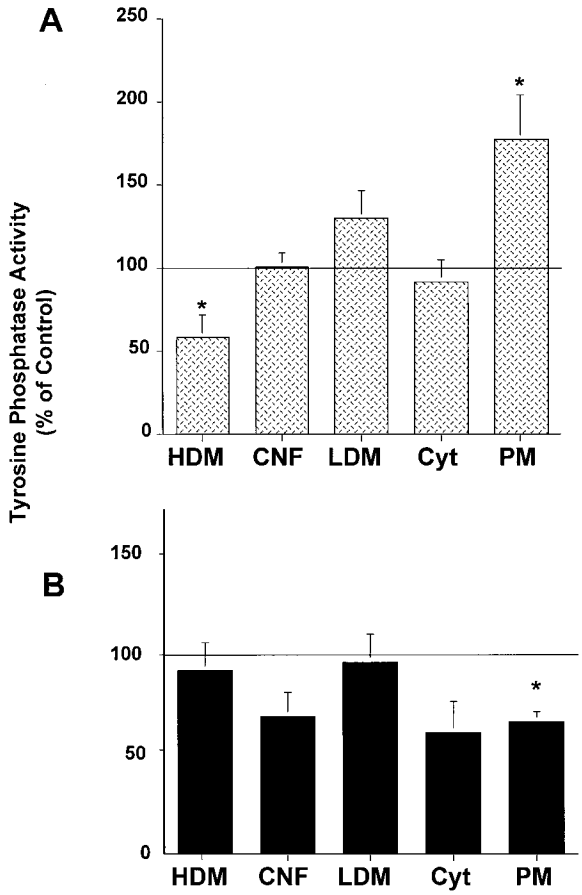

Fig. 1. Effects of (**A**) insulin, and (**B**) palmitate, on basal subcellular tyrosine phosphatase activity. Rat adipocytes were incubated with FAF or 1 mM palmitate buffer, followed by treatment without or including insulin, fractionated, and subcellular fractions were assayed for tyrosine phosphatase activity as described in the Materials and Methods. Fractions assayed were high-density microsomes (HDM), crude nuclear fractions (CNF), low-density microsomes (LDM), cytosol (Cyt), and plasma membranes (PM). All values are derived from duplicate measurements of four or five separate experiments. Data are presented as percentage tyrosine phosphatase activity of untreated, FAF-incubated fractions (control). Error bars = ±SEM. *Significantly different from untreated, FAF-incubated control cells in the respective fractions ($P < 0.006$).

(n = 5, $P < 0.004$) (Fig. 1B). Activities in cytosolic and crude nuclear fractions (CNF) showed similar decreases but were not statistically significant, while PTPase activities in the LDM and HDM were unaltered by palmitate treatment (Fig. 1B).

There was no statistical difference between the control plus insulin group and the palmitate plus insulin-treated group (data not shown). Thus, although basal inhibition of PTPase by palmitate indicates that increased protein tyrosine phosphorylation may occur, the lack of difference with insulin treatment indicates that insulin-stimulated PTPase was unaffected by palmitate.

To determine whether measured changes in subcellular PTPase activity were indicative of altered phosphatase activity within the cell, we examined the tyrosine phosphorylation status of proteins in subcellular fractions. While most of the subcellular fractions demonstrated little change in protein tyrosine phosphorylation, immunoblots of PM proteins demonstrated similar patterns of increased protein tyrosine phosphorylation in insulin-stimulated cells, with or without palmitate treatment (Fig. 2A). In addition, certain proteins displayed reductions in tyrosine phosphorylation upon treatment with both palmitate and insulin, as compared with insulin alone, as seen for the approximately 40-kDa PM protein (Fig. 2A), indicating a nonuniform effect of palmitate on protein tyrosine phosphorylation. Furthermore, a protein of similar molecular weight as the insulin receptor (~95 kDa) was hyperphosphorylated after insulin treatment of palmitate-incubated cells (Fig. 2A). The most significant changes in tyrosine phosphorylation among the subcellular fractions occurred on a protein of approximately 60-kDa (p60) found predominantly in PM fractions (Fig. 2B). While insulin treatment increased its phosphorylation 10-fold, palmitate alone increased the phosphorylation of a protein at the identical molecular weight more than 16-fold. The effects of insulin and palmitate on the tyrosine phosphorylation of this protein were not additive (data not shown). While p60 displays the most significant alteration in tyrosine phosphorylation, specificity can be seen by comparison with an insulin-sensitive 62-kDa protein in the same sample, which was unaffected by palmitate (Fig. 2B). By inducing the tyrosine phosphorylation of an insulin sensitive protein

