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INSULIN-DEPENDENT PHOSPHATIDYLINOSITOL 3'-KINASE ACTIVITY CO-PRECIPITATES WITH INSULIN RECEPTOR IN HUMAN CIRCULATING MONONUCLEAR CELLS

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SUMMARY : In order to establish a working cellular model for the study of post-receptor signaling events, insulin-dependent phosphatidylinositol 3' (PtdIns 3')-kinase activity was investigated in circulating mononuclear cells from normal subjects. The p85α regulatory subunit of PtdIns 3'-kinase co-precipitated with activated insulin receptor as revealed by immunoblotting. Whereas insulin receptor substrate-1 was weakly detected, insulin increased 5.5±1.5-fold (mean±SD) PtdIns 3'-kinase activity in receptor immunoprecipitates. We conclude that insulin-stimulated PtdIns 3'-kinase activity is measurable in circulating mononuclear cells which may constitute an easily available cellular model for the detection of post-receptor defects in insulin-resistant states.

Insulin signaling is initiated at the cellular level by activation of the insulin receptor tyrosine kinase and tyrosine phosphorylation of intracellular protein substrates (1, 2). A 165-185 kDa protein, initially called pp185 and more recently renamed insulin receptor substrate-1 (IRS-1), has been identified as a major insulin receptor substrate which undergoes tyrosine phosphorylation immediately after insulin stimulation (3, 4). Several of the twenty-one potential tyrosine phosphorylation sites of IRS-1 are followed by specific sequence motifs which are capable of specific binding to *src* homology domains 2 (SH2) of various intracellular proteins (5-7). Among these, phosphatidylinositol 3' (PtdIns 3')-kinase, composed of a 85kDa regulatory subunit (p85α) and a 110 kDa catalytic

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subunit (p110), has been the most studied, both in intact cells and in cell free systems (8-12). PtdIns 3'- kinase plays an important role in the regulation of cell growth, insulin- or IGF-1-induced membrane ruffling, p $70^{S6kinase}$ activation and translocation of glucose transporters (13, 14). Insulin appears to stimulate the PtdIns 3'-kinase activity in most intact cells by binding of SH2 domains of p85α to phosphorylated Tyr-(Met/Xaa)-Xaa-Met motifs of IRS-1 (9-12). Both phosphorylated IRS-1 and tyrosine phosphopeptides corresponding to IRS-1 phosphorylation sites are able to stimulate *in vitro* the activity of PtdIns 3'-kinase (11, 15). In addition, direct activation of PtdIns 3'-kinase through the interaction of p85α with the [Tyr^{1322}]-Thr-His-Met motif at C-terminal end of the β-subunit of insulin receptor has also been reported as an alternative pathway of insulin signaling (16). IRS-1 expression and phosphorylation, as well as PtdIns 3'-kinase activation, have shown to be severely affected in liver and muscle from both genetic and acquired insulin resistant animal models (17-19). Similar investigations in humans have been limited, mainly because major insulin target tissues are not easily accessible (20).

Circulating mononuclear cells (CMC) are easily available from blood samples and have been extensively used in human studies. These cells contain both insulin and IGF-1 receptors and reflect *in vivo* alterations of insulin receptor number, affinity and kinase activity in various pathological situations (21-23). However, post-receptor signaling mechanisms have not been studied in monocytes and little is known about the specificity of insulin transduction in these cells. In the present study, we have investigated PtdIns 3'-kinase activity in insulin-stimulated freshly isolated CMC from normal subjects. We report here for the first time that PtdIns 3'-kinase association with insulin receptor and enzymatic activity can be measured in monocytes.

MATERIALS AND METHODS

Materials

Polyclonal antiphosphotyrosine antibodies (αPY) and anti-β-subunit of the insulin receptor (C-terminal) antibodies were kindly provided by M.F. White (Joslin Diabetes Center, Boston, MA). Monoclonal antibodies (mouse IgG, clone 29B4) against insulin receptor were from Oncogene Science (Manhasset, NY, USA). Polyclonal rabbit antiserum (or purified IgG) against PtdIns 3'-kinase (N-terminal SH2 domain of p85α subunit) were from UBI (Lake Placid, NY, USA). Anti-rat IRS-1 antibodies used for immunoprecipitation were from UBI (chromatographically purified and immobilized sequence). Protein G- and A-Sepharose (4 Fast Flow) were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Protein A IgG purification kit was from Pierce (Rockford, IL, USA). All other reagents were of analytical or sequence grade.

