Glucocorticoids control phosphoenolpyruvate carboxykinase gene expression in a tissue specific manner

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ABSTRACT
Cytosolic Phosphoenolpyruvate carboxykinase is a key gluconeogenic enzyme which is expressed in a tissue specific manner in the liver, kidney and adipose tissue and is under hormonal control. The effect of glucocorticoids on expression of the gene coding for phosphoenolpyruvate carboxykinase in adipose tissue has been studied in vivo in rats and in vitro in adipose tissue organ culture and mouse 3T3 L1 adipocytes. Glucocorticoids, both in vivo and in vitro, repress the steady state level of phosphoenolpyruvate carboxykinase mRNA in the adipose tissue while increasing it in the kidney. The size of the mRNA and its 5' end are identical in adipose tissue and kidney, thus the same promoter is used in all tissues. The inhibitory effect of glucocorticoids on phosphoenolpyruvate carboxykinase gene expression was located at the level of transcription. As glucocorticoids are known to stimulate transcription of phosphoenolpyruvate carboxykinase gene in the liver and kidney, the inhibitory effect on its transcription in adipose tissue suggests that tissue specific transcription factors may modulate the effect of glucocorticoids.

INTRODUCTION
Features inherent to expression of the gene encoding cytosolic phosphoenolpyruvate carboxykinase (GTP) (EC. 4.1.1.32), make this gene an attractive model to study tissue specificity and hormonal control of gene expression in mammalian tissues. Thus, cytosolic phosphoenolpyruvate carboxykinase (PEPCK) is a key gluconeogenic enzyme which is specifically expressed in the gluconeogenic tissues (liver and kidney) and in the glyceroneogenic adipose tissue (1,2). In addition, alterations in the rate of synthesis of the enzyme in all three tissues are modulated by many hormones (1,2). Recent studies in the liver and kidney of the rat have clearly documented that those changes occurred through corresponding changes in the abundance of PEPCK mRNA levels by modulating transcription of the gene (3-7). Thus, it constitutes a suitable model to unravel the mechanisms underlying multi-hormonal control of gene expression.
A unique feature of PEPCK gene expression is exhibited by the observation that hormones regulate the rates of synthesis of PEPCK in a tissue specific
manner (1,2,8). We have previously shown that glucocorticoids repress the synthesis of PEPCK in the adipose tissue while enhancing it in the liver and kidney (8). The inhibitory effect of PEPCK synthesis by glucocorticoids has been demonstrated both in vivo in adipose tissue of the rat (8) and in vitro in rat adipose tissue organ culture (9). Whereas studies in the liver and kidney have clearly attributed the hormone stimulatory effect to the transcription level, the nature of the negative effect in the adipose tissue has hitherto remained unknown. Such reciprocal effect of glucocorticoids is intriguing especially bearing in mind that PEPCK is encoded by a single copy gene (10,11) and that positive glucocorticoid responsive elements were identified in its promoter region (12,13). We therefore set out to elucidate the nature of the negative effect of glucocorticoids on adipose tissue PEPCK gene expression.

In the present study we provide evidence that glucocorticoids repress PEPCK mRNA steady state level in the adipose tissue while increasing it in the kidney. Both these reciprocal effects of glucocorticoids on PEPCK gene expression are at the level of transcription initiated from the same promoter. These findings lead to the conclusion that glucocorticoids modulate PEPCK gene transcription in a tissue specific manner, thereby adding another aspect to the hierarchy of tissue specific control of gene expression.

**EXPERIMENTAL PROCEDURES**

**Animals:** Adult Sabra male rats (Wistar origin) 6-8 weeks old, from Hebrew University breeding farm, were adrenalectomized and used 4-7 days post operation. Synthetic glucocorticoids, dexamethasone phosphate (Ikaphram, Israel) or triamcinolone acetonide (Lederle) (2.5mg/100 g body weight), were injected intraperitoneally 4 or 17 h before sacrifice, respectively. Fasting when indicated, was for 24 h.

