Hormonal Regulation of Hepatic Fatty Acid Synthetase in Chick Embryo

ROLE OF INSULIN*

VasuDeV C. JosHi AND SalIH J. WAKIL

From the Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030

Hepatic fatty acid synthetase activity is low in chick embryo and precociously increases 16-, 20-, and 12-fold upon administration of insulin, glucagon, and dibutyryl adenosine 3':5'-monophosphate (Bt,cAMP), respectively. Immunoprecipitations of different enzyme preparations from induced and uninduced embryos against anti-synthetase gamma-globulin gave identical equivalence points, indicating that the changes in enzyme activity after hormonal induction are due to changes in the amount of enzyme protein. The increase in hepatic synthetase content after administration of insulin, glucagon, and Bt,cAMP can be accounted for by a maximal increase in the rate of synthetase synthesis of due to changes in enzyme protein. The increase in hepatic synthetase content after administration of insulin, glucagon, and Bt,cAMP can be accounted for by a maximal increase in the rate of synthetase synthesis of

The induction of fatty acid synthetase by glucagon, in ovo, is related to elevated serum insulin after glucagon injection. The neonatal period is characterized by marked changes in lipogenesis and activities of enzymes involved in fatty acid synthesis. In contrast to the active lipogenesis in mammalian fetal liver (1, 2), de novo fatty acid synthesis in chick embryo liver is low but detectable (3, 4). Hepatic lipogenesis and fatty acid synthetase activity are stimulated when newly hatched chicks are fed a high carbohydrate diet (4, 5). The synthetase activity increased 3-fold on hatching and thereafter by 35- to 50-fold over the basal embryonic activity (4). The factors underlying the changes in synthetase activity of developing avian liver have been determined by immunochemical techniques and it was shown that the increase in hepatic synthetase activity after hatching was a consequence of an increase in the rate of enzyme synthesis, resulting in a net increase in the amount of fatty acid synthetase (6). We have recently reported that the fatty acid synthetase activity in chick embryo liver is precociously increased by administration of insulin, glucagon or N',O'-dibutyryl adenosine 3':5'-monophosphate (Bt,cAMP) and that the induced enzyme was immunochemically equivalent to the enzyme in fasted-refed chickens (7). However, it has been reported that a portion of the fatty acid synthetase in the liver of fasted chicks is present as an inactive form which can be activated upon refeeding (8). Hence, we have investigated the possible presence of an inactive form of synthetase in chick embryos by immunochemical methods. In this communication we present evidence that the increase in fatty acid synthetase caused by all three inducers is a consequence of an increase in enzyme concentration resulting from an elevated rate of synthesis of the enzyme. The induction of fatty acid synthetase by glucagon, in ovo, is related to elevated serum insulin after glucagon injection.

EXPERIMENTAL PROCEDURES

Materials—Acetyl-CoA, malonyl-CoA, NADPH, and dithiothreitol were obtained from P-L Biochemicals, Inc., Milwaukee; glucagon, bovine insulin (24.3 iu/mg) and Bt,cAMP from Sigma Chemical Co., St. Louis; [1,3-14C]malonyl-CoA from New England Nuclear Corp., Bedford; L-[4,5-3H]leucine (6 Ci/mmol) from Schwarz/Mann. Chicken insulin was a gift from Dr. R. L. Hazelwood, University of Houston. All other chemicals were of reagent grade.

Animals—Embryonated eggs from White Leghorn chickens were

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1 The abbreviation used is: Bt,cAMP, N',O'-dibutyryl adenosine 3':5'-monophosphate.
Regulation of Fatty Acid Synthetase by Insulin

incubated in an electric forced-draught incubator at 37.5° and 60% relative humidity. Insulin, glucagon, and Bt-CAMP solutions were sterilized by filtration through Millipore filters (0.45 μm) and injected into chick embryos as described previously (11) except that column dimensions and buffer volumes were scaled down 15-fold and Dowex 1 column chromatography was omitted. The supernatant fraction (150,000 g supernatant) was prepared as described previously (4) and utilized in the assay for fatty acid synthetase and in the quantitative immunoprecipitin reaction.

Assay of Fatty Acid Synthetase—The synthetase activity was assayed by the NADPH-dependent incorporation of 13C-malonyl-CoA into fatty acids as described previously (7) except that NADPH was present at a final concentration of 0.2 mM and incubation was carried out at 37°. The enzyme activity is expressed as nanomoles of NADPH oxidized/min and is based on the relationship of 2 mol of NADPH oxidized to malonyl-CoA incorporated into fatty acids.