Preparation of anti-peptide antibodies to IRS-1

Peptides corresponding to putative phosphorylation sites of IRS-1 were obtained by solid phase peptide synthesis as previously described (10). IgG against peptides corresponding to sites Tyr ^{46} (near the N-terminus) and Tyr ^{658} (in the

middle of the molecule) were raised in rabbits and purified from sera using protein A IgG purification kit. The reactivity of anti-peptides antibodies in immunoblotting and immunoprecipitation was investigated using baculovirus-expressed IRS-1 (manuscript in preparation). Preimmune IgGs were used as controls for immunoprecipitation.

Cell preparation

Mononuclear cells from peripheral blood (200 ml drawn into 12 IU/ml heparin) of normal adult donors were separated by gradient centrifugation through diatrizoate-ficoll as previously described (24). The separation procedure yielded enriched population of monocytes up to 15 to 20% with more than 90% viability as determined by Trypan blue exclusion test.

Cell solubilization and immunoprecipitation procedures

Samples containing $4x10^6$ monocytes in RPMI 1640 medium were incubated with insulin (10 μg/ml) at 37°C. Cells were then separated by centrifugation, washed with ice-cold PBS and solubilized for 30 min in 200 μl ice-cold extraction buffer containing 50 mM HEPES (pH 7.4), 10 mM Na_3VO_4, 2mM EDTA, 135 mM NaCl, 2 μg/ml aprotinin and 5 μg/ml leupeptin, 34 μg/ml PMSF and 1% Nonidet P-40 (final concentrations). The cell lysate was cleared by centrifugation at 15,000 x g for 15 min at 4°C and incubated with monoclonal anti-insulin receptor antibodies (1 μg) and protein G-Sepharose for 8 h at 4°C or, alternatively, with antibodies against IRS-1 (10 μg) or p85α (5 μg) and protein A-Sepharose. Immune complexes were washed three times with 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 100 μM Na_3VO_4 and 150 mM NaCl.

Dissociation and re-immunoprecipitation of p85α from insulin receptor immunoprecipitates

Insulin receptor immunoprecipitates were dissociated in 25 μl ice-cold extraction buffer without NP-40 but containing 2% SDS and incubated for 20 min with agitation. Samples were then diluted up to 0.1% SDS (final concentration), BSA was added to a final concentration of 0.1%, and the protein G-Sepharose beads were pelleted by centrifugation. Cleared supernatants were incubated with anti-p85α antibodies for 8 h at 4°C. Immune complexes were precipitated with protein A-Sepharose, washed and processed as described below.

Western blotting

Immunoprecipitated proteins were separated in SDS-PAGE (7.5% acrylamide) and transfered to nitrocellulose. Nitrocellulose strips were incubated for 8 h at 4°C in blocking solution containing 20 mM Tris, 150 mM NaCl, 0.01% (v/v) Tween 20 and 3% BSA. The primary antibodies were incubated in the blocking solution at 2 μg/ml for 8 h at 4°C and further steps were followed for ECL detection as recommended by the manufacturer (Amersham).

Phosphatidylinositol 3 '(PtdIns 3') -kinase assay

Cell lysates obtained after insulin stimulation of $4x10^6$ monocytes were incubated with monoclonal anti-insulin receptor antibodies (1 μg) and protein G-Sepharose or anti-IRS-1 antibodies (10 μg) and protein A-Sepharose for 8 h at 4°C. Immunoprecipitates were washed and the PtdIns 3'-kinase reaction was performed as previously described (25). The samples obtained after PtdIns 3'-kinase reaction were centrifuged and the lower organic phase was removed and applied to silica gel TLC plates coated with 1% potassium oxalate. TLC plates were developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (60 : 47 : 11.3 : 2), dried and visualized by autoradiography. Activity was measured by scanning densitometry of spots that co-migrated with a PI-4 standard and was expressed in arbitrary units.

