Reversible inactivation by noradrenaline of long-chain fatty acyl-CoA synthetase in rat adipocytes

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1. Incubation of rat adipocytes with the same range of noradrenaline concentrations that stimulate lipolysis caused a rapid and stable decrease in the activity of fatty acyl-CoA synthetase. 2. Corticotropin, glucagon and dibutyryl cyclic AMP also decreased the activity of the enzyme. 3. The effect of noradrenaline was apparent over a wide range of concentrations for the three substrates of the enzyme. 4. A novel fluorescence assay of fatty acyl-CoA synthetase using (1,*N*⁶-etheno)-CoA is described. 5. The effect of noradrenaline was not abolished by inclusion of albumin in homogenization buffers, persisted through subcellular fractionation and isolation of microsomes (microsomal fractions) and even survived treatment of microsomes with Triton X-100. 6. The effect of noradrenaline was rapidly reversed within cells by the subsequent addition of insulin or propranolol. 7. The inclusion of fluoride in homogenization buffers did not alter the observed effect of noradrenaline. 8. Additions of cyclic AMP-dependent protein kinase to adipocyte microsomes caused considerable phosphorylation of microsomal protein by [γ -³²P]ATP, but did not affect the activity of fatty acyl-CoA synthetase.

The reaction catalysed by ATP-dependent long-chain fatty acyl-CoA synthetase (FAS; EC 6.2.1.3) occupies a potentially important position in the triacylglycerol-synthesis process in adipose tissue, in that it acts as a feeder of fatty acyl-CoA thioesters for the acyl-transfer reactions. The activity of FAS may be altered either *in vivo* or *in vitro* by nutritional or hormonal manipulation, being decreased by factors likely to direct adipose metabolism towards diminished synthesis and increased mobilization of triacylglycerol. Conversely, its activity may rise under conditions favouring increased triacylglycerol synthesis. *In vivo*, FAS activity in rat adipose tissue is decreased in starvation (Lawson *et al.*, 1981) and rapidly, under certain circumstances, after injection of corticotropin (Pollard, 1983). *In vitro*, treatment of adipocytes with insulin has been shown to increase microsomal FAS activity by as much as 100% within 2 min (Jason *et al.*, 1976), whereas adrenaline causes up to 40% decreases in activity within 10 min (Sooranna & Saggerson, 1978). These rapid hormonal effects *in vitro* are relatively persistent, and the effect of adrenaline is opposed

by insulin or by β -adrenoceptor blockade (Sooranna & Saggerson, 1978). The mechanism underlying these changes in activity are unknown. In the present study we have investigated the conditions under which the activity decrease owing to the catecholamine hormones remains persistent. We have demonstrated that this effect is rapidly reversible within cells and we have partially explored the possibility that the mechanism of inactivation by catecholamines might involve cyclic AMP-mediated protein phosphorylation.

Materials and methods

Chemicals were obtained and treated as described by Rider & Saggerson (1983). In addition, *N*⁶,*O*^{2'}-dibutyryl 3',5'-cyclic AMP was from Boehringer Corp. (London) Ltd. (Lewes, East Sussex, U.K.), (1,*N*⁶-etheno)-CoA was from Pharmacia P-L Biochemicals (Milton Keynes, Bucks., U.K.), palmitoyl-(etheno-CoA) was a gift from Dr. M. R. Edwards (Department of Biochemistry, University of New South Wales, Sydney, Australia) and [9,10-³H]palmitic acid was from Amersham International (Amersham, Bucks., U.K.). Cyclic AMP-dependent protein kinase holoenzyme was prepared from bovine heart by the method of

Abbreviation used: FAS, ATP-dependent long-chain fatty acyl-CoA synthetase (EC 6.2.1.3).

Brostrom *et al.* (1969), except that the fractionation stages using Sepharose 6B and calcium phosphate gel were omitted. The activity of this preparation was determined by monitoring the phosphorylation of histone IIA by [γ - 32 P]ATP (Reimann *et al.*, 1971). A unit of protein kinase represents the amount of enzyme required to incorporate 1 nmol of 32 P into histone/min at 30°C.

