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Studies of the interactions between experimental inflammation and adaptive synthesis of fatty acid synthetase in rat liver.

by

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"STUDIES OF THE INTERACTIONS BETWEEN EXPERIMENTAL INFLAMMATION AND ADAPTIVE SYNTHESIS OF FATTY ACID SYNTHETASE IN RAT LIVER"

by

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To my wife Carolyn and my parents.

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ABSTRACT

During the course of adaptive synthesis in the rat, it is known that hepatic fatty acid synthetase activity increases significantly (2, 41, 42) and that this is due to an increase in enzyme content (2, 3, 43, 44). It is also known that the level of the liver synthesized, serum acute phase reactant, α_1-acid glycoprotein increases in response to experimentally-induced inflammation (96, 111, 164-166, 175). The studies within this thesis show that experimentally-induced inflammation during the earlier stages of adaptive synthesis (less than 10 h after refeeding with a fat free diet), interferes with the rise of rat liver fatty acid synthetase activity normally observed during adaptive synthesis. Conversely, it was found that adaptive synthesis in its intermediate stages interfers with the rise in the levels of α_1-acid glycoprotein normally observed in response to experimentally-induced inflammation.

It has been established that fatty acid synthetase is primarily synthesized on "free" ribosomes (32) and α_1-acid glycoprotein on "membrane-bound" ribosomes (175).

The distribution of hepatic RNA between "free" and "membrane-bound" ribosome fractions was determined and suggests that the mutual interference between the synthesis of fatty acid synthetase and α_1-acid glycoprotein is dependent on the state of committment shown by the protein synthesizing mechanism towards the synthesis of either protein. The observation that hepatic glycogen levels are decreased with the onset of inflammation regardless of the stage of adaptive synthesis, suggests that the synthesis of α_1-acid glycoprotein, fatty acid synthetase, and glycogen are controlled by distinct but related mechanisms. However, the nature of the controlling mechanisms remains obscure.

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ABBREVIATIONS

ATP	-	adenosine 5'-triphosphate
EDTA	-	ethylenediaminetetraacetate
c-AMP	-	cyclic adenosine 3':5'-monophosphate
CoA	-	Coenzyme A
NADP	-	nicotinamide adenine dinucleotide phosphate
NADPH	-	reduced nicotinamide adenine dinucleotide phosphate
DTT	-	dithiothreitol
GDP	-	guanosine 5'-diphosphate
UDP	-	uridine 5'-diphosphate
CMP	-	cytidine 5'-monophosphate
Tris	-	tris-hydroxymethylamino-methane
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
FAS	-	Fatty Acid Synthetase
α_1-AGP	-	α_1-acid glycoprotein

INTRODUCTION

Fatty Acid Synthetase; The Intracellular Protein

Rat liver fatty acid synthetase (FAS), a multienzyme complex which catalyzes the de novo synthesis of long chain fatty acids, has been shown to be one of several lipogenic enzymes in the mammalian liver to have it's activity regulated by nutritional factors (1-3). Starvation results in the depletion of several lipogenic enzyme activities while refeeding restores activity to normal or in some cases supranormal levels (4). Hormonal factors have also been implicated in hepatic lipogenesis (5-11).

The Reaction Catalyzed by FAS

The overall reaction catalyzed by FAS is as follows;

$$\text{Acetyl CoA} + 7\ \text{Malonyl CoA} + 14\ \text{NADPH} + 14\ H^{+} \longrightarrow \text{palmitic acid} + 14\ NADP^{+} + 8\ \text{CoA-SH} + 7\ CO_2 + 6\ H_2O$$

The covalent binding of acetyl and malonyl groups to the enzyme, in the presence of NADPH, initiates their conversion to palmitic acid (27). The mechanism of this reaction proposed by Porter for pigeon liver (28) and Lynen for

yeast FAS (29) consists of a number of sequential reactions elongating acyl chain intermediates bound to the 4'-phosphopantetheine (4'-pp) prosthetic group of the enzyme. This prosthetic group transports the intermediates to various catalytic sites on the enzyme. The enzyme is reputed to have seven sites at which the different reactions leading to the synthesis of fatty acids occur (30). These site reactions are as follows;