**Organ and Tissue Culture:** Adipose tissue organ culture, from epididymal fat pads of 5 weeks old intact rats, was prepared as previously described (9). Dexamethasone phosphate (1 µM) was added to the medium for the last 4 or 17 h of incubation. 3T3 L1 cells (originally from Dr. H. Green) were converted to adipocytes at confluence (following Rubin et al. (14)) and kept in the presence of insulin (4 milliunits/ml, Lilly Research Laboratories) for 8 days post-confluence, when the adipocytes constituted the majority of the cell population. Insulin was removed 48 h prior to cell harvesting. Dexamethasone phosphate (1µM) was added to the medium for the last 17 h of incubation.

**RNA Extraction and Analysis:** Total RNA from tissues or cells was extracted with guanidinium thiocyanate and centrifuged through CsCl cushion according to
Chirgwin et al. (15). The RNA was analysed by Northern blot or dot-blot hybridization as described (16). Densitometry of the autoradiographic images was performed using the Helena Quick-scan R&D densitometer (Helena Laboratories, Beaumont, TX).

**Molecular Probes:** pPCk10 is a rat cDNA clone (10). pGSRK-1 is a rat glutamine synthetase cDNA clone (17). p53 is a cDNA encoding mouse ribosomal L19 (18). pBH1.2 is a BamHI – HindIII fragment of the 5' region of rat PEPCK gene cloned into pBR322 (10). GA3PD is a cDNA containing the sequence coding for mouse glyceraldehyde 3 phosphate dehydrogenase, kindly provided by P. Curtis. L18, L19 (19) and L32 are cDNAs containing the sequences coding for the corresponding rat ribosomal proteins provided by O. Meyuhas and D. Peleg.

The probes were $^{32}$P - labeled by nick translation (20) for hybridization of RNA blots.

**S1 mapping:** 20 μg of total kidney or adipose tissue RNA were hybridized with 5' $^{32}$P - end labeled Bgl II-restricted pBH1.2 DNA and digested with S1 nuclease (Boehringer) as described (21). The protected probe was analysed by size on an urea polyacrylamide gel together with a size marker.

**Primer extension assay:** The assay was performed essentially as described by Beale et al. (22) by using a 5' $^{32}$P-labeled 24 nt synthetic oligomer (+69 to +46 of the PEPCK gene), as a primer. The radioactive primer was ethanol precipitated overnight, with the RNA, and solubilized in 20 μl hybridization buffer containing 0.13M Tris HCl pH 8.3 and 0.18M KCl. Hybridization was carried out, in the presence of 10 units of RNase inhibitor (Rnasin, Promega) for 1.5h at 42⁰C, after initial heating of the samples for 5 min. at 85⁰C. At the end of the annealing period the mixture was adjusted to 2mM dXTP, 12mM MgCl$_2$, 4mM dithiotreitol and 30 units of AMV reverse transcriptase (New England Biolabs) and incubated for additional 1.5h at 42⁰C. After phenol-chloroform extraction and ethanol precipitation, the precipitate was dissolved and loaded on to a sequencing gel along with a ladder of sequenced DNA ($^{32}$P-labeled DNA at the 3' end of an Eco RI digested pUC 18, sequenced according to Maxam & Gilbert (23).

