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Stimulation of F_A and phosphatase-1 activities by insulin in 3T3-L1 cells

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The phosphatase-1 activator F_A and phosphatase-1 were assayed in 3T3-L1 cells exposed to insulin The cytosolic F_A activity was transiently stimulated (7–8-fold) 1 and 2 min after exposure to 10^{-8} M insulin and returned to control values within 5–10 min Cytosolic phosphatase-1 (assayed after trypsin treatment) was activated (120–140% of controls) between 2 and 5 min and returned to control values within 10 min Insulin effects were dose-dependent, with maximum stimulation of both activities at 10^{-8} M insulin The possibility that F_A and other kinases mediate phosphatase activation by insulin is discussed

Protein phosphatase, Phosphorylation, Insulin, Hormone

1. INTRODUCTION

The anabolic effects of insulin are accompanied by decrease in phosphorylation of various target proteins (reviewed in [1]). However, only recently it was shown that insulin can activate soluble protein phosphatase 1 (Pase-1) in 3T3-D1 cells [2] and in Swiss mouse 3T3 cells [3]. In some cells, but not in others [3] Pase-1 is also activated by PDGF [2] or EGF [2,4].

The cytosolic Pase-1 is purified as inactive complex with I-2 (reviewed in [5]). Such complex is activated either by trypsin and Mn^{2+} [6] or by the kinase F_A in a reaction that involves phosphorylation of I-2 and conversion of inactive catalytic subunit into active conformation [6,7]. Pase-1 activation by F_A is enhanced if I-2 has been previously phosphorylated by casein kinase II (CK-II) [8], while casein kinase I (CK-I) may have an inhibitory role on such activation (reviewed in [9]).

The mechanism by which insulin activates Pase-1 is poorly understood. It is possible that F_A is part of a cascade mechanism triggered by insulin. Also the other casein kinases [9] might contribute to such activation, and specifically CK-II, which is also activated by insulin [10,11]. In the present work the ability of insulin to stimulate F_A and Pase activities was tested in 3T3-L1 cells, a line chosen because of the abundance in insulin receptors.

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Dedicated to the memory of Steve McNall as ideal continuation of his work

Abbreviations. Pase, protein phosphatase; Pase-1, protein phosphatase type-1, I-2, phosphatase inhibitor 2, also called modulator, F_A, kinase that activates Pase-1, identical to glycogen synthase kinase 3, CK-II, casein kinase II, CK-I, casein kinase I; PDGF, platelet-derived growth factor, EGF, epidermal growth factor, IGF-1, insulin-like growth factor 1, IBMX, 3-isobutyl-1-methylxanthine, PMSF, phenylmethylsulfonyl fluoride, βME, β-mercaptoethanol; BSA, bovine serum albumin

2. MATERIALS AND METHODS

2 1 *Materials*

Tissue culture media and supplements were from Gibco Laboratories IGF-1 was from Collaborative Research Dexamethasone and IBMX were from Aldrich. Insulin (cell culture grade) and Triton X-100 were from Boehringer The other chemical reagents were from Sigma

2 2 *Cell culture and insulin treatment*

3T3-L1 cells were grown to confluence on 150-mm culture plates [2] and differentiated into adipocytes by supplementing the medium for 3 days with 1 μM dexamethasone, 0.5 mM IBMX and 0 25 mM IGF-1 [12] On day 5 the cells were deprived of serum for 12 h and then exposed to fresh serum-deprived medium with or without insulin

2 3 *Cell homogenate and fractionation*

After washing the plates twice with 5 ml of cold saline the cells were collected by scraping with a rubber policeman at 4°C in 1 ml 5 mM EDTA, 2 mg/ml deionized oyster glycogen, 2 mM benzamidine, 0.2 mM PMSF, 15 mM βME, 4 mg/ml leupeptin, pH 7 5 and disrupted with 20 strokes of a tight Dounce homogenizer Each homogenate used one plate and was immediately frozen in dry ice/ethanol mixture and stored at −70°C Homogenates from each experiment were thawed together and centrifuged at 100000 × g for 20 min at 4°C The supernatant thus obtained, defined as cytosolic fraction, was assayed immediately for F_A and Pase activities The particulate fraction was prepared by resuspending the pellet in extraction buffer added with 0 5% Triton X-100 and 0 2 M NaCl, sonicating for 10 s and recentrifuging as above