The enzyme activity was linear with time and added protein. The values reported are average values of duplicate assays which differed from each other by less than 5%.

The assays were carried out on pooled livers from three to six embryos. As shown previously (7), the values on pooled livers agree well with those obtained on individual livers. In experiments where partially purified liver extracts were used, the synthetase activity was assayed at 25° by monitoring the malonyl-CoA-dependent oxidation of NADPH at 340 nm (9). The synthetase activity is 2.5 times higher at 37° than at 25°. Protein concentration was measured as described by Lowry et al. (10).

Purification of Fatty Acid Synthetase—Chicken liver fatty acid synthetase was purified according to a previously published procedure (11). Hepatic fatty acid synthetase from chick embryos was also prepared as described in a similar fashion except that column dimensions and buffer volumes were scaled down by about 15-fold and Dowex 1 column chromatography was omitted.

Two groups of 20-day-old embryos (70 per group) were injected with chick embryo serum was calculated from the standard curve made with insulin, glucagon, or Bt-CAMP. Insulin, glucagon, and Bt-CAMP administration at optimal dose level increased synthetase activity 35-, 44-, and 24-fold, respectively, in 20-day-old chick embryos, at 12 h after injection of inducers (7). We considered first the question whether the elevated synthetase activity in inducer-treated embryos is related to changes in the amount of the enzyme. Earlier studies in this laboratory had demonstrated that highly purified fatty acid synthetase and partially purified liver extracts from hormone-treated embryos and fasting-refed chickens had identical equivalence points, suggesting that the enzyme in the induced embryos and adult chicken were equivalent (7). However, without a knowledge of the equivalence point of the extract from control (saline-treated) embryo, it cannot be concluded that the change in enzyme activity was a function of a change in enzyme protein concentration. Hence, quantitative immunoinhibition reactions were carried out with liver extracts from saline-, insulin-, glucagon, and Bt-CAMP-treated embryos. As shown in Fig. 1, the equivalence points for all four preparations were identical, indicating that there was a constant and equivalent amount of immunoprecipitable enzyme per unit of activity in these liver extracts and that the increase in the enzyme activity following insulin, glucagon, and Bt-CAMP administration reflected differences in content and not in the catalytic properties of the synthetase. This conclusion is based on the premise that there is no form of the synthetase that is enzymatically inactive and immunologically unreactive. Such a form of the enzyme has not yet been

RESULTS

As reported previously (1), the hepatic fatty acid synthetase activity in chick embryo is induced by the administration of insulin, glucagon, or Bt-CAMP. Insulin, glucagon, and Bt-CAMP administration at optimal dose level increased synthetase activity 35-, 44-, and 24-fold, respectively, in 20-day-old chick embryos, at 12 h after injection of inducers (7). We considered first the question whether the elevated synthetase activity in inducer-treated embryos is related to changes in the amount of the enzyme. Earlier studies in this laboratory had demonstrated that highly purified fatty acid synthetase and partially purified liver extracts from hormone-treated embryos and fasting-refed chickens had identical equivalence points, suggesting that the enzyme in the induced embryos and adult chicken were equivalent (7). However, without a knowledge of the equivalence point of the extract from control (saline-treated) embryo, it cannot be concluded that the change in enzyme activity was a function of a change in enzyme protein concentration. Hence, quantitative immunoinhibition reactions were carried out with liver extracts from saline-, insulin-, glucagon, and Bt-CAMP-treated embryos. As shown in Fig. 1, the equivalence points for all four preparations were identical, indicating that there was a constant and equivalent amount of immunoprecipitable enzyme per unit of activity in these liver extracts and that the increase in the enzyme activity following insulin, glucagon, and Bt-CAMP administration reflected differences in content and not in the catalytic properties of the synthetase. This conclusion is based on the premise that there is no form of the synthetase that is enzymatically inactive and immunologically unreactive. Such a form of the enzyme has not yet been detected.
Regulation of Fatty Acid Synthetase by Insulin

Uninduced embryos.

