Endocrine (1995) 3, 439-444

Prolonged infusion of epinephrine down-regulates expression of the fatty acid synthase gene in adipocytes

Lotan Shilo[1], Jane H. Chin, Salman Azhar & Brian B. Hoffman

Department of Medicine, Stanford University School of Medicine and Geriatric Research, Education and Clinical Center, VA Medical Center, Palo Alto, California, USA

Fatty acid synthase is a key enzyme in the *de novo* synthesis of fatty acids. Expression of fatty acid synthase mRNA in adipocytes is inhibited by beta adrenergic agonists. We wondered if prolonged exposure to high concentrations of catecholamines might inhibit expression of the fatty acid synthase gene in fat cells. In this study we investigated the effects of adrenergic stimulation on the regulation of fatty acid synthase in fat cells. Rats were infused continuously with epinephrine (60 μg/kg/hr) or vehicle for 48 h and fat pads were isolated. The infusion of epinephrine down-regulated expression of the fatty acid synthase mRNA as well as decreasing enzyme activity. Both epinephrine and isoproterenol inhibited fatty acid synthase mRNA expression when adipocytes were activated *in vitro*. These effects were blocked by the β adrenergic antagonist propranolol. In contrast, incubation with the adenosine receptor agonist phenylisopropyladenosine which decreases cAMP accumulation in fat cells, caused an increase in accumulation of fatty acid synthase mRNA. These results indicate that prolonged exposure to catecholamines, acting via β adrenergic receptors, inhibit expression of the fatty acid synthase gene possibly by increasing intracellular concentrations of cAMP.

Keywords: fatty acid synthase; beta adrenergic; lipolysis; adipocytes; gene expression; adenosine

Introduction

Fatty acid synthase (FAS) is a key enzyme in fatty acid synthesis, catalyzing the synthesis of fatty acids using the substrates malonyl-CoA and acetyl-CoA. The regulation of FAS in hepatocytes is primarily at the transcriptional level and is affected by nutritional and hormonal state (Iritani, 1992). For example, a several fold increase in mouse liver FAS mRNA and enzyme activity was found after refeeding of fasted animals with carbohydrates (Paulauskis & Sul, 1988). A similar increase in FAS mRMA was observed after treating diabetic mice with insulin (Paulauskis & Sul, 1989). Incubation of hepatocytes with triiodothyronine mimics the effect of carbohydrate refeeding, causing an increase of up to five times in mRNA and enzyme activity. Addition of insulin or insulin-like growth factor amplify this effect (Stapleton *et al.*, 1990). On the other hand a decrease in FAS mRNA can be seen after a fatty meal or after incubation of hepatocytes with fatty acids, especially polyunsaturated fatty acids (Clarke *et al.*, 1990). Glucagon and cyclic AMP also cause a decrease in FAS mRNA and enzyme activity when added to primary hepatocyte culture (Stapleton *et al.*, 1990). Administration of dibutyryl cAMP to mice prior to refeeding also prevented the expected increase in mRNA values (Paulauskis & Sul, 1989).

In view of the capacity of cAMP to inhibit expression of FAS in hepatocytes, we wondered if enhanced stimulation of beta adrenergic receptors in fat cells might over time lead to a significant decrement in expression of FAS in these cells. Only a few studies have examined the regulation of FAS in fat tissue or differentiated 3T3-L1 cells. FAS mRNA values have been shown to be markedly increased during differentiation of 3T3-L1 cells to adipocytes due to both increased gene transcription and increase in mRNA stability (Moustaid & Sul, 1991). In mature adipocytes thyroid hormone induces an increase in FAS gene transcription (Moustaid & Sul, 1991). It is interesting that fatty tissue FAS mRNA values were fivefold increased in genetically obese JCR:LA-corpulent rats and the elevated FAS mRNA levels were not affected by diet (Shillabeer *et al.*, 1992). We have found that stimulation of beta adrenergic receptors in isolated fat cells leads to a decrement in expression of FAS mRNA within 1 h *in vitro* (Shilo *et al.*, 1994). We wondered if prolonged elevated concentrations of catecholamines *in vivo* could also result in a decrease of FAS mRNA. In the current study we examined the effects of an infusion of epinephrine into rats on FAS gene expression, enzyme activity and also on lipolysis. Additionally, we examined the effect of the anti-lipolytic adenosine receptor agonist phenylisopropyladenosine (PIA) on FAS gene expression in isolated adipocytes. Together, these studies demonstrate that hormones which regulate cAMP accumulation in adipocytes modify expression of this key enzyme involved in fatty acid synthesis.