2 4. *Enzyme and protein assays*

Pase was assayed by the release of $^{32}P_i$ from [^{32}P]phosphorylase *a* (2–4 × 10^5 cpm/nmol) with or without trypsin-treatment (20 μg/ml trypsin for 5 min at 30°C) [6] F_A was assayed by reactivation of inactive Pase-1 Each assay contained 20 μl of cytosolic or particulate

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fraction, the amount of totally inactive Pase-1 (purified catalytic subunit-I-2 complex [6]) that would release 1 nmol of P_i/min when assayed after trypsin and Mn^{2+} activation [6], 0.5 mM ATP and 1 mM $MgCl_2$ diluted in phosphatase assay buffer [6] (25 μl final volume) After 2 min preincubation at 30°C 25 μl of 3 mg/ml labelled phosphorylase *a* were added for a 2 min phosphatase assay Blanks containing all but inactive Pase-1 were run in parallel with each assay F_A activity was calculated from the P_i release per min after subtracting blanks. Protein was determined by the method of Bradford [13] using BSA as standard

3. RESULTS AND DISCUSSION

The first question addressed was whether insulin was able to activate Pase in differentiated 3T3-L1 cells. Pase was assayed as spontaneous and trypsin-stimulated activity on cytosolic and particulate fractions. In such fractions Pase-1 represented 85–90% of the total Pase activity, as detected by assaying Pase in the presence of I-2. It was found that when the cells were exposed to 10^{-8} M insulin the cytosolic trypsin-stimulated Pase activity was increased (average 122% of control value) between 2 and 5 min and returned to basal level within 10 min (fig.1). 10^{-8} M insulin was the concentration that gave maximum Pase stimulation (145% in fig.2). Pase in the particulate fraction was not stimulated by insulin (not shown). Trypsin-treatment of Pase was required to detect significant insulin effect. It is known that trypsin would remove I-2 from purified Pase-1, allowing to assay the active catalytic subunit that was previously inhibited, but would not convert inactive catalytic subunit into active [6,7]. Consequently the results might indicate that insulin induced activation of catalytic subunit, which however was still inhibited by being bound to I-2 until this was removed by trypsin. The insulin effect became undetectable when I-2 was added to the assay mixture after trypsin treatment (not shown), thus confirming that the Pase stimulated by insulin is of type 1 [2,4]. The present results show that also in 3T3-L1 cells cytosolic Pase-1 is transiently activated by insulin. Such activation is similar to that reported for 3T3-D1 cells (140% of control value 5 min after exposure to ~10^{-8} M insulin [2]). On the other hand it seems to be different from the activation of S6 Pase (in Swiss mouse 3T3 cells [3]), which required less insulin (10^{-11} M) and 2 h for maximum stimulation.

The next question addressed was whether also F_A was activated by insulin at the same time as Pase-1. F_A was assayed on the same fractions used for Pase assay by its ability to reactivate exogenous inactive Pase-1. Such functional assay does not allow to discriminate between F_A and casein kinases that might contribute to or interfere with Pase-1 activation (see section 1). Such caveat must be kept in mind when interpreting the results. It was found that cytosolic fractions from cells exposed to 10^{-8} M insulin for 1 and 2 min were able to reactivate exogenous Pase-1 7–8-fold better than the corresponding fractions from untreated cells (fig.3).

