Biochem. J. (1985) **226**, 123–130
Printed in Great Britain

The effect of ethanol, alone and in combination with the glucocorticoids and insulin, on glucose-6-phosphate dehydrogenase synthesis and mRNA in primary cultures of hepatocytes

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(*Received 23 July 1984/Accepted 18 October 1984*)

The hormonal regulation of the relative rate of synthesis and mRNA of glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) was studied in primary cultures of adult-rat liver parenchymal cells maintained in a chemically defined medium. Maintenance of hepatocytes from starved animals in a culture medium devoid of any hormones resulted in a 4-fold increase in the relative rate of G6PDH synthesis in 48 h. Parallel cultures treated with glucocorticoids alone exhibited a rate of G6PDH synthesis comparable with that in the control cultures, whereas insulin alone caused a 6.5-fold increase in the rate of synthesis in 48 h. However, if the cultures were treated with glucocorticoids and insulin simultaneously, a 13-fold increase in the rate of synthesis was observed. The effect of ethanol, alone and in combination with the hormones, on the relative rate of G6PDH synthesis was studied also. Ethanol alone caused an 8-fold increase in the rate of synthesis in 48 h, whereas the combination of ethanol, glucocorticoid and insulin caused a 25-fold increase. The amount of functional mRNA encoding G6PDH, as measured in a cell-free translation system, was compared with enzyme activity and relative rate of enzyme synthesis. The increases in G6PDH activity and relative rate of synthesis in primary cultures of hepatocytes treated with ethanol, alone and in combination with the glucocorticoids and insulin, were paralleled by comparable increases in G6PDH mRNA. The results of this study show that the glucocorticoids acted in a permissive manner to amplify the insulin stimulation of G6PDH synthesis and that insulin, glucocorticoids and ethanol interact to stimulate synthesis of G6PDH primarily by increasing the concentration of functional G6PDH mRNA.

G6PDH is the key regulatory enzyme of the pentose phosphate pathway. One of the main functions of this pathway is to supply reducing equivalents in the form of NADPH. The NADPH generated by the metabolism of glucose through the pentose phosphate pathway is used in reductive biosynthetic processes such as the synthesis of fatty acids and steroids.

Hepatic G6PDH has been shown to be under both nutritional and hormonal long-term regulation. Intact animal studies have demonstrated that G6PDH activity is decreased in the livers of animals which have been starved or starved and subsequently re-fed with a high-fat diet, and is markedly increased during re-feeding of high-carbohydrate diets to previously starved animals (Glock & McLean, 1955; H. M. Tepperman & J. Tepperman, 1958, 1963; J. Tepperman & H. M. Tepperman, 1958; Johnson & Sassoon, 1967; Yugari & Matsuda, 1967; Rudack *et al.*, 1971; Miksicek & Towle, 1982). Enzyme activity is increased by injection of insulin (Weber & Convery, 1966; Rudack *et al.*, 1971; Nepokroeff *et al.*, 1974), glucocorticoids (Berdanier *et al.*, 1976; Wurdeman *et al.*, 1978; Berdanier & Shubeck, 1979) and thyroid hormone (Glock & McLean, 1955; Miksicek & Towle, 1982), and decreased by injection of glucagon (Garcia & Holton, 1975).

Results obtained from intact-animal studies cannot differentiate between direct and indirect effects of hormones on G6PDH activity, and thus

Abbreviations used: G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); SDS, sodium dodecyl sulphate.

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several investigators have utilized primary cultures of adult hepatocytes to examine the hormonal regulation of G6PDH further. Conflicting results have been reported by several laboratories using cultured hepatocytes to study G6PDH regulation. Winberry *et al.* (1980) and Kurtz & Wells (1981) have observed that the glucocorticoids and insulin each increased G6PDH activity individually, and when present together they had no additive effect. Nakamura *et al.* (1982), on the other hand, reported that the glucocorticoids had no effect on basal or insulin-stimulated G6PDH activity. These three groups used serum either to coat culture dishes or to inoculate and maintain the hepatocytes in culture, and thus the interaction of the hormones present in the serum with those added to the culture medium obscures the results. In a more recent report Kelley & Kletzien (1984), using hepatocytes inoculated on to collagen-coated culture dishes and maintained in the complete absence of serum, demonstrated that the glucocorticoids by themselves had no effect on G6PDH activity, but, when they were present along with insulin, an amplification of the insulin stimulation of enzyme activity was observed.