1. The Acetyl transacylase site

   Acetyl-CoA + E-4'-pp ⟶ Acetyl-4'-pp-E + CoA-SH

2. The Malonyl transacylase site

   Malonyl-CoA + E-4'-pp ⟶ Malonyl-4'-pp-E + CoA-SH

3. The Condensation-decarboxylation site

   Acetyl-4'-pp-E + E-Scys-H ⟶ Acetyl-Scys-E + 4'-pp-E

   or

   Acyl-4'-pp-E + E-Scys-H ⟶ Acyl-Scys-E + 4'-pp-E

3.' β - ketoacyl synthetase

   Acetyl-(or acyl-) Scys-E + Malonyl-4'-pp-E

   ⟶ Acetoacetyl-(or β-ketoacyl-) 4'-pp-E + CO_2 +

   E-Scys-H

4. The β-Ketoacyl reductase site

   β-Ketoacyl-4'-pp-E + NADPH + H^+

   ⟶ D (-)-β-Hydroxyacyl-4'-pp-E + $NADP^+$

5. The β-Hydroxyacyl dehydrase site

   D(-)-β-Hydroxyacyl-4'-pp-E ⟶ trans - α, β Unsaturated acyl-4'-pp-E + H_2O

6. The Enoyl reductase site

   trans - α, β-Unsaturated acyl-4'-pp-E + NADPH + H^+ ⟶ Acyl-4'-pp-E + $NADP^+$

7. The Palmityl deacylase site

   palmityl-4'-pp-E ⟶ palmitic acid + 4'-pp-E

Structural Properties of FAS

Generally FAS has been found to exist either as a multienzyme complex such as for animals and other higher organisms or as a system of separate enzymes such as for Escherichia coli. The latter system consists of at least six separable enzymes and an acyl carrier protein containing the 4'-pp prosthetic group. The investigations on FAS from animal tissues and yeast have revealed multienzyme complexes with molecular weights of about 500,000 and 2,300,000 respectively (13-18). Using chicken, rat and yeast FAS complexes as model systems, investigators are currently attempting to solve the question of whether the multienzyme complex is composed of seven individual enzyme peptides tightly held together by non-covalent interactions or if in fact it is an aggregate of two polypeptide chains as proposed by Schweizer et al (19).

Initial investigation of the purified yeast FAS complex identified seven different N-terminal amino acids and the isolation of a 4'-pp containing acyl carrier protein component by guanidine-HCl treatment has been accomplished (20). Studies on avian synthetase using affinity chromatographic and preparative disc-gel electrophoretic techniques have led to the isolation of two half-size subcomplexes which dissociate upon aging into individual proteins some retaining their enzymatic activity (21-23). This is the point where the controversy occurs as Stoops et al (24) claims that contamination by proteolytic enzymes is the cause of the apparent spontaneous dissociation despite the contrary claims of Bratcher and Hsu (25). The laboratories of Schweizer et al (19) and Stoops et al (24), using mild conditions of low ionic strength to dissociate chicken and rat liver FAS complexes found that only when proteolytic activity was detected did the subcomplexes dissociate into smaller components. Furthermore, the discovery of proteolytic activity in FAS prepared by the normally used purification methods and it's subsequent elimination in modified procedures (24) spurred a reassessment of the 4'-pp content of chicken on rat liver FAS by Arslanian et al (26). Preparations of FAS in which proteolytic activity was absent had a higher content of 4'-pp per mol of complex than protease containing preparations. The data indicated the presence of two prosthetic groups per FAS complex. Previously one 4'-pp per mol FAS was the accepted value.

Either value is consistent with the observed lack of synthetase activity of the monomeric subunits and the dependence of the β-Ketoacyl synthetase (the condensing reaction) on the existence of the dimer. This dual prosthetic group hypothesis, one binding acetyl-CoA (or acyl-CoA) and the other malonyl-CoA does not significantly alter the accepted mechanism of the condensing reaction or any other step as previously described.

In addition to the two polypeptide hypothesis there are three other significant properties of the purified FAS preparation. The first was reported by Burton et al (2) indicating the presence of a protein that accompanied the synthetase complex through each step of it's purification from fasted rats. This contaminating protein was shown to be immunologically unrelated to FAS and acetyl-CoA carboxylase, it could not bind acetyl nor malonyl groups and has no known function (31). More recently, Yu and Burton (32, 33) have demonstrated that a holo-apo enzyme relationship exists between FAS and it's biosynthetic precursors. Confirmation of this was achieved through the separation of pigeon liver apo- and holo-FAS by affinity gel chromatography (34). Thirdly, the observed variation in specific activity of avian and mammalian liver FAS with varying nutritional or hormonal states, coupled with the observation that an apo-enzyme-less FAS preparation showed variation in specific activity at two different places in the protein peak eluted from the

affinity chromatographic column, led to a search for the existence of more than one form of holo-enzyme (35). As a result, a high specific activity holo- a form and a low specific activity holo- b form of FAS, interconvertible by phosphorylation-dephosphorylation, were isolated.

