Effects of glucagon and dibutyryl adenosine 3′,5′-cyclic monophosphate on oxidative desaturation of fatty acids in the rat

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Abstract The present work was undertaken to study the effect of anti-insulinic and glycogenolytic factors on the oxidative desaturation of fatty acids. The effects of glucagon and dibutyryl cyclic AMP on the desaturation of linoleic acid to γ-linolenic acid, α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid, stearic acid to oleic acid, and eicosa-8,11,14-trienoic acid to eicosa-5,8,11,14-tetraenoic acid by rat liver microsomal preparations were investigated. Fasted rats had low desaturating activity, but refeeding a fat-free diet enhanced the activity. Administration of glucagon or dibutyryl cyclic AMP abolished the increase of the 6-desaturase activity elicited by refeeding. However, a similar effect on the 9-desaturase and 5-desaturase activity was not observed. The relationship between these effects and glucose metabolism is discussed.

Supplementary key words linoleic acid · α-linolenic acid · stearic acid · eicosa-8,11,14-trienoic acid · glucose metabolism · liver microsomes · fasting · refeeding

The regulatory effect of nutritional factors on several enzymes involved in fatty acid oxidative desaturation is well established (1–6). Feeding a high protein diet to rats evokes a significant activation in liver of 6-desaturation of linoleic acid to γ-linolenic acid and of α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid (3, 4, 7), although the activity of the 9-desaturase that converts stearic acid to oleic acid (3, 7) is not substantially modified. In addition, the feeding of a high protein diet evokes a small increase in the 5-desaturation of eicosa-8,11-dienoic acid to eicosa-5,8,11-trienoic acid (8). The activating effect of proteins is attributed to enzymatic induction of the 6-desaturase or related enzymes (7, 9). High carbohydrate diets produce an effect different from that of protein. They cause a decrease of the 6-desaturase activity and an increase of the 9-desaturase activity (4, 7, 10).

Insulin has also been considered to be involved in the regulation of 9-, 6-, and 5-desaturations (2, 7, 8, 11). In diabetes, the activity of the 9-desaturase is markedly decreased and that of the 6-desaturase falls to a lesser degree, but the activity of the 5-desaturase is only slightly decreased. The activities are restored by parenteral injection of insulin. One of the mechanisms attributed to the action of insulin is enzyme induction (2, 12). However, apart from these similarities there are some differences in the way 9- and 6-desaturases respond to insulin (6). These differences have been tentatively explained by the effect of insulin on glycolysis (13). Some of the glycolytic intermediates presumably enhance 9-desaturation (5) and interfere with 6-desaturation (7, 10). Because glucagon is an antagonist of insulin in many physiological reactions and enhances glycogenolysis through an increase of cyclic AMP, it is very important to investigate the effect of glucagon and its second messenger, cyclic AMP, on the 6-desaturation of linoleic and α-linolenic acids in liver. In the present work we have addressed this problem as well as a study of the effect of these compounds on 9-desaturation of stearic acid and the 5-desaturation of eicosa-8,11,14-trienoic acid to eicosa-5,8,11,14-tetraenoic acid.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Stearic acid (56 mCi/mmole, 99% radiochemical purity), [1-^{14}C]linoleic acid (56.2 mCi/mmole, 99% radiochemical purity), and [1-^{14}C]eicosa-8,11,14-trienoic acid (58.9 mCi/mmole, 98% radiochemical purity) were purchased from New England Nuclear Corp., Boston, Mass. α-[1-^{14}C]Linolenic acid (41.5 mCi/mmole, 99% radiochemical purity) was obtained from the Radiochemical Centre, Amersham, England. N^6-2′-O-Dibutyryl 3′,5′-

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cyclic AMP (sodium salt), glucagon, and theophylline were purchased from Sigma Chemical Co., St. Louis, Mo.

Animals

Adult female Wistar rats weighing 250–300 g and maintained on standard Purina chow were used. The rats were divided into five groups of three animals each. All rats were fasted for 48 hr and then refed a fat-free diet for 48 hr. The distribution of calories in this diet was 30% protein and 70% carbohydrate. From the beginning of the refeeding period the different groups were administered glucagon, dibutyryl cyclic AMP, dibutyryl cyclic AMP plus theophylline, or theophylline. Water was given ad lib. A group of rats maintained on the fat-free diet without treatment and a sixth group of rats fasted for 48 hr were used as controls. Glucagon was administered at a dose of 200 μg/8 hr/100 g of body weight (each dose was 500–600 μg). Dibutyryl cyclic AMP was administered at a dose of 5 mg/8 hr/100 g of body weight. Theophylline was given at a dose of 2 mg/8 hr/100 g of body weight. All compounds were injected intraperitoneally. The doses chosen for glucagon and dibutyryl cyclic AMP were the same as those used by Lakshmanan, Nepokroeff, and Porter (14), who demonstrated an inhibition of fatty acid synthetase induction.