In the study reported here, we have examined the influence of the glucocorticoids and insulin on the relative rate of G6PDH synthesis in primary cultures of hepatocytes maintained in a chemically defined medium. Our results establish that the glucocorticoids by themselves did not increase the relative rate of G6PDH synthesis, but that they amplified the insulin stimulation of G6PDH synthesis. The hormone-mediated effects on the relative rate of G6PDH synthesis paralleled the effects on cellular G6PDH activity. In addition, the effect of ethanol on the hormone-mediated changes in G6PDH synthesis was examined, since an earlier study (Kelley & Kletzien, 1984) has shown that ethanol, alone and in combination with the glucocorticoids and insulin, increased G6PDH activity. Our results demonstrate that the effects of ethanol on G6PDH activity are paralleled by changes in the relative rate of G6PDH synthesis. In addition, our results shows that the stimulatory effect of ethanol, alone and in combination with the glucocorticoids and insulin, on G6PDH activity and relative rate of synthesis occurs primarily at the pre-translational level by modulating amounts of functional G6PDH mRNA.

Experimental

Materials

Male Sprague–Dawley rats were obtained from Hilltop Farms (Scottdale, PA, U.S.A.). The following tissue-culture media were purchased from GIBCO: Waymouth's 752, Swim's S-77, and MEM Select-Amine kit (catalogue no. 300-9050). Crystalline pig insulin was generously given by Dr. W. W. Bromer of Eli Lilly and Co. L-[^{35}S]-Methionine (1000Ci/mmol), Biofluor scintillation fluid and En^{3}Hance were from New England Nuclear. X-Omat AR-2 film was purchased from Kodak, and guanidine thiocyanate (purum grade) was from Fluka. Pansorbin cells (heat-killed fomaldehyde-fixed *Staphylococcus aureus*, Cowan I strain) were from Calbiochem–Behring, Corp. Dexamethasone sodium phosphate was kindly given by Dr. K. Bohra of Organon Inc. Calf liver tRNA was from Boehringer Mannheim, and oligo(dT)–cellulose (type 7) was purchased from P-L Biochemicals. Nuclease-treated rabbit reticulocytelysate was obtained from Bethesda Research Laboratories. All other chemicals, including guanidine hydrochloride (grade 1), were supplied by Sigma Chemical Co.

Methods

Animal preparation, cell isolation and culture. Adult-rat liver parenchymal cells were isolated and maintained in culture as described previously (Kelley & Kletzien, 1984). For studies of the relative rate of enzyme synthesis, cells were maintained in Waymouth medium until initiation of the assay of this relative rate, at which time the medium was replaced with a low-methionine (10 μM) custom medium (6ml/100mm-diam. dish). This custom medium was prepared from a MEM Select-Amine kit purchased from GIBCO. The kit contained minimal essential medium supplemented with Earle's balanced salts. Insulin (46.7munits/ml final concn.), dexamethasone (0.5 μM) and ethanol (43.5mM) were added 2h after plating, re-added with each change of medium and present until the time of cell harvest or mRNA isolation. Previous work has demonstrated that these concentrations were well above that needed to obtain the maximum effect on G6PDH activity (Kelley & Kletzien, 1984).

Relative rate of G6PDH synthesis. At 2, 4 or 6h before cell harvest, the Waymouth medium was changed to the low-methionine custom medium, and the appropriate hormones or ethanol were re-added along with 100 μCi of [^{35}S]methionine, After incubation with the label the cells were harvested, homogenized and centrifuged, and G6PDH activity in the supernatant was determined as described previously (Kelley & Kletzien, 1984). G6PDH activity is expressed as munits/mg of protein, where 1 munit is defined as the amount required to reduce 1 nmol of $NADP^+$/min at 30°C. In addition to measuring enzyme activity, a sample of the enzyme supernatant was removed to determine the total amount of protein synthesis (Pelham & Jackson, 1976). Briefly, radioactivity incorpora-

tion into total protein was determined by first deacylating the ^{35}S-labelled proteins in the supernatant with a solution containing 1M-NaOH, 0.5M-H_2O_2 and 1mg of L-methionine/ml, followed by precipitation with trichloroacetic acid. The precipitated proteins were collected on glass-fibre filters and the amount of radioactivity was determined by scintillation counting.

