RESEARCH COMMUNICATION

Inhibitors of signalling identify differential control processes responsible for selective effects of insulin on the expression of phosphoenolpyruvate carboxykinase and gene 33 in rat H4 hepatoma cells

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Gene 33 and phosphoenolpyruvate carboxykinase (PEPCK) present excellent model systems for the analysis of the differential control of hepatic gene expression by the insulin-regulated signal-transduction pathway(s). We have analysed the importance of specific components in the insulin-regulated transduction pathway(s) towards enhanced gene expression (gene 33) and inhibited gene expression (PEPCK) by examination of the influence of selective inhibitors. Rapamycin, which inhibits the 70 kDa S6 kinase (p70^{rsk}) does not influence the actions of insulin on gene 33 or PEPCK; thus the kinase p70^{rsk} appears to play no direct role in the regulation of expression of these two

hepatic genes. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase, differentiates between processes involved in insulin regulation of gene 33 and PEPCK mRNA expression. Although the actions of insulin on gene 33 expression are abolished by wortmannin, the actions of insulin on PEPCK expression are insensitive to wortmannin. The existence of wortmannin-sensitive and rapamycin/wortmannin-insensitive pathways for transducing insulin signals to factors controlling gene expression, and the differential actions on specific genes, presents an initial step towards deciphering the linkage between signalling components and selective control of gene expression.

INTRODUCTION

At least 20 genes are known to be regulated at transcriptional level in response to insulin, and such changes are selective, rapid and reversible [1]. Phosphoenolpyruvate carboxykinase (PEPCK) and gene 33 represent two of the best characterized insulin-regulated genes; within H4 hepatoma cells insulin stimulates gene 33 expression and inhibits PEPCK expression [2–4]. Insulin response elements (IREs) have been proposed for several genes, but no consensus IRE has been defined, and the functional involvement of putative IREs has been shown in only a few cases [5–9]. PEPCK presents the best characterized insulin-regulated promoter, but even in this case there remains no complete definition of the promoter-associated proteins which facilitate insulin regulation of transcription or of their interaction with components of the insulin signal-transduction pathway(s) [5]. It is envisaged that insulin will regulate transcription by changes in the phosphorylation state or conformation of protein(s) which can interact with the promoter-regulatory regions of target genes. Although some components of insulin signalling cascades are localized to the nucleus, or can move there upon insulin activation [10,11], the complexity of insulin signalling has hampered analysis of the linkage between signalling and transcriptional control. Thus it is uncertain at present if gene-selective effects of insulin are generated through distinct ‘branches’ of signalling cascades or if stimulatory and inhibitory actions arise from the same signalling components.

To define the components required for selective effects of insulin on PEPCK and gene 33 expression in H4 hepatoma cells, we have examined the influence of rapamycin and wortmannin on insulin action. Rapamycin blocks one branch of the insulin

signalling cascade, involving the 70 kDa S6 kinase (p70^{rsk}), but does not influence a second branch, the mitogen-activated protein kinase (MAPK)/MAPK-activated protein kinase 1 (MAPKAP kinase 1 or p90^{rsk}) pathway [12]. Wortmannin, a potent inhibitor of phosphatidylinositol 3-kinase (PI 3-K) [13], has been used to define the role of p70^{rsk} and other putative upstream components in that branch of mitogen signalling [14]. Recent work has shown that wortmannin may, in addition, block activation of the MAPK pathway by inhibition of signalling events between Ras and Raf [15].

In the present paper we show that wortmannin prevents the stimulation of gene 33 mRNA in response to insulin, but does not prevent the effects of this hormone on PEPCK mRNA. Rapamycin, in contrast, does not influence the actions of insulin on either mRNA. Although the phorbol ester, PMA (phorbol 12-myristate 13-acetate), produces insulin-mimetic effects on gene 33 and PEPCK, it shows a distinct sensitivity to wortmannin. Effects of PMA on PEPCK are inhibited by wortmannin, but the effects of PMA on gene 33 are insensitive to wortmannin. These findings indicate that multiple pathways generate or control the selective effects of insulin and phorbol esters on gene 33 and PEPCK mRNA expression in H4 hepatoma cells.

MATERIALS AND METHODS

Sources of materials

Rat hepatoma cells [H4-II-E-C3] were obtained from the European Collection of Animal Cell Cultures (E.C.A.C.C.) (register no. 85061112). Rapamycin and wortmannin were obtained from Calbiochem, Nottingham, U.K.; guanidine thiocyanate, insulin and PMA were purchased from Sigma, Poole,

Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase; PMA, phorbol 12-myristate 13-acetate; IRE, insulin response element; MAPK, mitogen-activated protein kinase; MAPKAP kinase 1 ($\equiv$ p90^{rsk}), mitogen-activated-protein-kinase-activated protein kinase 1; p70^{rsk}, 70 kDa S6 kinase; PI 3-K, phosphatidylinositol 3-kinase; α MEM, α minimal essential medium; GSK-3, glycogen synthase kinase 3.