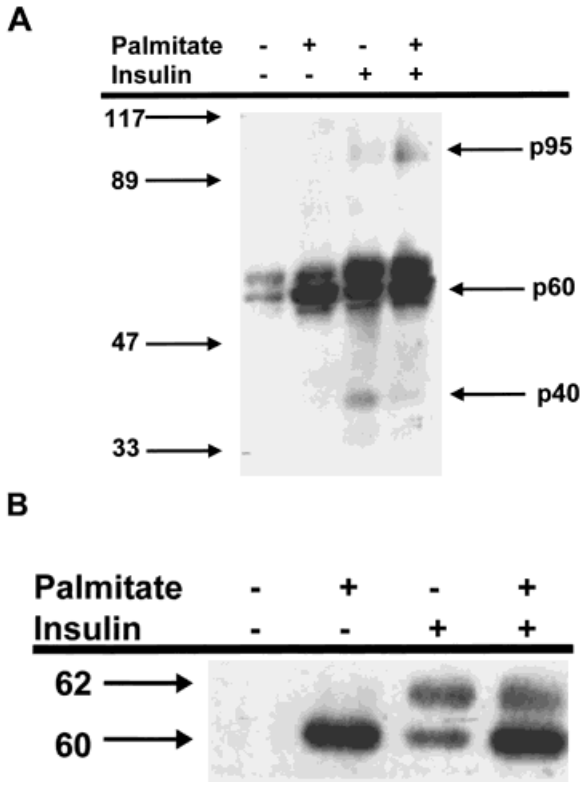

Fig. 2. Effects of palmitate and insulin on PM protein tyrosine phosphorylation. Rat adipocytes were incubated with FAF or 1mM palmitate (Palm) buffer followed by treatment with or without (±) insulin (1nM), and fractionated as described in the Materials and Methods. Data are representative of seven separate experiments. The above blots were exposed for (**A**) 10 min, and (**B**) 30 seconds, respectively. The molecular weights listed on the left panel A correspond to molecular weight markers.

(p60), and/or reducing the tyrosine phosphorylation of an insulin sensitive protein (p40), it is possible that palmitate is adversely affecting the insulin signaling cascade in such a way as to promote resistance.

Subcellular Serine/Threonine Phosphatase Activity

The majority of intracellular phosphorylation occurs on serine and threonine residues. In these experiments insulin alone significantly increased PM and CNF S/T phosphatase activity 59 ± 19% (n = 4, $P < 0.005$), and 21 ± 7% (n = 4, $P < 0.007$), respectively (Fig. 3A).

Although palmitate did not appear to affect cumulative basal adipocyte S/T phosphatase activity (see above), it specifically affected phosphatase activity in subcellular fractions. While there were no significant changes seen