The amount of newly synthesized G6PDH was measured by carrying out immunoadsorption of the remaining ^{35}S-labelled proteins in the supernatant. The supernatant was adjusted to 10mM/HCl, pH7.5, 140mM-NaCl, 2.5mM-EDTA, 5mM-L-methionine, 0.5% (v/v) Triton X-100, 0.05% (w/v) sodium deoxycholate, 0.5% (w/v) SDS (Immunoadsorption Buffer) and pre-cleared by adding 50μl of a 10% (w/v) suspension of heat-killed formaldehyde-fixed *Staphylococcus aureus*, Cowan I strain (SaC), and incubating at 4°C for 15min, followed by centrifugation at 4°C for 2min in an Eppendorf Microfuge (12800*g*). Supernatant fractions were immunoadsorbed by adding rabbit antibody to rat liver G6PDH (50% in excess of the amount of enzyme activity present) for 60min at 4°C, and then 10% (w/v) SaC (5 times the volume of antibody added) for 15min at 4°C. Immunoadsorbed proteins were collected by centrifugation (12800*g*, 2min, 4°C) and washed five times by resuspension in 0.8ml of Immunoadsorption buffer. Immunoadsorbed proteins were extracted for 2min at 100°C in 50μl of electrophoresis sample buffer [50mM-Tris/HCl, pH6.8, 1% (w/v) β-mercaptoethanol, 10% (v/v) glycerol]. Samples were centrifuged (12800*g*, 4min, 4°C) to remove SaC, Bromophenol Blue was added, and analysis was performed by SDS/polyacrylamide-gel electrophoresis. Verification that the immunoadsorption procedure quantitatively and specifically binds rat liver G6PDH is discussed under 'Determination of radioactivity associated with immunoadsorbed proteins'.

Values obtained for the relative rate of G6PDH synthesis were the same with the three different radioisotope pulse times (2, 4 and 6h). A 4h pulse time was routinely used to generate the data presented in this paper.

Isolation of mRNA. mRNA was isolated from primary cultures of hepatocytes by guanidine thiocyanate extraction, as previously described (Stumpo & Kletzien, 1984).

Translation in vitro and immunoadsorption of translational products. mRNA was translated in a nuclease-treated rabbit reticulocyte lysate system. Assays were carried out in a total volume of 30μl containing 10μl of lysate, 80mM-K^+, 1.17mM-Mg^{2+}, 100μg of calf liver tRNA/ml, 10mM-phosphocreatine, 10μM each of 19 amino acids (except methionine), 100μCi of [^{35}S]methionine, and 0.5μg of rat liver mRNA, and incubated at 30°C for 60min. At the end of the incubation period, the samples were placed on ice; 100μl of a solution containing 0.1M-Tris/HCl, 2.4mM-$NADP^+$, 4.8mM-β-mercaptoethanol and 1mM-EDTA was added, and the mixture was centrifuged for 20min at 170000*g* (Beckman SW-60) to remove microsomal-bound products and nascent polypeptide chains. The resulting supernatant represented released proteins. The incorporation of radioactivity into released proteins was determined by trichloroacetic acid precipitation as described by Pelham & Jackson (1976). The incorporation of radioactivity in the absence of added rat liver mRNA was determined in parallel assays and subtracted from the amount of radioactivity incorporated into total protein caused by the addition of mRNA. The incorporation of labelled amino acid into total proteins synthesized was linear up to the addition of 0.5μg of mRNA (results not shown). There were no differences observed in the total translated proteins as directed by equal amounts of mRNA isolated from hepatocytes subjected to different treatments. In addition, the relative abundance of an unregulated protein synthesized in the reticulocyte system in response to mRNA isolated from hepatocytes subjected to different treatments remained unchanged under all treatments (results not shown). Thus the effects observed in response to ethanol or hormones cannot be attributed to generalized effects on overall protein synthesis.

The remaining 170000*g* supernatant was immunoadsorbed as described for assays of relative rate of G6PDH synthesis with the exception that 5μl of antibody to rat liver G6PDH was used. Immunoadsorbed proteins were extracted into 50μl of electrophoresis sample buffer and analysed on SDS/polyacrylamide gels.

Determination of radioactivity associated with immunoadsorbed proteins. Immunoadsorbed proteins from the ^{35}S-labelled enzyme supernatants and cell-free translational products were electrophoresed on SDS/9%-polyacrylamide gels by the method of Laemmli (1970). After electrophoresis the gels were stained, destained and impregnated with En^{3}Hance according to the manufacturer's instructions, dried and exposed to X-Omat AR film at −70°C.

