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Long-chain fatty acids suppress the induction of urea cycle enzyme genes by glucocorticoid action

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Abstract In order to test the possibility that free fatty acids are the mediator of the abnormal expression of urea cycle enzyme genes in carnitine-deficient juvenile visceral steatosis (JVS) mice, the effects of fatty acids on urea cycle enzyme, carbamoylphosphate synthetase (CPS) and argininosuccinate synthetase (ASS), mRNA levels were examined in rat primary cultured hepatocytes. Addition of a synthetic glucocorticoid hormone, dexamethasone, caused increases in CPS and ASS mRNAs. Further addition of oleic acid suppressed the induction of CPS and ASS mRNAs by dexamethasone. In contrast, the phosphoenolpyruvate carboxykinase (PEPCK) mRNA level induced by dexamethasone was enhanced in the presence of oleic acid. The effects were reversed on further addition of carnitine. The mRNA levels of these enzymes induced by dibutyryl cAMP were not affected by the addition of oleic acid. A study of the specificity of fatty acids revealed that long-chain fatty acids of more than 16 carbons chain length had a suppressive effect on the CPS mRNA level induced by dexamethasone and that the presence of double bonds enhanced the effect. The changes in gene expression of CPS, ASS and PEPCK caused by the fatty acids in the cultured hepatocytes were very similar to those observed in the liver of JVS mice. The AP-1 DNA binding activity in the presence of dexamethasone was slightly enhanced by the addition of oleic acid. These results suggest that the long-chain fatty acids not metabolized in JVS mice are mediators of the abnormal gene expression in the liver which results in hyperammonemia.

Key words: Fatty acid; Carnitine; Argininosuccinate synthetase; Carbamoylphosphate synthetase; Glucocorticoid; Rat cultured hepatocyte

1. Introduction

Fatty acids are normal metabolites with serum concentration of about 1 mM. They are metabolized to fatty acyl-CoA in the cells, and further to acetyl-CoA by β-oxidation, to triacylglycerol or to phospholipid. The degradative metabolism of long-chain fatty acids by β-oxidation needs carnitine as a cofactor for transport into the mitochondria. In hereditary carnitine-deficient JVS mice, the major symptoms in the infantile period are fatty liver, hypoglycaemia and hyperammonemia [1,2]. We have shown that the hyperammonemia is caused by the suppression of urea cycle enzyme genes [3–5], which is effectively treated by the administration of carnitine [6] and may be related to enhanced c-jun and c-fos expression and AP-1 DNA binding activity [5,7]. The concentration of free fatty acid in serum is very high in infant JVS mice and in starved adult JVS mice where the induction of the urea cycle enzyme genes is suppressed [8].

We postulate that fatty acid is a mediator causing the suppression of gene expression of urea cycle enzymes in carnitine deficiency. In the present paper, we examine the effect of fatty acids on the mRNA levels of carbamoylphosphate synthetase (CPS; EC 2.7.2.5) and argininosuccinate synthetase (ASS; EC 6.3.4.5) induced by a synthetic glucocorticoid hormone, dexamethasone, in rat primary hepatocytes.

2. Materials and methods

2.1. Primary culture of rat hepatocytes

Parenchymal hepatocytes were isolated from male Wistar rats weighing 180–300 g by in situ perfusion of the liver with collagenase [9]. Cells at a density of 2×10^6 cells per 60 mm dish were cultured with Williams E (WE) medium containing 10% calf serum and 1 nM insulin. After 4–6 h of culture, the medium was replaced by fresh WE medium containing 5% calf serum. Before treatments, cells were cultured in serum-free WE medium containing 0.33% fatty acid-free bovine serum albumin for 8 h. Treatment started 24 h after plating in serum-free WE medium. Hepatocytes were exposed to various treatments for 3–24 h, as indicated in the text and figure legends. Control cells were exposed to 0.33% bovine serum albumin in WE medium. Dexamethasone and fatty acids were dissolved in ethanol, and clofibrate and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in dimethylsulfoxide. The viability of all cell preparations was greater than 85% as estimated by the trypan blue exclusion assay. Cytotoxicity was determined by assaying the release of lactate dehydrogenase into the medium using the method of Cabaud and Wroblewski [10]. No significant increase of the enzyme released into the medium was observed.

