Induction of mRNA for phosphoenolpyruvate carboxykinase (GTP) by dexamethasone in cultured rat hepatocytes requires on-going protein synthesis

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Dexamethasone is necessary and sufficient to induce mRNA for phosphoenolpyruvate carboxykinase (GTP) (PEPCK) by 19-fold in rat hepatocytes cultured in serum-free medium. However, the time required for maximum induction is 16 h. The slow induction suggested that glucocorticoids regulate the expression of an intermediate gene product(s) which is required for glucocorticoid stimulation of PEPCK-gene expression. Consistent with this notion was the finding that cycloheximide completely blocked the response to dexamethasone. In contrast, cycloheximide did not block the response to a cyclic AMP analogue.

INTRODUCTION

Amounts of phosphoenolpyruvate carboxykinase (GTP) (PEPCK; EC 4.1.1.32), the rate-limiting enzyme in gluconeogenesis, are regulated by a variety of hormones, including glucagon (acting via cyclic AMP), glucocorticoids, insulin and thyroid hormone (reviewed in ref. [1]). Hormonal regulation of the expression of the PEPCK gene occurs primarily at the level of transcription, and 5' flanking regions of the gene which are involved in regulation by cyclic AMP and glucocorticoids have been identified [2–4]. However, a detailed molecular description of the hormonal regulation of expression of the native PEPCK gene in liver cells has not yet been obtained. For example, the issue of whether glucocorticoids are necessary and sufficient to regulate the expression of this gene in liver cells has not been clearly resolved. The suggestion has been made that the action of glucocorticoids is primarily permissive for cyclic-AMP-mediated increases in PEPCK amount (e.g. [5–10]). In these particular studies, little or no change in PEPCK activity, synthesis rate or mRNA amount occurred after brief exposure to glucocorticoid alone. However, various experiments with isolated rat hepatocytes or hepatoma-cell lines have yielded conflicting results ([7–12] versus [13–16]). Some of the apparent conflicts were probably due to differences in culture media which contained different concentrations of other hormones or serum. The present study has used rat hepatocytes cultured exclusively in a chemically defined medium to address the question of whether glucocorticoids are necessary and sufficient to increase PEPCK mRNA abundance.

The long time course of PEPCK mRNA response to dexamethasone observed in these experiments led us to address a second question, i.e. whether induction of PEPCK mRNA by glucocorticoids requires on-going protein synthesis. Cycloheximide was found to block induction of PEPCK mRNA by dexamethasone. This indicates that dexamethasone-stimulated expression of

the PEPCK gene depends on the expression of some other glucocorticoid-regulated gene(s).

MATERIALS AND METHODS

Materials

Guanidine hydrochloride, dexamethasone (9 α -fluoro-16 α -methylprednisolone), bovine serum albumin (fatty acid free) and soya-bean trypsin inhibitor were purchased from Sigma Chemical Co. Gene Screen membranes and [α -³²P]dCTP were from New England Nuclear. Oligo-labelling kits were obtained from Pharmacia or International Biotechnologies Inc. Other supplies included guanidine thiocyanate (EM Science), collagenase (Cooper Biomedical), tissue-culture dishes (Corning), 8-(4-chlorophenylthio)adenosine cyclic phosphate (CPT-cAMP) and cycloheximide (Boehringer Mannheim), and Hepes buffer (Research Organics). The cDNA clone pPCK10 [17] was generously provided by Dr. Richard W. Hanson (Case Western Reserve University, Cleveland, OH, U.S.A.).

Isolation and culture of hepatocytes

Male Sprague–Dawley rats (200–300 g) were obtained from Zivic–Miller (Allison Park, PA, U.S.A.). Preparation of animals and isolation and culture of hepatocytes were carried out by a modification [18] of the method of Berry & Friend [19]. Hepatocytes were cultured in Waymouth's 752/1 medium containing 0.2% bovine serum albumin (fatty-acid-free), 0.02 mM-oleate, 10 mM-Hepes (pH 7.4), and 20 μ g of gentamycin/ml [20]. Medium was replaced 4 h and 22 h after plating. At 28 h, a portion of a concentrated stock solution of dexamethasone was added to the medium to yield a final concentration of 1 μ M.