With the aid of the fluorogram, labelled bands that migrated with the same relative mobility as purified G6PDH were cut from the dried gels and solubilized in 0.2ml of 30% H_2O_2 for 2h at 80°C. The solubilized gel slices were counted for radioactivity in 4ml of Biofluor scintillation fluid. Protein specific radioactivity was corrected for background by subtracting out the average radioactivity from adjacent gel slices. The relative rate

of G6PDH synthesis and amount of functional mRNA coding for G6PDH were expressed as 100 × (specific immunoadsorbed radioactivity in G6PDH/total trichloroacetic acid-precipitable radioactivity in the enzyme supernatant or released proteins), and were corrected for the 27% efficiency with which radioactivity was recovered from polyacrylamide gels.

The degree of recovery of radioactivity from polyacrylamide gels was determined by electrophoresing ^{35}S-labelled rat liver cytosolic proteins on a SDS/polyacrylamide gel cross-linked with *NN'*-diallyltartardiamide instead of *N'N*-methylenebisacrylamide. The final concentrations of acrylamide and cross-linker in the gel were 9% and 2.43% respectively. Labelled proteins were recovered as described by Spath & Koblet (1979). Labelled protein bands were excised and solubilized for 30min at room temperature in 88mM-$NaIO_4$ (0.5ml/gel slice). Solubilized proteins were precipitated with trichloroacetic acid for 20min on ice [3vol. of 10% (w/v) trichloroacetic acid (0.75ml) was added to 2vol. of solubilized gel (0.5ml) to give a final trichloroacetic acid concentration of 6%] and collected by centrifugation at 195000*g* for 90min at 4°C. The precipitated proteins were washed once with ice-cold water, dissolved in 50μl of electrophoresis sample buffer, heated for 2min at 100°C and Bromophenol Blue was added. The amount of radioactivity present was determined in a 2μl sample. A known amount of radioactivity was electrophoresed on a SDS/polyacrylamide-gel cross-linked with *NN'*-methylenebisacrylamide (final concentrations of acrylamide and cross-linker were 9% and 0.24% respectively). Labelled protein bands were solubilized with H_2O_2 as described above, and the degree of recovery of radioactivity from polyacrylamide gels was determined. The percentage recovery was calculated as 100 × [total radioactivity (c.p.m.) obtained by H_2O_2 solubilization/total radioactivity (c.p.m.) applied].

Verification of the specificity and quantitativeness of the immunoadsorption method used to assess the amount of functional mRNA encoding G6PDH has been presented elsewhere (Stumpo & Kletzien, 1984). The results from those experiments demonstrated that when the released proteins from a translation assay *in vitro* programmed with hepatic mRNA isolated from a carbohydrate-induced rat (i.e. 3-day-starved/3-day-carbohydrate-refed) were immunoadsorbed with antibody directed against G6PDH and electrophoresed on a SDS/polyacrylamide gel, a single radioactive band was obtained which migrated with the same relative mobility as purified G6PDH. When the supernatant fraction from this immunoadsorption was immunoadsorbed a second time with antibody, only a small amount of labelled G6PDH was obtained (some 5–10% of the radioactivity observed in the first immunoadsorption). No radioactive bands were observed when immunoadsorption was performed on the translated mixture if mRNA was omitted from the assay or when non-immune rabbit serum was used for immunoadsorption. Finally, when immunoadsorption was done in the presence of 25μg of purified G6PDH, the added unlabelled protein competed completely with newly synthesized labelled G6PDH for binding to the antibody. From these results we concluded that the amount of functional mRNA coding for G6PDH could be determined by our assay procedures.

Statistical analysis. Statistical evaluation of the data was performed by Student's *t* test.

Results

Effect of dexamethasone, insulin and ethanol on G6PDH activity in cultured hepatocytes

The ability of dexamethasone and insulin to induce G6PDH activity as a function of time in primary cultures of hepatocytes is shown in Fig. 1. Cells were maintained in culture in either the absence (control group) or the presence of dexamethasone and insulin (hormone group) for the indicated lengths of time. The maximum observed change in G6PDH activity in both the control and hormone groups was at 48h. At this time there was about a 3-fold increase in the control group and a 5–6-fold increase in the hormone group compared with the activity found in cells that had been in culture for 2h in the absence of hormones. In subsequent experiments cells were treated with hormones for 48h.