2.2. Isolation and analysis of RNA

Total cellular RNA from the cultured hepatocytes 24 h after treatments was isolated according to Chomczynski and Sacchi [11] and used for analyses of mRNAs for CPS, ASS, phosphoenolpyruvate carboxykinase (PEPCK; EC 6.4.1.1), tyrosine aminotransferase (EC 2.6.1.5) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) by Northern and slot-blot hybridization with specific cDNA probes [4].

2.3. Gel mobility-shift assay

Nuclear extracts from cultured hepatocytes were prepared as described by Schreiber et al. [12]. Double-stranded synthetic oligonucleotides were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and used as probes. The binding reaction was carried out as described by Murakami et al. [13].

3. Results and discussion

We examined the effect of oleic acid on CPS, ASS, PEPCK and GAPDH mRNAs in primary cultured hepatocytes with and without dexamethasone and dibutyryl cAMP (Fig. 1). Addition of 0.5 mM oleic acid (OA) to the medium caused

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Abbreviations: AP-1, activator protein 1; ASS, argininosuccinate synthetase; CPS, carbamoylphosphate synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

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almost no changes in the amounts of mRNA. Addition of 10 nM dexamethasone (Dex) led to approx. 30-, 5- and 2-fold increases in CPS, ASS and PEPCK mRNAs, respectively. Further addition of oleic acid (Dex+OA) suppressed the induction of CPS and ASS mRNAs by dexamethasone, and enhanced that of PEPCK mRNA. The presence of 1 mM carnitine in the system (Dex+OA+Cn) relieved the suppression and enhancement by oleic acid of CPS and ASS induction and of PEPCK induction, respectively. Oleic acid showed no effect on the induction of CPS, ASS and PEPCK by 10 μM dibutyryl cAMP. In the case of GAPDH, dexamethasone and dibutyryl cAMP resulted in a slight decrease and increase in the mRNA content, respectively, and oleic acid caused a slight increase in the content irrespective of dexamethasone and dibutyryl cAMP. Tyrosine aminotransferase mRNA was changed like CPS and ASS mRNAs (data not shown). Thus, oleic acid specifically suppresses the induction by glucocorticoid of a group of genes. These changes in the mRNA of the enzymes caused by oleic acid seem to mimic those in the liver of JVS mice [4,14]. Increase in the oleic acid concentration dose-dependently increased the suppressive effect on dexamethasone induction of CPS mRNA, with 0.5 mM oleic acid reducing induction to about 50% (Fig. 2).