Adipocytes were obtained by collagenase treatment of epididymal-fat-pad pieces (Rodbell, 1964) taken from male Sprague-Dawley rats weighing 160–180 g. Portions of cells (equivalent to two-thirds of a fat-pad) were incubated with shaking at 37°C in a final volume of 4 ml of Krebs-Henseleit (1932) saline containing 1.27 mM- Ca^{2+} , fatty acid-poor albumin (40 mg/ml), 5 mM-glucose and adenosine deaminase (4 munits/ml). The gas phase was O_2/CO_2 (19:1). Incubation times and other additions to flasks are indicated in the legends to Tables and Figures. Before use in incubations, adenosine deaminase (EC 3.5.4.4) was dialysed overnight at 4°C against 0.9% NaCl to remove $(\text{NH}_4)_2\text{SO}_4$. The dialysed enzyme was standardized spectrophotometrically at 265 nm at 25°C by the method of Kalckar (1947) on the morning of experiments. A unit of adenosine deaminase is the amount of enzyme needed to deaminate 1 μmol of adenosine/min at 25°C in this assay.

Adipocyte extracts were prepared in a medium consisting of 0.25 M-sucrose, 10 mM-Tris/HCl buffer, pH 7.4, 1 mM-EDTA and 1 mM-dithiothreitol. This is referred to as 'sucrose medium'. 'Sucrose/albumin medium' was sucrose medium containing fatty acid-poor albumin (20 mg/ml). 'Sucrose/ F^- medium' contained 0.21 M-sucrose, 10 mM-Tris/HCl buffer, pH 7.4, 20 mM-NaF, 1 mM-EDTA and 1 mM-dithiothreitol.

After incubation, adipocytes were recovered by centrifugation at 200 g_{av} for 20 s, and the underlying medium was removed by aspiration. Fat-free homogenates were prepared after freeze-stopping of the cells with liquid N_2 by using an Ultra-Turrax tissue disintegrator (Janke and Kunkel, Staufen, Germany) as described by Sooranna & Saggerson (1976). Microsomes (microsomal fractions) were obtained by differential centrifugation (Saggerson *et al.*, 1980) of extracts obtained by breaking unfrozen adipocytes by agitation on a vortex mixer (Martin & Denton, 1970).

Unless stated otherwise, fatty acyl-CoA synthetase was assayed radiochemically at 25°C by measuring the incorporation of [^3H]palmitate into the acid-precipitable, ether-insoluble, reaction product [^3H]palmitoyl-CoA as described by Jason *et al.* (1976). The reaction mixture consisted of 0.35 M-Tris/HCl buffer, pH 7.4, 8 mM- MgCl_2 , 5 mM-dithiothreitol, 10 mM-ATP, 0.5 mM-CoA, 60 μM -[^3H]palmitic acid (1.2 μCi) and Triton WR