Kelley & Kletzien (1984) reported that the inclusion of ethanol in control and insulin-plus-dexamethasone-treated primary cultures of hepatocytes caused a 50% increase in enzyme activity. Therefore we examined the effect of ethanol on the induction of G6PDH in control and hormone-treated cells. Fig. 1 demonstrates that cells treated with ethanol (43.5mM) in combination with insulin and dexamethasone had 8–10-fold more activity than did control cells in culture for 2h, 3–4-fold more activity than control cells cultured for 48h and 1.5–2-fold more activity than that seen in cells treated with dexamethasone and insulin for 48h.

The results in Fig. 2 demonstrate that effect of treating primary cultures of hepatocytes with ethanol and hormones, alone and in combination, for 48h. When the synthetic glucocorticoid dexamethasone was added alone, there was no stimulation of G6PDH activity above that present in the control group. Insulin caused an approx. 1.5-fold increase in G6PDH activity, and when insulin and

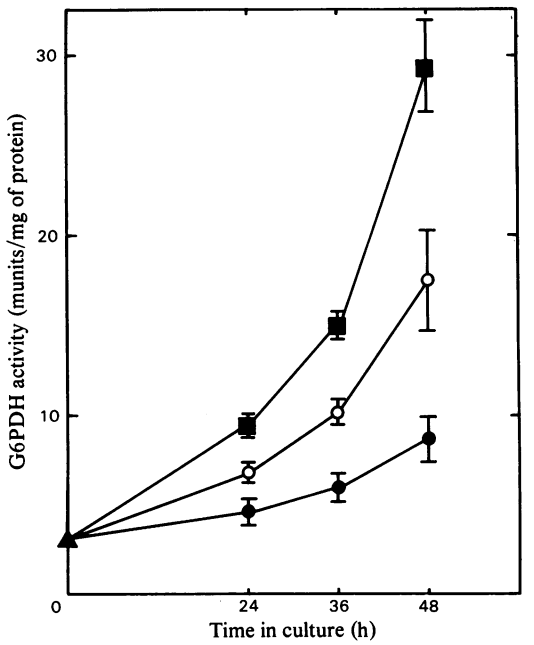

Fig. 1. *Time course of ethanol effect on G6PDH induction by dexamethasone and insulin in cultured hepatocytes*
Hepatocytes were maintained in Waymouth medium, with hormones and ethanol being added 2h after plating and re-added with each change of medium. Cells were harvested at the indicated times and enzyme activity was determined. The control group (●) received the hormone and ethanol diluting solutions; the hormone-treated group (○) received insulin (46.7munits/ml) and dexamethasone (0.5 μM) and the ethanol+hormone-treated group (■) received ethanol (43.5mM), insulin (46.7munits/ml) and dexamethasone (0.5 μM). ▲, Enzyme activity present in cells in culture for 2h in the absence of hormones, ethanol or diluting solutions. Data shown are the means ±S.E.M. from three different experiments, except for the 48h time point, where the control group represents four experiments, the hormone-treated group represents five, and the ethanol+hormone-treated group represents seven. The S.E.M. for the 2h control time point was within the symbol. Significant differences were observed between 2h control and 36h or 48h control cultures ($P \leqslant 0.05$), between control and hormone-treated cultures at 36h ($P \leqslant 0.01$) and 48h ($P \leqslant 0.05$), between control and ethanol+hormone-treated cultures at 24h, 36h ($P \leqslant 0.01$) and 48h ($P \leqslant 0.001$), and between hormone-treated and ethanol+hormone-treated cultures at 24h ($P \leqslant 0.05$), 36h and 48h ($P \leqslant 0.02$).