RESULTS AND DISCUSSION

Since monocytes are known to contain insulin receptors, we first attempted to identify the phosphorylation of the insulin receptor and its substrate IRS-1. CMC lysates were immunoprecipitated with either anti-insulin receptor, anti-rat IRS-1 or anti-(Tyr^{46}) IRS-1 antibodies and then alternatively immunoblotted with αPY, anti-insulin receptor, anti-rat IRS-1 or anti-(Tyr^{658}) IRS-1 antibodies. Immunoblots with αPY antibodies revealed tyrosine phosphorylation of a 95 kDa protein in receptor precipitates after insulin stimulation (Figure 1, lane b) whereas no clear tyrosine phosphorylated 185 kDa protein could be detected in IRS-1 precipitates (data not shown). The 95 kDa phosphoprotein represents the β-subunit of the insulin receptor, as confirmed by immunoblotting of the same precipitates with anti-insulin receptor antibodies (Figure 1, lane c). A weak 185 kDa protein band could be detected by immunoblotting the anti(Tyr^{46}) IRS-1 precipitates with anti-(Tyr^{658}) IRS-1 antibodies (Figure 1, lane d). Control immunoprecipitations with equivalent amounts of rabbit non-specific immunoglobulins G did not precipitate either the insulin receptor or any 185 kDa protein (Figure 1, lane e).

To investigate the association of the p85α subunit of PtdIns 3'-kinase with insulin receptor and IRS-1, intact monocytes were stimulated with insulin, lysed, immunoprecipitated with either anti-insulin receptor or anti-(Tyr^{46})IRS-1 antibodies and then immunoblotted with anti-p85α antibodies (Figure 2). The amount of p85α co-precipitated with the insulin receptor was increased 7-fold after insulin treatment (lane b). The receptor-associated 85 kDa protein was further confirmed as being PtdIns 3'-kinase regulatory subunit by an

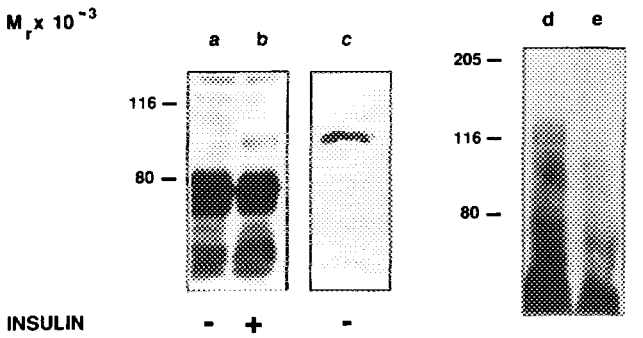

Figure 1. **Identification by Western blotting of insulin receptor and IRS-1 in monocytes.** Samples of 4 x 10^6 monocytes were stimulated by insulin for 5 min as described in Materials and Methods. Insulin receptor was immunoprecipitated with monoclonal anti-insulin receptor antibodies and protein G-Sepharose (lanes a, b, c) and IRS-1 with anti-(Tyr^{46})IRS-1 antibodies (lane d) or preimmune IgG (lane e) and protein A-Sepharose. Immunoblotting was performed alternatively with αPY (lanes a, b), anti-insulin receptor antibodies raised against C-terminal end of the β-subunit (lane c) or anti-(Tyr^{658})IRS-1 antibodies (lanes d, e).

