Induction of Hepatic ATP-Citrate Lyase by Insulin in Diabetic Rat

—Effects of insulin on the contents of enzyme and its mRNA in cytosol, and the transcriptional activity in nuclei—

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The effects of insulin on ATP-citrate lyase, its mRNA in cytosol, and the transcriptional activity in nuclei of diabetic rat liver were studied. Experimental diabetes was induced by an intraperitoneal injection of streptozotocin, and livers were removed from rats at 0, 1, 3, 6, 16, and 72 hours after the administration of insulin. ATP-citrate lyase began to increase at 16 hours, and continuously increased until 72 hours. The amount of mRNA encoding ATP-citrate lyase increased abruptly at 16 hours, then decreased to near basal level in 72 hours. No change in the transcription rate was observed until 3 hours after insulin administration. However, the activity increased 4-fold at 6 hours and 7-fold at 16 hours, 16-fold at 6 hours and 28-fold at 16 hours when pGACL1 and pGACL2 were used as probes, respectively, preceding the increase in the amounts of mRNA and the enzyme. It is suggested that the increase in the amount of ATP-citrate lyase by insulin is primarily due to the increase in the transcriptional activity of the gene in nuclei, which results in the subsequent increase in the amount of mRNA for the biosynthesis of ATP-citrate lyase in cytosol.

Key Words: ATP-citrate lyase, transcription, insulin, mRNA

ATP-citrate lyase (EC 4.1.3.8) is a cytosolic enzyme that catalyzes the formation of acetyl CoA and oxaloacetate from citrate and CoA with a concomitant hydrolysis of ATP to ADP and phosphate (Kornacker and Lowenstein, 1965). The product, acetyl CoA, serves several important biosynthetic pathways, including lipogenesis and cholesterogenesis (Srere 1972; Sullivan et al. 1973). The enzyme is a tetramer (molecular weight about 440,000) of four apparently identical subunits (Singh et al. 1976; Linn and Srere 1979; Pierce et al. 1981). In the nervous tissue, ATP-citrate lyase may be involved in the biosynthesis of acetylcholine (Fetal and Owen 1976).

ATP-citrate lyase contains three phosphorylation sites on two tryptic peptides (peptide A and B). These phosphorylation sites are under hormonal control (Ramakrishna and Benjamin 1979; Pucci et al. 1983; Ramakrishna et al. 1989). In adipocytes and hepatocytes the enzyme is phosphorylated in response to catecholamines or glucagon (Alexander et al. 1979; Janski et al. 1979; Swergold et al. 1982). Insulin acts to increase the phosphorylation of ATP-citrate lyase at the tryptic peptide A by cAMP-dependent protein kinase (Ramakrishna et al. 1989) while decreasing the phosphorylation at the peptide B (Pucci et al. 1983; Ramakrishna et al. 1983; Ramakrishna et al. 1989). Insulin also increases the biosynthesis of ATP-citrate lyase.

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in the primary cultures of adult rat hepatocytes (Spence et al. 1979). It is also suggested that insulin reduces the leakage of ATP-citrate lyase from the digitoxin-permeabilized cells, and that the effect of phosphorylation is to target the enzyme to different cell loci (Straifors 1987). Although the phosphorylation at the A-site decreases enzyme activity under certain conditions (Hourston and Nimmo 1985), the physiological function of this regulatory phosphorylation is not yet clear, and the major regulation of ATP-citrate lyase activity is probably not by phosphorylation or dephosphorylation but by altering the amount of enzyme.

The activity and concentration of ATP-citrate lyase in hepatocytes and adipocytes can vary over a wide range under the control of nutrients, hormones, and the expression of cellular programs for differentiation and development (Kornacker and Lowenstein 1965: Mackall et al. 1976; Elshourbagy et al. 1990). When rats are starved, the level of hepatic ATP-citrate lyase declines rapidly and when the animals are subsequently fed a high carbohydrate-low fat diet ATP-citrate lyase is induced 20 to 30-fold to supranormal levels (Gibson et al. 1971; Whang and Kim 1978). The changes in ATP-citrate lyase activity and the content were reported to be directly proportional to alterations in the rate of ATP-citrate lyase biosynthesis. Sul et al. (1984) reported that the increased amount of enzyme in cytosol is due to the increase in the amount of cytosolic mRNA for ATP-citrate lyase. Kim et al. (1992) showed that the early phase of mRNA increase is correlated to the increased transcription rate of the gene in nuclei, however, the late discrepancy between the elevated levels of enzyme, mRNA in cytosol, and the transcription rate of genes in nuclei represents the presence of an additional unknown mechanism modulating the content of ATP-citrate lyase mRNA and the transcription rate of the ATP-citrate lyase gene.