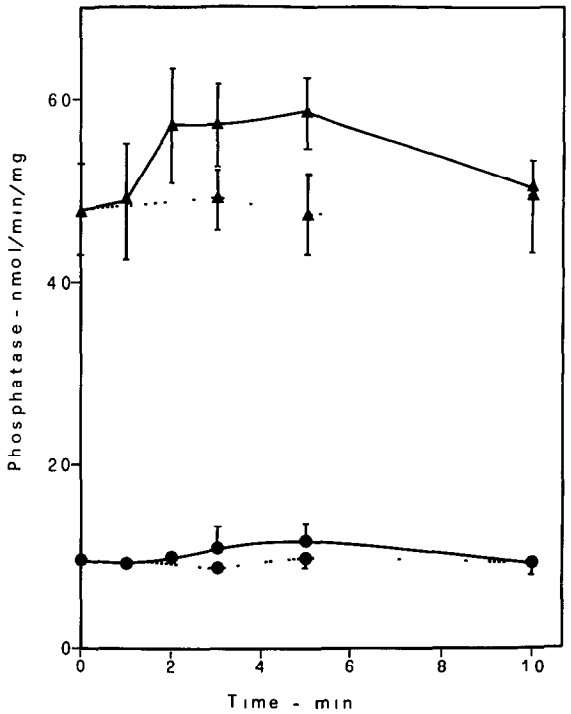

Fig.1 Time course of the effect of insulin on cytosolic Pase activity in 3T3-L1 cells. Serum-starved cells were exposed to serum-free medium with (——) or without (– – –) 10^{-8} M insulin at 37°C for the time indicated Pase was assayed as spontaneous (●) or as trypsin-stimulated (▲) activity. Values are means of 4 independent experiments ± SE

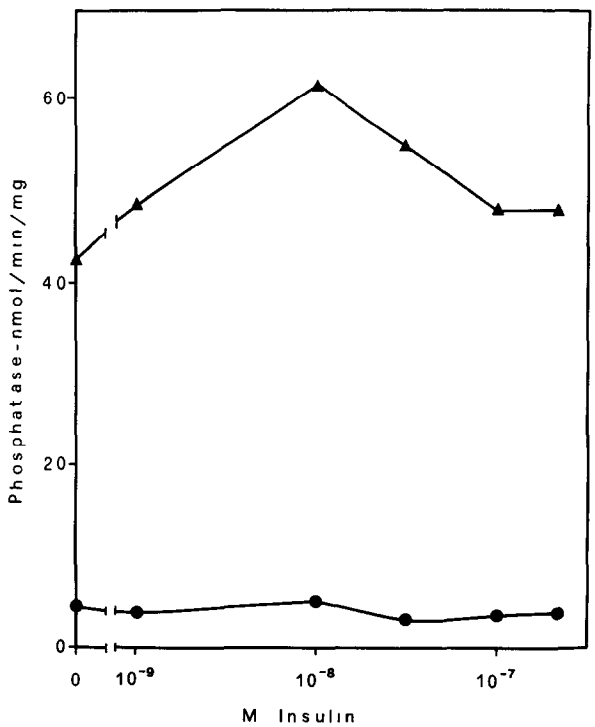

Fig.2. Concentration dependence of insulin stimulation of cytosolic Pase activity in 3T3-L1 cells Serum-starved cells were exposed for 3 min at 37°C to serum-free medium containing the indicated amounts of insulin Pase was assayed as spontaneous (●) or trypsin-stimulated (▲) activity.