1339 (1 mg/ml) in a final volume of 0.2 ml. The reaction was started by addition of 10 μl of fat-free homogenate or of the quantities of microsomal protein indicated in legends to Tables. A novel fluorimetric assay was also developed. This was used in one set of experiments (see Table 3) and involved measuring the incorporation of the fluorescent analogue (1, N^6 -etheno)-CoA into the acid-precipitable reaction product palmitoyl-(etheno-CoA). The reaction mixture consisted of 0.2 ml containing 0.35 M-Tris/HCl buffer, pH 7.4, 8 mM- MgCl_2 , 5 mM-dithiothreitol, Triton WR 1339 (1 mg/ml) and indicated concentrations of ATP, palmitic acid and etheno-CoA. The reaction was performed at 25°C and was initiated by addition of either 10 μl of fat-free homogenate or up to 4 μg of microsomal protein. Assays were conducted for a maximum of 7 min, at the end of which the reaction was terminated by addition of 0.2 ml of fatty acid-poor albumin (6.25 mg/ml) followed immediately by 2 ml of ice-cold 0.3 M-trichloroacetic acid. After standing in ice for at least 5 min, unchanged etheno-CoA was removed by centrifugation (200 g_{av} for 5 min). The pellet was resuspended in a further 2 ml of 0.3 M-trichloroacetic acid and re-centrifuged as above. This washing procedure was carried out three times. Finally, the precipitate was dissolved in 3 ml of 25 mM-NaOH and the fluorescence of each sample measured in a Perkin-Elmer 3000 fluorescence spectrometer with an excitation wavelength of 275 nm and an emission wavelength of 410 nm. Fluorescence measurements were compared with a standard curve constructed using various amounts of palmitoyl-(etheno-CoA) dissolved in 25 mM-NaOH. With 10 mM-ATP, 60 μM -palmitic acid and 0.1 mM-etheno-CoA the assay was linear with incubation time for up to 10 min and with amount of microsomal protein or fat-free homogenate. This assay is cheaper and/or more convenient than assays using radioactive palmitic acid or CoA (Jason *et al.*, 1976) and, unlike a previously reported spectrophotometric assay (Tanaka *et al.*, 1979), can be used with crude tissue extracts. When 0.1 mM-etheno-CoA was substituted for 0.1 mM-CoA in the radiochemical assay using [^3H]palmitic acid (see above), similar FAS activities were observed with microsomes or with fat-free homogenates. However, when the radiochemical and the fluorescence assays were compared by using equivalent amounts of fat-free homogenates or of microsomes, approx. 3-fold higher FAS activities were obtained with the fluorescence assay (five experiments in each case; results not shown). It is presumed that there is loss of the reaction product in the radiochemical assay. FAS activities in fat-free homogenates are expressed relative to lactate dehydrogenase activity (Sooranna & Saggerson,

1978), where 1 unit of lactate dehydrogenase reduces 1 μ mol of pyruvate/min at 25°C. FAS activities in microsomal fractions are expressed relative to protein determined by the method of Lowry *et al.* (1951), with bovine albumin as standard.

[32 P]P_i bound to microsomal proteins by enzyme phosphorylation was measured by the method of Walsh *et al.* (1971). ATP was measured in deproteinized extracts obtained after incubation of microsomes by the method of Greengard (1963). Glycerol was assayed enzymically in deproteinized samples of adipocyte incubation media by the method of Garland & Randle (1962).

Statistical significance was determined by Student's *t* test for paired samples.

Results and discussion

In agreement with previous observations (Sooranna & Saggerson, 1978), exposure of adipocytes to noradrenaline for 60 min decreased the FAS activity in homogenates by approx. 50% (Tables 1, 2 and 5; Figs. 1 and 2), and even 10 min of treatment with the catecholamine caused a significant decrease, of approx. 25% (Tables 2 and 5; Fig. 2a). This effect was not confined to the catecholamines, since other lipolytic agents, i.e. glucagon, corticotropin and dibutyryl cyclic AMP, also decreased FAS activity (Table 1). Also, in accord with previous findings (Evans & Denton, 1977; Sooranna & Saggerson, 1978), insulin alone had no significant effect in the experiment shown in Table 1. In subsequent separate sets of experiments (results not shown) we have observed a significant

(22%) increase in FAS activity in homogenates or a non-significant (34%) increase in FAS activity in isolated microsomes after incubation of cells with insulin (400 μ units/ml) for 45 min. The conclusion reached in this laboratory is that insulin can cause some degree of activation of FAS, but that this effect is extremely variable and is difficult to control. This may be contrasted with the findings of Jason *et al.* (1976), who reported a doubling of microsomal FAS activity after exposure of adipocytes to insulin for a few minutes.

It was shown previously that adrenaline or noradrenaline in the concentration range that stimulates lipolysis also caused inactivation of glycerolphosphate acyltransferase (Sooranna & Saggerson, 1976) and phosphatidate phosphohydrolase (Cheng & Saggerson, 1978a). Fig. 1 demonstrates that the dose-response curve for inactivation of FAS by noradrenaline was very similar to the curve for stimulation of lipolysis. It is therefore concluded that 'physiological' concentrations of noradrenaline can also cause modification of FAS activity.