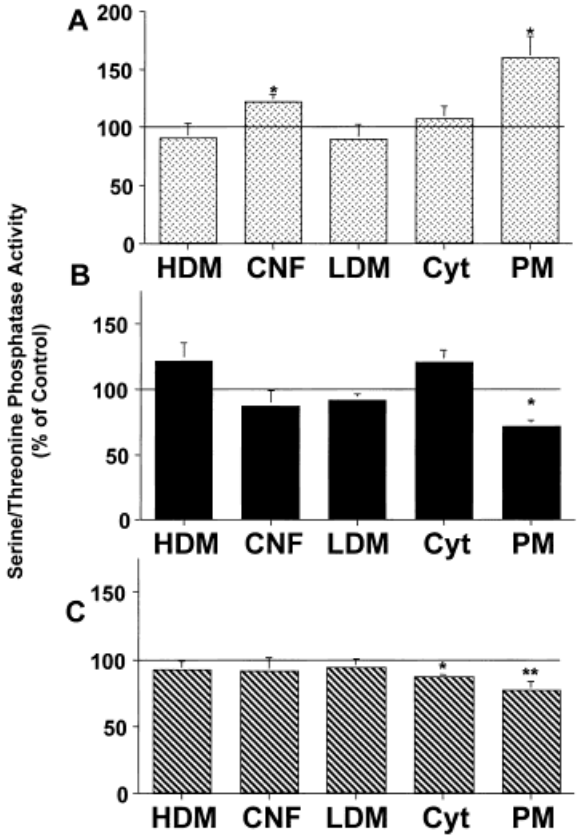

Fig. 3. Effects of (**A**) insulin and (**B**) palmitate on basal, and (**C**) palmitate on insulin-treated subcellular serine/threonine phosphatase activity. Rat adipocytes were incubated with FAF or 1 mM palmitate buffer, followed by treatment without or including insulin, fractionated, and subcellular fractions were assayed for S/T phosphatase activity as described in the Materials and Methods. Fractions assayed were high-density microsomes (HDM), crude nuclear fractions (CNF), low-density microsomes (LDM), cytosol (Cyt), and plasma membranes (PM). All values are derived from duplicate measurements of four or five separate experiments. Data are presented as percentage S/T phosphatase activity of (**A,B**) untreated, or (**C**) insulin-treated, FAF-incubated fractions (control). Error bars = ±SEM. *Significantly different from (**A,B**) untreated, or (**C**) insulin-treated, FAF-incubated cells in the respective fractions ($P < 0.009$). **($P = 0.045$).

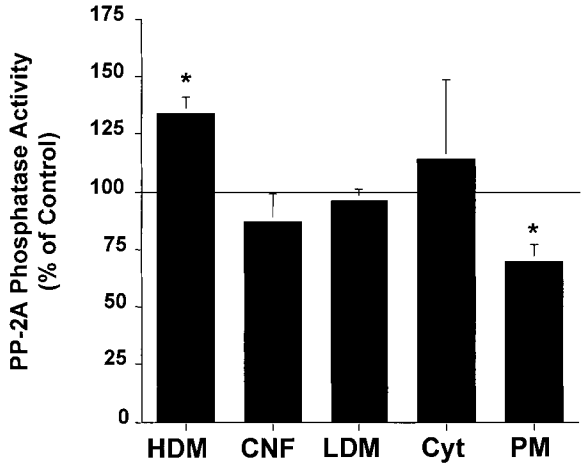

Fig. 4. Effects of palmitate alone on PP-2A activity in adipocyte subcellular fractions. Alterations in PP-2A activity were calculated from S/T phosphatase assays performed in the presence of 0 or 3 nM OA as described in the Materials and Methods. Fractions assayed were high-density microsomes (HDM), crude nuclear fractions (CNF), low-density microsomes (LDM), cytosol (Cyt), and plasma membranes (PM). Resulting values are derived from duplicate measurements of four separate experiments. Data are presented as percentage of PP-2A activity in untreated, FAF-incubated cells in similar fractions. Error bars = ±SEM. *Significantly different from untreated, FAF-incubated cells in their respective fractions ($P < 0.05$).

in S/T phosphatase activity in palmitate-treated LDM, HDM, cytosolic, and CNF fractions (Fig. 3B), basal PM phosphatase activity was significantly inhibited 28 ± 4% by palmitate (n = 5, $P < 0.004$) (Fig. 3B). Moreover, palmitate inhibited insulin-treated cytosolic and PM serine/threonine phosphatase activity by 14 ± 3% (n = 5, $P = 0.008$) and 23 ± 7% (n = 4, $P = 0.04$), respectively (Fig. 3C).