In this report, it is demonstrated that the administration of insulin into diabetic rats substantially increase the amount of ATP-citrate lyase in liver cytosol, and it is suggested that this increase in the amount of enzyme is due to the increase in the transcriptional activity of the ATP-citrate lyase gene in liver nuclei, which results in the subsequent increase in ATP-citrate lyase mRNA in cytosol.

**MATERIALS AND METHODS**

**Animals and chemicals**

Male Sprague-Dawley rats (weighing 150 to 200 g) were used in all experiments. Multiprime DNA-labeling kits, [α-32P]dCTP (3000 Ci/mmole), and [α-32P]UTP (800 Ci/mmole) were purchased from Amersham (Buckinghamshire, U.K.). Restriction enzymes and nucleotides were purchased from Boehringer Mannheim (Mannheim, Germany). A nitrocellulose membrane was purchased from Hoeffer Scientific Inst. (San Francisco, U.S.A.), and the other chemicals were obtained from Sigma (St. Louis, U.S.A.).

**Induction of diabetes and insulin treatment**

Rats were made diabetic by the intraperitoneal injection of streptozotocin (6 mg/100 g body weight) dissolved in 0.1 M sodium citrate, pH 4.5, after starvation for 20 hours (Bar-On et al. 1976). The rats were given free access to a high-carbohydrate diet (casein 18%, carbohydrate 78%, salt mixture 4%, Kim and Lambooy 1967) for three days, then the level of glucose in the urine was determined by Diastix (Miles Inc. Elkhart). The animals whose urinary glucose exceeded 1000 mg% were considered diabetic. Each rat of the insulin-treated group was given 0.5 unit per 100 g body weight of regular insulin intraperitoneally, with the simultaneous injection of 1 unit per 100 g body weight of NPH (Neutral Protamine Hagedon) insulin, subcutaneously. Thereafter, NPH insulin (2.5 units per 100 g body weight, subcutaneously; 1 unit in the morning and 1.5 units in the evening) was injected daily. The rats were sacrificed by decapitation at 0, 1, 3, 6, 16 and 72 hours after the first injection of insulin. Livers were quickly removed and aliquots of each were homogenized with 5-volume of 0.25 M sucrose to measure the amount ATP-citrate lyase in the supernatant after centrifugation at 15,000 x g. Liver nuclei were isolated as described below. The remaining liver were frozen in liquid nitrogen and stored at -70°C for subsequent experiments.

**Western blot analysis**

The amounts of total protein in liver
homogenates prepared as above were determined by the method of Bradford (Bradford 1976). Each 100 µg of protein prepared from liver homogenates was subjected to electrophoresis in a 7% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane in 25 mM Tris-HCl buffer, pH 8.3, containing 190 mM glycine, 20% (v/v) methanol (Towbin et al. 1979). The blots were immunostained with affinity-purified antibody against ATP-citrate lyase (Park et al. 1991) and alkaline phosphatase-conjugated anti-rabbit IgG (Promega, Madison, U.S. A.).

Northern hybridization

For Northern blot analysis of ATP-citrate lyase mRNA, the total RNA was extracted from the livers by the method as described by Chirgwin et al. (1979). The total RNA (30 µg) was denatured in 50% (v/v) formamide, 2.2 M formaldehyde, and 20 mM MOPS, pH 7.0 at 55°C for 15 minutes, and separated by 1% agarose-formaldehyde gel electrophoresis (Lehrach et al. 1977). Following electrophoresis, RNA was blotted onto a nitrocellulose membrane in 10× SSC (15 M NaCl, 0.15 M sodium citrate, pH 7.0). The blots were hybridized with 32P-labeled probes prepared from the EcoRI/KpnI fragment (1.62 kb) of pGACL1 in 5× SSC, 50% formamide, 0.5% SDS, 5× Denhardt’s, 100 µg/ml herring sperm DNA at 42°C for 24 h. The plasmid, pGACL1 encodes the sequence homologous to the coding region of ATP-citrate lyase cDNA (Kim et al. 1992) and the probe was labeled with [α-32P]dCTP using Multiprime DNA-labeling kits. The front 0.12 kb EcoRI/BglII fragment of cDNA is derived from pGACL5 (Park et al. 1991) and the rear 1.5 kb BglII/KpnI fragment was made by polymerase chain reaction (Kim et al. 1992). The blots were washed twice with 2× SSC, 0.1% SDS for 15 minutes at room temperature, and once with 0.1× SSC, 0.1% SDS for 30 minutes at 55°C, and then autoradiographed using intensifying screens on Fuji X-ray film, at −70°C.