It was considered possible, though unlikely, that noradrenaline could penetrate into the cell and directly inactivate FAS. To eliminate this possibility, 1 μ M-noradrenaline was added to assays of FAS using fat-free homogenates or microsomes. The hormone had no effect on FAS activity in these assays.

As stated above, both Mg²⁺-dependent phosphatidate phosphohydrolase and microsomal glycerolphosphate acyltransferase also undergo a relatively stable inactivation when adipocytes are

Table 1. *Effect of lipolytic agents and insulin on FAS activity in fat-free homogenates*

Adipocytes were incubated for 1 h, followed by freeze-stopping and preparation of homogenates in sucrose medium. FAS activity was measured radiochemically. The values are means $\pm$ S.E.M. for four separate experiments in each case. The values in parentheses indicate the percentage changes relative to the control. **P* < 0.025, ***P* < 0.005, for comparison of incubations with hormones versus the control.

Additions to cell incubations	FAS activity (nmol/min per unit of lactate dehydrogenase)	Glycerol accumulation in incubation media (mM)
None	18.4 $\pm$ 1.4	0.04 $\pm$ 0.01
Noradrenaline (1 μ M)	8.9 $\pm$ 0.6** (−52 $\pm$ 2%)	1.27 $\pm$ 0.16**
Glucagon (0.1 μ g/ml)	13.6 $\pm$ 2.3* (−27 $\pm$ 8%)	0.71 $\pm$ 0.18*
Corticotropin (0.1 μ g/ml)	8.5 $\pm$ 0.7* (−53 $\pm$ 6%)	1.20 $\pm$ 0.07**
Insulin (1 munit/ml)	19.2 $\pm$ 0.6 (+6 $\pm$ 8%)	0.05 $\pm$ 0.01
None	15.5 $\pm$ 1.8	0.09 $\pm$ 0.00
Noradrenaline (1 μ M)	7.1 $\pm$ 1.1** (−54 $\pm$ 7%)	1.37 $\pm$ 0.06**
Dibutyryl cyclic AMP (2mM)	6.0 $\pm$ 1.6** (−62 $\pm$ 8%)	1.12 $\pm$ 0.14**

Fig. 1. Comparison of dose-response curves for inactivation of fatty acyl-CoA synthetase and stimulation of lipolysis by noradrenaline

Adipocytes were incubated for 1 h, followed by freeze-stopping and preparation of homogenates in sucrose medium. FAS activity was measured radiochemically. The values are from four separate experiments, except for those with zero and $1 \mu\text{M}$ -noradrenaline, which are from eight experiments. The control value for FAS activity was $12 \pm 1.6 \text{ nmol/min per unit of lactate dehydrogenase}$. This was decreased by $55 \pm 3\%$ to $5.4 \pm 0.8 \text{ nmol/min per unit of lactate dehydrogenase}$ with $1 \mu\text{M}$ -noradrenaline. Glycerol release was increased from 0.07 ± 0.01 to $1.53 \pm 0.07 \text{ mM}$ by $1 \mu\text{M}$ -noradrenaline. The values are normalized by expressing the changes with $1 \mu\text{M}$ -noradrenaline as 100 and expressing other changes as means $\pm$ S.E.M. of this value. FAS activity was significantly decreased ($P < 0.005$) by $0.1 \mu\text{M}$ -, $0.3 \mu\text{M}$ -, $1 \mu\text{M}$ - and $10 \mu\text{M}$ -noradrenaline. $\circ$, FAS; $\bullet$, glycerol release.

