

Inhibition of MAP kinase blocks insulin-mediated DNA synthesis and transcriptional activation of *c-fos* by Elk-1 in vascular smooth muscle cells

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Abstract Insulin-stimulated DNA synthesis, MAP kinase (MAPK) activity and *c-fos* expression in vascular smooth muscle cells (VSMCs) was blocked by the MAPK inhibitor PD 98059. Regulation of *c-fos* expression by the transcription factor Elk-1 at the serum response element (SRE) is dependent on its phosphorylation by MAPK. PD 98059 also suppressed insulin-induced Elk-1 transcriptional activity through the SRE. These data show that MAPK plays a critical role in both insulin-mediated growth and Elk-1-dependent induction of *c-fos* in VSMCs.

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Key words: Vascular smooth muscle cell; Insulin; *c-fos*; Serum response element; Elk-1; Mitogen-activated protein kinase

1. Introduction

Hyperinsulinemia is an independent risk factor for coronary artery disease [1]. Insulin induces vascular smooth muscle cell (VSMC) proliferation, migration, and the production of extracellular matrix which are critical processes for neointima formation in injured vessels [2]. Activation of the ERK1 and ERK2 mitogen-activated protein kinases (MAPK) is required for mitogenic signaling through a number of tyrosine kinase growth factor receptors [3,4]. ERKs transduce mitogenic signals to the nucleus by phosphorylating and activating specific transcription factors, such as Elk-1, which induce expression of *c-fos* and other early growth response genes that control the transition from quiescence to proliferation [5,6].

No study to date has examined the role of the MAPK pathway in insulin-stimulated VSMC growth. We have recently shown that MAPK plays a central role in PDGF- and AII-directed migration [6,7] and bFGF-induced proliferation [8] in rat VSMCs. These growth factors, like insulin, have been implicated in promoting lesion development by enhancing VSMC growth, migration, and matrix production [9,10]. In the present study, the importance of MAPK in VSMCs is further extended by our findings that this pathway is required for insulin-stimulated DNA synthesis and Elk-1-mediated *c-fos* activation in VSMCs.

2. Materials and methods

2.1. Cell culture

Rat aortic smooth muscle cells were prepared from thoracic aorta of 2–3 month old Sprague-Dawley rats using the explant technique [11]. The cells were cultured in DMEM containing 10% FBS, 150 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 200 mM glutamine (Sigma, St. Louis, MO). The purity and identity of the smooth muscle cell cultures were verified using a monoclonal antibody against smooth muscle α -actin (Sigma). For all experiments, early passaged (8 or less) rat VSMCs were grown to 60–70% confluence and made quiescent by serum starvation for at least 24 h, when MAPK activity was assayed. For all data shown, each individual experiment represented in the *n* value was performed using an independent preparation of VSMC.

2.2. VSMC DNA synthesis

Incorporation of the thymidine analogue, bromodeoxyuridine (BrdU), was measured to determine the effect of insulin on DNA synthesis as described previously [8]. VSMCs were plated out at 3.0×10^4 cells on 24 well plates (Falcon Primaria) in DMEM with 10% FBS for 48 h. After serum starvation for 24 h in DMEM containing 0.1% FBS, cells were incubated with insulin (Eli Lilly, Indianapolis, IN) in the presence or absence of PD 98059 (1–30 µM), for the next 20 h. When present, PD 98059 was added 30 min prior to insulin stimulation and maintained throughout the incubation. Then BrdU at 15 µM (Sigma) was added, and the incubation continued for another 4 h. BrdU-positive cells were visualized using BrdU monoclonal antibody (Zymed, San Francisco, CA) followed by the ABC method. BrdU-positive cell nuclei were counted in 4–6 different high power fields/well and related to total cell number/high power field.

2.3. MAP in-gel kinase assay

MAPK activity was measured by the in-gel kinase assay as described previously [8]. PD 98059 (New England Biolabs) was given 30 min before inducing MAPK activation with insulin for 10 min and was present throughout the incubation period. For the data shown, each individual experiment represented in the *n* value was performed using an independent preparation of VSMCs. Densitometric analysis was performed using NIH Image 1.60 software on a Macintosh PC.