Preparation of nuclei and transcription elongation assay

Liver nuclei were isolated by a modification of the method as described by Goldfine and Smith (1976). Two g of liver were homogenized in 20 ml of STM buffer (0.25 M sucrose, 10 mM MgCl₂, and 20 mM Tris-HCl, pH 7.5) using a Teflon homogenizer in ice-water bath. The homogenate was centrifuged at 800 × g for 10 minutes at 4°C. The pellets were suspended in 6 ml of 2 mM sucrose, 1 mM MgCl₂, and centrifuged at 53,000 × g for 45 minutes. The pellet was then suspended in 1 ml of STM buffer with 0.5% (v/v) Triton X-100 and centrifuged at 800 × g for 10 minutes. The precipitated nuclei were dissolved in storage buffer [40% (v/v) glycerol, 50 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA], and stored at −70°C. The number of nuclei was determined by microscopic examination. Transcription elongation assay was carried out by a modification of the procedure described by Linial et al. (1985). The transcription mixtures contained 1× 10⁶ nuclei in a final volume of 300 µl with 5 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 150 mM KCl, 25% (v/v) glycerol, 0.25 mM each of ATP, GTP, and CTP, and 200 µCi of [α-32P]UTP. The mixtures were incubated at 30°C for 30 minutes and the reaction was stopped by adding 15 µl of DNase I (1 mg/µl, Boehringer Mannheim) at 30°C for 5 minutes. Then 10× SET buffer (10% SDS, 50 mM Tris HCl, pH 7.4) and proteinase K were added to the reaction mixtures to a final concentration of 1× SET and 200 µg/ml, respectively, and incubated at 37°C for 45 minutes. The mixtures were extracted once with phenol/chloroform, and RNA was precipitated by adding 10 M ammonium acetate to make 2.3 M and an equal volume of isopropyl alcohol. The RNA was collected by centrifugation and dissolved in 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The dissolved RNA was alkaline-denatured, ethanol-precipitated, and collected by centrifugation. The final RNA precipitates were dissolved in 500 µl of hybridization solution (10 mM TES, pH 7.4, 0.3 M NaCl, 1× Denhardt’s solution, 250 µg/ml herring sperm DNA). These RNA transcripts (1× 10⁶ cpm) were hybridized to each 5 µg of pGACL1, pGACL2 (Kim et al. 1992), and β-actin probes slot-blotted onto a nitrocellulose membrane in hybridization solution at 65°C for 72 hours. The plasmid, pGACL2 contains 1.6 kb cDNA from pGACL1 and 0.73 kb EcoRI fragment from pGACL9 (Park et al. 1991). After hybridization, the filter was washed at 65°C for 1 hour in 2× SSC containing 0.1% SDS, and then incubated at 37°C for 30 minutes in 2× SSC containing RNases A (10 µg/ml). The filter was washed at
37°C for 1 hour in 2×SSC, dried and then autoradiographed using an intensifying screen for 72 hours at −70°C. The autoradiographic signals of each slot were scanned using a densitometer.

RESULTS

Induction of ATP-citrate lyase enzyme in cytosol by NPH insulin

The changes of the amounts of ATP-citrate lyase in cytosol after insulin injection were determined by western blot analysis with antibody against ATP-citrate lyase (Fig. 1). In diabetic rats, hepatic ATP-citrate lyase was not detected and maintained at this low level for 6 hours. The amount of the enzyme rapidly increased at 16 hours, and increased continuously thereafter until 72 hours after the first insulin administration.