exposed to noradrenaline (Cheng & Saggerson, 1980; Rider & Saggerson, 1983). In these instances the effect of noradrenaline can be abolished by inclusion of albumin (10 mg/ml) in homogenization buffers, and it has been suggested that the effect of noradrenaline is brought about by the tight binding of some (presumably hydrophobic) regulatory ligand(s) to these enzymes. Table 2 shows that homogenization in buffer containing albumin (20 mg/ml) did not abolish the effect of

noradrenaline on FAS activity. In this respect it is noteworthy that albumin also did not abolish the effect of noradrenaline to inactivate the mitochondrial form of glycerolphosphate acyltransferase in adipocytes (Rider & Saggerson, 1983). However, albumin increased FAS activity in extracts prepared from cells incubated either with or without noradrenaline (Table 2). A similar effect of albumin to increase glycerolphosphate acyltransferase activity was also observed by Rider & Saggerson (1983). Table 2 also shows that inclusion of fluoride in homogenization buffers did not enhance the persistent inactivation of FAS brought about by noradrenaline and did not significantly alter the absolute activity of FAS.

Inactivation of glycerolphosphate acyltransferase in adipocytes by noradrenaline persists throughout the isolation of microsomes (Rider & Saggerson, 1983). The same phenomenon is observed with FAS. The decrease in the microsomal FAS activity was similar whether expressed relative to protein or the activity of the microsomal marker NADP-cytochrome *c* reductase (results from five experiments; not shown).

The persistent effect of the hormone is demonstrated in Table 3, which also shows that the inactivation is observed over a wide range of concentrations for the three substrates of the enzyme. Obviously the situation is complex with this number of substrates, but it appears unlikely that the hormone effect can be overcome by increasing the substrates to saturating concentrations.

Persistence of the effect of noradrenaline on FAS activity through microsomal isolation is again demonstrated in Table 4, which also shows that the effect of the hormone persisted even after treatment of the microsomes with Triton X-100 for 1 h. These conditions in fact resulted in a substantial degree of 'solubilization' of the microsomal FAS. This was seen in two experiments (results not shown) in which freshly isolated microsomes were incubated for 1 h in ice with or without Triton X-100, followed by re-centrifugation for 1 h at $105000g_{\text{av}}$. Assays were performed after this treatment, and it was found that 74% of the FAS activity was not sedimented after Triton treatment, whereas only 22% was not sedimented when Triton was omitted.

The experiment summarized in Table 5 and Fig. 2(a) demonstrates that inactivation of FAS by noradrenaline is rapidly reversible within the cells. This reversal was achieved by addition of either insulin or the β -adrenoceptor blocker propranolol. Blockade of β -adrenoceptors was rapidly achieved, as evidenced by an almost immediate cessation of lipolysis (Fig. 2b), and resulted in restoration of FAS activity back to control values within 5 min.

Table 2. *Effect of inclusion of albumin and fluoride in homogenization media on inactivation of FAS by noradrenaline*
Adipocytes were incubated for 10 or 60 min as indicated, followed by freeze-stopping and preparation of fat-free homogenates in the indicated media. FAS activity was measured radiochemically. The values are means $\pm$ S.E.M. The values in parentheses indicate the number of separate experiments in each case. * $P < 0.05$, ** $P < 0.005$.

Incubation time	Homogenization medium	Noradrenaline (1 μ M) added to cell incubation	FAS activity (nmol/min per unit of lactate dehydrogenase)	Change in FAS activity caused by noradrenaline (%)	Change in FAS activity caused by albumin or fluoride (%)
10min	Sucrose	—	11.1 $\pm$ 2.0 (8)	{ -25 $\pm$ 3 (8)** -23 $\pm$ 6 (4)*	+66 $\pm$ 3 (4)** +69 $\pm$ 22 (4)*
	Sucrose	+	8.5 $\pm$ 1.7 (8)		
	Sucrose/albumin	—	15.2 $\pm$ 2.5 (4)		
	Sucrose/albumin	+	11.4 $\pm$ 1.6 (4)	-23 $\pm$ 7 (4)*	
60min	Sucrose	—	10.8 $\pm$ 4.8 (3)	-48 $\pm$ 4 (3)**	+6 $\pm$ 8 (3) +4 $\pm$ 14 (3)
	Sucrose	+	5.2 $\pm$ 2.0 (3)		
	Sucrose/F ⁻	—	12.1 $\pm$ 6.2 (3)		
	Sucrose/F ⁻	+	5.2 $\pm$ 1.8 (3)	-47 $\pm$ 13 (3)*	