2.4. Isolation and analysis of RNA

Total RNA was isolated from VSMCs using guanidinium-isothiocyanate followed by phenol-chloroform extraction [12]. RNA was size-fractionated by electrophoresis through a denaturing 1% agarose gel, transferred to nitrocellulose membranes, and hybridized with cDNA probes labeled with [³²P]dCTP (3000 Ci/mmol) by random priming. The cDNA for *c-fos* was from Jill Norman (UCLA School of Medicine). The hybridization signals of the specific mRNAs of interest were normalized to those of CHOB to correct for differences in loading or transfer. CHOB cDNA was originally isolated from Chinese hamster ovary cells and corresponds to a mRNA ubiquitously expressed in mammalian tissues which does not exhibit regulation as a function of growth or development [13]. Quantitation of Northern blots was performed by densitometric analysis using NIH Image 1.60 software for Macintosh personal computers. Several autoradiographic film exposures were used to ensure that the density of the signals were linear on each film.

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2.5. Transfections

VSMCs were transfected with 15 μ g of SRE-CAT reporter plasmid, or 7.5 μ g of the Gal-Elk-1 expression vector and 7.5 μ g Gal4-CAT reporter plasmid, or 7.5 μ g of the mutant Gal-Elk1 Δ A expression vector and 7.5 μ g Gal4-CAT reporter plasmid and 5 μ g of pCH110 (SV40 β -galactosidase reference plasmid) using a calcium phosphate transient transfection kit (Stratagene, La Jolla, CA) as reported elsewhere [8]. After transfection, cells were starved for 24 h before stimulation with insulin for 6 h. PD 98059 was added 30 min prior to the addition of insulin. Lysates were prepared and normalized for protein content using the Lowry method. Chloramphenicol acetyltransferase (CAT) and β -galactosidase assays were performed using standard methods [14,15]. All experiments were performed in duplicate and repeated at least three times to ensure reproducibility. CAT activities were corrected for transfection efficiency by assaying extracts for β -galactosidase activity.

2.6. Statistical analysis

Analysis of variance, paired or unpaired *t*-test, were performed for statistical analysis, as appropriate. *P* values less than 0.05 were considered to be statistically significant. Data are expressed as mean $\pm$ S.E.M.

3. Results

3.1. Inhibition of MAPK activation with PD 98059 blocks insulin-induced DNA synthesis

MAPK is phosphorylated and activated by MEK (MAPK-extracellular signal regulated kinase kinase), a dual specificity kinase that phosphorylates serine and tyrosine residues [3,4].

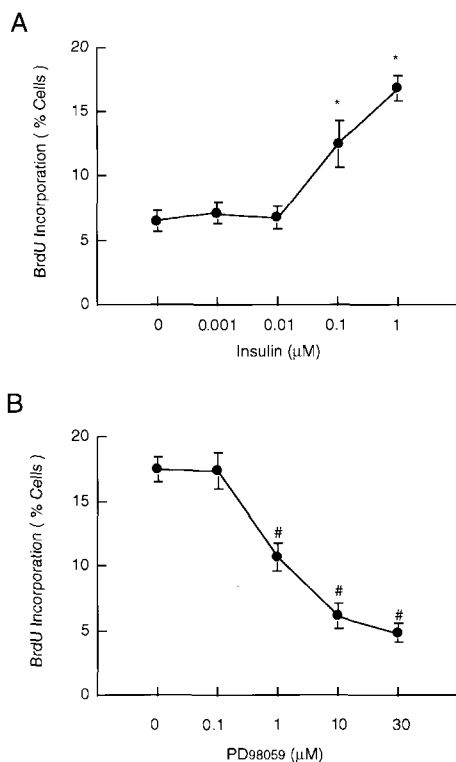


Fig. 1. The MAPK pathway inhibitor PD 98059 blocks insulin-stimulated DNA synthesis in VSMCs. Cells were made quiescent by incubation for 24 h in DMEM containing 0.1% FBS. To measure DNA synthesis, cells were stimulated with insulin for 24 h; 15 μ M BrdU was present for the final 4 h. The percentage of cells incorporating BrdU is expressed as the mean $\pm$ S.E.M. A: Dose response for insulin-stimulated DNA synthesis ($n=4$, * $P<0.01$ vs. unstimulated control). B: Dose-dependent inhibition of DNA synthesis by PD 98059 in VSMCs stimulated with 1 μ M insulin ($n=4$, $P<0.01$ vs. 1 μ M insulin alone).