Isolation of microsomes

After 48 hr of fasting or refeeding, the rats were killed by decapitation without anesthesia. Livers were rapidly excised and immediately placed in ice-cold homogenizing medium (8). After homogenization the microsomes were separated by differential centrifugation by a procedure described in a previous study (8).

Assay for oxidative desaturation of fatty acids

Desaturation of the fatty acids by liver microsomes was measured by estimation of the percentage conversion of [1-^{14}C]stearic acid to oleic acid, [1-^{14}C]linoleic acid to γ-linolenic acid, α-[1-^{14}C]linolenic acid to octadeca-6,9,12,15-tetraenoic acid, and [1-^{14}C]eicosa-8,11,14-trienoic acid to eicosa-5,8,11,14-tetraenoic acid. The same liver microsomes were used for studying linoleic acid and α-linolenic acid desaturation whereas different groups of rats were used for the stearic acid and eicosatrienoic acid desaturation experiments.

3 nmoles of labeled acid and 97 nmoles of unlabeled acid were carefully measured and then incubated with 5 mg of microsomal protein in a Dubnoff shaker at 35°C for 20 min in a total volume of 1.5 ml of 0.15 M KCl, 0.25 M sucrose. The solution contained 4 μmoles of ATP, 0.1 μmole of CoA, 1.25 μmoles of NADH, 5 μmoles of $MgCl_2$, 2.25 μmoles of glutathione, 62.5 μmoles of NaF, 0.5 μmole of nicotinamide, and 62.5 μmoles of phosphate buffer (pH 7). Under such conditions, it was found that the rates of the reactions were independent of fatty acid concentration (15, 16). The reaction was started by injection of the microsomes with a Hamilton syringe and stopped by addition of 2 ml of 10% KOH in methanol. At the end of the incubation, the fatty acids were recovered by saponification of the incubation mixture. The acids were esterified with methanolic 3 M HCl (3 hr at 68°C), and the distribution of radioactivity between substrate and product was determined by gas–liquid radiochromatography in an apparatus equipped with a Packard proportional counter (8). The specific activity, expressed as nanomoles of product per milligram of microsomal protein per minute, was calculated from these data.

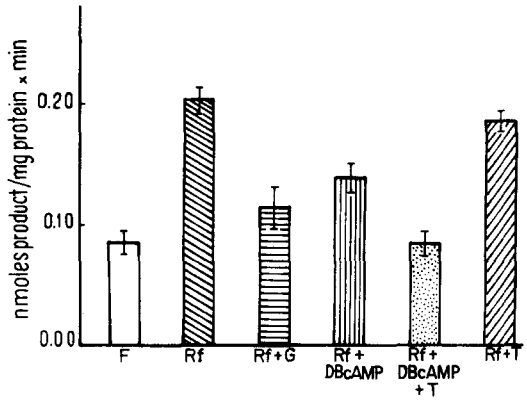

Fig. 1. Effect of fasting (F), refeeding (Rf), and administration of glucagon (Rf + G), dibutyryl cyclic AMP (Rf + DBcAMP), and theophylline (Rf + T) on the oxidative desaturation of [1-^{14}C]linoleic acid to γ-linolenic acid. Rats were fasted for 48 hr and then refed a fat-free diet for 48 hr. Results are means of analyses of three animals (each analysis was performed in duplicate). Vertical lines represent 1 SEM. All results are significantly different from those from refed animals ($P < 0.01$) except for Rf + T.

The fatty acid methyl esters were identified by equivalent chain length determination and comparison with authentic standards. The protein content of the microsomal fraction was determined by the biuret method of Gornall, Bardawill, and David (17), using crystalline bovine serum albumin as a standard.