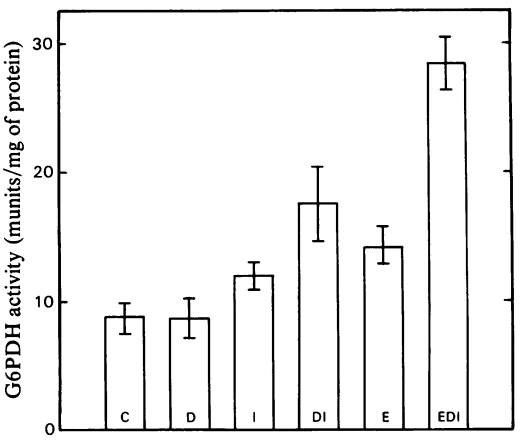

Fig. 2. *Effect of ethanol alone and in combination with dexamethasone and insulin on G6PDH activity*
Cells were maintained in Waymouth medium; ethanol and hormones were added after the 2h plating period and re-added with each change of medium. Cells were harvested after 48h and enzyme activity was determined. Cells were treated with the hormone- and ethanol-diluting solutions (C), dexamethasone (D), insulin (I), dexamethasone and insulin (DI), ethanol (E), or ethanol, dexamethasone and insulin (EDI). Hormone and ethanol concentrations are as indicated in Fig. 1. Data shown are the means ±S.E.M., from four (C), three (D), six (I), five (DI), six (E) and seven (EDI) different experiments. Significant differences were observed between C and DI ($P \leqslant 0.05$), between C and E ($P \leqslant 0.05$), and between C and EDI ($P \leqslant 0.001$). Significant differences were also seen between DI and EDI ($P \leqslant 0.02$).

dexamethasone were added together the glucocorticoid amplified the insulin stimulation of G6PDH activity. Ethanol potentiated both basal and insulin-plus-dexamethasone-stimulated G6PDH activity 1.5–2-fold. Treatment of cultures with ethanol plus dexamethasone or ethanol plus insulin did not significantly increase G6PDH activity above that found in cultures treated with ethanol alone (results not shown).

Relative rates of G6PDH synthesis in response to dexamethasone, insulin and ethanol

To determine whether or not the increases in G6PDH activity observed in response to treatment with insulin alone or in combination with dexamethasone were due to increases in enzyme protein synthesis, we measured the rate of G6PDH synthesis relative to total cytosolic protein synthesis in control and hormone-treated cells. Cell cultures were pulsed with [^{35}S]methionine for 4h before cell harvest, and newly synthesized labelled G6PDH was immunoadsorbed with antibody specific for rat liver G6PDH. Immunoadsorbed proteins were analysed and quantified by SDS/polyacrylamide-gel electrophoresis. Results presented in Table 1 show that dexamethasone treatment for 48h did not affect the relative rate of

Table 1. *Effects of dexamethasone and insulin alone and in combination on the relative rates of G6PDH synthesis*
Relative rates of G6PDH synthesis were measured, as described in the Experimental section, in hepatocytes maintained in culture in the absence (control) or presence of hormones during the 48h induction period. Relative rates of G6PDH synthesis were calculated as 100 × (specific immunoadsorbed radioactivity in G6PDH/trichloroacetic acid-precipitable radioactivity in the enzyme supernatant) and are expressed as means ± S.E.M., except for the 36h time point. The numbers in parentheses represent the numbers of determinations carried out to obtain the value presented. Significant differences from corresponding control cultures are shown by $^{a}P \leqslant 0.01$ and $^{b}P \leqslant 0.05$.

	Length of hormone treatment (h)	10^3 × Relative rate of G6PDH synthesis (%)
Control (2h in culture)	—	0.76 ± 0.43 (3)
Control (24h)	—	1.53 ± 0.50 (3)
Control (36h)	—	1.57 ± 0.47 (3)
Control (48h)	—	3.48 ± 1.02 (6)
Dexamethasome	48	3.66 ± 0.83 (4)
Insulin	48	4.89 ± 0.83 (4)
Dexamethasone + insulin	24	3.81 ± 1.43 (3)
Dexamethasone + insulin	36	6.66 (2)[a]
Dexamethasone + insulin	48	10.10 ± 3.28 (4)[b]

Table 2. *Effects of ethanol, alone and in combination with dexamethasone and insulin, on the relative rates of G6PDH synthesis*
Relative rates of G6PDH synthesis were measured in hepatocytes maintained in culture in the absence (control) or presence of ethanol and hormones during the 48h induction period. Relative rates of G6PDH synthesis were calculated as in Table 1, and are expressed as means ± S.E.M. The numbers in parentheses represent the numbers of determinations carried out to obtain the value presented. Significant differences from control cultures are shown by $^{a}P \leqslant 0.001$, $^{b}P \leqslant 0.01$, $^{c}P \leqslant 0.02$, $^{d}P \leqslant 0.05$. Significant differences from ethanol-treated cultures are shown by $^{e}P \leqslant 0.02$.

