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Insulin activates myelin basic protein (p42 MAP) kinase by a protein kinase C-independent pathway in rat adipocytes

Dissociation from glucose transport

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Myelin basic protein kinase (MBPK) activity of rat adipocytes was measured directly or in gels after purification of p42 microtubule-associated protein kinase (MAPK). Insulin and phorbol esters provoked 2- to 3-fold increases in MBPK/MAPK activity within 5–10 min. Whereas phorbol ester effects were blocked by protein kinase C (PKC) depletion or inhibition, insulin effects were fully intact, indicating that insulin activates MBPK/MAPK independently of PKC. In contrast, PKC depletion or inhibition markedly inhibited insulin effects on [^{3}H]2-deoxyglucose uptake, suggesting that this effect requires PKC, rather than a factor within the ras/MAPK cascade.

Insulin; Adipocyte; MAP kinase; Phorbol ester; Protein kinase C; Glucose transport

1. INTRODUCTION

Insulin activates serine/threonine PK, including MAPK [1] and PKC [2,3] in rat adipocytes. Frequently, these PK pathways are interdependent, e.g. phorbol esters may activate MAPK via PKC-mediated activation of *ras* or a PK upstream of MAPK [4,5], or, *ras* may activate phosphatidylcholine hydrolysis [6,7] and thus PKC. Studies in NIH3T3 cells suggest that insulin activates the ras/MAPK pathway independently of PKC [8], but, it is uncertain if insulin activates PKC significantly in these cells. Since insulin translocates or activates PKC in rat adipocytes [2,3,9,10], we questioned whether insulin effects on MAPK were sensitive to PKC depletion or inhibition in these cells. This question seemed important since hexose uptake in rat adipocytes is stimulated by activation of PKC and/or other serine/threonine protein kinases [11–14], is inhibited by PKC depletion [12,13] and PKC inhibitors [15,16], and is rescued by electroporation of purified PKC into PKC-depleted adipocytes [17].

2. EXPERIMENTAL

As described [2,13], adipocytes were prepared from epididymal fat pads of 200 g male Holtzman rats, equilibrated in glucose-free Krebs–Ringer phosphate (KRP) buffer containing 1% BSA, and treated with or without insulin (Elanco) or tetradecanoyl phorbol-13-acetate (TPA; Sigma). After incubation, adipocytes were sonicated in buffer containing 40 mM β-glycerophosphate (pH 7.3), 0.5 mM dithiothreitol, 0.75 mM EGTA, 0.15 mM sodium vanadate, 5 μg/ml leupeptin, 0.1 mM PMSF, 5 μg/ml trypsin inhibitor and 5 μg/ml aprotonin, as described [1]. Cytosolic extracts were obtained by centrifugation for 30 min at 100,000 × g and directly assayed for MBPK activity, (see [1]), or after SDS-PAGE in MBP-containing gels (see [18]). In the direct MBPK assay, 5 μg cytosolic protein was incubated at 30°C for 10 min in 50 μl buffer containing 25 mM β-glycerophosphate (pH, 7.3), 0.5 mM dithiothreitol, 1.25 mM EGTA, 0.5 mM sodium vanadate, 10 mM $MgCl_2$, 1 mg/ml BSA, 1 μM okadaic acid, and 0.1 mM [γ-^{32}P]ATP (NEN; sp. act. 1,500,000 dpm/nmol), with or without 50 μg MBP (Sigma) (see [1]). After incubation, aliquots of reaction mixtures were applied to P81 Whatman filter paper, washed with 1% phosphoric acid, and counted for radioactivity. Labelling observed in the absence of MBP was subtracted from that observed in the presence of MBP. In the MBP-containing gel assay, cytosolic extracts were subjected to SDS-PAGE in gels containing 0.5% MBP, and, after denaturation and subsequent renaturation of the gels, MBPK activity was measured in the presence of [γ-^{32}P]ATP and 10 mM $MgCl_2$ (see [18]). The gels were analyzed by autoradiography and laser densitometry scanning for determination of MBPK activity of 42 and 45 kDa proteins, i.e. p42/45 MAP kinases [18,19], or ERK-2/ERK-1. In some experiments, adipocytes were cultured overnight in the Dulbecco's Modified Eagles Medium (DMEM) containing 1% BSA with or without 1 μM TPA, as described [13,17,20]. These 'TPA-downregulated' adipocytes are partially depleted (50–80%) of PKC enzyme activity and PKC isoforms, α, β, γ, δ and ζ, but there are no changes in insulin receptor binding and tyrosine kinase activities [12,13,17,20]. In some experiments, bisindolylmaleimide (Calbiochem) was used as a PKC inhibitor (see [21]). The uptake of [^{3}H]2-deoxyglucose [^{3}H]2-DOG) was determined, as described [13,17].