Biosynthesis of FAS and Fatty Acids

The synthesis of fatty acids is of major metabolic importance as they can be utilized via oxidation for energy in liver, heart, skeletal muscle, and other tissues, and also may be incorporated into various lipids for energy storage or cellular structures. Thus the control of FAS is a critical process as demonstrated by the marked changes in fatty acid synthesis in various nutritional states and developmental stages. It has been well established that starvation and insulin deficiency in animals are associated with impaired lipogenesis in the liver (1, 2, 36). Refeeding after starvation or insulin therapy after insulin deficiency restores the ability to convert carbohydrate to triglycerides (1, 2, 36). These fatty acid-synthesizing reactions occur in the soluble cell sap portion of the hepatocyte excepting the generation of acetyl groups from mitochondria and fatty acyl chain desaturation or elongation. Several lipogenic enzymes have been shown to have activities which fluctuate with

lipogenesis according to the nutritional status of the animal. These lipogenic enzymes include acetyl-CoA carboxylase (37), citrate cleavage enzyme (38), malic enzyme (38), glucose-6-phosphate dehydrogenase (39), 6-phosphogluconate dehydrogenase (40) and of course fatty acid synthetase (3).

The effect of the nutritional state on lipogenic enzymes of the liver is exemplified by the reduction of FAS activity in fasting rats and it's subsequent return to normal upon refeeding of a normal balanced diet. However, if a high carbohydrate, fat-free diet is used for refeeding then the activity rises to even higher than normal, or supranormal, levels (2, 41, 42). Initial indications that this increase in activity was due to an increase in de novo synthesis as opposed to activation of pre-existing enzyme were observed by Hicks et al (43) who used puromycin or actinomycin D at the beginning of the refeeding period and found it to prevent the increase in activity. Burton et al (2) demonstrated by direct purification that the level of FAS was considerably lower in livers of fasted rats as opposed to fat-free fed rats. Also ^{14}C-leucine was shown to be readily incorporated in vivo into purified FAS formed six hours after refeeding commenced. Tweto and Larrabee (44), studying the rate of synthesis of rat liver FAS with U-^{14}C-amino acid pulse labelling, found that after sixteen hours of fasting the

values were the lowest. Immunological techniques, or more specifically quantitative precipitin analysis, indicated that equivalence points were the same for FAS obtained from rats fed a normal diet or starved then refed a fat-free diet (3). Thus the differences in activity after changes in the diet of the animal are accompanied by proportionate changes in the quantity of immunoprecipitable FAS as opposed to a change in catalytic activity.

Craig and Porter (6) used rat liver cells, in a basic maintenance medium, which were isolated from rats under various nutritional states to investigate the adaptive synthesis of FAS in a cell suspension. They found the cells to retain the same properties in respect to the adaptive synthesis of FAS as if the experiments were done using the whole animal. Nepokroeff et al (45) demonstrated the presence of apo- and holo- forms of FAS for an in vitro cell-free rat hepatic polysome system. Alberts et al (46) also used a cell free system when rat liver polysomes were isolated and ^{125}I-labelled antibody against FAS was used to identify the FAS polysomes isolated from various nutritional states. Their results also conformed to those found from in vivo experiments as the level of FAS polysomes was the highest for the starve-refeed condition. Following this work, Strauss et al (47) used in vitro protein synthesis to demonstrate that the levels of previously isolated FAS polysomes correspond to

proportionate enzyme content.