	Length of ethanol or hormone treatment (h)	10^3 × Relative rate of G6PDH synthesis (%)
Control	—	3.48 ± 1.02 (6)
Ethanol	48	6.44 ± 1.21 (3)[d]
Ethanol + dexamethasone + insulin	24	9.55 ± 1.93 (3)[c]
Ethanol + dexamethasone + insulin	36	11.18 ± 1.89 (3)[b]
Ethanol + dexamethasone + insulin	48	19.13 ± 3.02 (4)[a,e]

G6PDH synthesis compared with that seen in control cells in culture for 48h. Treatment of cells with insulin or insulin plus dexamethasone for 48h stimulated the relative rate of G6PDH synthesis approx. 1.5-fold and 3-fold respectively. Also presented in Table 1 are the relative rates of G6PDH synthesis measured in control and insulin-plus-dexamethasone-treated cells as a function of time of hormonal treatment. Cells maintained in the absence or presence of hormones showed increasing relative rates of G6PDH synthesis over the 48h culture period. The data presented in Table 1 do not preclude the possibility that alterations in enzyme-degradation rates may be occurring, but they do demonstrate that the increases observed in G6PDH activity (Fig. 1) in control and hormone-treated cells can be fully accounted for by parallel increases in the relative rates of G6PDH synthesis.

Kelley & Kletzien (1984) suggested that ethanol was exerting its effect on G6PDH activity by affecting the synthesis of new enzyme protein. This was based on their observation that ethanol had to be present for at least 12h before a stimulatory effect on G6PDH activity could be elicited. We measured the relative rates of G6PDH synthesis in both ethanol and ethanol + insulin + dexamethasone-treated cells to determine if increased enzyme synthesis was responsible for the observed ethanol stimulation of G6PDH activity. Table 2 shows that ethanol treatment for 48h caused a 1.5–2-fold increase in the relative rate of G6PDH synthesis compared with 48h control cells, and 48h treatment with ethanol in combina-

tion with insulin and dexamethasone enhanced G6PDH synthesis to a value 1.5–2 times that in the presence of just insulin and dexamethasone (Table 1). As was observed from control and insulin+dexamethasone-treated cell cultures (Table 1), the relative rates of G6PDH synthesis in ethanol+insulin+dexamethasone-treated cells increased as a function of time of ethanol and hormone treatment (Table 2). The data presented in Table 2 demonstrate that the stimulatory effect of ethanol, either alone or in combination with insulin and dexamethasone, on G6PDH activity can be fully accounted for by commensurate increases in the relative rates of enzyme synthesis.

Effect of ethanol on G6PDH mRNA activity

The observed effects of ethanol on G6PDH activity and relative rate of synthesis in 48 h control cultures and cells treated with glucocorticoid and insulin for 48 h could result from an increase in a translationally directed process. On the other hand, the increased activity and synthesis rate could occur as a result of an increase in a pre-translational event, which would result in an increase in G6PDH mRNA. To distinguish between these mechanisms, we measured functional G6PDH mRNA activity in primary cultures of hepatocytes treated with ethanol and/or glucocorticoid and insulin. mRNA was isolated from primary cultures of hepatocytes, translated in a mRNA-dependent reticulocyte-lysate system and the products were analysed by quantitative immunoadsorption using protein A-containing *Staphylococcus aureus*, followed by SDS/polyacrylamide-gel electrophoresis and fluorography (Stumpo & Kletzien, 1984). Table 3 shows that treatment of hepatocytes with ethanol for 48 h caused a 4–5-fold increase in G6PDH mRNA compared with 48 h control cells, and treatment for 48 h with ethanol in combination with dexamethasone and insulin stimulated mRNA activity to a value 1.3 times that observed in hepatocytes treated with dexamethasone and insulin. The data presented in Fig. 2 and Tables 2 and 3 demonstrate that ethanol, alone and in combination with dexamethasone and insulin, stimulates G6PDH activity through an increased relative rate of enzyme synthesis as a result of an increase in functional mRNA. Thus ethanol appears to regulate G6PDH at the pre-translational level.