**Run-on Transcription:** Epididymal fat pads from 25 rats were crushed by tissue press at 4⁰C, homogenized quickly (using a tight teflon homogenizer) with 10 volumes of ice cold heptane and spun at 1000 x g for 5 min. The fat free nuclei were resuspended in 4 volumes of a solution containing 3mM MgCl$_2$, 0.01 M NaCl, 5mM dithiotreitol, 0.01 M Tris buffer pH 7.4 and 0.5% Nonidet P40 and centrifuged at 4⁰C for 10 min at 1000 x g. The pellet was again resuspended in the above solution (without Nonidet P40), containing 0.3 M sucrose and 25% glycer-
Figure 1. Effect of glucocorticoids on the steady state level of PEPCK transcripts in the adipose tissue. 6–8 weeks old male rats used 4–7 days post adrenalectomy. Fasting when indicated was for 24 h. Triamcinolone acetonide (Lederle) (2.5 mg/100 g b.w.) was injected intraperitoneally 17 h before sacrifice. RNA was extracted (15) from epididymal pads, pooled from 7 rats equally treated, and from 2 kidneys of each such group. The abundance of specific transcripts was determined by dot hybridization assay (3–18 µg total RNA per dot), using the following molecular probes: pPCK10, a PEPCK cDNA containing plasmid (10); pGSRK-1, a glutamine synthetase cDNA (17); and p53, a cDNA encoding mouse ribosomal L19 (18). Densitometric scans of the autoradiographic films of a representative experiment, in arbitrary units, are presented in histograms showing mRNA’s abundance without (empty boxes) and with (stippled boxes) triamcinolone treatment.

ol, and centrifuged at 2000xg for 10 min. The nuclear pellet was resuspended in nuclear storage buffer (5) at 10^8 nuclei/ml and frozen at -70°C. Nuclei run on assays and extraction of the 32P-RNA was according to McKnight and Palmiter (24) as described by Meisner et al., (5) except that 15 x 10^6 nuclei and 0.25 milliCi 32P-UTP were used per assay. The extracted 32P-RNA consistently yielded 20 x 10^6 trichloroacetic acid precipitable cpm per assay which were hybridized with immobilized plasmid DNA probes.

RESULTS

Previously, we have shown that glucocorticoids treatment repressed synthesis of PEPCK in the adipose tissue while stimulating it in the liver and kidney
Figure 2. Effect of glucocorticoids on PEPCK mRNA levels in rat adipose tissue organ culture and in mouse 3T3 L1 adipocytes. (A) Adipose tissue organ culture, from epididymal fat pads of rats was prepared as previously described (9). Dexamethasone phosphate (1 μM) was added to the medium for the last 4 or 17 h incubation. RNA from control dishes (−) and dexamethasone containing dishes (+) was assayed for PEPCK transcripts by dot blot hybridization. 2, 4, 8 and 12 indicate the amounts of RNA in μg per dot. (B) 3T3 L1 cells were converted to adipocytes at confluence (see Experimental Procedures). The effect of glucocorticoids on the abundance of PEPCK transcripts in adipocytes was tested by the addition of 1 μM dexamethasone for the last 17 h. RNA extracted from the cells, as described in (A) was assayed for PEPCK mRNA by blot hybridization with rat PEPCK cDNA as probe (We have previously shown homology of cytosolic PEPCK transcripts in rodents (37). 60 μg of total RNA from adipocytes incubated without dexamethasone (lane 1) and with dexamethasone (lane 2). 50 μg of total RNA from rat liver (lane 3). rRNA size markers are indicated. GAPDH – The abundance of glyceraldehyde 3-P dehydrogenase mRNA, revealed by reprobing the same blot with a complementary cDNA probe, is shown in the bottom of the figure as an internal control. Note that these cells are rich in GAPDH mRNA as has been previously reported (25).

(8). To delineate the nature of this reciprocal effect, we initially determined the steady state level of PEPCK mRNA in the adipose tissue and kidney. Thus, total RNA was extracted from adipose tissue and from kidneys of adrenalecto-mized rats, with or without triamcinolone treatment for 17 h. The abundance of the specific mRNA was determined by dot blot hybridization (3–18 μg RNA per dot) with 32P-labeled PEPCK cDNA (10) as a probe. Densitometric scanning of the autoradiograms demonstrated a progressively increasing autoradiographic signals, proportionately linear with respect to increased amounts of RNA per dot. The resulting slopes of these signals are presented as histograms (Fig. 1). These demonstrated comparable baseline levels of PEPCK mRNA in adipose tissue and kidney. However, 17 h after triamcinolone administration,
PEPCK transcripts in the adipose tissue markedly decreased while they increased by 2-3 fold in the kidney (Fig 1).