3. RESULTS

Insulin-induced increases in cytosolic MBPK activity were maximal (2-fold) at 10 minutes and decreased at later times: in comparison, TPA provoked 2- to 3-fold

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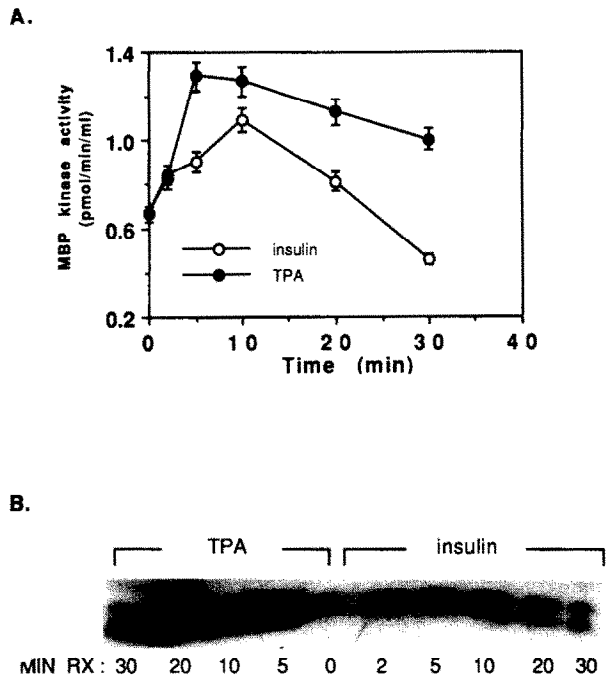

Fig. 1. Time-dependent effects of insulin (10 nM, ○) and TPA (500 nM, ●) treatment on MBPK activity in rat adipocytes. Cytosolic fractions were assayed for MBPK activity directly (Panel A), or after SDS-PAGE in MBP-containing gels (Panel B). Panel A shows mean (± S.E.) values of 3 experiments. Panel B portrays a representative autoradiogram of MBP labeling by p42/45 MAPK (in most experiments, only p42 MAPK was apparent). Note that the control (0 min) is shown in the center of the autoradiogram.

increases at 5 min and these were more persistant (Fig. 1). As in other studies [18,19], increases in cytosolic MBPK activity observed in the direct assay were reflective (albeit not solely) of p42/45 MAPK activation, as shown in MBP-containing gel assays (Figs. 1–3). In four experiments, the mean (± S.E.) insulin-induced increase in p42 MAPK activity (as per gel assay) was 68 ± 8% ($P < 0.005$; paired t-test).

As in previous studies [12,13,17,20], overnight TPA-pretreatment diminished PKC (not shown) and downregulated both insulin- and TPA-stimulated, but not basal, hexose transport (Fig. 2). In contrast, in these downregulated adipocytes, insulin provoked 2-fold increases in cytosolic MBPK/MAPK activity, as measured in direct and gel assays (Fig. 2). In three separate experiments, insulin effects on MBPK/MAPK kinase were virtually the same in downregulated and non-downregulated cells. In contrast, TPA-induced activation of MBPK was blocked in PKC-downregulated adipocytes (Fig. 2). Note that basal MBPK/MAPK activity was not altered significantly by overnight TPA pre-treatment.

As a PKC inhibitor [21], 100 μM bisindolylmaleimide (BIM), markedly inhibited insulin-stimulated hexose uptake and TPA-stimulated MBPK, but only mildly (20–30%) inhibited basal MBPK, and had no effect on insulin-induced activation of MBPK/MAPK (Fig. 3). If anything, insulin effects on MBP/MAPK were more prolonged by BIM.