Results

Effect of in vivo *treatment with epinephrine on fatty acid synthase mRNA in fat pads*

After 48 h continuous infusion of epinephrine (60 μg/kg/hr from a mini pump), FAS mRNA in fat pads was decreased (Figure 1A) when measured by a sensitive RNase protection assay. Based on densitometry measurements of the hybridized protected fragments, expression of FAS relative to β-actin was about two times greater in fat pads from control than that from epinephrine-treated animals ($n = 5-6$, $P < 0.05$) (Figure 1B). In the same animals, fatty acid synthase activity tended to decrease in fat pads from the epinephrine-treated animals. Enzyme activity expressed as the ratio of FAS to lactate dehydrogenase (LDH) in fat pads from epinephrine-treated animals was about 66% that from control animals due mainly to a change in FAS activity with essentially no difference in LDH activity between the two groups (Table 1). Because of the high variability in FAS enzyme activity among animals, power analysis of the data indicated the sample size of five animals per group was insufficient to allow an 80% chance of detecting a difference of 34% with an alpha error of 0.05 for a two-tailed test.

Effect of epinephrine in vitro *on fatty acid synthase mRNA in isolated adipocytes*

To determine whether the down-regulation of FAS observed in fat pads by epinephrine *in vivo* could be mimicked in a

Correspondence: B.B. Hoffman
[1]Current address: Meir Hospital, Kfar-Saba, Israel
Received 21 October 1994; accepted 7 March 1995

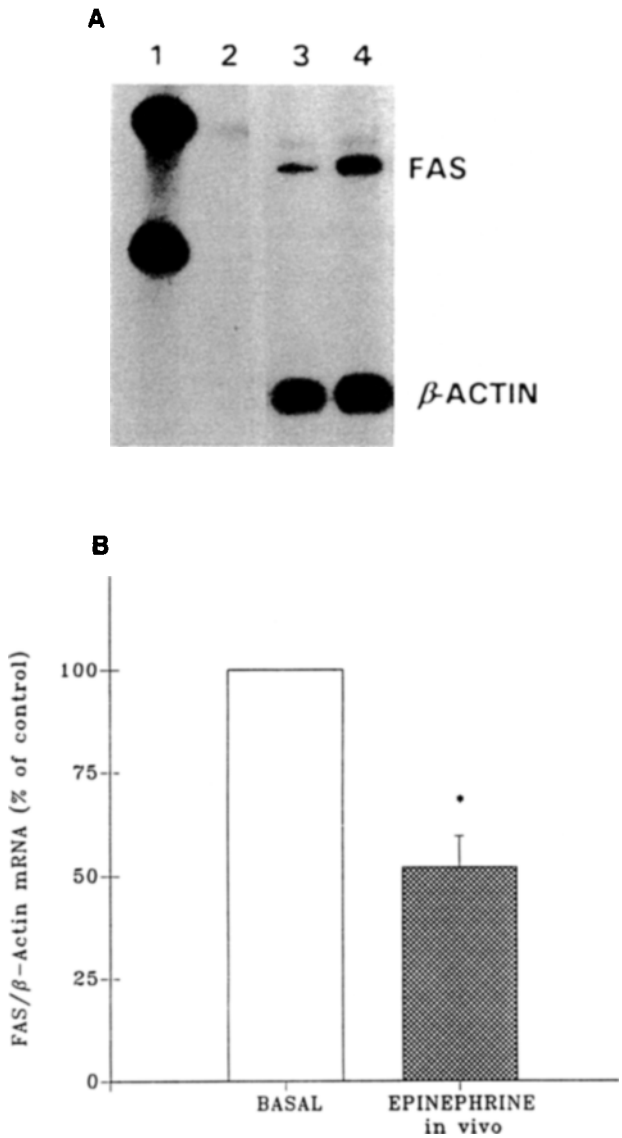

Figure 1 Inhibition of expression of FAS mRNA in fat pads from epinephrine-infused rats. (**A**) Autoradiogram of RNase-protected fragments of riboprobe-hybridized fatty acid synthase and β-actin mRNA in epididymal fat pads taken from rats infused with epinephrine (60 μg/kg/hr from minipump) for 48 h. Radiolabeled riboprobes for FAS and β-actin alone (lane 1), with 10 μg of yeast tRNA (lane 2) or with fat pad total RNA from epinephrine-infused (lane 3) and control (lane 4) rats. The samples were digested with RNase A and T1 and the protected fragments resistant to digestion were resolved by gel electrophoresis. (**B**) The peak area of the RNase-protected fragments were determined by densitometry. FAS mRNA densitometry bands were normalized to those of β-Actin to correct for differences in the amount loaded. Each bar represents the mean of 5–6 animals ± SEM. $^*P<0.05$ *vs* control

Table 1 Fatty acid synthase (FAS) activity in fat pads from rats infused with epinephrine

	Control	*Infused*	*Infused/Con*
FAS activity (nmol/mg protein)	30.54 ± 6.69	20.09 ± 4.21	0.658
LDH (mmol NADH ox/min/mg protein	1.57 ± 0.03	1.60 ± 0.07	1.020
FAS/LDH	19.35 ± 4.02	12.82 ± 2.98	0.663

Values are means ± SEM of five animals in each group. FAS and LDH enzyme activity were assayed using the 12000*g* supernatant as described in Methods

more controlled environment *in vitro*, we exposed isolated adipocytes to epinephrine (2.5 μM) for 14 h in culture. Epinephrine markedly reduced FAS mRNA to about 50% of the control value ($P<0.01$, $n=6$) similar to that observed *in vivo* and the inhibition induced by epinephrine was completely blocked by the β-adrenergic antagonist propranolol (10 μM) (Figure 2). In the same experiments epinephrine-stimulated lipolysis ($P<0.01$, $n=6$) was blocked by propranolol (Figure 3).