RESULTS

Fasting causes a decrease of the desaturation activity of rat liver microsomes to convert stearic acid to oleic acid (3) and linoleic acid to γ-linolenic acid (4). The reduction of the activity is greater for stearic acid than for linoleic acid (2). Refeeding enhances the activity of both enzymes (2, 4). **Fig. 1,** Fig. 2, and Table 1 show the enhancing effect of a fat-free diet on the oxidative desaturation of linoleic, α-linolenic, and stearic acids by liver microsomes from rats that had been previously fasted for 48 hr. In addition, Fig. 1 shows that the administration of glucagon during the refeeding period abolishes the reactivation of the microsomal

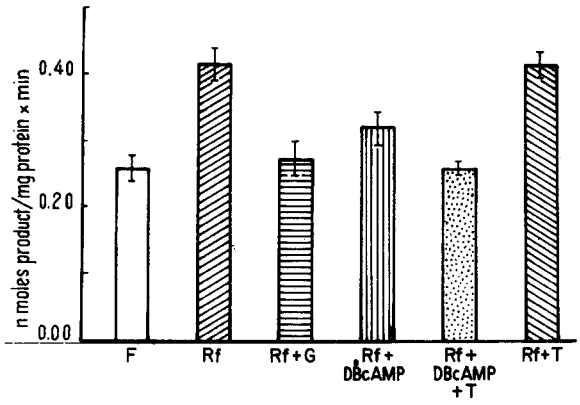

Fig. 2. Effect of fasting (F), refeeding (Rf), and treatment with glucagon (Rf + G), dibutyryl cyclic AMP (Rf + DBcAMP), and theophylline (Rf + T) on the oxidative desaturation of α-[1-^{14}C]linolenic acid to octadecatetraenoic acid. Rats were fasted for 48 hr and then refed a fat-free diet for 48 hr. See Materials and Methods for treatment. Results are means of analyses of three animals (each analysis was performed in duplicate). Vertical lines represent 1 SEM. All results are significantly different from those from refed animals ($P < 0.01$) except for Rf + T and Rf + DBcAMP.

conversion of linoleic acid to γ-linolenic acid evoked by the food intake. Injection of dibutyryl cyclic AMP and dibutyryl cyclic AMP plus theophylline also significantly decreased the reactivation. Theophylline increased the effect of the dibutyryl cyclic AMP but theophylline injection alone did not alter the effect of the food intake.

Fig. 2 illustrates the effect of glucagon and dibutyryl cyclic AMP on the oxidative desaturation of α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid. The liver microsomes were obtained from the same animals used in the previous study (Fig. 1). Glucagon and dibutyryl cyclic AMP inhibited the enhancing effect of the food intake on the desaturation activity by the liver of fasted rats. However, the decrease due to dibutyryl cyclic AMP is not statistically significant. The simultaneous injection of theophylline and dibutyryl cyclic AMP again enhanced the effect of the cyclic AMP, and this effect was significant. Therefore, glucagon and dibutyryl cyclic AMP antagonize the effect of refeeding on the 6-desaturase activity of the fasted rats.

A similar experiment was carried out in other groups of rats to investigate the effect of the same drugs on the oxidative desaturation of stearic acid (**Table 1**). In this case neither glucagon nor dibutyryl cyclic AMP nor dibutyryl cyclic AMP plus theophylline inhibited the activating effect of refeeding on the 9-desaturase.

Table 2 indicates that under the same experimental conditions glucagon, dibutyryl cyclic AMP, and dibutyryl cyclic AMP plus theophylline do not alter the effect of refeeding on the specific activity of eicosa-8,11,14-trienoic acid desaturation to eicosa-5,8,11,14-tetraenoic acid. In order to determine whether this group of rats responded to linoleic acid desaturation in the same way as shown in Fig. 1, the desaturation of this acid was studied simultaneously. It was found that the conversion of linoleic acid to γ-linolenic acid decreased the same as in Fig. 1. However, the specific activity of the enzyme was 50% less than in the previous experiment. Therefore, it is evident that glucagon and dibutyryl cyclic AMP evoke similar effects, but the 6-desaturase responds in a different way than the 9-desaturase and 5-desaturase.

DISCUSSION

This work has once more confirmed that fasted animals have less hepatic activity to desaturate linoleic, α-linolenic, and stearic acids than refed animals. The increase of 9- and 6-desaturase activity in different tissues produced by refeeding is thought to result from an increase of blood insulin (2, 4, 6). However, the different responses of 6- and 9-desaturases to dietary conditions, which have been discussed by several authors (3, 6), indicate the existence of different regulatory mechanisms for the two enzymes.

Several studies have demonstrated that cyclic AMP may modify lipid metabolism in liver. Thus, it was shown that cyclic AMP increases the production of ketone bodies in perfused liver (18) and liver homogenates (19) and stimulates the oxidation of fatty acids (20). Cyclic AMP decreases the incorporation of acetate into fatty acids and cho-

TABLE 1. Effect of glucagon and dibutyryl cyclic AMP on the microsomal oxidative desaturation of [1-^{14}C]stearic acid

Condition	Treatment	Specific Activity[a]
Fasted	None	Not measurable
Refed[b]	None	0.057 ± 0.003
Refed	Glucagon	0.061 ± 0.002
Refed	Dibutyryl cyclic AMP	0.053 ± 0.002
Refed	Dibutyryl cyclic AMP + theophylline	0.053 ± 0.002
Refed	Theophylline	0.048 ± 0.003

[a] nmoles product/mg protein/min. Results are means of analyses of three animals (each analysis performed in duplicate) ± 1 SEM.
[b] All animals were fasted for 48 hr and then refed a fat-free diet for 48 hr.