PP-2A is the phosphatase largely responsible for the regulation of hormone sensitive lipase activity [Wood et al., 1993], while PP-1 has been proposed to regulate insulin-stimulated glycogen synthesis [Skurat and Roach, 1996]. To determine which S/T phosphatase(s) was affected by palmitate, OA was used to selectively inhibit PP-2A alone, or both PP-2A and PP-1 together, as described in the Materials and Methods. PP-2A accounted for 80 ± 4% (n = 4) of the S/T phosphatase activity in each subcellular fraction except the cytosol, regardless of treatment. In cytosolic fractions, basal PP-2A activity was only 31 ± 1% (n = 4) of adipocyte S/T phosphatase activity. Insulin alone increased CNF PP-2A activity 70 ± 29% (n = 3, $P < 0.04$) and completely inhibited cytosolic PP-2A activity independent of palmitate treatment (not represented graphically). Palmitate had no significant effects on PP-2A activity in subcellular fractions from insulin-treated cells, except to slightly inhibit the insulin-induced increase in CNF, by 10 ± 2% (n = 3, $P < 0.05$, not shown). Palmitate significantly increased basal HDM PP-2A activity 34 ± 7% (n = 3, $P < 0.04$), while decreasing PM PP-2A activity 29 ± 7% (n = 4, $P < 0.025$) (Fig. 4).

Subcellular localization has been suggested to alter phosphatase activity or availability

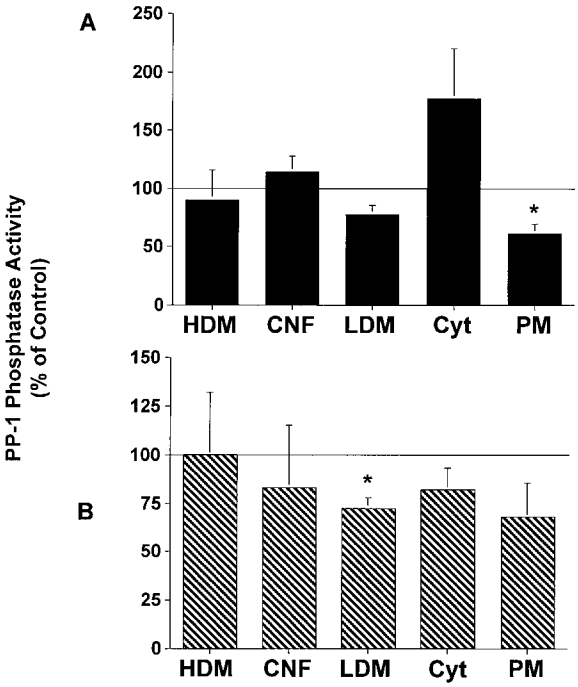

Fig. 5. Effects of palmitate on PP-1 activity in (**A**) basal and (**B**) insulin-treated adipocyte subcellular fractions. Alterations in PP-1 activity were calculated from S/T phosphatase assays performed in the presence of 3 or 300 nM OA as described in the Materials and Methods. Fractions assayed were high-density microsomes (HDM), crude nuclear fractions (CNF), low-density microsomes (LDM), cytosol (Cyt), and plasma membranes (PM). Resulting values are derived from duplicate measurements of four separate experiments. Data are presented as percentage of PP-1 activity in (**A**) untreated, or (**B**) insulin-treated, FAF-incubated cells in similar fractions. Error bars = ±SEM. *Significantly different from (**A**) untreated, or (**B**) insulin-treated, FAF-incubated cells in their respective fractions ($P < 0.014$).

[Begum and Draznin, 1992; Lester and Scott, 1997]; therefore, it was determined whether changes in PP-2A activity were due to subcellular redistribution. Western blots for PP-2A in the cytosolic fraction confirmed that insulin-stimulated inhibition of PP-2A activity was not due to translocation of PP-2A to another subcellular location (data not shown).