Effect of isoproterenol and adenosine agonist on FAS gene expression in isolated adipocytes

Isoproterenol, known to stimulate cAMP production and lipolysis, significantly reduced FAS mRNA in adipocytes at 1 ($P<0.05$, $n=4$), 3 (Figures 4 and 5, $P<0.01$, $n=5$), and 6 (Figures 4 and 5, $P<0.01$, $n=6$) h after addition of the β-adrenergic agonist. Isoproterenol (1 μM) also activated lipolysis of isolated adipocytes at each of the incubation times. In contrast, the adenosine receptor agonist, PIA (phenylisopropyladenosine), which blunts the cAMP and lipolytic response induced by isoproterenol, increased FAS mRNA to 124% of control at 3 h ($n=4$, $P<0.05$, paired *t* test) and 185% of control at 6 h (Figure 5).

Discussion

The aim of this study was to investigate the regulation of fatty acid synthase by adrenergic agonists. Catecholamines activate beta adrenergic receptors in adipocytes which increases release of fatty acids via activation of lipolysis while simultaneously repressing *de novo* synthesis of triglycerides in adipose tissue. We also determined that exposure of adipocytes to the anti-lipolytic adenosine receptor agonist PIA resulted in the increased expression of FAS as well as the well-known inhibition of lipolysis.

There is very limited information on regulation of expression of FAS in adipocytes. We have found in this study that a prolonged, continuous infusion of epinephrine *in vivo* leads to a decrease in FAS mRNA and enzymatic activity in epididymal fat pads. Similarly, epinephrine and isoproterenol added *in vitro* increased cAMP accumulation and lipolysis in isolated adipocytes and also decreased the abundance of FAS mRNA as we have found previously (Shilo *et al.*, 1994). These actions of catecholamines were both blocked by the beta adrenergic antagonist propranolol indicating that these responses were mediated via beta adrenergic receptor activation. In contrast, the antilipolytic adenosine receptor agonist PIA, which inhibits cAMP accumulation, protein kinase A activation and lipolysis in adipocytes (Hoffman *et al.*, 1984b, 1989), increased FAS mRNA abundance in fat cells.

It is well known that the expression of many genes, such as somatostatin and phosphoenolpyruvate carboxykinase, may be markedly induced by cAMP via cAMP dependent protein kinase-mediated phosphorylation of cAMP response element binding proteins (CREBs) (Lee and Masson, 1993; Meyer and Habener, 1993). However, the mechanism underlying suppression of gene expression by hormones which increase cAMP concentrations is still unclear. Several recent investigations suggest that the repression may be caused by CRE modulating proteins (CREMs). The CREM gene has been shown to encode for both antagonists (CREM alpha/beta/gamma) and activators (CREM tau) of cAMP induced transcription by alternative splicing (Laoide *et al.*, 1993).

In addition to effects of protein kinase A on transcription factors, other mechanisms may be involved in the decrement of FAS mRNA induced by epinephrine and isoproterenol. For example, free fatty acids released by lipolysis of catecholamines may secondarily inhibit FAS gene expression. Polyunsaturated fatty acids in the diet for 8 to 9 days have been shown to decrease FAS mRNA in rat hepatocytes (Clarke *et al.*, 1990). In contrast, long chain fatty acids