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Figure 1 Influence of insulin and PMA on gene 33 and PEPCK mRNA of H4 hepatoma cells: interactions with rapamycin and wortmannin

H4 Hepatoma cells were cultured in serum-free medium for 18 h prior to challenge with insulin (100 nM), PMA (1 μ M), rapamycin (10 nM) and wortmannin (100 nM), as specified. At different times of incubation, RNA was isolated and analysed for gene 33, PEPCK and 18 S rRNA content as described in the Materials and methods section. All results are presented after standardization of gene 33 and PEPCK mRNA for 18 S rRNA loading. Changes in mRNA contents in response to insulin (●, ■) and PMA (▲, ▼) are shown in (a); values for gene 33 (●, ▲) and PEPCK (■, ▼) mRNA are presented relative to the appropriate value at zero time (zero-time value = 1.0). Each value is the mean $\pm$ S.E.M. for four separate experiments (* P < 0.05 versus the respective time-point control). (b) Presents the data from three independent experiments which illustrate the influence of rapamycin on changes in gene 33 (●, ■) and PEPCK (▲, ▼) mRNA contents in response to insulin. Each value is the mean $\pm$ S.E.M. and is presented in relation to the control (no rapamycin) at 0.5 h (●, ▲) or plus rapamycin (■, ▼). (* P < 0.05 versus the respective control at each time point). (c) Presents the data from three independent experiments which illustrate the influence of wortmannin on changes in gene 33 (●, ■) and PEPCK (▲, ▼) mRNA contents in response to insulin. Each value is the mean $\pm$ S.E.M., and is presented in relation to the control (no wortmannin) at 0.5 h (●, ▲) or plus wortmannin (●, ▼). (* P < 0.05 versus the respective control at each time point).

Dorset, U.K. Caesium chloride (ultra-pure, optical grade) was obtained from Gibco BRL (Uxbridge, Middx., U.K.). [α - 32 P]dCTP was purchased from ICN (High Wycombe, Bucks., U.K.). cDNA probes for PEPCK (pPCK10), gene 33 (p33) and 18 S rRNA (p100-D9) were generously given by Professor R. Hanson (Case Western Reserve University, Cleveland, OH, U.S.A.), Professor F. Kenney (Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.) and Dr. D. Edwards (University of Calgary, Calgary, Alberta, Canada) respectively.

Cell-culture procedures

Hepatoma cells were maintained in α minimal essential medium (α MEM) supplemented with 10% (v/v) foetal-calf serum. For RNA isolation, 60–70% confluent cell sheets were washed four times in PBS and cells were incubated in serum-free α MEM for 18 h prior to challenge with insulin, PMA, rapamycin or wortmannin. When present, rapamycin and wortmannin were added to cultures 10 min prior to addition of insulin. After the appropriate incubation period, cells were harvested for RNA isolation.

RNA isolation and quantification of specific mRNA species

RNA was isolated using a guanidine thiocyanate/caesium chloride centrifugation procedure as described previously [16]. RNA recovered was quantified by A_{260} measurements, and 15 μ g of each RNA sample was subjected to agarose-gel electrophoresis to separate RNA species. mRNA for gene 33 and PEPCK were localized by sequential Northern hybridization using 32 P-labelled cDNA produced by nick translation with a specific radioactivity of at least 10^8 c.p.m./ μ g of DNA. Quantification of each RNA species was performed using phosphorimage analysis and by reference to loading standardization using hybridization with 18 S rRNA cDNA. Within each set of data, the values for PEPCK and gene 33 mRNA have been normalized to the 18 S rRNA loading and then expressed relative to the appropriate

control (which was either a basal or insulin-treated sample as specified for each experimental condition).

RESULTS

Preliminary investigations identified the time course of action of insulin on gene 33 and PEPCK mRNA; in agreement with the work of others [2–4] we found that insulin produced a transient stimulation of gene 33 mRNA, with the maximal effects exerted around 2 h of treatment (Figure 1a). As the effects of insulin on PEPCK mRNA content are significant at 2 h, this time interval offers a suitable treatment regime for a detailed examination of the influence of wortmannin and rapamycin on mRNA contents.