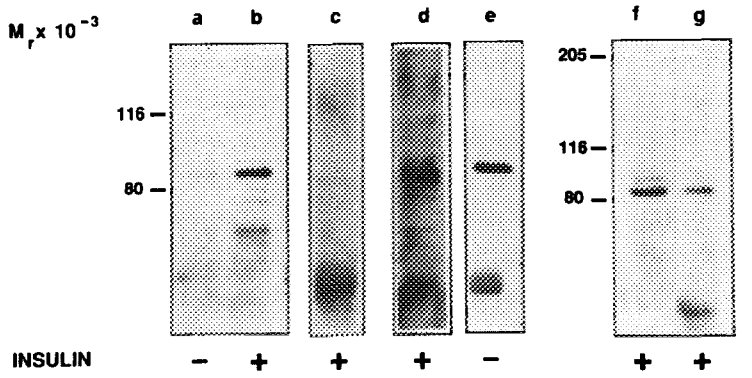

Figure 2. **Association of p85α subunit of PtdIns 3'-kinase with insulin receptor and IRS-1 in intact human monocytes.** 4 x 10^6 monocytes were stimulated for 5 min as described in Materials and Methods. PtdIns 3'-kinase was revealed in immunoprecipitates by Western blotting with anti-p85α antibodies. Homogenates were immunoprecipitated with monoclonal anti-insulin receptor antibodies (lanes a, b) or pre-immune IgG (lane c) and protein G-Sepharose or anti-p85α antibodies and protein A-Sepharose (lane e). Western blotting was also performed after dissociation of proteins from insulin receptor immunoprecipitates and re-immunoprecipitation with anti-p85α antibodies (lane d). Immunoblotting was also performed after sequential immunoprecipitations with anti-insulin receptor (lane f) and anti-IRS-1 antibodies (lane g).

immunoprecipitation with anti-p85α antibodies of SDS-dissociated immunoprecipitates obtained in the presence of insulin (lane d) and also by comparison with the 85 kDa band obtained by p85α immunoprecipitation of total cell lysates (lane e). Very low amounts of p85α were detected in IRS-1 precipitates of stimulated cells (data not shown).

To confirm the prevailing co-precipitation of p85α with insulin receptor, a sequential immunoprecipitation of cell lysate with anti-insulin receptor and then with anti-IRS-1 antibodies was performed. This experiment showed that after insulin stimulation a much larger amount of p85α was detected in insulin receptor than in IRS-1 precipitates (Figure 2, lanes f and g).

To assess whether p85α association with insulin receptor and IRS-1 is followed by the activation of the catalytic subunit of PtdIns 3'-kinase, phosphorylation of phosphatidylinositol was assayed in immunoprecipitates with anti-insulin receptor or anti-IRS-1 antibodies. PtdIns 3'-kinase activity in insulin receptor precipitates from insulin stimulated cells was 5.5±1.5-fold (mean±SD) increased as compared to basal level, whereas that in IRS-1 precipitates was weakly detectable (Figure 3). This is consistent with the much greater amount of PtdIns 3'-kinase p85α associated with insulin receptor as compared to IRS-1 in extracts of monocytes (see Figure 2, lanes f and g).

In this report we provide evidence for the formation of molecular complexes associating the activated insulin receptor and p85α subunit of PtdIns 3'-kinase and for the PtdIns 3'-kinase activation in human CMC following insulin

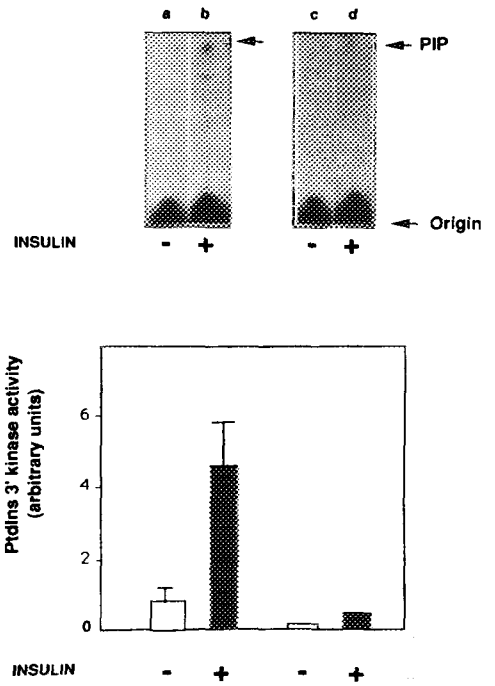

Figure 3. **Insulin-stimulated PtdIns 3'-kinase activity in monocytes.** 4 x 10^6 monocytes were stimulated for 5 min and the PtdIns 3'- kinase activity was measured as described in Materials and Methods in immunoprecipitates using anti-insulin receptor and protein G-Sepharose (lanes a, b) or anti-IRS-1 antibodies and protein A-Sepharose (lanes c, d). Upper panels are autoradiographies of TLC plates. PIP refers to PtdIns-P. Lower panel represents values (mean±SD of 4 experiments) obtained after scanning densitometry and are expressed in arbitrary units.