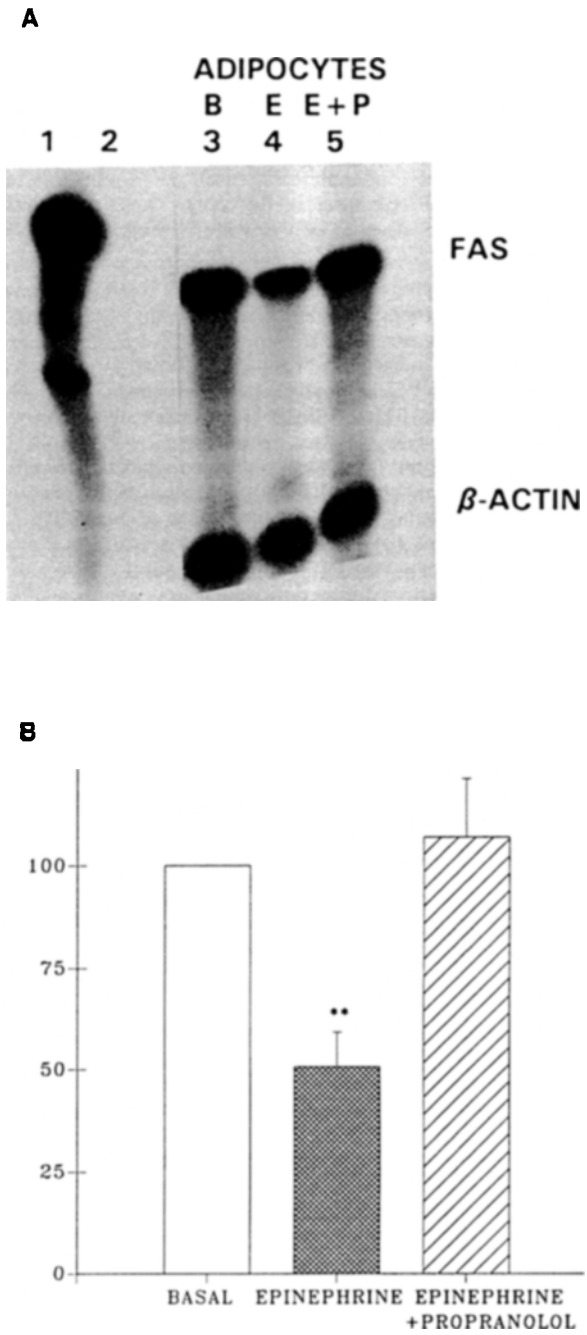

Figure 2 Propranolol antagonizes inhibition of expression of FAS mRNA in isolated adipocytes incubated with epinephrine *in vitro*. (**A**) Representative autoradiogram of RNase-protected fragments of riboprobe-hybridized fatty acid synthase mRNA and β-actin mRNA in adipocytes from 6–10 weeks rats. Adipocytes were incubated with ascorbate alone (100 μM) as basal (B, lane 3), epinephrine (2.5 μM dissolved in 100 μM ascorbate) alone (E, lane 4) or combined with l-propranolol (10 μM, E + P, lane 5) for 14 h at 37°C. Total RNA was extracted from adipocytes. Radiolabeled riboprobes for FAS and β-Actin mRNA were added together in solution either alone (lane 1), with 10 μg of yeast tRNA (lane 2), or with adipocyte total RNA (lanes 3–5). The samples were digested with RNase A and T1 and the protected fragments resistant to digestion were resolved by gel electrophoresis. FAS mRNA values in adipocytes treated with epinephrine alone (2.5 μM) or combined with l-propranolol (10 μM) for 14 h at 37°C were measured from the peak area of the RNase-protected fragments by densitometry (**B**). FAS mRNA densitometry bands were normalized to those of β-actin to correct for differecnes in the amount loaded. The ratio of FAS/β-Actin in treated cells was expressed as the percentage of the control FAS/β-Actin ratio within the same preparation. Each bar represents the mean of six independent experiments ± SEM. $^{**}P < 0.01$ *vs* control

activate aP2 gene expression in preadipose cells (Amri *et al.*, 1991) and are involved in differentiation of preadipose cells to adipose cells (Amri *et al.*, 1994).

It is well-known that prolonged activation of beta adrenergic receptors in many cells leads to receptor sequestration or down-regulation, as well as blunted capacity of beta agonists to activate adenylyl cyclase activity and enhance cAMP accumulation. We have found previously that prolonged exposure to catecholamines in rats harboring pheochromocytoma, a tumor which secretes enormous quantities of norepinephrine, leads to marked down-regulation of beta adrenergic receptors and attenuated isoproterenol-stimulated cAMP accumulation in adipocytes isolated from these rats (Tsujimoto *et al.*, 1984). Interestingly, these markedly

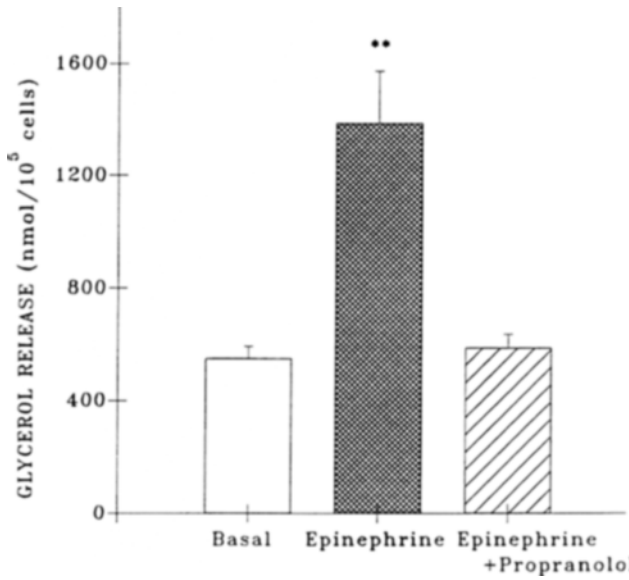

Figure 3 Epinephrine-stimulated lipolysis, measured as glycerol release. Lipolysis induced by epinephrine alone (2.5 μM) or combined with l-propranolol (10 μM) for 14 h at 37°C. Each bar represents the mean of six independent experiments ± SEM. $^{**}P \leqslant 0.01$ *vs* control