Influence of rapamycin on gene control by insulin

Throughout cultures of up to 4 h, rapamycin (10 nM) had no significant effect on the normal pattern of insulin action on gene 33 and PEPCK mRNA expression (Figure 1b). In a subsequent series of experiments presented in Figure 2, insulin produced a 300–400% increase in gene 33 mRNA and a 52–65% decrease in PEPCK mRNA. Rapamycin (at concentrations of 1–50 nM) did not alter relative amounts of either mRNA species under basal conditions. Throughout this concentration range rapamycin did not influence insulin action on gene 33 and PEPCK mRNA (Figures 2a and 2b). Under the conditions of these experiments, rapamycin (at 10 nM or greater concentration) inhibited insulin-stimulated proliferation and DNA synthesis of H4 hepatoma cells [C. E. Budgell, S.-H. Yang and A. J. Dickson, unpublished work].

Influence of wortmannin on gene control by insulin

Wortmannin (100 nM) blocked insulin-stimulated gene 33 expression, and this effect was observed consistently throughout a 4 h culture period (Figure 1c). In contrast, there were no significant changes to the effects of insulin on PEPCK mRNA.

Figure 2 Concentration-dependent influences of rapamycin and wortmannin on basal and insulin-induced changes of gene 33 and PEPCK mRNA in H4 hepatoma cells

H4 Hepatoma cells were cultured in serum-free medium for 18 h prior to challenge with insulin (100 nM), rapamycin (1–50 nM) and wortmannin (5–100 nM), as specified. After 2 h of incubation, RNA was isolated and analysed for gene 33, PEPCK and 18S rRNA content as described in the Materials and methods section. All results are presented after standardization of gene 33 and PEPCK mRNA for 18 S rRNA loading [$P < 0.05$ versus the respective control (i.e. basal or insulin-treated in the presence of no rapamycin or wortmannin)]. The influence of rapamycin on gene 33 and PEPCK mRNA contents is shown in (a) and (b) respectively. Results for basal (●) and insulin-challenged (■) incubations represent the means $\pm$ S.E.M. of the results from three separate experiments. For presentation of gene 33 mRNA the content in the presence of insulin (at zero rapamycin) has been taken as 100% (insulin produces a 3.01 ± 0.24 -fold increase in gene 33 mRNA over basal values) and for PEPCK mRNA the content in the absence of insulin (at zero rapamycin) has been taken as 100% (insulin decreases PEPCK mRNA to $48 \pm 12\%$ of basal values). The influence of wortmannin on gene 33 mRNA content is shown in (c). Results for basal (●) and insulin-challenged (■) incubations represent the means $\pm$ S.E.M. of the results from four separate experiments. For presentation the mRNA content in the presence of insulin (at zero rapamycin) has been taken as 100% (insulin produces a 4.16 ± 0.14 -fold increase in gene 33 mRNA over basal values).

Table 1 Interaction of wortmannin with control of gene 33 and PEPCK mRNA by the phorbol ester PMA and insulin

H4 Hepatoma cells were cultured in serum-free medium for 18 h prior to challenge with insulin (100 nM), PMA (1 μ M) and wortmannin (100 nM), as specified. After 2 h incubation, RNA was isolated and analysed for gene 33, PEPCK and 18 S rRNA content as described in the Materials and methods section. All results are presented after standardization of gene 33 and PEPCK mRNA for 18 S rRNA loading and represent means $\pm$ S.E.M. for five to eight independent experiments. The values for gene 33 or PEPCK mRNA content for cells in the absence of any treatment are given as 1. [$P < 0.05$ versus the respective control (basal or wortmannin alone); $**P$, 0.05 between with or without wortmannin for each condition].

Conditions of culture	Standardized gene expression	
	Gene 33	PEPCK
Basal (serum-free)	1	1
Wortmannin	1.34 ± 0.11	0.86 ± 0.05
Insulin	$3.76 \pm 0.36^*$	$0.45 \pm 0.06^*$
Wortmannin + insulin	$1.99 \pm 0.19^{***}$	$0.46 \pm 0.06^*$
PMA	$2.24 \pm 0.65^*$	$0.56 \pm 0.07^*$
Wortmannin + PMA	$1.81 \pm 0.25^*$	$1.38 \pm 0.21^{***}$

To define the potency of wortmannin, this agent was used over a concentration range 5–100 nM; this encompasses the concentrations shown by others to inhibit PI 3-K and to inhibit some of the biological responses of insulin, other growth factors and hormones [14,15,17]. Expression of gene 33 mRNA under basal conditions was unaltered by the presence of wortmannin, but there was a concentration-dependent inhibition of insulin action on gene 33 mRNA (Figure 2c). There was no significant change to PEPCK mRNA content in the presence of wortmannin, and the ability of insulin to decrease PEPCK mRNA was not influenced over the range of wortmannin concentrations tested (results not shown).