stimulation. Although the CMC preparation used in this study was not exclusively composed of monocytes, it has been previously established that, in mixed mononuclear preparations, monocytes account for more than 90 per cent of the expressed insulin receptors (24). Since the formation of PtdIns 3'-kinase molecular complexes was predominantly detected in the experiments using precipitation of the insulin receptor, the signaling events described in this study most likely occurred in monocytes.

Insulin-dependent association of tyrosine phosphorylated insulin receptor and PtdIns 3'-kinase can be regarded as functionally important considering that PtdIns 3'-kinase has been involved in glucose uptake which is insulin-stimulated in monocytes (26, 27). By such association, the PtdIns 3'-kinase is presumably recruited from the cytosol to a location near the plasma membrane, i.e. close to its phosphatidylinositide substrates (6). Although PtdIns 3'-kinase has been shown to be involved in the translocation of GLUT 4 glucose transporters (14) which are not expressed in monocytes, we may speculate that PtdIns 3'-kinase could play a role in glucose uptake mediated by GLUT 1 or GLUT 3 isoforms which are present in these cells and have been shown insulin-responsive in other cell types (28).

Our data suggest that in CMC the preferential partition of PtdIns 3'-kinase is in complexes with insulin receptor rather than IRS-1. The very low concentration of IRS-1 detected in CMC, consistent with previously reported results (29), may promote the association of p85α with receptor complexes. One may speculate that p85α directly associates with the insulin receptor via the [Tyr^{1322}]-Thr-His-Met motif of C-terminal domain of the β-subunit of insulin receptor as previously reported (6, 16). More recently, the role of a "third component" other than IRS-1 facilitating p85α association with insulin receptor through its binding to phosphorylated tyrosine residues of the receptor has been suggested (30).

In conclusion, the identification of PtdIns 3'-kinase co-precipitation with insulin receptor and the possibility to quantify PtdIns 3'-kinase activity in human monocytes upon insulin stimulation indicate that these cells may represent a novel cellular model for the investigation of insulin signaling in humans and for the study of post-receptor signaling defects in insulin resistant states.

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REFERENCES

1. Kasuga, M, Karlson, FA, Kahn, CR (1982) Science 215, 185-187.
2. White, MF, Kahn, CR (1994) J. Biol. Chem. 269, 1-4.
3. White, MF, Maron, R, Kahn, CR (1985) Nature (Lond) 318, 183-186.
4. Sun, XJ, Rothenberg, P, Kahn, CR, Backer, JM, Araki, E, Wilden, PA, Cahill, DA, Goldstein, BJ, White, MF (1991) Nature 352, 73-77.
5. Myers, MG, White, MF (1993) Diabetes 42, 643-650.
6. Cantley, LC, Auger, KR, Carpenter, C, Duckworth, B, Graziani, A, Kapeller, R, Soltoff, S (1991) Cell 64, 281-302.
7. Songyang, Z, Schoelson, SE, Chaudhuri, M, Gish, G, Pawson, T, Haser, WG, King, F, Roberts, T, Ratnofsky, S, Lechleider, RJ, Neel, BG, Birge, RB, Fajardo, JE, Chou, MM, Hanafusa, H, Schaffhausen, B, Cantley, LC (1993) Cell 72, 767-778.
8. Carpenter, LC, Duckworth, BC, Auger, KR, Cohen, B, Schaffhausen, BS, Cantley, LC (1990) J. Biol. Chem. 265, 10704-10711.
9. Backer, JM, Myers, MG, Jr, Shoelson, SE, Chin, DJ, Sun, XJ, Miralpeix, M, Hu, P, Margolis, B, Skolnik, EY, Schlessinger, J, White, MF (1992) EMBO J. 11, 3469-3479.
10. Yonezawa, K, Ueda, H, Hara, K, Nishida, K, Ando, A, Chavanieu, A, Matsuba, H, Shii, K, Yokono, K, Fukui, Y, Calas, B, Grigorescu, F, Dhand, R, Gout, I, Otsu, M, Waterfield, MD, Kasuga, M (1992) J. Biol. Chem. 267, 25958-25966.
11. Myers, MG, Jr, Backer, JM, Sun, XJ, Shoelson, S, Hu, P, Schlessinger, J, Yoakin, M, Schaffhausen, B, White, MF (1992) Proc. Natl. Acad. Sci. (USA) 89, 10350-10354.