Acid synthetase from insulin-, glucagon-, Br2cAMP-, and normal saline-injected chick embryos. Four groups of 20-day-old embryos (five per group) were injected with insulin (50 μg/embryo), glucagon (50 μg/embryo), Br2cAMP (4 mg/embryo), and normal saline (0.1 ml/embryo). The embryos which received glucagon, insulin, and normal saline were killed after 12 h and those that received Br2cAMP were killed after 5 h. The liver extracts were prepared for immunoinhibition reaction as described under "Experimental Procedures." Increasing amounts of the liver supernatant from embryos treated with saline (○), insulin (◆), glucagon (□), and Br2cAMP (▲) were incubated with a constant amount of antibody and the remaining synthetase activity was assayed. Liver extracts from fasted-refed chickens also gave the same equivalence point as the induced and uninduced embryos.

described. The fatty acid synthetase in liver extracts of fasted-refed chickens has also the same equivalence point as the liver extracts from inducer- and saline-injected embryos (data not shown).

Further support for immunological similarity of the hepatic synthetase in normal and induced embryos and in fasted-refed chickens was provided by Ouchterlony double-diffusion analysis and by immunoelectrophoresis (Fig. 2). The immunodiffusion analysis showed a reaction of complete identity, a single precipitin band without spurring, between the preparation of liver synthetase from fasted-refed chickens and partially purified preparations of liver synthetase from saline-, insulin-, glucagon-, and Br2cAMP-treated chick embryos. Immunoelectrophoresis of the purified fatty acid synthetase and partially purified liver extracts from insulin-, glucagon-, and Br2cAMP-treated embryos with anti-synthetase y-globulin in the troughs, gave a single precipitin line at the same distance from the antigen well. This indicated that the antibody reacted with a single antigen in the liver extracts from insulin-, glucagon-, and Br2cAMP-treated embryos and that this antigen had a similar electrophoretic mobility to that of the purified fatty acid synthetase.

Preparations of fatty acid synthetase from livers of embryos treated with insulin and glucagon were purified to a specific activity of about 1,300 milliunits/mg of protein at 25° and had chromatographic properties on DEAE-cellulose and Sepharose 6B similar to the preparation from fasted-refed chicken (data not shown), i.e., the enzyme activity was eluted at 0.15 M NaCl from a DEAE-cellulose column and from a Sepharose 6B column with a Vp/Vo = 1.2. Furthermore, experiments on the thermal inactivation of the enzyme preparations from fasted-refed chickens and from insulin- and glucagon-treated chick embryos demonstrated that the synthetase activity in all three preparations had identical time courses of inactivation, following first order kinetics with t1/2 value of 6.2 min at 52° (data not shown). These results clearly demonstrate similarity of enzyme preparations from induced embryos to that from fasted-refed chickens.

The degree to which changes in hepatic synthetase content

FIG. 1. Quantitative immunoinhibition reactions of hepatic fatty acid synthetase from insulin-, glucagon-, Br2cAMP-, and normal saline-injected chick embryos. Four groups of 20-day-old embryos (five per group) were injected with insulin (50 μg/embryo), glucagon (50 μg/embryo), Br2cAMP (4 mg/embryo), and normal saline (0.1 ml/embryo). The embryos which received glucagon, insulin, and normal saline were killed after 12 h and those that received Br2cAMP were killed after 5 h. The liver extracts were prepared for immunoinhibition reaction as described under "Experimental Procedures." Increasing amounts of the liver supernatant from embryos treated with saline (○), insulin (◆), glucagon (□), and Br2cAMP (▲) were incubated with a constant amount of antibody and the remaining synthetase activity was assayed. Liver extracts from fasted-refed chickens also gave the same equivalence point as the induced and uninduced embryos.

of inducer-treated embryos reflect changes in enzyme synthesis was evaluated by measuring the rate of incorporation of [3H]leucine using immunoochemical techniques. Initial experiments with normal and inducer-treated embryos indicated that the incorporation of [3H]leucine into liver supernatant proteins and fatty acid synthetase was linear up to 2 h after injection of leucine. Hence embryos were pulsed for 1 h with [3H]leucine and the rate of incorporation of radioactivity into
Regulation of Fatty Acid Synthetase by Insulin

The rate of synthesis of fatty acid synthetase (FAS) after injection of insulin, glucagon, and Bt,cAMP in chick embryo. Chick embryos (20-day-old; five/time interval) were injected with insulin (30 μg/embryo; solid symbols in Panel A), glucagon (50 μg/embryo; solid symbols in Panel B), Bt,cAMP (2.5 mg/embryo; solid symbols in Panel C), or normal saline (0.1 ml/embryo; open symbols). One hour before the indicated time intervals all embryos received [3H]leucine (40 μCi/embryo). The livers were processed at the indicated time intervals as described under *Experimental Procedures*.