Interaction between PMA and wortmannin on gene control

The phorbol ester PMA produced insulin-mimetic effects on expression of gene 33 and PEPCK mRNA in H4 cells (Figure 1a), in agreement with the work of others [2–4]. Under the conditions of our experiments, the effects of PMA were less dramatic than those of insulin. In the presence of wortmannin, PMA activation of gene 33 mRNA expression was unimpaired, but the inhibition of PEPCK mRNA contents by PMA was abolished in the presence of wortmannin (Table 1).

DISCUSSION

The use of inhibitors of specific steps/branches of insulin signalling pathway(s) can permit definition of the importance of signalling components towards specific cell responses. Hence rapamycin and, more recently, wortmannin, have been used to indicate the importance of a PI 3-K/p70^{rsk} 'arm' of growth-factor signalling cascades [14,15,17]. Although there are concerns over the selectivity of 'specific' inhibitors when applied to the interactive environment of intact cells, effects of rapamycin appear to localize to p70^{rsk} exclusively [14]. For example, rapamycin does not influence the activation of MAPKAP kinase 1 or MAPK by insulin [15,17]. Our studies with rapamycin indicate that p70^{rsk} is not involved in the basal control of gene 33 or PEPCK mRNA expression. In addition, the effects of insulin on gene 33 and PEPCK mRNA were unaltered by rapamycin at concentrations reported to inhibit p70^{rsk} [14] and which inhibited H4 cell proliferation. Although we can make no statements about other insulin-regulated genes, it appears that p70^{rsk} is not required for insulin regulation of gene 33 and PEPCK mRNA, and it is feasible that p70^{rsk} controls translation but not transcription [18].

When we initiated our studies with wortmannin, we hoped it would distinguish the contribution of (unknown) components 'upstream' of p70^{rsk} to regulation of gene 33 and PEPCK mRNA. Our finding that wortmannin inhibits insulin stimulation of gene 33 mRNA has two possible interpretations: first, that

there are signalling components downstream of PI 3-K which are essential for the ability of insulin to stimulate gene 33 mRNA expression and, secondly, that components of the Ras/MAPK/MAPKAP kinase 1 pathway are required for insulin action on gene 33 mRNA. Wortmannin has been shown to inhibit insulin activation of p74^{raf-1} (by interfering with an unknown event in recruitment of p74^{raf-1} to the plasma membrane), MAPK kinase, MAPK, MAPKAP kinase and inactivation of glycogen synthase kinase 3 (GSK-3) [15,17]. Whether this occurs in H4 cells is unknown at present. Finally it should be noted that insulin activation of glycogen synthase in PC12 cells can operate through a wortmannin-sensitive pathway, independently of activation of Ras/MAPK or p70^{ras} [19]. Although the nature of the components which participate in this putative pathway remains to be determined, they may present a further target for wortmannin action in regulation of gene 33 expression.

Having shown that wortmannin inhibited the actions of insulin on gene 33 mRNA expression, analysis of the effects on PEPCK mRNA were important for deciphering whether (i) common pathways and (ii) common final target proteins were involved in regulation of expression of two genes showing reciprocal control in response to insulin. In contrast with gene 33, the response of PEPCK mRNA to insulin was unaltered by wortmannin. The results suggest that insulin controls PEPCK mRNA through a wortmannin-insensitive pathway. Our present understanding of wortmannin action implicates the Ras/MAPK signalling pathway in the actions of insulin on PEPCK mRNA contents. There are precedents that modulation of the Ras/MAPK pathway may alter transcription as GSK-3 can regulate transcription through AP-1 elements [20] and the cyclic AMP-response-element-binding protein [21]. Whatever signalling pathways may be responsible for the effects of insulin on PEPCK and gene 33 mRNA, the differential sensitivities of these mRNA to insulin in H4 cells may underlie the distinctive effects of wortmannin [4].

The relationships between wortmannin and PMA regulation of gene 33 and PEPCK mRNA expression present difficulties for interpretation. Although insulin and PMA provoke similar effects on gene 33 and PEPCK mRNA contents, wortmannin inhibits insulin actions on gene 33 and is without effect on PEPCK. In contrast, wortmannin is without effect on the PMA-sensitivity of gene 33, but it abolishes the action of PMA on PEPCK. At present we are unable to explain these results. The interaction of phorbol esters/protein kinase C with growth-

factor signalling pathways is complex; thus protein kinase C has been shown to activate MAPK by Ras-dependent and Ras-independent mechanisms [22] and, in addition, phorbol esters have been shown to cause inactivation of GSK-3 [17]. This latter effect may be direct as protein kinase C can phosphorylate and inactivate GSK-3 *in vitro* [23]. In CHO.T cells, wortmannin does not influence inhibition of GSK-3 by PMA, and it alters MAPK activation by PMA only marginally [17].

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