4. DISCUSSION

As in NIH3T3 cells [8,22], insulin activated MAPK in rat adipocytes independently of PKC. This conclu-

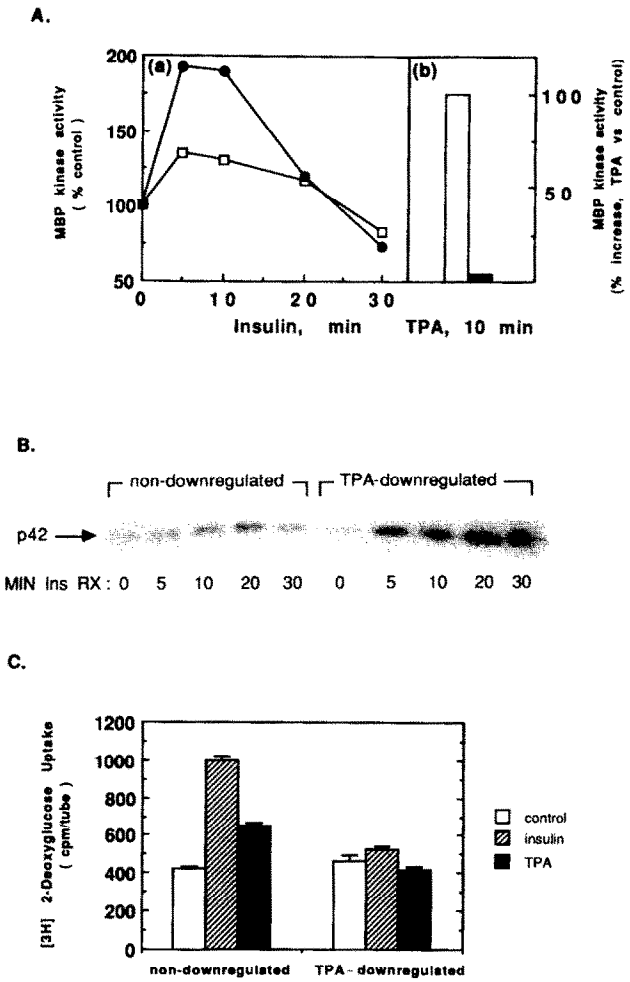

Fig. 2. Effects of TPA-induced downregulation of PKC on insulin- or TPA-induced activation of MBPK/MAPK (Panels A and B) and [^{3}H]2-deoxyglucose uptake (Panel C) in rat adipocytes. Cells were cultured overnight with ('TPA-downregulated') or without ('non-downregulated') 1 μM TPA, washed, equilibrated in glucose-free KRP medium and treated without or with 10 nM insulin or 500 nM TPA for the indicated times. Cytosolic fractions were assayed for MBPK activity directly (Panel A) or after SDS-PAGE in MBP-containing gels (Panel B). Results at the left (a) of Panel A are expressed as the percent of the control (closed circles, TPA-downregulated cells; open squares, non-downregulated cells). Results at the right (b) of Panel A are expressed as the percent increase due to acute 10-min TPA treatment over the non-downregulated (open bar) or the TPA-downregulated (solid bar) control. (Note: overnight TPA pre-treatment did not alter basal MBPK or p42MAPK activity.) Panel B shows autoradiographic results of insulin effects on p42 MAPK in non-downregulated (left) or TPA-downregulated (right) cells. Pancel C shows acute effects of insulin (hatched bars). TPA (solid bars), or vehicle alone (controls, open bars) on [^{3}H]2-deoxyglucose uptake in non-downregulated (left) and TPA-downregulated (right) cultured cells. Values are mean ± S.E. of 4 determinations. Comparable result were observed in 3 experiments.