Effects of NPH insulin treatment on ATP-citrate lyase mRNA content in cytosol

ATP-citrate lyase mRNA contents were ana-
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Fig. 2. Northern blot analysis of ATP-citrate lyase mRNA after the insulin administration. The cytoplasmic RNA was isolated from diabetic rat livers at indicated times after the insulin administration, and 30 μg of each was subjected to 1% formaldehyde-agarose gel electrophoresis. After electrophoresis, RNA was blotted onto the nitrocellulose membrane and the blot was hybridized with 3P-labeled random primed ATP-citrate lyase cDNA from pHACL1, and autoradiographed. The position of ATP-citrate lyase transcript is indicated by the arrow with ACL, and the positions of 18 S and 28 S ribosomal RNA are indicated by the arrows.

lyzed by Northern hybridization with pGACL1 as a probe. The total RNA (30 μg) was fractionated on 1.0% agarose-formaldehyde gel. The discrete 18 S and 28 S ribosomal RNA bands were shown with the same intensity in all groups by ethidium bromide staining (data not shown). The RNA was blotted onto a nitrocellulose membrane and hybridized with 3P-labeled pGACL1 cDNA. Northern hybridization revealed the presence of an ATP-citrate lyase transcript of about 4.3 kb in size, which was consistent with that of other studies (Sul et al. 1984; Elshourbagy et al. 1990). Messenger RNA for ATP-citrate lyase was not detected until 6 hours, but significantly increased at 16 hours after the insulin treatment (Fig. 2). The increment of hepatic mRNA for ATP-citrate lyase correlated well with the increment of the enzyme in cytosol for 16 hours. However, the mRNA content declined rapidly toward the initial value by 72 hours after the insulin administration, despite the continuous increments of the enzyme in cytosol.

Effects of NPH insulin treatment on the transcriptional activity of ATP-citrate lyase gene in isolated nuclei

The transcription elongation assay was performed in isolated nuclei with [α-32P]UTP to elucidate whether the increased amount of mRNA in cytosol is related to the transcription rate of the ATP-citrate lyase gene. The amount of transcripts for the ATP-citrate lyase gene decreased to nearly undetectable levels in liver nuclei from diabetic rats compared to that for the 3-actin gene (Fig. 3A). No change in the transcription rate was found within 3 hours; however, the transcription rate increased 4-fold at 6 hours and 7-fold at 12 hours, 15-fold at 6 hours and 28-fold at 16 hours when pGACL1 and pGACL2 were used as probes, respectively (Fig. 3B). The increased transcription rate preceded the accumulation of mRNA in cytosol, suggesting that the increase in the amount of mRNA by insulin is primarily due to the increase in the transcription rate in nuclei.

DISCUSSION

The majority of studies on the regulation of ATP-citrate lyase have dealt with the influence of insulin treatment on phosphorylation of the enzyme. It is known that insulin stimulates the incorporation of 32P into ATP-citrate lyase in isolated rat hepatocytes and adipocytes (Avruch et al. 1976; Alexander et al. 1979; Ramakrishna and Benjamin 1979; Ranganathan et al. 1982). However, the physiological significance of the phosphorylation of ATP-citrate lyase remains to be elucidated, since the phosphorylated and dephosphorylated forms of the enzyme are not distinguishable by the kinetic criteria (Janski et al. 1979; Ranganathan et al. 1980; Guy et al. 1981). Rat liver ATP-citrate lyase activity is depressed in starved diabetic animals and highly elevated (20- to 30-fold) when these animals are fed a fat-free high carbohydrate diet or by insulin administration. The long term regulation of ATP-citrate lyase activity by hormones has also been studied in
Fig. 3. The transcriptional activity of ATP-citrate lyase gene in diabetic rat liver nuclei. Nuclei were isolated from diabetic rat liver at indicated times after the insulin administration, and nuclear run-on transcription was performed as described under "Materials and Methods" (A) The transcripts isolated after the transcription in the presence of [α-32P]UTP were hybridized for 72 hours to β-actin, pGACL1, and pGACL2 biotinylated onto the nitrocellulose membrane. The time course is indicated on the top. (B) The autoradiographic signals were scanned with a densitometer, and the transcription rates were calculated and represented as the ratio of ATP-citrate lyase intensities standardized by β-actin. Closed bars and open bars represent the transcription ratio for pGACL1 and pGACL2, respectively.