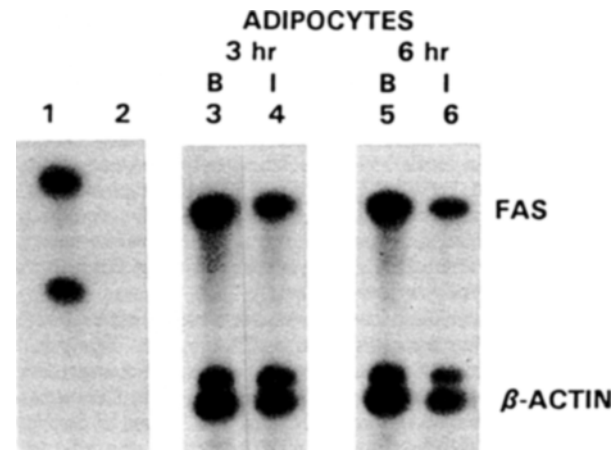

Figure 4 Representative autoradiogram of RNase-protected fragments of riboprobe-hybridized fatty acid synthase mRNA and β-actin mRNA in adipocytes from 6–10 weeks rats. Adipocytes were incubated with ascorbate alone (100 μM) as basal (B, lanes 3 and 5), isoproterenol (1 μM) combined with ascorbate (100 μM) (lanes 4 and 6) for 3 or 6 h at 37°C. Total RNA was extracted from adipocytes. Radiolabeled riboprobes for FAS and β-actin mRNA were added together in solution either alone (lane 1), with 10 μg of yeast tRNA (lane 2) or with adipocyte total RNA (lanes 3–6). The samples were digested with RNase A and T1 and the protected fragments resistant to digestion were resolved by gel electrophoresis

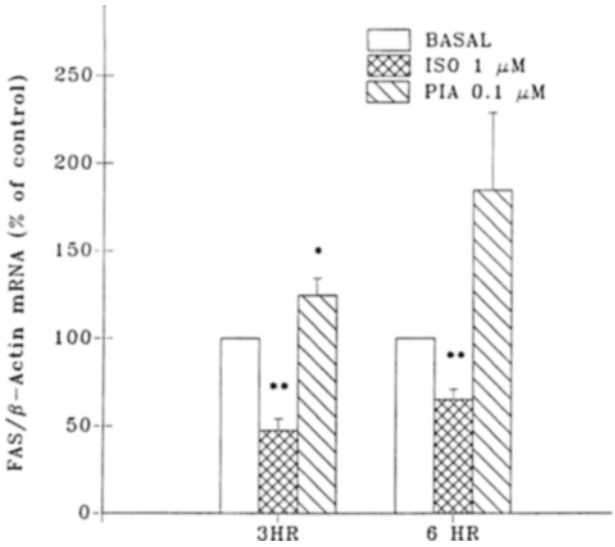

Figure 5 FAS mRNA levels in isolated adipocytes decreased by isoproterenol but increased by phenylisopropyladenosine at incubation times of 3 and 6 h. The peak area of the RNase-protected fragments were determined by densitometry. FAS mRNA densitometry bands were normalized to those of β-actin to correct for differences in the amount loaded. The ratio of FAS/β-Actin in treated cells was expressed as the percentage of the control FAS/β-Actin ratio within the same preparation. Each bar represents the mean of 4–6 independent experiments ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ *vs* control

attenuated responses do not lead to a decreased maximal lipolytic response to the beta adrenergic agonist isoproterenol although there is a considerable loss in potency of this drug in the adipocytes isolated from rats harboring pheochromocytoma (Prokocimer *et al.*, 1988). The reason that the maximal lipolytic response is preserved in the desensitized adipocytes is that there is enormous reserve in the cAMP signaling pathway in these cells. Only a modest increment in protein kinase A activity is required to maximally activate lipolysis. Consequently, even though isoproterenol activation is desensitized, there remains a large enough increment in cAMP concentration to activate protein kinase A sufficiently to maximally activate lipolysis (Prokocimer *et al.*, 1988). While we did not measure desensitization of beta adrenergic receptors in the current study, it is very plausible that desensitization of beta adrenergic receptors would have occurred in this infusion model. Nonetheless, we found quite substantial decrements in FAS mRNA fat pads from these rats in this study. Consequently, it would seem that any desensitization of beta receptors which may have occurred was not sufficient to 'desensitize' the capacity of these receptors to inhibit FAS mRNA abundance.

Catecholamines activate lipolysis in isolated rat adipocytes while adenosine agonists such as PIA inhibit lipolysis by decreasing adenylyl cyclase activity and suppressing plasma levels of free fatty acids and triglycerides. Since the response to beta adrenergic agonists declines with aging in Sprague-Dawley rats (Hoffman *et al.*, 1984a), while the effects of adenosine are potentiated (Hoffman *et al.*, 1984b), our results on FAS mRNA gene expression suggest the possibility that FAS activity could increase with aging in these rats which also become obese. This possibility requires direct experimental testing.