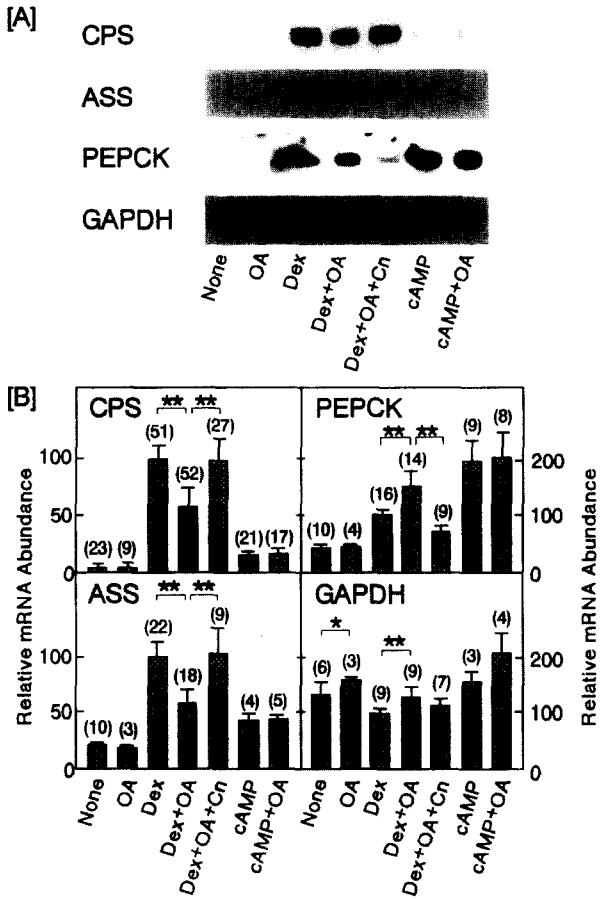

Fig. 1. Effect of oleic acid (OA) and carnitine (Cn) on induction by dexamethasone (Dex) and dibutyryl cAMP (cAMP) of carbamoylphosphate synthetase (CPS), argininosuccinate synthetase (ASS), phosphoenolpyruvate carboxykinase (PEPCK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs in cultured hepatocytes. (A) Total RNA (20 μg/lane) isolated from cultured hepatocytes was analyzed by Northern blot hybridization as described under Section 2. (B) mRNA contents were quantitated by slot-blot hybridization analysis and values are presented as means ± S.D. in relative absorbance with dexamethasone as 100. Experimental details are given in Section 2. Concentrations of reagents used were 0.5 mM oleic acid, 10 nM dexamethasone, 1 mM carnitine, and 10 μM dibutyryl cAMP. Statistically significant differences between the groups with and without oleic acid or carnitine: $^{*}P<0.05$ and $^{**}P<0.01$.

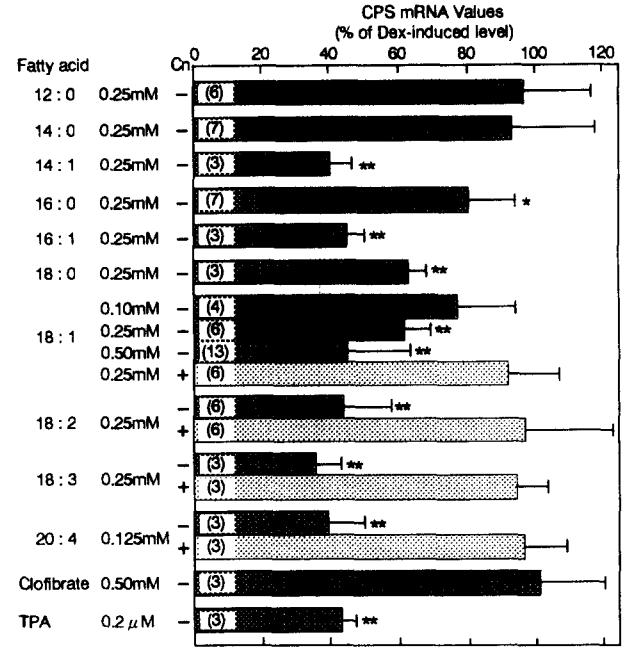

Fig. 2. Effect of various fatty acids, TPA, clofibrate and carnitine on induction by dexamethasone of CPS mRNA in the cultured hepatocytes. Fatty acids indicated with numbers of carbons and double bonds, TPA or clofibrate were added to the medium with 10 nM dexamethasone at the concentration indicated without (−) or with (+) 1 mM carnitine. Values are presented as means ± S.D. Numbers in columns denote numbers of experiments. Statistically significant differences from the value of dexamethasone only ($n=8$; 100 ± 11.7): $^{*}P<0.05$ and $^{**}P<0.01$.

Furthermore, we assessed the specificity of fatty acids, as shown in Fig. 2. The induction of CPS mRNA by dexamethasone was not influenced by the addition of saturated medium-chain fatty acids (carbon-chain length less than 14). Saturated fatty acids having a carbon-chain length greater than 16 demonstrated suppressive effects and the presence of double bonds enhanced the suppressive effect. Myristic acid (14:0) displayed no influence but myristoleic acid (14:1) showed a strong effect. Arachidonic acid (20:4) was the strongest suppressor we tested. With all fatty acids, addition of 1 mM carnitine reversed the effect almost completely. This suggests that the effect of fatty acids results partly from a shortage of carnitine in primary cultured hepatocytes [15] causing the accumulation of fatty acids and that the addition of carnitine stimulates the metabolism by converting the fatty acids into fatty acylcarnitine. Addition of 0.5 mM lauric acid (12:0) or salicylic acid, which were not effective but may compete with an effective fatty acid for CoA to be further metabolized, did not have any effect on the suppression (data not shown). All these results suggest that the mediators are free fatty acids, but not their metabolites. The spectrum of fatty acids affecting the induction by dexamethasone seems to be similar to that for protein kinase C activation. Unsaturated long-chain fatty acids such as oleic acid and arachidonic acid have been reported to activate protein kinase C [16,17]. TPA (0.2 μM), a