Consistent with previous findings on the rate of synthesis of PEPCK (8) we observed that 24 h fasting of adrenalectomized rats increased the steady state level of PEPCK transcripts in the adipose tissue, but glucocorticoid treatment reduced it as well (Fig 1). The magnitude of the glucocorticoids-mediated repressive effect, in several independent experiments of fasted or fed adrenalectomized rats, ranged between 3-7 fold.

As an internal control we reprobed the same blot with cloned ribosomal protein L 19 cDNA (18). The abundance of the corresponding mRNA was similar in both tissues and did not change significantly with any of the experimental treatments (Fig. 1). Furthermore, reprobing the blot with glutamine synthetase cDNA (17) revealed that administration of triamcinolone increased, rather than decreased, the steady state level of the corresponding mRNA in the adipose tissue of both fed and fasted adrenalectomized rats (Fig. 1). These findings essentially agree with those observed by Burns et al. (17) for glutamine synthetase mRNA in 3T3 L1 adipocyte cells. Thus, the down-regulation mediated by glucocorticoids appears to be specific for PEPCK mRNA rather than a result of a general effect on the content of mRNA in this tissue.

To ascertain that glucocorticoids directly affect the steady state level of PEPCK mRNA in the adipose tissue we have examined the glucocorticoid effect in two in vitro experimental systems. First, rat adipose tissue organ culture was utilized. This system, as we have previously shown, allows a steady state maintenance of tissue proteins for 96 h, and the addition of glucocorticoids specifically inhibit PEPCK synthesis (9). Figure 2A shows that incubation of the adipose tissue organ culture with 1 μM dexamethasone leads to a parallel decrease in the level of PEPCK mRNA, and that the effect was readily noticeable after 4 h incubation. The second in vitro system used was the mouse 3T3 L1 cell line which is converted from preadipocytes to adipocytes. The abundance of the PEPCK mRNA level is lower in 3T3 L1 adipocytes than it is in rat epididymal fat pads; nevertheless, the repression of PEPCK mRNA level by glucocorticoids in these adipocytes is likewise marked (Fig. 2B). Conversely, the level of glycer-aldehyde-3-P dehydrogenase mRNA which was reported to be high in these cells (25), did not change under these conditions and thus served as a control for the amount of RNA blotted (Fig. 2B). Hence, it is evident that glucocorticoids directly and rapidly down-regulate the level of PEPCK mRNA in adipocytes.