Discussion

The results of this study establish that the relative rate of G6PDH synthesis in primary cultures of hepatocytes is influenced by insulin and the glucocorticoids. Treatment of cultures with glucocorticoid alone did not significantly elevate the rate of synthesis above that observed in the control cultures, whereas insulin alone caused a consistent but modest increase. Simultaneous treatment of the cultures with insulin and glucocorticoid for 48 h resulted in an increase that was 3-fold greater than control and 12–15-fold greater than the rate of synthesis observed in hepatocytes from starved animals. The glucocorticoids are known to amplify hormone-mediated effects on metabolism, although the precise mechanisms, for this are not understood. Since our results show that the increase in the relative rate of synthesis in paralleled by and can account for the increased G6PDH activity elicited by the hormones, the glucocorticoids must be amplifying insulin effects on the enzyme by either increasing transcription or increasing translation of pre-existing mRNA encoding G6PDH. Measurement of the amount of functional mRNA encoding G6PDH demonstrated that treatment with glucocorticoid and insulin resulted in an increase in G6PDH mRNA, which could account for the observed increase in enzyme activity and synthesis. Thus the

Table 3. *Effects of ethanol, alone and in combination with dexamethasone and insulin, on G6PDH mRNA activity*

mRNA isolated from 48 h control and treated hepatocytes was translated in a reticulocyte-lysate system, and newly synthesized G6PDH was analysed and quantified as described in the Experimental section. The amounts of functional mRNA coding for G6PDH were calculated as 100 × (specific immunoadsorbed radioactivity in G6PDH/total trichloroacetic acid-precipitable radioactivity in released proteins) and are expressed as means ± S.E.M. The numbers in parentheses represent the numbers of translation assays *in vitro* performed to obtain the value presented. Significant differences from control cultures are shown by $^{a}P \leqslant 0.001$, $^{b}P \leqslant 0.002$. Significant differences from ethanol-treated cultures are shown by $^{c}P \leqslant 0.01$.

	$10^3 \times$ G6PDH mRNA activity (%)
Control	0.97 ± 0.26 (4)
Dexamethasone + insulin	7.39 ± 0.88 (4)[a]
Ethanol	4.27 ± 0.48 (5)[a]
Ethanol + dexamethasone + insulin	9.95 ± 1.13 (5)[a,c]

glucocorticoids and insulin are affecting G6PDH by regulating pre-translational events which may involve any one or a number of transcriptional and post-transcriptional events.

Hepatic fatty acid synthesis is known to be regulated in response to the hormonal and nutritional state of the animal. Rats that are subjected to several days of starvation followed by feeding *ad lib* of a high-carbohydrate diet synthesize fat at an elevated rate. This phenomenon is called adaptive hyperlipidaemia, and it has been shown that the hepatic lipogenic enzymes, including G6PDH, exhibit greatly increased activity under these conditions. Several groups have shown that in adaptive hyperlipidaemia the relative rate of G6PDH synthesis increases (Rudack *et al.*, 1971; Winberry & Holten, 1977; Miksicek & Towle, 1982), although suggestions have been made that the enzyme is synthesized at a constant rate (Kelley *et al.*, 1975). Previous work has demonstrated that the increase in G6PDH activity in adpative hyperlipidaemia was not seen if the animals were either diabetic or adrenalectomized (Berdanier & Shubeck, 1979). Insulin- or glucocorticoid-replete animals did exhibit the increased G6PDH activity (Berdanier & Shubeck, 1979). Our studies in primary cultures of hepatocytes support the notion that the increase in G6PDH activity in adaptive hyperlipidaemia is the result of increased enzyme synthesis, and furthermore demonstrate that the glucocorticoids and insulin are directly involved in causing this effect in liver.

The development of alcoholic fatty liver is one of the major outcomes of alcohol abuse. Although the precise etiology of alcoholic fatty liver is not clear, increased hepatic lipogenesis may be a contributing factor (Baraona & Lieber, 1979). Our observation that ethanol, alone or in combination with insulin and glucocorticoid, increases the relative rate of G6PDH synthesis as a result of increased G6PDH mRNA activity is the first demonstration that ethanol can modulate the amount of a specific enzyme mRNA and subsequently cause an effect on the rate of synthesis of that enzyme. Since G6PDH is one of the key enzymes effecting the reduction of $NADP^+$, an increased catalytic capacity of G6PDH may contribute to some extent to the development of alcoholic liver damage. It is also tempting to speculate that ethanol in combination with the glucocorticoids may increase the synthesis of other hepatic lipogenic enzymes.

This study was supported by a grant from the Juvenile Diabetes Foundation. R. F. K. is the recipient of a Career Development Award (NIH AM-00733).

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