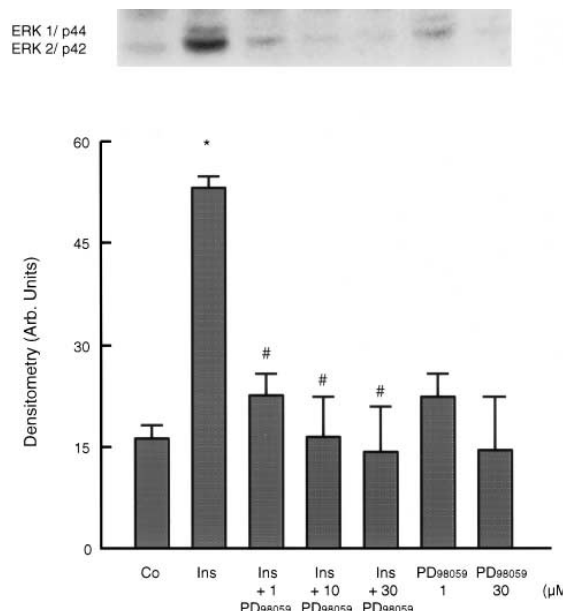


Fig. 2. Insulin stimulates MAPK in VSMCs which is inhibited by PD 98059. Quiescent cells were stimulated with 1 μ M insulin for 10 min prior to stimulation. Cell extracts were prepared and equal amounts of protein (10 μ g/lane) were separated on a 10% PAGE-SDS gel with 0.4 mg/ml myelin basic protein (MBP). In-gel kinase renaturation assays were performed and bands corresponding to the 44-kDa (ERK1) and 42-kDa (ERK2) MAPKs were visualized by the phosphorylation of MBP. A representative autoradiogram is depicted. Densitometric analysis was performed on in-gel kinase assays from four separate experiments and are expressed as arbitrary units $\pm$ S.E.M. ($n=4$, * $P<0.01$ vs. unstimulated control; $P<0.01$ vs. insulin alone).

PD 98059 is a highly selective inhibitor of MEK commonly used to block MAPK activation [16]. VSMCs were made quiescent by 16 h treatment in medium containing 0.1% serum. Exposure to insulin for 20 h induced a concentration-dependent increase in DNA synthesis ($P<0.01$ at 0.1 and 1 μ M, Fig. 1A). The addition of PD 98059 30 min prior to insulin treatment (1 μ M) and its presence throughout the incubation period resulted in a dose-dependent inhibition of insulin-stimulated DNA synthesis. The insulin-mediated increase in DNA synthesis was completely suppressed by 30 μ M PD 98059 ($P<0.01$ vs. 1 μ M insulin alone, Fig. 1B). These data demonstrate that mitogenic signaling by insulin in VSMCs is totally MAPK-dependent.

3.2. Insulin-induced MAPK activity in VSMCs is inhibited by PD 98059

Quiescent VSMCs exhibited very low MAPK activity, as evidenced by the faint signals produced by the phosphorylation of substrate myelin basic protein by ERK1 (p44) and ERK2 (p42) MAPKs in the in-gel kinase assay (Fig. 2). VSMC treated with 1 μ M insulin for 10 min displayed a strong induction of ERK1 and ERK2 activity (~ 4 -fold, Fig. 2). Insulin-induced MAPK activity was inhibited in a concentration-dependent manner by PD 98059. At 30 μ M PD 98059, the insulin-induced activation of ERK1 plus ERK2 MAPKs was abolished ($n=4$, $P<0.01$ vs. insulin alone). These data corroborate that mitogenic signaling by insulin in VSMCs is MAPK-dependent since both insulin-induced DNA synthesis and MAPK activation are completely

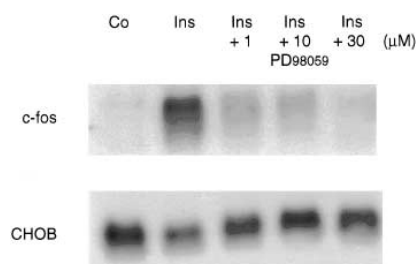


Fig. 3. PD 98059 inhibits induction of *c-fos* mRNA by insulin (1 μ M). Quiescent cells were stimulated in the presence or absence of PD 98059 as in Fig. 2. RNA was isolated 30 min after stimulation with insulin and subjected to Northern analysis. 20 μ g of RNA were loaded per lane and blots were hybridized with 32 P-labeled DNAs for *c-fos* and CHOB, a mRNA from a housekeeping gene used to normalize for equivalent RNA loading and transfer. Depicted is an autoradiogram which is representative of four independent experiments that were performed.

inhibited by the MAPK pathway inhibitor PD 98059 (compare Figs. 1 and 2).