Experiments on animals were approved by the Animal Care and Use Committee of the University of Pittsburgh.

Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase (GTP); CPT-cAMP, 8-(4-chlorophenylthio)adenosine cyclic phosphate.

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Isolation of RNA and quantification of mRNA

Total RNA was isolated by the method of Chirgwin *et al.* [21]. Samples (5 μ g) of total RNA were subjected to Northern-blot analysis, and quantification of specifically hybridized PEPCK mRNA was performed by liquid-scintillation spectrometry as previously described [22].

RESULTS AND DISCUSSION

In the absence of any added hormones, PEPCK mRNA declined to 3% of the amount found in liver of a fed adult rat (Table 1). Determinations of dose-response established that maximal induction of PEPCK mRNA in cultured rat hepatocytes was obtained at 0.1–1.0 μ M-dexamethasone (results not shown). Overnight exposure of the hepatocytes to 1 μ M-dexamethasone resulted in a 19-fold increase in abundance of PEPCK (Table 1). Thus, dexamethasone alone was sufficient to produce increases in PEPCK mRNA in these cells.

A clue as to why some investigators may have previously failed to observe a glucocorticoid response is found in the slow time course of the response. In contrast with the rapid increase in PEPCK mRNA after addition of CPT-cAMP to the medium, maximal response to dexamethasone was achieved only after 16 h of exposure to the hormone (Fig. 1). This is much longer than expected if the response kinetics were dictated slowly by the 30–40 min half-life of PEPCK mRNA [23–25]. Thus examination of hepatocytes up to 4 h after addition of dexamethasone would have failed to detect increases in PEPCK mRNA. A similar difference in kinetics of induction of PEPCK activity by dibutyryl cyclic AMP and dexamethasone was previously noted in serum-deprived Reuber H-35 cells [13], although the time difference was not as pronounced as in Fig. 1.

The relatively long lag period and slow induction kinetics for the dexamethasone induction of PEPCK mRNA suggested that glucocorticoids are required for the expression of some labile gene product(s), which is necessary for glucocorticoid induction of PEPCK-gene expression. This hypothesis suggested that inhibition of protein synthesis would block the PEPCK-mRNA

response to dexamethasone. Addition of cycloheximide simultaneously with dexamethasone resulted in complete inhibition of the normal response to dexamethasone at either of the time points examined (Table 2). While our studies were in progress, similar results were reported for dexamethasone-stimulated expression of genes for albumin [26] and tryptophan 2,3-dioxygenase [27] in cultured rat hepatocytes. Thus a group of genes in rat liver shares the requirement of on-going protein synthesis for glucocorticoid response.

To eliminate the possibility that cycloheximide somehow impaired the capacity of the hepatocytes to express high amounts of PEPCK mRNA, the effect of cycloheximide on the response to CPT-cAMP was also determined (Table 2). In this case, addition of cyclo-

Table 1. Induction of PEPCK mRNA by dexamethasone in cultured rat hepatocytes

Hepatocytes were prepared from adult male Sprague-Dawley rats and cultured in serum-free medium as described in the Materials and methods section. At 28 h of culture, dexamethasone (1 μ M) was added to one set of plates and incubation was continued for 16 h. Total RNA was extracted and subjected to quantitative Northern-blot analysis as described in the Materials and methods section. The average radioactivity (c.p.m.) in the PEPCK mRNA band is shown, corrected for filter background. Amounts of mRNA are expressed relative to that found in RNA pooled from three livers of fed adult rats. Relative amounts of PEPCK mRNA in control hepatocytes cultured for 28 or 44 h were identical (results not shown). Values represent means $\pm$ S.E.M. for four separate hepatocyte preparations.