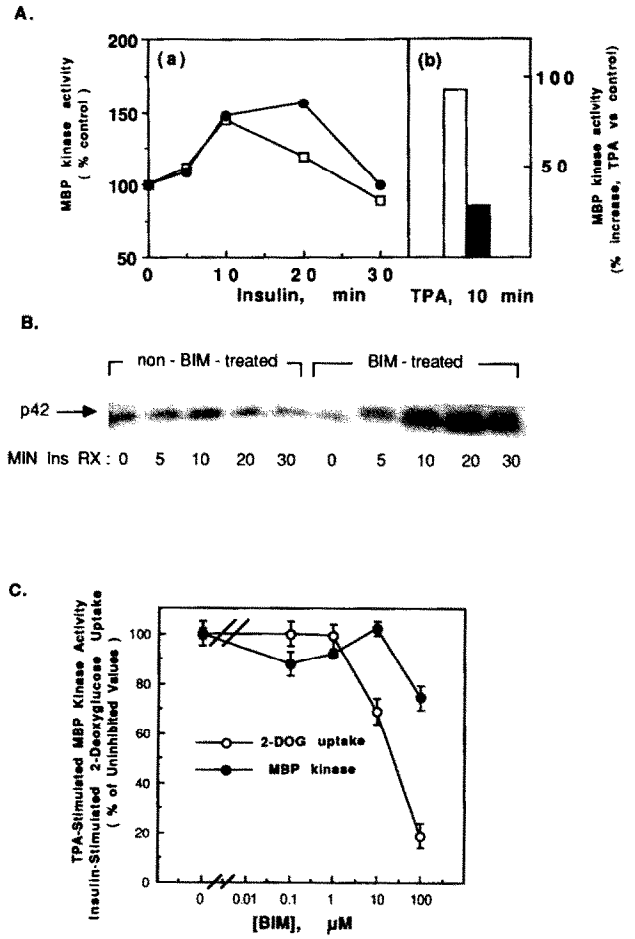

Fig. 3. Effects of bisindolylmaleimide (BIM) on insulin- or TPA-induced activation of MBPK/MAPK and [^{3}H]2-deoxyglucose uptake in rat adipocytes. Cells were treated for 10 min with 100 μM BIM in 0.5% DMSO (solid circles and bars), or with 0.5% DMSO alone (open squares and bars), and then treated with 10 nM insulin or 500 nM TPA for times indicated in Panels A and B. Cytosolic extracts were assayed for MBPK activity directly (Panel A) or after SDS-PAGE in MBP-containing gels (Panel B). Panel C shows dose-related inhibitory effects (mean values ± S.E. of 4 determinations) of BIM on insulin-stimulated [^{3}H]2-deoxyglucose uptake (○) in intact adipocytes, and in vitro MBPK activity (●) of TPA-stimulated cytosol. Comparable results were in repeat experiments.

sion seems clear, since insulin-induced activation of MAPK was fully intact in adipocytes that were functionally deficient in PKC, due to either TPA-induced PKC depletion or BIM-induced PKC inhibition. Thus, although PKC may be activated by insulin in rat adipocytes (see [2,3,9,10]), this is clearly not necessary for insulin-induced activation of MAPK. Further, if insulin activates MAPK via PKC, this would appear to be a redundant mechanism.

That insulin fully activated MAPK in PKC-depleted and PKC-inhibited adipocytes indicates that all relevant steps proximal to MAPK were functionally intact, presumably including the activation of insulin receptor tyrosine kinase, *ras*, and intervening PK's that precede MAPK (see [1,4,5,8,22]). These findings complement previous studies in which insulin receptor-dependent insulin-binding and tyrosine kinase activities were not altered by TPA-induced PKC depletion in rat adipocytes [13].

Of note, hexose transport effects of insulin were markedly compromised by PKC depletion and inhibition, despite full activation of MBPK/MAPK. Thus, insulin-stimulated hexose uptake seems to require PKC or a related factor extrinsic to the *ras*/MAPK pathway. A similar conclusion was reached by Osterof et al. [23], who found that dominant positive or negative mutant-induced alterations in *ras* activity had little or no effect on the activation of hexose transport by insulin in NIH3T3 cells, CHO cells, and rat-1 fibroblasts. On the other hand, the *ras*/MAPK pathway seems to play an important role in insulin-regulated gene expression and cellular proliferation [24].

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