Relative rate of synthetase synthesis denotes per cent radioactivity of the total soluble enzyme in insulin-treated embryos which is equivalent to porcine and bovine insulin in cross-reaction with antibodies to [3H]leucine-labeled liver extracts of induced embryos. The foregoing results clearly demonstrate that the increase in the liver synthetase activity after administration of insulin, glucagon, and Bt,cAMP is directly related to an increase in the rate of synthetase synthesis.

The inductive effect of glucagon and Bt,cAMP on chick hepatic synthetase, *in vivo*, is contrary to their inhibitory effect on lipogenesis (16-20). It is known that administration of glucagon in mammals increases plasma insulin levels (21) and this forms the basis for the principle of the insulin-glucagon negative feedback system, *i.e.* glucagon stimulates insulin while insulin suppresses glucagon secretion (22). Direct support for this possibility in the chick embryo was provided by simultaneous measurement of hepatic synthetase activity and serum insulin levels in chick embryos at different times after injection of glucagon. In radiolmmunoassay, human insulin is equivalent to porcine and bovine insulin in cross-
Regulation of Fatty Acid Synthetase by Insulin

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitate isolated from liver extract of insulin-treated chick embryos. Immunoprecipitate isolated from liver extracts at 8 h after administration of insulin in the experiment of Fig. 3 was dissolved in 1% sodium dodecyl sulfate containing 1% /-mercaptoethanol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out on 5% acrylamide gels according to the method of Weber and Osborn (14). Homogenous preparation of chicken liver fatty acid synthetase was also electrophoresed for comparison. Stained gels were scanned for absorbance with a model 2410 linear transport attached to a Gilford model 240 recording spectrophotometer. After scanning for absorbance, the gel was sectioned and the radioactivity in the gel slices was measured after dissolution of slices in 30% H₂O₂ at 45°C. Stained protein bands with migration distance of 1.2, 5, and 7.5 cm are due to fatty acid synthetase, immunoglobulin heavy chain, and immunoglobulin light chain, respectively.

reactivity with the antibody made against porcine insulin (13). Chicken insulin can competitively displace human ¹²⁵I-insulin from the antigen antibody complex, although to a slightly lesser extent than human insulin² (23). These results suggest that the antibody against porcine insulin can be used to quantitate chicken insulin by radioimmunoassay.

Administration of glucagon in chick embryos significantly increased hepatic synthetase activity at 2 h and thereafter increased the enzyme activity in a linear fashion up to 8 h (Fig. 5). Serum insulin levels in these embryos increased 3-fold at 0.5 h and up to 10-fold at 3 h after glucagon injection. Thereafter the serum insulin level declined to about 3 times the value of saline-treated embryos at 8 h. These results clearly establish that the rise in serum insulin level precedes the rise in hepatic fatty acid synthetase after glucagon injection and strongly support the possibility that the rise in serum insulin level following glucagon injection is responsible for hepatic synthetase induction.

FIG. 5. Serum insulin levels and hepatic fatty acid synthetase (FAS) activity following injection of glucagon in chick embryos. Chick embryos (20-day-old; eight/time interval) received glucagon (100 μg/embryo, ●) or normal saline (0.1 ml/embryo, ○). At various time intervals after injection blood was withdrawn from the vitelline artery after breaking the shell at the narrower, conical end of the egg. Simultaneously, embryos were killed and livers were removed to measure synthetase activity. Serum insulin levels were measured, in triplicate, on pooled samples at various time intervals. The vertical bars at each time interval give the standard error of the mean.