3.3. Insulin-induced *c-fos* expression is inhibited by PD 98059

Induction of *c-fos* transcription is a paradigm for the nuclear effects of MAPK signaling that lead to cell proliferation [17]. Quiescent VSMCs do not express detectable *c-fos* mRNA by Northern analysis (Fig. 3). Treatment with 1 μ M insulin for 30 min produces a strong induction of *c-fos* mRNA. Pretreatment of VSMCs with PD 98059 for 30 min and its inclusion throughout the incubation period resulted in a dose-dependent inhibition of insulin-induced *c-fos* expression (Fig. 3). At 30 μ M PD 98059 insulin-induced *c-fos* expression was almost completely suppressed.

3.4. PD 98059 blocks insulin-induced transactivation of the serum response element (SRE) by Elk-1

Insulin-induced transcriptional activation of the *c-fos* gene is mediated by transcription factors which bind to the SRE located in the *c-fos* 5'-flanking DNA [18]. The transcription factor Elk-1 activates *c-fos* expression through the SRE and this activity is dependent on its phosphorylation by MAPK [5]. The data in Fig. 4 demonstrate that insulin-stimulated transactivation of SRE is through the MAPK pathway. VSMCs were transiently transfected with 2x-SRE-CAT reporter plasmids. Addition of 1 μ M insulin resulted in an increase in CAT activity relative to unstimulated transfectants. Insulin-stimulated SRE-CAT activity was reduced to levels detected in unstimulated cells by the addition of either 10 μ M or 30 μ M PD 98059 to suppress MAPK signaling.

MAPK-dependent Elk-1-specific transactivation can be quantitatively measured by transfection of an expression vector which produces a chimeric transcription factor composed of the N-terminal binding domain of the yeast GAL4 protein and the C-terminal transactivation domain of Elk-1 [5]. The GAL-Elk-1 expression vector is cotransfected with a reporter construct containing four copies GAL4 DNA-binding sites to which the GAL-Elk-1 fusion protein binds and directs CAT transcription from a thymidine kinase minimal promoter.

Stimulation of quiescent VSMCs cotransfected with GAL-Elk-1 and GAL-CAT plasmids with 1 μ M insulin resulted in a 5-fold increase in GAL-CAT activity ($P < 0.01$ vs. unstimulated cells, Fig. 5). Insulin-induced GAL-CAT activity was completely abolished by 30 μ M PD 98059. The presence of

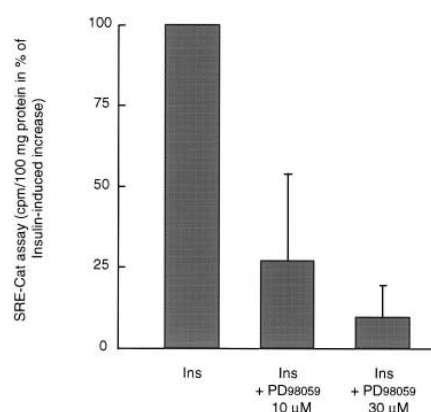


Fig. 4. PD 98059 inhibits insulin-induced transcriptional activation of a SRE. VSMCs were transfected with SRE-CAT plasmid and made quiescent as described in Section 2. CAT activity was measured 6 h after stimulation with 1 μ M insulin in the presence or absence of PD 98059. Data are expressed as % of insulin-stimulated CAT activity (100%), after normalization to co-transfected pCH110 β -galactosidase activity. Data are from three separate experiments $\pm$ S.E.M.

PD 98059 alone had no effect on basal GAL-CAT activity. The MAPK dependence of insulin-induced GAL-CAT activity was verified by transfecting the expression plasmid GAL-Elk-1 Δ A in which the C-terminal MAPK phosphorylation sites have been deleted [19]. Treatment with 1 μ M insulin failed to stimulate GAL-CAT activity in VSMCs cotransfected with GAL-Elk-1 Δ A.

In combination, the data in Figs. 4 and 5 demonstrate that insulin-induced *c-fos* expression through the SRE is dependent on the phosphorylation of Elk-1 by MAPK.