Table 3. *Effect of substrate concentration on the inactivation of fatty acyl-CoA synthetase by noradrenaline*
Adipocytes were incubated for 1 h with or without 1 μ M-noradrenaline followed by isolation of microsomes in sucrose medium. FAS activity was measured by the fluorescence assay and is given as the mean of the control values (nmol/min per mg of microsomal protein) from three to five experiments. The values in parentheses indicate the percentage decrease in FAS activity due to noradrenaline. * $P < 0.05$, ** $P < 0.01$ for effect of noradrenaline.

[ATP] (mM)	[Etheno-CoA] (mM)	[Palmitate] (μ M)	FAS activity		
			6	30	60
1	0.1	—	—	—	38.0 (-72 $\pm$ 20%)*
5	0.1	—	—	—	63.4 (-45 $\pm$ 16%)*
10	0.01	—	—	—	19.0 (-33 $\pm$ 30%)
	0.05	—	—	—	54.3 (-53 $\pm$ 18%)*
	0.1	—	25.5* (-48 $\pm$ 14%)	65.1 (-33 $\pm$ 9%)**	55.7 (-34 $\pm$ 14%)*
	0.5	—	—	—	67.7 (-50 $\pm$ 13%)**
	1.0	—	—	—	73.2 (-52 $\pm$ 14%)*
	—	—	—	—	—

Table 4. *Effect of Triton X-100 on the persistence of the effect of noradrenaline on fatty acyl-CoA synthetase activity in microsomes*

Adipocytes were incubated for 1 h with or without 1 μ M-noradrenaline, followed by isolation of microsomes in sucrose medium. Portions of microsomes (0.1–0.35 mg of protein/ml) in sucrose medium were then kept on ice for 1 h with or without Triton X-100 (0.48 mg/ml), followed by radiochemical assay of the enzyme. The values are means $\pm$ S.E.M. for five separate experiments. * $P < 0.025$, ** $P < 0.01$.

Noradrenaline (1 μ M) added to cell incubation	Treatment of microsomes with Triton X-100	Specific activity of fatty acyl-CoA synthetase (nmol/min per mg of microsomal protein)	Decrease in activity caused by noradrenaline (%)	Increase in activity caused by Triton X-100 (%)
—	—	100 $\pm$ 16	—	—
+	—	63 $\pm$ 11*	36 $\pm$ 6%**	—
—	+	108 $\pm$ 18	—	8 $\pm$ 6%
+	+	74 $\pm$ 10*	28 $\pm$ 7%**	21 $\pm$ 6%*

Table 5. *Reversal of noradrenaline-induced inactivation of fatty acyl-CoA synthetase in fat-free homogenates by insulin and propranolol*

Adipocytes were incubated with or without noradrenaline. At 20 min further additions of insulin or propranolol were made to some flasks. The cells were freeze-stopped at the indicated times and homogenates prepared in sucrose medium. FAS activity was measured radiochemically. The earliest sampling time (approx. 1 min) represents the time taken for addition of noradrenaline, mixing, separation of cells from incubation media and freeze-stopping. The values represent FAS activity as nmol/min per unit of lactate dehydrogenase and are means $\pm$ S.E.M. for five separate experiments. Results for testing of statistical significance were as follows. (a) Versus 20 min value with noradrenaline: $P < 0.1$ for 25 min value (+insulin and noradrenaline); $P < 0.05$ for 30 min value (+insulin and noradrenaline); $P < 0.025$ for 25 min value (+propranolol and noradrenaline); $P < 0.025$ for 40 min value (+propranolol and noradrenaline). (b) Versus 20 min control value: $P < 0.1$ for 25 min value (+insulin and noradrenaline); $P < 0.25$ for 30 min value (+insulin and noradrenaline); $P > 0.25$ for 25 min value (+propranolol and noradrenaline); $P > 0.25$ for 40 min value (+propranolol and noradrenaline). (c) Versus 60 min value with noradrenaline: $P < 0.02$ for 60 min value (+insulin and noradrenaline); $P < 0.05$ for 60 min value (+propranolol and noradrenaline). (d) Versus 60 min control value: $P < 0.25$ for 60 min value (+insulin and noradrenaline); $P < 0.1$ for 60 min value (+propranolol and noradrenaline).