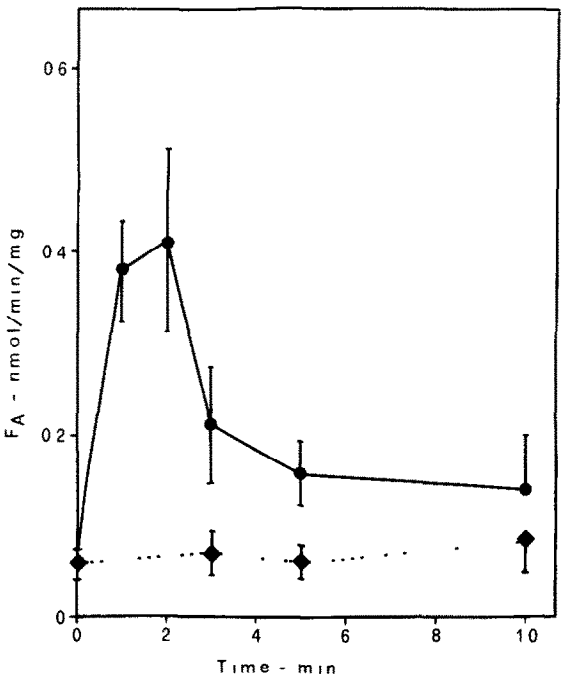

Fig 3. Time course of the effect of insulin on cytosolic F_A activity in 3T3-L1 cells. The same extracts as in fig 1 were used Serum-starved cells were exposed to serum-free medium with (●—●) or without (♦---♦) 10^{-8} M insulin at 37°C for the time indicated. Values are means of 4 independent experiments ± SE

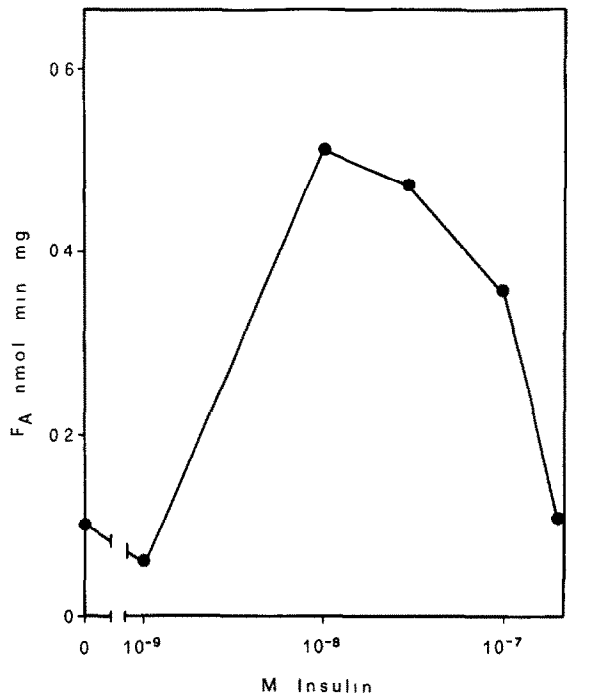

Fig.4 Concentration dependence of insulin stimulation of cytosolic F_A activity in 3T3-L1 cells. Serum-starved cells were exposed for 3 min at 37°C to serum-free medium containing the indicated amounts of insulin

With longer exposure to insulin the F_A activity dropped (3 min) and returned to basal values within 10 min. The best F_A response was obtained with 10^{-8} M insulin (fig.4). Some F_A activity was also detected in the particulate fraction, but it was not affected by insulin (not shown) and there was no indication of the F_A translocation reported in other systems [13,14].

The above results show that insulin induced transient increase in the ability of cytosolic fractions to activate Pase-1. This may be due to the presence of activated kinase F_A, with or without the contribution of the casein kinases. The relevance of such contributions cannot be decided on the basis of the present results. The detected increase in F_A activity seems to be related to Pase activation in several ways: (i) as with Pase activation, the increase in F_A activity was reversible and insulin dose-dependent, with coincident optimal insulin concentrations for both activities; (ii) the increase in F_A activity preceded Pase activation by 1 min and was lost earlier; this would agree with the hypothesis that such activity is involved in the activation of Pase; (iii) although F_A activity was increased much more (7–8-fold) than Pase (122% average), in terms of absolute activity (compare figs 1 and 3) the increase in Pase is more than one order of magnitude higher than the increase in F_A; such 'signal amplification' would be in agreement with the hypothesis that F_A is part of a cascade triggered by insulin to activate Pase.

In conclusion it was found that activation of Pase-1 by insulin was preceded and accompanied by an increase in the ability of the cytosolic fraction to activate Pase-1. The respective contribution of F_A and of other kinases to such activation is presently under investigation.

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