DISCUSSION

The present investigation has established the role of insulin in the hormonal regulation of hepatic fatty acid synthetase in chick embryos. In a previous study, from this laboratory, it was shown that administration of insulin, glucagon, or Bt,cAMP to chick embryos induced synthetase activity in liver (7). In the present study it has been demonstrated that the increase in synthetase activity after injection of inducers in chick embryo is due to an increase in the amount of the enzyme and not an activation of pre-existing enzyme since identical equivalence points were obtained in immunoinhibitory reactions of enzyme extracts from control and induced embryos. Immunochemical analysis of liver extracts after pulse-labeling with [³H]leucine showed that increased amounts of synthetase in insulin-, glucagon-, and Bt,cAMP-treated embryos resulted from a rapid increase in the rate of synthesis of the enzyme as early as 2 to 4 h after administration of inducers. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the radioactive immunoprecipitate isolated from inducer-treated embryos pulsed with [³H]leucine showed that all of the radioactivity in the immunoprecipitate was associated with the synthetase. This eliminated the possibility of nonspecific precipitation of other radioactive proteins from liver extracts. These observations established that the increased synthetase activity subsequent to the administration of inducers results from an increase in the rate of synthesis of fatty acid synthetase. It is particularly noteworthy that in induced embryos, the hepatic synthesis of fatty acid synthetase can account for 4 to 7.6% of the total overall soluble protein synthesis.

A common feature of this type of premature induction is the rapid decay of enzyme activity after elevation to higher levels. The t₁/₂ of embryonic liver synthetase activity, as calculated from the first order decay after elevation to higher levels by administration of insulin, glucagon, or Bt,cAMP was found to be 4 h in the present study as well as in previous investiga-

² V. C. Joshi, unpublished results.
Regulation of Fatty Acid Synthetase by Insulin

The half-life of hepatic synthetase activity in chick embryo is very much shorter than that of the enzyme in neonatal chick (t₁/₂ = 32 h) (6), adult chicken (t₁/₂ = 70 h), and rat (24–26). The difference in energy source of the chick embryo (primarily fat) from that of adult chickens (primarily carbohydrate) may well be a determining factor in shortening the half-life of the fatty acid synthetase in embryos. It is interesting to note that the degradation of the synthetase in chick embryos is reminiscent of the accelerated degradation of the enzyme in fasted rats (26).

The present studies have attempted to delineate the mechanism by which glucagon induces fatty acid synthetase in chick embryos. Administration of glucagon in chick embryos resulted in a marked increase in the plasma insulin level followed by an increase in hepatic fatty acid synthetase. Glucagon, suggesting that elevated serum insulin after glucagon injection may increase the synthetase in liver. The rise in serum insulin level after glucagon administration in chick embryos is consistent with marked stimulation of insulin secretion by glucagon in perfused chicken pancreas (27). Since glucagon injection in chick embryos increases the endogenous insulin level in the blood, the blood insulin level after glucagon injection may be much higher than that after intraperitoneal injection of porcine insulin in the embryos. In addition, chicken insulin may be more physiological in inducing hepatic fatty acid synthetase in chick embryo than exogenous porcine insulin. These suggestions are consistent with our observations that the synthetase induction after glucagon injection is higher than that after porcine insulin administration. The increase in hepatic fatty acid synthetase following Bt,cAMP injection in chick embryos may also be related to increased endogenous serum insulin since insulin release induced by glucose is mediated by cyclic AMP (28–30). The potentiation of β-adrenergic receptor-mediated insulin secretion (28) and of glucagon- and Bt,cAMP-mediated hepatic fatty acid synthetase induction in chick embryo (7) by the phosphodiesterase inhibitor theophylline is consistent with the above suggestion. The suggestion that glucagon and Bt,cAMP induce hepatic synthetase, in vivo, by indirect means is further supported by recent studies in our laboratory which show that both glucagon and Bt,cAMP do not induce fatty acid synthetase in cultured liver explants' but physiological concentrations of insulin increase synthetase by 10-fold in these explants (31).

The involvement of insulin in the induction of hepatic fatty acid synthetase in chick embryo is consistent with several other investigations in the rat. For example, the rapid rise in hepatic synthetase activity upon refeeding rats after a fast is paralleled by a similar increase in levels of plasma insulin (32). Hepatic lipogenesis and fatty acid synthetase activity are reduced in experimental alloxan diabetes and are restored by injection of insulin (33, 34). The decrease in synthetase activity in diabetic rats is caused by a diminution in synthesis of the enzyme and administration of insulin to diabetic rats restores activity and synthesis of the enzyme (25, 35). Although insulin administration is known to correct the diminution of synthesis of synthetase in diabetic rat liver, it may not be directly involved in the regulation of the synthetase activity since, in the absence of insulin administration, fructose feeding to diabetic rats increases the rate of synthesis of the enzyme, possibly through changes in the concentration of certain intermediates of glycolysis or beyond (35).

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