The reciprocal effect of glucocorticoids on PEPCK gene expression in adipose tissue and kidney could involve tissue specific differences in the gene
Figure 3. RNA blot hybridization and S1 protection assay of adipose tissue PEPCK transcripts. Adrenalectomized rats fasted for 24h were used in all these experiments. Triamcinolone (2.5 mg/100 bg.w) was administered, as indicated, by a single intraperitoneal injection 17 h before sacrifice. (a) blot hybridization assay of PEPCK mRNA using 100 μg total kidney RNA (lanes 1, 3) and 50 μg of RNA from epididymal fat pads (lanes 2, 4) without (lanes 1, 2) and with (lanes 3, 4) triamcinolone for 17 h. (b) - S1 mapping of the 5' PEPCK mRNA in total RNA from kidney (lane 1) and adipose tissue (lanes 2, 3) without (lanes 1,2) and with (lane 3) triamcinolone for 17 h. The cloned Bam HI-HindIII fragment (pBH1.2 (10), harboring the 5' end and flanking region of the PEPCK gene, was digested by Bgl II at nucleotide +70 and 32P-end labeled by polynucleotide kinase. After hybridization with 20 μg total RNA and S1 digestion as described (21), the size of the protected probe was analyzed on a urea polyacrylamide sequencing gel. The map of the probe and protected fragment with respect to PEPCK mRNA is described below.
transcripts. Northern blot hybridization analysis revealed a PEPCK mRNA of the same size in both tissues, before and after hormone treatment (Fig. 3a). To precisely map the 5' end of PEPCK mRNA in adipose tissue and examine whether it differed from that in the kidney we have used S1 protection assay, utilizing the same 32P-end labeled probe of the PEPCK gene as has been used previously by Wynshaw-Boris et al. (21). Results of this analysis, using 20 μg total RNA from adipose tissue, revealed a 70 nt protected DNA fragment regardless of the hormonal treatment. The size of the protected fragment was identical to that obtained by using total RNA from the kidney (Fig. 3b), suggesting that the 5' end of PEPCK mRNA is identical in both tissues. However to rule out the possibility that the PEPCK transcripts might have initiated at a further 5' exon, which could not be revealed by the protected probe that we have used for the S1 analysis, we have performed a primer extension analysis. The primer was a synthetic oligonucleotide of the sequence which covers the 3' end of the probe used for the S1 protection assay. Results of the analysis (Fig. 4) clearly show an extended fragment of 69 nt with adipose tissue, kidney and liver RNA regardless of the hormonal treatment. Furthermore, the size of the fragment was identical to that recently reported for PEPCK transcripts in the kidney and liver (22). This indicates an identical start site of transcription of the PEPCK gene in the adipose tissue, kidney and liver and implicates the existence of only one promoter for this gene in all tissues.

Glucocorticoids, as already mentioned above, stimulate transcription of the PEPCK gene in the kidney (5), liver (3) and in cultured hepatoma cells (26), and this stimulation accounts for changes observed in the steady state level of PEPCK mRNA in those tissues. Thus, it was intriguing to examine whether the negative effect in adipose tissue might also be exerted at the transcription level. Therefore, we attempted to determine, by run on assay, the relative transcription rate of PEPCK gene in isolated nuclei from adipose tissue. Due to the high fat content in this tissue, we developed a method to isolate nuclei after homogenizing the tissue in ice cold heptane (see Experimental Procedures). The relative low yield of nuclei extracted from adipose tissue (1-2x10^6/g) made it necessary to use 25 adrenalectomized rats per each assay. PEPCK transcription rate was assessed by the amount of radioactive RNA specifically hybridized with immobilized cloned PEPCK cDNA. As shown in two independent experiments (Fig. 5), treatment of fasted adrenalectomized rats with glucocorticoids for 17 h or even for a shorter period of 4 h, repressed transcription of the PEPCK gene by 3-4 fold while that of ribosomal protein genes remained unaltered. Thus, the repression of adipose tissue PEPCK mRNA level by
Figure 4. Transcription start site mapping, by primer extension assay, of the PEPCK gene in different rat tissues. A synthetic primer containing nucleotides +69 to +46 of the PEPCK gene was 5'-end P-labeled and annealed with RNA from adipose tissue, kidney and liver. A1 - 1 μg of poly A+ RNA from adipose tissue. A2 - 70 μg and A3 - 35 μg respectively of total RNA from adipose tissue of adrenalectomized rats with (A3) and without (A2) treatment with dexamethasone (2.5 mg per 100 g body weight) for 4 h. L - 1 μg poly A+ RNA from liver and K - 10 μg of poly A+ RNA from kidney of adrenalectomized rats. K' is a shorter exposure of K. The ladder sequence is a Maxam & Gilbert, (23) sequenced Eco RI digested pUC 18.
glucocorticoids can be attributed to an inhibition of transcription of the corresponding gene. The reciprocal control of transcription of the PEPCK gene by glucocorticoids in the adipose tissue (Fig. 5) and kidney (5) is of the same magnitude (3-4 fold) and is readily observed within 4 h.