Time (min)	Control incubations			Incubations with noradrenaline (1 μ M)		
	No other addition	Insulin (400 μ units/ml) added at 20 min	($\pm$)-Propranolol (10 μ M) added at 20 min	No other addition	Insulin (400 μ units/ml) added at 20 min	($\pm$)-Propranolol (10 μ M) added at 20 min
0	17.7 $\pm$ 4.1					
1 (approx.)				14.2 $\pm$ 3.6		
5				10.8 $\pm$ 2.7		
10				11.3 $\pm$ 3.2		
20	15.0 $\pm$ 2.5			9.3 $\pm$ 2.2		
25					11.9 $\pm$ 3.5	14.3 $\pm$ 3.5
30					13.7 $\pm$ 3.5	
40						14.3 $\pm$ 3.3
50					11.6 $\pm$ 2.8	13.0 $\pm$ 2.5
60	14.8 $\pm$ 2.4	16.2 $\pm$ 2.8	14.1 $\pm$ 2.3	7.9 $\pm$ 2.6	11.1 $\pm$ 2.8	10.9 $\pm$ 2.7

Insulin (400 μ units/ml) was slower acting, in that a partial suppression of lipolysis was only observed after approx. 10 min (Fig. 2b). Nevertheless, insulin restored FAS activity to a value that was not significantly different from the control within 10 min (Table 5). At longer times (30 and 40 min) after the addition of propranolol or insulin there was some decrease in FAS activity (Fig. 2a). The reason for this is unclear. Nevertheless, even at the end of the incubation period these FAS activities were still significantly greater than that in cells incubated with noradrenaline alone (Table 5).

Four further experiments were performed (results not shown) to establish the dose-dependence of this effect of insulin. Cells were incubated with 1 μ M-noradrenaline, which decreased FAS activity by $30 \pm 7\%$ relative to the control after 30 min. Various insulin concentrations were added at 20 min, and it was found that 100 μ units of insulin/ml restored FAS activity such that it was $87 \pm 11\%$ of the control value by the 30 min time point (i.e. not significantly different from the control). Higher insulin concentrations (400, 1000 or 4000 μ units/ml) were no more effective than 100 μ units/ml (measurements also made at the 30 min time point). At 10 μ units/ml, insulin caused

a partial restoration to $80 \pm 10\%$ of the control value under the same experimental conditions. It is concluded that physiological concentrations of insulin reverse the effect of noradrenaline under the experimental conditions employed here.

Time courses of inactivation of glycerolphosphate acyltransferase and Mg^{2+} -dependent phosphatidate phosphohydrolase by noradrenaline, together with reversal of these effects by insulin and propranolol, have been reported previously (Rider & Saggerson, 1983; Cheng & Saggerson, 1978a,b). The initial rate of inactivation of FAS by noradrenaline is quite considerable [approx. 4%/min, as shown by Sooranna & Saggerson (1978), and possibly even higher in the present study; see Fig. 2(a)]. This is more rapid than the decreases in phosphatidate phosphohydrolase (approx. 2%/min; Cheng & Saggerson, 1978a) or glycerolphosphate acyltransferase (approx. 1%/min; Rider & Saggerson, 1983); i.e. the more rapidly one of these enzymes is inactivated by noradrenaline, the more rapidly the effect can be reversed.