The Regulation of FAS Biosynthesis

The study of the conditions of diabetes and starvation has led to the implication of hormonal factors, especially insulin, in the control of hepatic lipogenesis (2, 48, 49). Burton et al (2), demonstrated that insulin restored rat liver FAS activity to normal after depression of activity in the alloxan-diabetic condition. Lakshmanan et al (5), showed that insulin is essential for the dietary induction or adaptive synthesis of FAS by increasing the rate of synthesis while studying the streptozotocin-induced diabetic condition. Glucagon and dibutyryl c-AMP were also shown to inhibit the increase in FAS activity. Dietary induction of FAS, acetyl-CoA carboxylase, citrate cleavage enzyme, malic enzyme, glucose-6-phosphate dehydrogenase and glucokinase in diabetic rats were all shown to be dependent on insulin treatment (7). The dietary inductions of these enzymes in normal rats were shown to be inhibited by glucagon treatment. In addition Nepokroeff et al (7) demonstrated that serine dehydrase, selected as a model gluconeogenic enzyme, was shown to increase in diabetic rats but decrease upon insulin treatment.

With the implication of insulin and glucagon, two physiological antagonists, in the control of hepatic lipogenesis, control mediated via c-AMP is a logical possibility. Several investigators have found that glucagon and dibutyryl-c-AMP inhibit the adaptive synthesis of FAS under *in vivo* conditions using intact rats (5,7, 11). It is known that the hepatic levels of c-AMP can be increased by glucagon (55) and various studies *in vitro* with slices of rat or avian liver or with cultured liver cells have shown a decrease in fatty acid synthesis from acetate or glucose in the presence of c-AMP (50, 51, 54). Raskin *et al* (53) questioned the significance of c-AMP inhibition studies because a higher than physiological range of concentrations were used (52). Using the perfused, intact rat liver, they demonstrated that glucagon-induced changes in intracellular c-AMP caused negligible effects on fatty acid and cholesterol biosynthesis. Using this line of thought, McGarry *et al* (56) have given evidence to support the idea of an extra hepatic factor which acted to stimulate carnitine acyl transferase in glucagon, c-AMP or anti-insulin serum induced ketogenesis. The β-oxidation sequence was found to be governed, at least in part, by the concentration of carnitine.

Recently, Okajima and Ui (59) have outlined evidence concerning the lack of correlation between hormonal effects on c-AMP and glycogenolysis in the rat liver. They have found that by using a more sensitive method to assay for c-AMP, a radioimmunoassay, they could

detect changes in the concentration of c-AMP at doses of glucagon which were previously thought not to stimulate adenylate cyclase. Whether or not this has any implications regarding the observations of Raskin et al (53) remains to be seen, since they used a dose of glucagon known to give a fifty-fold increase in the level of c-AMP. They found glycogenolysis to be affected but not fatty acid oxidation, cholesterol biosynthesis or fatty acid biosynthesis.

Nevertheless, the finding that there exist two differently phosphorylated forms of holo FAS, suggests strongly a phosphorylation-dephosphorylation form of control inevitably influenced by insulin and glucagon levels (35). This introduces the concept of short-term and long-term regulation of hepatic lipogenesis. Short-term would imply changes in FAS activity or other lipogenic enzyme activities for immediate and short time intervals. There is much evidence to implicate acetyl-CoA carboxylase as being under the influence of short-term control (63-70). The enzyme is activated by citrate and inhibited by long chain fatty acyl-CoA (63, 67, 68). Phosphorylation catalyzed by a c-AMP-independent protein kinase was shown to inactivate the carboxylase while a Mg^{+2} requiring phosphatase was shown to reactivate it (69,70) which is similar to the findings for FAS phosphory-

lation-dephosphorylation (35). Long-term would imply changes in the rates of synthesis of FAS or other lipogenic enzymes for extended time periods such as the diabetic and starvation-refeeding conditions (1-11).

Despite all the evidence indicating an absolute requirement of insulin for the adaptive synthesis of FAS, Volpe and Vagelos (8) claim that insulin is not directly required for the regulation of FAS synthesis. Insulin administration to normal rats causes a fluctuation in their food consumption which correlates to fluctuations in FAS activity but not of the same magnitude as for dietary induction. Instead Volpe & Vagelos suggest that a number of glycolytic pathway intermediates may play a role. This idea was based on observations of an experiment using glucose or fructose as sole source of carbohydrate for normal and diabetic rats. FAS activity in livers of diabetic rats was found to be restored by fructose to the same level as for normal rats and thus indicates insulin has a secondary effect on FAS synthesis. This observation was not found with adipose tissue. Volpe also suggests that alterations by c-AMP of activities of glycolytic pathway enzymes, such as shown for glucokinase (8), would be expected to reduce the hepatic concentration of glycolytic pathway intermediates and lead to a decrease in the synthesis of FAS (11). The proposed mechanism of stimulation by fructose is that in the diabetic state,