**DISCUSSION**

Experiments presented in this work using adrenalectomized rats, rat adipose tissue organ culture and mouse 3T3 L1 adipocytes demonstrate that glucocorticoids negatively affect PEPCK gene expression in the adipose tissue. This hormonal effect is carried out at least in part by controlling the rate of transcription. The results document that glucocorticoids inhibit transcription of the PEPCK gene in the adipose tissue. However, to account for the marked reduction in the level of PEPCK mRNA by glucocorticoids, one can presume that these hormones exert in addition some posttranscriptional effects, such as destabilization of the mRNA. Decreased transcription rate, together with destabilization of a mRNA, can result in dramatic reduction of the mRNA level as has been reported in the case of histone mRNA (38). We have also presented evidence that the inhibitory effect is not of a general nature since other genes examined are either unaffected or induced by glucocorticoids, like glutamine synthetase. In contrast, previous studies in liver, kidney and in cultured hepatoma cells, have shown that glucocorticoids stimulate transcription of the PEPCK gene (3,5,26). Thus, it is evident that this single gene is reciprocally modulated by glucocorticoids in the various tissues.
One plausible explanation for such a reciprocal effect on the transcription of this single copy gene might be the utilization of alternative promoters as has been previously shown to underly tissue specific expression of other genes (27,28). A putative promoter might function specifically in the adipose tissue and be negatively modulated by glucocorticoids. It is worth noting that the chicken cytosolic PEPCX gene is indeed transcribed from two promoters 70 bp apart from each other (11). Furthermore, an additional weak promoter has also been suggested for the rat cytosolic PEPCX gene (22). By using S1 protection and primer extension assays we have established that transcription of the PEPCX gene utilizes a single promoter in all three tissues regardless of the hormonal treatment.

Thus, while glucocorticoids modulate transcription of the PEPCX gene in all three PEPCX-expressing tissues, the direction of the effect - whether it is stimulation or repression - must be determined by factor/s specific to the tissue. It may be that the glucocorticoids receptor is different between tissues or that other tissue specific factors which interact with the PEPCX gene modulate the reciprocal effect in the presence of glucocorticoids.

The vast majority of the genes affected by glucocorticoids are induced by the hormone. In many such cases defined functional glucocorticoids responsive elements (GRE), residing near the promoter, have been identified by introducing chimeric genes into cells (29). In a few instances, like the genes encoding proopiromelanocortin, alpha-feto protein and prolactin, glucocorticoids repress rather than stimulate transcription (30-32). A sequence containing a consensus GRE, residing upstream to the promoter, has been identified in these genes as well (32-34). Recently, by performing similar experiments, Hanson and colleagues have identified two functional GREs in the region of the PEPCX promoter (13). These are responsible for the stimulatory effect of glucocorticoids on the expression of chimeric genes, driven by the PEPCX promoter, in rat hepatoma cells. The elements raise the interesting question of whether such GREs can mediate both the positive effects and the negative effects we observe in adipose tissue. If this is true, it implies that there must be different proteins in the cells whose interaction with the GREs results in very different effects on transcription. Whether one of these different proteins could be the receptor itself, remains to be determined. Alternatively the identical glucocorticoid receptor could interact with different consensus GREs, such that one kind of interaction leads to a stimulation of transcription while the other leads to a repression. We have recently developed a transfection assay in our laboratory to test these two possibilities.
Reciprocal tissue specific hormonal control of transcription may be a rather general phenomenon. It has been shown that expression of one of the genes encoding myosin heavy chain was reciprocally controlled by thyroid hormones in different muscles (35). In this case the effects were observed in vivo after 8 days treatment of the rats and the steady state level of the RNA, rather than transcription, was determined. Transcription is generally considered as an important target for tissue specific control of gene expression (36). The phenomenon we describe, involving hormonal modulation of gene transcription in a tissue specific manner, may imply an additional step in the hierarchy underlying tissue specific transcription of genes.

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