	Radioactivity (c.p.m.)	Relative mRNA amount
Control (28 h)	59 $\pm$ 15	0.03 $\pm$ 0.008
+ Dexamethasone	1112 $\pm$ 141	0.58 $\pm$ 0.07
Increase (fold) . . .		19

Fig. 1. Time course of PEPCK mRNA response to dexamethasone (●) and CPT-cAMP (○)

Dexamethasone (1 μ M) or CPT-cAMP (50 μ M) was added to cultured rat hepatocytes as described in the legend to Table 1. RNA was extracted from the cultures at the times indicated and analysed by quantitative Northern-blot analysis. (a) Relative mRNA amounts have been normalized to the 16 h value within each time course for purposes of comparison. Maximum amounts of PEPCK mRNA in the presence of CPT-cAMP or dexamethasone are equivalent to within $\pm$ 50%. Values represent the averages and range of two separate experiments for each time course. (b) Values represent means $\pm$ S.E.M. for three separate experiments. Error bars are not shown when exceeded by dimensions of the symbol.

Table 2. Effect of cycloheximide on induction of PEPCK mRNA by dexamethasone and CPT-cAMP

Rat hepatocytes were prepared as described in the Materials and methods section. At 28 h of culture, dexamethasone (1 μ M) or CPT-cAMP (50 μ M), in the presence or absence of cycloheximide (10 μ g/ml), was added, and incubation was continued for 8 h or 16 h. This concentration of cycloheximide blocks protein synthesis in cultured rat hepatocytes by more than 90% [26,27]. Increases in PEPCK mRNA in the presence of cycloheximide are shown relative to those observed in the absence of cycloheximide. Values represent means $\pm$ S.E.M. for three separate experiments; those in parentheses are mRNA amounts relative to those in untreated controls at time of addition.

Addition to medium	Increase (% over that in absence of cycloheximide)	
	8 h	16 h
Cycloheximide	0 (0.33 $\pm$ 0.03)	0 (0.23 $\pm$ 0.20)
Dexamethasone + cycloheximide	0 (1.01 $\pm$ 0.26)	0 (0.20 $\pm$ 0.05)
CPT-cAMP + cycloheximide	190 $\pm$ 45 (11.8 $\pm$ 2.6)	116 $\pm$ 23 (5.30 $\pm$ 0.85)

heximide actually augmented the response to CPT-cAMP. Cycloheximide alone decreased PEPCK mRNA below the value in untreated controls. These results demonstrate that on-going protein synthesis is not required for induction of PEPCK mRNA by a glucocorticoid-independent pathway. This is in agreement with results of similar experiments using rat hepatoma cells [28].

Cycloheximide was previously shown to block induction of PEPCK mRNA and synthesis rates by dexamethasone in kidney of intact rats [29,30]. These results led to the proposal that the response to dexamethasone required an intermediate step involving the synthesis of a regulatory protein [30]. However, since these studies were carried out in intact animals, it could not be determined whether or not the results reflected direct responses to either dexamethasone or cycloheximide. The use of rat hepatocytes cultured in serum- and hormone-free medium was designed to rule out the possibility of secondary responses to added hormones or other agents.

Glucocorticoid stimulation of PEPCK-gene expression resembles that of at least three other rat liver genes (albumin, tryptophan 2,3-dioxygenase and α_1 -acid glycoprotein) in that increases in transcription rate or in mRNA require on-going protein synthesis [26,27,31–33]. This contrasts with the glucocorticoid induction of mouse mammary-tumour virus RNA, which does not require on-going protein synthesis in any cell types tested, including rat hepatoma cells [31,32,34]. The regulatory step at which cycloheximide blocked the dexamethasone-dependent increase in PEPCK mRNA was not identified in the present study. However, since glucocorticoid regulation of PEPCK-gene expression occurs primarily at the level of transcription (e.g. [1–4,35,36]), this is likely the regulatory step affected by cycloheximide.

The precise role of the proposed regulatory protein which is required for glucocorticoid induction of PEPCK mRNA is not known. Further studies are required to determine, e.g., whether it interacts directly with specific DNA sequences or only with the glucocorticoid-hormone-receptor complex or other transcription factors.

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