primary cultures of rat hepatocytes. Spence et al. (1979) reported that the addition of insulin to hepatocyte culture increased ATP-citrate lyase activity 2.6-fold, whereas the addition of glucagon decreased the activity by 68%. They reported the increase in the enzyme activity was the result of new enzyme synthesis, however, the mechanism of regulation on the changes of the amount of ATP-citrate lyase by insulin was not clearly identified on the gene level. Katsurada et al. (1999a; 1999b) investigated the changes of the amounts of fatty acid synthase and acetyl-CoA carboxylase after the insulin administration in diabetic rats. They reported that the increased enzyme activity is due to the increase in the content of cytoplasmic mRNA, following the increase in the transcription rate of genes in nuclei from diabetic rat liver. While ATP-cit-
rate lyase is one of the key enzymes in lipogenesis, the discrete mechanisms of regulation by hormones, nutrients, and during the development of the organism remain undefined.

In this report, the changes of amount of the enzyme, mRNA, and the transcription rate were studied in diabetic rat liver after the insulin administration. ATP-citrate lyase began to increase in cytosol at 16 hours and continuously increased by 72 hours after insulin administration. The change of ATP-citrate lyase mRNA was not observed until 6 hours after the insulin administration. However, there was an abrupt increase at 16 hours, followed by the rapid decrease of mRNA in cytosol. The increase in the amounts of the enzyme even after the rapid decrease in the amount of mRNA may represent the fact that the half-life of the enzyme is longer than that of mRNA even though it was not studied in this paper. However, it could not simply rule out some other mechanisms governing the regulation of the enzyme after the transcription in the induction of ATP-citrate lyase, and the post-transcriptional regulatory mechanism has to be studied.

In the isolated nuclei of rat liver, the transcription rate of the ATP-citrate lyase gene was measured in the presence of [α-32P]UTP. The transcription rate began to increase at 6 hours and reached the maximum level at 16 hours after the insulin administration. For pGACL2, the transcription rate increased 16-fold at 6 hours, 28-fold at 16 hours for pGACL1, 4-fold at 6 hours, 7-fold at 16 hours after the insulin administration. The difference between the elevated level of the transcription rates according to the probes, such as pGACL1 and pGACL2 used in this study was not clearly demonstrated. pGACL2 contains 2.3 kb cDNA which was constructed by joining the additional sequences spanning downstream of 1.6 kb cDNA in pGACL1. It is supposed that the amount of [α-32P]UTP incorporated in the 3’ portion of ATP-citrate lyase transcripts is larger than that of the transcripts in the 5’ portion, because the lengths of nascent RNA labeled with [α-32P]UTP at the 5’ portion of the transcripts are different. The resulting transcripts could show more strong radioactivity when more downstream sequences of cDNA were used as probes. With these results, it is proposed that insulin primarily induces the transcriptional activity of the ATP-citrate lyase gene in liver nuclei, and as a result, the cytosolic mRNA for ATP-citrate lyase increased. These results correspond to those of Katsurade et al. (1990a; 1990b) they reported that the fatty acid synthase and acetyl-CoA carboxylase were induced by insulin in the same manner in diabetic rat liver. These patterns of changes are also similar to that of changes by starvation and refeeding a high carbohydrate regimen (Kim et al. 1992). However, the mechanism controlling the discrepant time course of changes on enzyme, mRNA, and transcriptional activity of ATP-citrate lyase is not clear until now. It could be explained by the other mechanisms, e.g., the increased rate of mRNA decay and the increased stability of mRNA in cytosol. Messina (1989) proposed the mechanisms in the regulation of P33-mRNA in H4IE hepatoma cell culture by insulin: increase in the rate of transcription; decrease in the content of enzyme degrading the mRNA; increase in the efflux of mRNA from nuclei, increase in the processing of the nascent transcript. The other possible mechanism to be clarified remains to be studied, regarding the pretranscriptional and posttranscriptional regulation of the ATP-citrate lyase gene.

REFERENCES


Goldfine ID, Smith GF: Binding of insulin to isolated nuclei. *Proc Natl Acad Sci USA* 73: 1427-1431, 1976


Ranganathan NS, Srere PA, Linn TC: Comparison of phospho- and dephospho-ATP citrate lyase. *Arch Biochem Biophys* 204: 52-58, 1980


Stralfors P: Isoproterenol and insulin control the cellular localization of ATP citrate lyase through its phosphorylation in adipocytes. *J Biol
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Chen 262: 11486-11489, 1987