As shown above, the rapid effect of noradrenaline on FAS activity is surprisingly persistent, which might suggest modification of the enzyme

Fig. 2. Reversal of noradrenaline-induced inactivation of fatty acyl-CoA synthetase in fat-free homogenates by insulin and propranolol

The values are obtained from the experiment shown in Table 5. Control incubations: ○, no additions; △, +insulin; □, +propranolol. Incubations with noradrenaline: ●, no other additions; ▲, +insulin; ■, +propranolol. (a) Values for fatty acyl-CoA synthetase activity expressed as percentages of the zero-time control. (b) Glycerol accumulation in incubation media. The bars indicate S.E.M. Where not shown, these lie within the symbol. The arrows indicate the time of addition of insulin or propranolol.

by a phosphorylation/dephosphorylation mechanism. Since the inactivating effect of noradrenaline appears to be mediated through β -adrenoceptors (Sooranna & Saggerson, 1978; also see Table 5 and Fig. 2a), it was considered that cyclic AMP-dependent protein kinase might cause modification and inactivation of FAS. Adipocyte microsomes were therefore incubated with bovine heart cyclic AMP-dependent protein kinase under conditions which resulted in considerable phosphorylation of microsomal proteins (Fig. 3), similar to that observed previously in comparable experiments (Nimmo & Houston, 1978; Rider & Saggerson, 1983). No inactivation of FAS was observed. This lack of effect could not be attributed to depletion of the added ATP, since approx. 70% still remained after 30 min (Fig. 3). One possible reason for the lack of effect of the protein kinase on FAS activity in microsomes could be inaccessibility of FAS to the protein kinase. However, treatment of 100 μ g of adipocyte microsomal protein with 1.5 μ g of trypsin in a volume of 50 μ l for 10 min at 30°C caused an 80% loss of FAS activity (results not shown). Similarly, Bell *et al.* (1979) found that treatment of liver microsomes with chymotrypsin or Pronase caused a substantial loss of FAS activity. These findings suggest, but do not prove, that the catalytic site of FAS is on the cytoplasmic side of the endoplasmic reticulum. Further support for this hypothesis comes from studies using inhibitors which do not penetrate the microsomal membrane (Bell *et al.*, 1979). However, the orientation in the membrane of potential phosphorylation sites on FAS is unknown. It is suggested that FAS in adipocyte microsomes either is not a ready substrate for cyclic AMP-dependent protein kinase or that phosphorylation by the protein kinase does not cause inactivation. However, the possibility that phosphorylation might render the enzyme more susceptible to inactivation by other regulatory factors within the cell cannot be discounted at present. Nor can we at present discount the possibility that the enzyme might be phosphorylated by some other protein kinase or might undergo some other form of covalent modification.

In conclusion, noradrenaline causes a rapid, reversible, inactivation of adipocyte FAS which is extremely persistent in that it is not affected appreciably by albumin or detergent and survives through isolation of microsomes. The lack of any effects of fluoride or of cyclic AMP-dependent protein kinase does not at present give any support to the possibility that the effect of noradrenaline might be attributed to protein phosphorylation.

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Fig. 3. Effect of cyclic AMP-dependent protein kinase on microsomal fatty acyl-CoA synthetase activity. Adipocyte microsomes were incubated at a protein concentration of 0.3 mg/ml in 0.3 ml containing 0.25 M-sucrose, 10 mM-Tris/HCl, pH 7.4, 10 mM-MgCl₂, 20 mM-NaF, 1 mM-EDTA, 1 mM-dithiothreitol, 10 μ M-cyclic AMP and bovine heart protein kinase holoenzyme (10 units/ml) at 30°C. The incubation was initiated by the addition of ATP. When FAS activity was measured, this was 3.5 mM-ATP (unlabelled). When protein phosphorylation was measured this was 1 mM-[γ -³²P]ATP (5–20 mCi/mmol). Samples (20 μ l) were removed at the indicated times for measurement of FAS activity by the radiochemical assay (●), protein-bound ³²P (■) and ATP concentration (▲). Control experiments were performed in parallel. There were: measurement of FAS activity after incubation in the absence of ATP (○); measurement of protein-bound ³²P after incubation in the absence of MgCl₂ (□). Points represent means, and the bars S.E.M. of four separate experiments (three for ATP measurements).

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