Alterations of subcellular PP-1 activity were also apparent with both insulin and palmitate treatment. The only fraction in which PP-1 activity was significantly changed by insulin was the cytosol (Cyt) where insulin increased PP-1 activity 160 ± 50% (n = 3, $P < 0.015$, not shown). While palmitate alone also increased cytosolic PP-1 activity, the increase was not statistically significant (Fig. 5A). However, palmitate did significantly reduce insulin-treated LDM PP-1 activity by 28 ± 6% (n = 3, $P < 0.04$, Fig. 5B). Palmitate also reduced basal (Fig. 5A) and insulin-stimulated (Fig. 5B) PM PP-1 activity; however, it was only significant under basal conditions (39 ± 7%, n = 4, $P < 0.015$).

DISCUSSION

Several studies relate the importance of phosphatase activity to the modification of signaling cascades, including those involved in insulin signaling [Chan et al., 1988; Ahmad et al., 1995; Corvera et al., 1991; Meyerovitch et al., 1991; Schecter, 1990; Rondinone and Smith, 1996; Fischer et al., 1991; Hashimoto et al., 1992]. Because of the management of phosphatases by regulatory subunits and domains that may specify subcellular localization, it is important to determine the identity and exact subcellular location of affected phosphatases. This study represents a unique and thorough characterization of adipocyte subcellular phosphatase activity in response to insulin and fatty acid-induced insulin resistance. Furthermore, efforts were made to deduce the identity of affected subcellular phosphatases. The use of enzyme-specific phosphopeptides and divalent-cation chelators excluded the activity measurement of some phosphatases (i.e., PP-2B and PP-2C); however, these peptides provided adequate sensitivity and rapid phosphatase activity measurements [Cohen et al., 1990].

It has been demonstrated that insulin can regulate the activity of both protein kinases and phosphatases [Chan et al., 1988], and evidence relating alterations in phosphatase activity to the development of insulin resistance and diabetes is abundant [Ahmad et al., 1995; Zierath et al., 1997; Begum et al., 1991; Corvera et al., 1991; Meyerovitch et al., 1991; Begum and Ragolia, 1998]. High concentrations of dietary saturated fat are affiliated with obesity, insulin resistance, and type 2 diabetes mellitus [van Amelsvoort et al., 1986; Swislocki et al., 1987; Charles et al., 1997]. Furthermore, nonesterified fatty acids have been demonstrated to induce insulin resistance in muscle, liver, and adipocytes [Hunnicutt et al., 1994; Oakes et al., 1997]. Although induction of insulin resistance had been previously demonstrated in vitro with this method at 200 μM [Hunnicutt et al., 1994], 1 mM palmitate was used to ensure maximum effects. This is a high concentration for a single fatty acid; however, myristate and stearate have been shown to

have similar effects to palmitate. Therefore, if one considers the saturated fatty acids as approximately 30% of the total, and that diabetics have reported free fatty acid concentrations higher than 5mM [Malchoff et al., 1984], it is not unreasonable to predict saturated fatty acid concentrations of 1mM in a physiological setting.

PM PTPases LAR and LRP have been implicated in the dephosphorylation of the insulin receptor kinase, and likely alter other tyrosine phosphorylated proteins found in that region [Hashimoto et al., 1992; Kulas et al., 1995; Ren et al., 1998]. In the present study, insulin alone increased PTPase activity in the PM and reduced it in the HDM, confirming similar work by others [Meyerovitch et al., 1991; Begum and Draznin, 1992]. It is tempting to speculate that the insulin-induced decrease in HDM PTPase activity may be involved in potentiating intracellular insulin receptor signaling. Internalized insulin receptors retain their kinase activity [Kahn et al., 1989]; thus, it is possible that internalized signaling by the insulin receptor in endosomes, which separate into the HDM fraction, is an important aspect of insulin action. Increased tyrosine phosphorylation of a PM protein of similar molecular weight as the insulin receptor in palmitate-treated cells may reflect the observed reduction in PM PTPase activity.