Our results demonstrate that prolonged elevations in catecholamine concentrations lead to inhibition of expression of FAS, a key enzyme in the endogenous synthese of fatty acids. Since catecholamines also stimulate release of free fatty acids from adipocytes, our results suggest a possible contributory mechanism for the weight loss which occurs in patients with pheochromocytoma. Our results on the likely role of cAMP in catecholamine-mediated regulation of FAS expression may have implications for weight loss or gain in other settings, such as with amphetamines (which stimulate catecholamine release), theophylline-like drugs (which tend to increase cAMP in fat cells by blocking adenosine receptors or inhibiting cAMP phosphodiesterase) or aging (which diminishes cAMP responses in fat cells). These possibilities, while speculative, deserve experimental testing.

Materials and methods

Chemicals were purchased from the following sources: [α-^{32}P]dCTP and [2-^{14}C]malonyl CoA (Amersham Corp); RNA transcription kit, RNase Block II, (Stratagene); restriction enzymes, RNase-free DNAse, proteinase K, t-RNA, RNase A, RNase T1, and (−)N6-phenylisopropyladenosine (PIA) (Boehringer Mannheim and Biolabs); Molony Sarcoma Virus Reverse transcriptase and T4 DNA ligase (Gibco-BRl); formamide (Clontech); collagenase (161 U/mg, Cooper Biomedical); bovine serum albumin (fraction V, Armour); (−) isoproterenol (+) bitartrate, adenosine deaminase (Type VIII, A1030), triglyceride kit (320-UV) and all other chemicals (Sigma Chemical).

Infusion of epinephrine in vivo *in rats*

Male Sprague-Dawley rats weighing about 300 gm were infused continuously with epinephrine at a rate of 60 μg/kg/hr from an Alzet mini pump (model 2001) for 48 h. The mini pump was filled with (−)-epinephrine bitartrate dissolved in acidified isotonic saline (0.001 N HCl) or acidified isotonic saline alone and was then pre-incubated at 37°C for 3 hr prior to implantation. The mini pumps were implanted subcutaneously in the neck under light anesthesia.

Preparation of isolated adipocytes

Male 6–10 week Sprague-Dawley rats were decapitated and adipocytes were isolated from epididymal fat pads by the method of Rodbell (1964), with minor modifications. Fat pads were digested in a gyratory bath for 70–80 min with collagenase (1 mg/ml) dissolved in Krebs bicarbonate buffer with added glucose (2.5 mM) and 4% bovine serum albumin adjusted to pH 7.4 at 37°C. The cell suspension was washed three times with Medium 199 containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin (0.25 μg/ml). Upon standing, cells separated from the infranatant by flotation. Aliquots of 100 μl of diluted cells were fixed by 2% osmium tetroxide in collidine buffer and were counted in a Coulter counter.

Incubation of cells for fatty acid synthase RNA determination

Aliquots of adipocytes in supplemented M 199 were treated with hormones for 1, 3, 6 or 14 h in an incubator at 37°C in an atmosphere of O_2 (95%)–CO_2 (5%). Ascorbate (100 μM) was routinely included with isoproterenol and epinephrine to prevent oxidation during the incubation period. In some experiments adenosine deaminase (1 U/ml) was included to remove endogenously released adenosine in isoproterenol-activated adipocytes. We found the effects of isoproterenol on FAS mRNA and lipolysis were not significantly different in the presence or absence or adenosine deaminase in adipocytes from young animals used in this study. At the end of the incubation period, the infranatant was removed and used to measure lipolysis. Three to four ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.1 M β-mercaptoethanol, 0.5% β-lauroylsarcosine) was added to the adipocytes and the tubes were votexed briefly. Total RNA was isolated either immediately or after overnight storage at −70°C.

Lipolysis

Lipolysis was measured from aliquots of infranatant from adipocytes treated with drugs and used for RNA extraction, by use of a diagnostic kit that measures glycerol semi-enzymatically (Sigma Triglyceride kit 320 UV). The rate of lipolysis is expressed as glycerol released.

Assay of fatty acid synthase activity

One frozen fat pad from each rat was homogenized in 3 ml/gm wet weight of 20 mM potassium phosphate buffer pH 7.0 containing 2 mM dithiothreitol. Immediately after homogenization, 0.25 volume of 1 M potassium phosphate pH 7.0 was added to stabilize the enzyme. Fatty acid synthase (FAS) and lactate dehydrogenase (LDH) activity were assayed using the 12000 *g* supernatant. FAS activity was measured by the procedure of Arslanian and Wakil (1975) as modified by Weiss, Rosen and Rubin (1980). Thirty µl of enzyme preparation was mixed with 0.275 M potassium phosphate buffer pH 6.6 containing 8.3 mM EDTA, 2.75 mM dithiothreitol, 1.24 mM NADPH, 34.5 µM acetyl-CoA and 90 µg of albumin in a final volume of 145 µl and incubated for 20 min at 0°C to prime acetyl-CoA. The reaction mixtures were then warmed to 37°C and fatty acid synthesis was initiated by the addition of 55 µl of 0.5 mM [2-^{14}C] malonyl-CoA (2500 cpm/nmol). The reaction was terminated after 5 min by the addition of 50 µl of 2N NaOH. Samples were solubilized by heating for 20 min at 100°C, cooled, and acidified with 40 µl of 5N HCl and extracted three times with 3 ml of petroleum ether. The pooled petroleum ether extracts were evaporated and radioactivity in the residue was determined by scintillation spectroscopy.