4. Discussion

Hyperinsulinemia in diabetic patients may contribute to the development of atherosclerosis in this population through the

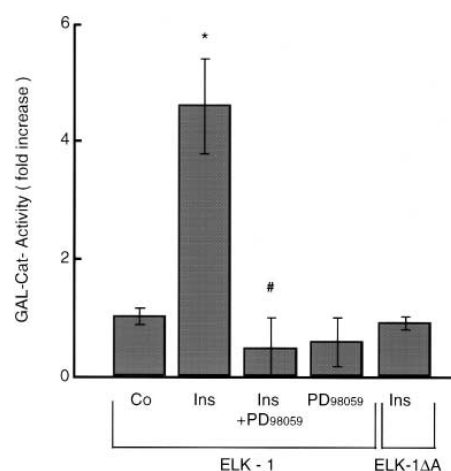


Fig. 5. Activation of Elk-1 by insulin is MAPK-dependent. Cells were co-transfected with GAL4-CAT reporter plasmid and either GAL-Elk-1 or GAL-Elk-1 Δ A vectors, which express chimeric transcription factors containing or lacking Elk-1 MAP phosphorylation sites, respectively. Quiescent transfected cells were stimulated with 1 μ M insulin in the presence or absence of PD 98059 for 6 h. Data are expressed as the fold increase of insulin-stimulated CAT activity over unstimulated controls after normalization to β -galactosidase activity $\pm$ S.E.M. ($n = 4$, * $P < 0.01$ vs. unstimulated controls; $P < 0.01$ vs. insulin alone).

mitogenic effect of insulin on VSMCs [20]. Understanding mitogenic signaling by insulin in VSMCs, therefore, is important since this may lead to identification of new targets for pharmacologic intervention to ameliorate diabetes-associated vascular disease.

Rat VSMCs express both insulin and insulin-like growth factor-I (IGF-I) receptors [21]. Insulin and IGF-I each promote VSMC growth, although the latter is a more potent mitogen. Mitogenic signaling by insulin has been studied primarily in non-vascular cells; rat fibroblast cells overexpressing transfected human insulin receptors being frequently studied as a model system [22]. In these cells, both insulin-induced DNA synthesis and *c-fos* expression were shown to be dependent on the activation of phosphatidylinositol 3-kinase (PI3-kinase), based on microinjection of neutralizing antibody [23,24] or treatment with the PI3-kinase inhibitors wortmannin [24]. Similarly, fibroblasts from insulin receptor substrate-1 (IRS-1) knockout mice fail to activate PI3-kinase in response to IGF-I which is paralleled by a decrease in IGF-I stimulated DNA synthesis and *c-fos* induction [25].

In combination, the above studies demonstrate that activation of PI3-kinase is necessary, at least in some cell types, for mitogenic signaling by insulin and IGF-1. Although the RAS → RAF → MEK → MAPK cascade has been implicated in tyrosine kinase growth factor receptor-mediated cell proliferation [3,4], no study has directly assessed the role of this pathway in insulin-stimulated mitogenesis. We have used PD 98059 to inhibit the phosphorylation and activation of MAPK by MEK. Treatment of VSMCs with PD 98059 resulted in a dose-dependent inhibition of both insulin-stimulated DNA synthesis and MAPK activation. Inhibition of insulin-induced mitogenic signaling is likely due to the disruption of MAPK-mediated nuclear events, which include the induction of the growth-associated gene *c-fos* by the MAPK-regulated transcription factor Elk-1. The observed effects of insulin may be mediated in part through IGF-1 receptors activated by the relatively high levels of hormone used in these experiments. Since both insulin and IGF-I have been shown to activate MAPK in non-vascular cells [26], we anticipate that PD 98059 will have a similar effect on either pathway.

Our data provide the first evidence that the MAPK pathway is necessary for insulin to function as a VSMC mitogen. This finding appears to be in conflict with prior studies in fibroblasts showing that insulin-stimulated growth is PI3-kinase-dependent, since that pathway is generally considered to be a post-receptor branch separable from the MAPK cascade. Our results can potentially be harmonized with findings made in fibroblasts for the following reasons: (1) signaling pathways critical for insulin-mediated mitogenesis may be cell-type specific and different in fibroblasts versus VSMCs; (2) studies with fibroblasts demonstrated only that the PI3-kinase pathway was necessary for insulin mitogenic signaling; they did not preclude a requirement for additional pathways; and (3) recent data have suggested potential cross-talk between the PI3-kinase and MAPK pathways, since phosphoinositide products of PI3-kinase increase activity of PKC ζ which can activate RAF or MEK [27,28].

MAPK has been previously shown to be required for PDGF- [29], bFGF- [8], and AII-stimulated growth [7], as well as for PDGF- and AII-directed migration [6,7] in VSMCs. The present finding that VSMC growth in response

to insulin is also MAPK-dependent provides additional support for the central role of this pathway in regulating VSMC growth and migration. Pharmacological intervention targeting MAPK, therefore, may represent a novel therapeutic strategy for the treatment of vascular disease driven by multiple growth factors, including insulin.

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