12. Backer, JM, Myers, MG, Jr, Sun, XJ, Chin, DJ, Shoelson, SE, Miralpeix, M, White MF (1993) J. Biol. Chem. 268, 8204-8212.
13. Kotani, K, Yonezawa, K, Hara, K, Ueda, H, Kitamura, Y, Sakaue, H, Ando, A, Chavanieu, A, Calas, B, Grigorescu, F, Nishiyama, M, Waterfield, MD, Kasuga, M (1994) EMBO J. 13, 2313-2321.
14. Cheatham, B, Vlahos, CJ, Cheatham, L, Wang, L, Blenis, J, Kahn, CR (1994) Mol. Cell. Biol. 14, 4902-4911.
15. Backer, JM, Myers, MG, Jr, Shoelson, SE, Chin, DJ, Sun, XJ, Miralpeix, M, Hu, P, Margolis, B, Skolnik, EY, Schlessinger, J, White, MF (1992) EMBO J. 11, 3469-3479.
16. Van Horn, DJ, Myers, MG, Backer, JM (1994) J. Biol. Chem. 269, 29-32.
17. Heydrick, SJ, Jullien, D, Gautier, D, Tanti, J F, Giorgetti, S, Van Obberghen, E, Le Marchand-Brustel, Y (1993) J. Clin. Invest. 91, 1358-1366.
18. Folli, F, Saad, MJA, Backer, JM , Kahn, CR (1993) J. Clin. Invest. 91 1787-1794.
19. Saad, MJA, Folli, F, Kahn, JA , Kahn, CR (1993)J. Clin. Invest. 91, 2065-2070.
20. Thies, RS, Molina, JM, Ciaraldi, TP, Freidenberg, GR, Olefsky, JM (1989) Diabetes 39, 250-259.
21. Grunberger, G, Zick, Y , Gorden, P (1983) Science 223, 932-934.
22. Kooijman, R, Willems, M, De Haas, CJC, Rijkers, G, Schuurmans, A, LG, Van Buul-Offers, SC, Heijnen, CJ, Zegers, BJ (1992) Endocrinology 131, 2244-2250.
23. Klein, HH, Kowalevski, B, Drenckhan, M, Neugebauer, S, Matthaei, S, Kotzke, G (1993) Diabetes 42, 883-890.
24. Schwartz, RH, Biano, AR, Handwerger, BS , Kahn, CR (1975) Proc. Nat. Acad. Sci. USA 72, 474-478.
25. Ruderman, N, Kapeller, R, White, MF, Cantley, LC (1990) Proc. Natl. Acad. Sci. USA 87, 1411-1415.
26. Bieger, W, Weicher, H, Michl, J (1980) J. Clin. Endocr. Metab. 50, 1121-1126.
27. Daneman, D, Zinman, B, Elliott, ME, Bilan, PJ, Klip, A (1992) Diabetes 41, 227-234.
28. Estrada, DE, Elliott, E, Zinman, B, Poon, I, Liu, Z, Klip, A, Daneman, D (1994) Metabolism 43, 591-598.
29. Zoppini, G, Galante, P, Zardini, M, Muggeo, M (1993) Exp. Clin. Endocrinol. 101(Suppl.2), 200-201.
30. Liu, R, Livingston, JN (1994) Biochem.J. 297, 335-342.