The most prominent change in tyrosine phosphorylated proteins in response to palmitate-induced insulin resistance is seen in p60. The phosphorylation of this protein appears to be insulin sensitive and is tyrosine phosphorylated more with palmitate than with insulin treatment. Multiple proteins of approximately 60 kDa have been indicated to be important in cell signaling cascades, including Src family kinases [Williams et al., 1998], p60-guanine nucleotide exchange factor-associated protein [Ogawa et al., 1995, Catipovic et al., 1996], and the insulin sensitive IRS-3 [Smith-Hall et al., 1997]. Elevated tyrosine phosphorylation is consistent with decreased PTPase activity. There was no significant effect of palmitate on PM PTPase activity, however there was a decrease of approximately 30%, which narrowly missed being significant ($P = 0.055$). Thus, it is possible that a specific PTPase is affected by palmitate. Another possibility is that p60 does not reside in the PM under basal conditions and that palmitate and insulin cause a translocation of this protein to the PM. It is not clear how palmitate-induced tyrosine phosphorylation of this insulin sensitive protein effects insulin signaling.

Type 1 protein phosphatases have been implicated in the insulin-stimulated dephosphorylation and subsequent activation of glycogen synthase, especially the glycogen associated phosphatase, PP-1G [Skurat and Roach, 1996]. PP-1 is located in the cytosol, LDM, which contains the glycogen pellet, and HDM fractions of rat adipocytes with smaller amounts found in the CNF and PM [Brady et al., 1997]. Interestingly, the only fraction that demonstrated significant stimulation of PP-1 activity with insulin treatment was the cytosol, where this stimulation was independent of palmitate treatment. This finding would suggest that palmitate is not altering insulin-stimulated glycogen synthesis, which is what is observed in this model [Van Epps-Fung et al., 1997]. Others have shown that PP-1 does not translocate in response to insulin in rat adipocytes [Brady et al., 1997]. How insulin regulates PP-1 activity in only one subcellular fraction is unknown. Nevertheless, palmitate significantly inhibited insulin-treated LDM PP-1 activity. This significant decrease is likely due to a combination of the nonsignificant decrease in PP-1 activity seen with palmitate alone and the nonsignificant increase in PP-1 observed with insulin alone (data not shown). Inhibition of glycogen synthesis by fatty acids in man is only seen after the intake of large amounts of exogenous fat for a long period of time [Saloranta and Groop, 1996]. It is possible that the PP-1 activity being affected by palmitate in the LDM is not associated with the glycogen granules, such that the long-term inhibitory effect of fatty acids on glycogen synthesis is due to an effect on an inhibitor or scaffolding protein associated with PP-1, or secondary to a decrease in glucose transport.

There are several potential mechanisms whereby palmitate may selectively inhibit phosphatase activity. It is possible that long-chain saturated fatty acids may compete for a reactive thiol-intermediate in specific cases in which they are able to access the active site. Another potential mechanism is the direct interaction of palmitate with specific motifs on phosphatases or substrates, which may subsequently protect the phosphorylated site from dephosphorylation. Spontaneous or insulin-inducible palmitoylation of specific proteins susceptible to such modifications may also pro-

vide this protection [Quesnel and Silvius, 1994; Jochen et al., 1991]. Careful biochemical studies must be done to confirm direct inhibition of PTPase activity by palmitate and determine the precise mechanism of inhibition.

In summary, the major effects of insulin on adipocyte phosphatase activity are to increase PM S/T and PTPase, CNF PP-2A and cytosolic PP-1 activities while inhibiting cytosolic PP-2A. Insulin resistance was characterized by reduced insulin-stimulated S/T phosphatase activity in the PM and LDM. Specific changes in phosphatase activity, such as those described above, may significantly alter insulin signaling, contributing to the development of insulin resistance.

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