Assay of lactate dehydrogenase

LDH was assayed as described by Rip *et al.* (1981). The enzyme was added to a final volume of 1 ml containing 0.1 M sodium phosphate buffer, pH 7.5, 0.2 mM sodium pyruvate and 0.25 mM NADH. LDH activity was determined as the rate of NADH oxidation in the presence of pyruvate. The decrease in absorbency at 340 nm at room temperature was recorded continuously and the initial linear portion of the slope was used to calculate the reaction rate.

RNA preparation

Total RNA from fat pads or adipocytes was prepared by the single-step RNA isolation method of Chomczynski and Sacchi (1987) as modified for adipocytes (Louveau *et al.*, 1991). For fat pads, 2 ml of denaturing solution was added to about 300 mg of frozen tissue and was homogenized with a Polytron for 15 s. For isolated adipocytes, the cells were shaken briefly in 5 ml of denaturing solution. For both preparations, lipids were initially removed by two extractions with equal volumes of chloroform. The aqueous layer was acidified with 0.1 volume of 2 M sodium acetate (pH 4) and total RNA was extracted free of DNA and protein by equal volumes of water-saturated phenol and chloroform. To facilitate precipitation of the small amounts of RNA 10–100 µg of t-RNA was added as carrier to each tube. The subsequent alcohol precipitation's were performed according to the standard protocol. RNA samples were stored in 70% ethanol at −70°C. Equal volumes of RNA solutions were used in the RNase protection assays, generally about 25% of the sample (between 0.25–1 microgram of total RNA).

Construction of plasmids expressing fragments of fatty acid synthase and β-actin

The fatty acid synthase gene was a generous gift from R. Smith (Oakland Children Hospital, Oakland, CA). A 400 bp EcoRI–HindIII fragment was subcloned into the transcription vector pBluescript KSII$^+$ (Stratagene) in 5′ to 3′ orientation to construct the plasmid pKSFAS. Rat β-actin was cloned using PCR as previously described (Shilo *et al.*, 1994). The 166 bp PCR product was subcloned into KSII$^+$ to create the plasmid pKSACT. The plasmid pKSFAS was linearized with HindIII and pKSACT was linearized with BamHI, extracted twice with phenol and twice with chloroform, precipitated in ethanol and redissolved in DEPC-treated water to a concentration of 250 µg/ml.

Preparation of riboprobe

Because of their high lability, the riboprobes were freshly prepared prior to hybridization with sample RNA by using a RNA transcription kit as described by the manufacturer (Stratagene). Unlabeled CTP was added to the reaction mix in a final concentration of 0.0175–0.050 M, to adjust the ^{32}P-labeled CTP to a specific activity of about 10^9 c.p.m./µg. RNA polymerase (T3 for β-actin or T7 for fatty acid synthase (FAS)) was added to the reaction mix and incubated for 30–60 min at 37°C. Then 25 µl of DEPC-treated water containing 10 units of DNase was added and the incubation was continued for 30 min at 37°C. At the end of this incubation 5 µl of proteinase K (10 mg/ml) and 5 µl of 10% SDS were added and the tubes were incubated for 15 min. After the DNA template and proteins were completely digested, the reaction mix was extracted with phenol/chloroform/isoamyl alcohol (25:24:1 v:v:v). The upper aqueous phase was transferred to another microfuge tube containing 10 µg of t-RNA as carrier. 200 µl of 2 M sodium acetate, pH 5.2 and 1 ml of ethanol were added and the tubes were incubated 15 min on dry ice and then centrifuged at maximum speed for 15 min at 4°C. The precipitation step was repeated two additional times. After the third precipitation, the pellets were dissolved in 100 µl of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 50 mM Pipes and 1 mM EDTA) and an aliquot was removed for liquid scintillation counting.