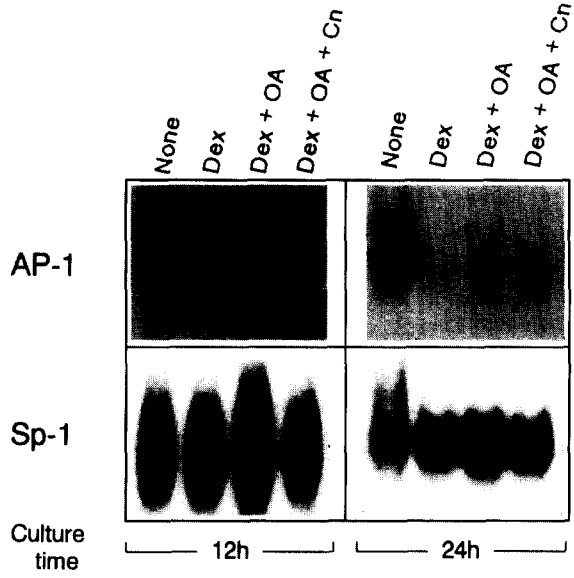

Fig. 3. Effect of oleic acid (OA) on AP-1 and Sp-1 DNA binding activities in the nuclear extract of cultured hepatocytes in the presence and absence of dexamethasone (Dex) and carnitine (Cn). Nuclear extract was prepared from hepatocytes cultured for 12 and 24 h after the additions indicated. Concentrations of reagents used were 10 nM dexamethasone, 0.5 mM oleic acid, and 1 mM carnitine.

potent protein kinase C activator, suppressed induction of CPS mRNA by dexamethasone (Fig. 2). We do not have conclusive data on this mechanism, because protein kinase C inhibitors showed no consistent results (data not shown) and as shown in Fig. 2, suppression was caused by saturated long-chain fatty acids such as palmitic acid and stearic acid which were reported not to activate protein kinase C [16,17]. Addition of clofibrate, a peroxisome proliferator, had no effect on the induction of CPS mRNA.

Finally, we assayed the AP-1 DNA binding activity by gel shift analysis of the nuclear fractions from the cultured hepatocytes under the conditions described in Fig. 3, since AP-1 DNA binding was highly activated in the liver of JVS mice [7]. AP-1 DNA binding activity observed under basal conditions was reduced by the addition of dexamethasone (Dex). Addition of oleic acid with dexamethasone (Dex+OA) enhanced the activity at 12 h but the effect was not clear at 24 h. Further addition of carnitine (Dex+OA+Cn) reduced the effect of oleic acid partially at 3 h (data not shown) and completely to the level of dexamethasone (Dex) at 12 h. Oleic acid only (OA) caused almost no change in the DNA binding activity. Sp-1 DNA binding activity was not affected by these treatments. These results suggest that the activated AP-1 DNA binding activity may enhance or mediate suppression of urea cycle enzyme genes by fatty acids in cultured hepatocytes, although the AP-1 DNA binding activity was not as markedly activated as found in JVS mice in vivo. The cross coupling between glucocorticoid receptor and AP-1, suggested by Yang-Yen et al. [18] and Schüle and Evans [19,20], may be a possible mechanism for the suppression of the glucocorticoid-responsive gene expression. The results showing enhanced glucocorticoid induction of PEPCK by fatty acids, however, cannot be explained by the cross coupling between glucocorticoid receptor and AP-1. Further analyses are under way to elucidate the mechanism of the fatty acid effect on gene expression and to examine its connection with the pathophysiology of JVS mice in vivo.

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