TABLE 2. Effect of glucagon and dibutyryl cyclic AMP on the microsomal oxidative desaturation of [1-^{14}C]eicosa-8,11,14-trienoic acid

Condition	Treatment	Specific Activity[a]
Refed[b]	None	0.17 ± 0.02
Refed	Glucagon	0.167 ± 0.005
Refed	Dibutyryl cyclic AMP	0.21 ± 0.01
Refed	Dibutyryl cyclic AMP + theophylline	0.18 ± 0.03
Refed	Theophylline	0.19 ± 0.01

[a] nmoles product/mg protein/min. Results are means of analyses of three animals (each analysis performed in duplicate) ± 1 SEM.
[b] All animals were fasted for 48 hr and then refed a fat-free diet for 48 hr.

lesterol in liver slices and isolated liver cells (21–23). Lakshmanan et al. (14) have also shown that glucagon antagonizes the induction of fatty acid synthetase produced by insulin and that dibutyryl cyclic AMP mimics the effect of glucagon. On the other hand, it has been reported that glucagon stimulates liver lipase, probably through the increase in cyclic AMP (19).

Glucagon not only inhibits the de novo synthesis of fatty acids (24) but also produces a decrease in the activity of the hepatic 6-desaturase of refed rats. The effect is shown in the conversion of linoleic and α-linolenic acids into γ-linolenic and octadecatetraenoic acids, respectively (Figs. 1 and 2). A similar effect is shown with the administration of dibutyryl cyclic AMP. Consequently, the intracellular level of cyclic AMP would play an important role in the activity of the 6-desaturase. This is confirmed with the administration of theophylline plus dibutyryl cyclic AMP. Theophylline increased the effect of dibutyryl cyclic AMP (Figs. 1 and 2), probably through an inhibition of cyclic AMP phosphodiesterase, which degrades cyclic AMP (25). Considering that it has also been proved that glucagon activates adenyl cyclase, which converts ATP to cyclic AMP, it is plausible to speculate that the inhibitory effect of glucagon on the 6-desaturase is produced through a rise in the intracellular level of the cyclic nucleotide. The data must be interpreted taking into account that glucagon was administered in pharmacological doses.

Neither glucagon nor dibutyryl cyclic AMP nor dibutyryl cyclic AMP plus theophylline altered the effect of refeeding on the 9- and 5-desaturases under our experimental conditions (Tables 1 and 2). This could suggest that these enzymes are insensitive or only slightly sensitive to the intracellular cyclic AMP concentration. However, this conclusion is not definitively proved, because the possibility exists that glucagon and cyclic AMP evoke an effect similar to that of refeeding on the activity of 9- and 5-desaturases. In this case, the effect of the drugs would very probably be masked by the effect of the food intake.

The results emphasize the possible importance of the endocrinological factors in controlling the 6-desaturase activity of the cell. The importance of this controlling function on the 6-desaturase would lie in the fact that this enzyme begins the synthesis of the polyethylenic fatty acids of the linoleic acid (n − 6) and linolenic acid (n − 3) series (6). Thus, the control of the activity of the 6-desaturase will at the same time regulate the synthesis of the polyethylenic acids such as arachidonic and eicosa-5,8,11,14,17-pentaenoic acids. The 6-desaturase has less specific activity than the 5-desaturase in rat liver (16). Consequently, as the 5-desaturase belongs to a later step in the synthesis of those acids and is more active than the 6-desaturase, it does not bear such an important function in regulation of synthesis. This fact is confirmed by the small response of the 5-desaturase to nutritional (26) and hormonal (8) factors (Table 2).

The possible mechanism of action of cyclic AMP on the 6-desaturase cannot be deduced with certainty from the present experiment. Nevertheless, all hypotheses must also consider the effects of 9- and 5-desaturases. The effect could be produced through a direct action of cyclic AMP on the desaturating enzyme, an action mediated through a regulatory protein, or through an indirect action. The indirect mechanism may be exerted through an anti-insulin effect and an increase of glucose metabolites. Research is in progress to understand better the relationship existing between insulin, cyclic AMP, and glucose metabolism on one side and fatty acid elongation and desaturation on the other.

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