RNase protection assay

A solution hybridization/RNase protection assay was used to measure relative amounts of fatty acid synthase and β-actin mRNA in fat pads and adipocytes. Total RNA from epididymal fat pads and adipocytes (0.25–1.0 µg) were dried under vacuum and redissolved in 25–30 µl of hybridization buffer containing 100 000 cpm of each probe (the radiolabeled RNA fatty acid synthase probe and the β-actin probe that was used as internal standard for quantification). The mixture was incubated for 5 min at 85°C to denature RNA and was then rapidly transferred to hybridization temperature of 42°C for incubation overnight (about 12 h). To digest unprotected probe, 350 µl of ribonuclease digestion buffer (10 mM Tris-HCl, 5 mM EDTA, 0.3 M NaCl) containing 40 µg/ml ribonuclease A and 2 µg/ml ribonuclease T1 was added to the hybridization reaction and incubated 1 hr at 30°C. The RNase digestion reaction was terminated by addition of proteinase K (2.5 µl of 20 mg/ml) and SDS (10 µl of 20% w/v) and incubation for 15 min at 37°C. After phenol-chloroform extraction (150 µl), the protected RNA-RNA hybrids were ethanol-precipitated using 10 µg yeast tRNA as carrier. The pellet was dissolved in 6–8 µl of loading buffer (80% formamide, 1 mM EDTA, pH 8, 0.1% bromophenol blue and 0.1% xylene cyanole) and heated for 3 min at 85°C. The protected fragments were separated on 6% acrylamide-urea denaturing gel. After electrophoresis, the gel was exposed to Kodak XAR-5 film at −70°C with intensifying screen. For strong signals, gels were usually exposed for 6–12 h and for weaker signals for up to 48 h. For quantification, the gels were analysed by densitometry. All treatments within an experiment were compared to the control basal value for each probe and the data were expressed as fraction of fatty acid synthase signal to that of β-actin in order to correct for differences in loading the small amounts of total RNA.

Acknowledgements
Dr Shilo was a Merck Foundation Fellow in clinical pharmacology. This work was supported by NIH (AG09597) and the Research Service of the Department of Veteran Affairs.

References

Amri, E.-Z., Bertrand, B., Ailhaud, G. & Grimaldi, P. (1991). *J. Lipid Res.*, **32,** 1449–1456.
Amri, E.-Z., Ailhaud, G. & Grimaldi, P.-A. (1994). *J. Lipid Res.*, **35,** 930–937.
Arslanian, M.J. & Wakil, S.J. (1975). *Methods Enzymol.*, **35,** 59–65.
Chomczynski, P. & Sacchi, N. (1987). *Anal. Biochem.*, **162,** 156–159.
Clarke, S.D., Armstrong, M.K. & Jump, D.B. (1990). *J. Nutrition*, **120,** 225–231.
Hoffman, B.B., Chang, H., Farahbakhsh, Z.T. & Reaven, G.M. (1984a). *Am. J. Physiol.*, **247,** E772–E777.
Hoffman, B.B., Chang, H., Farahbakhsh, Z. & Reaven, G.M. (1984b). *J. Clin Invest.*, **74,** 1750–1755.
Hoffman, B.B., Prokocimer, P., Thomas, J.M., Vagelos, R., Chang, H. & Reaven, G.M. (1989). *Endocrinology*, **124,** 2434–2442.
Iritani, N. (1992). *Eur. J. Biochem.*, **205,** 433–442.
Laoide, B.M., Foulkes, N.S., Schlotter, F. & Sassone-Corsi, P. (1993). *EMBO J.*, **12,** 1179–1191.
Lee, K.A.W. & Masson, N. (1993). *Biophys Acta*, **1174,** 221–233.
Louveau, I., Chauduri, S. & Etherton, T.D. (1991). *Anal. Biochem.*, **196,** 308–310.
Meyer, T.E. & Habener, J.F. (1993). *Endocrine Rev.*, **14,** 269–290.
Moustaid, N. & Sul, H.S. (1991). *J. Biol. Chem.*, **266,** 18550–18554.
Paulauskis, J.D. & Sul, H.S. (1988). *J. Biol. Chem.*, **263,** 7049–7054.
Paulauskis, J.D. & Sul, H.S. (1989). *J. Biol. Chem.*, **264,** 574–577.
Prokocimer, P.G., Maze, M., Vickery, R.G. & Hoffman, B.B. (1988). *Endocrinology*, **123,** 528–533.
Rip, J.W., Rupar, C.A., Chaudhary, N. & Carroll, K.K. (1981). *J. Biol. Chem.*, **256,** 1929–1934.
Rodbell, M. (1964). *J. Biol. Chem.*, **239,** 375–380.
Shillabeer, G., Hornford, J., Forden, J.M., Wong, N.C., Russell, J.C. & Lau, D.C. (1992). *J. Lipid Res.*, **33,** 31–39.
Shilo, L., Chin, J.H. & Hoffman, B.B. (1994). *Am. J. Physiol.*, **266,** E659–E665.
Stapleton, S.R., Mitchell, D.A., Salati, L.M. & Goodridge, A.G. (1990). *J. Biol. Chem.*, **265,** 18442–18446.
Tsujimoto, G., Manger, W.M. & Hoffman, B.B. (1984). *Endocrinology*, **114,** 1272–1278.
Weiss, G.H., Rosen, O.M. & Rubin, C.S. (1980). *J. Biol. Chem.*, **255,** 4751–4757.