# Mechanisms by which tumor necrosis factor stimulates hepatic fatty acid synthesis in vivo

**Carl Grunfeld,* Jennifer A. Verdier,* Richard Neese,† Arthur H. Moser,* and Kenneth R. Feingold***

Metabolism Section* and Cardiology Section,† Department of Medicine, University of California, San Francisco, and the Veterans Administration Medical Center, San Francisco, CA 94121

**Abstract** We have previously shown that bolus intravenous administration of tumor necrosis factor (TNF) to normal rats results in a rapid (within 90 min) stimulation of hepatic fatty acid synthesis, which is sustained for 17 hr. We now demonstrate that TNF stimulates fatty acid synthesis by several mechanisms. Fatty acid synthetase and acetyl-CoA carboxylase (measured after maximal stimulation by citrate) were not higher in livers from animals that had been treated with TNF 90 min before study compared to controls. In contrast, 16 hr after treatment with TNF, fatty acid synthetase was slightly elevated (35%) while acetyl-CoA carboxylase was increased by 58%. To explain the early rise in the hepatic synthesis of fatty acids, we examined the regulation of acetyl-CoA carboxylase. The acute increase in fatty acid synthesis was not due to activation of acetyl-CoA carboxylase by change in its phosphorylation state (as calculated by the ratio of activity in the absence and presence of 2 mM citrate). However, hepatic levels of citrate, an allosteric activator of acetyl-CoA carboxylase, were significantly elevated (51%) within 90 min of TNF treatment. TNF also induces an acute increase (within 90 min) in the plasma levels of free fatty acids. However, hepatic levels of fatty acyl-CoA, which can inhibit acetyl-CoA carboxylase, did not rise 90 min following TNF treatment and were 35% lower than in control livers by 16 hr after TNF. These data suggest that TNF acutely regulates hepatic fatty acid synthesis in vivo by raising hepatic levels of citrate. This is later followed by an increase in acetyl-CoA carboxylase and fatty acid synthetase and a decrease in hepatic fatty acyl CoA levels. — **Grunfeld, C., J. A. Verdier, R. Neese, A. H. Moser, and K. R. Feingold.** Mechanisms by which tumor necrosis factor stimulates hepatic fatty acid synthesis in vivo. *J. Lipid Res.* 1988. **29:** 1327–1335.

**Supplementary key words** acetyl-CoA carboxylase • fatty acid synthetase • citrate

In addition to the removal of infectious agents and development of immunity against future challenge, the host responds to infection with marked disturbances in intermediary metabolism (1–5). For example, hypertriglyceridemia due to the accumulation of very low density lipoproteins occurs during infection and in response to administration of endotoxin (2, 3). Infection produces both a decrease in clearance of triglyceride-rich lipoproteins (4) and an increase in hepatic lipid synthesis (5).

Cytokines, which mediate both the inflammatory and immune responses, have recently been shown to produce disturbances in intermediary metabolism (6–13). Pekala and co-workers (6) and Kawakami et al. (7) demonstrated that endotoxin-stimulated macrophages secrete a factor that decreases both the synthesis and storage of lipid in fat cells, while increasing lipolysis (8). This factor, when purified based on its ability to decrease lipoprotein lipase levels, was found to be identical to mouse tumor necrosis factor (TNF) by amino acid sequence analysis (9). Recombinant DNA-produced TNF also inhibits the synthesis and storage of lipid in fat cells (10, 11).

Recently we have demonstrated that administration of TNF to rats produces a rapid (within 90 min) increase in de novo hepatic fatty acid synthesis as measured by the incorporation of $^3H_2O$ into lipid in vivo (12). Hepatic lipogenesis remains increased in TNF-treated animals for more than 17 hr compared to control animals, although the absolute rates of lipogenesis decline in both, as animals were fasted during these experiments (12). The mechanism(s) by which TNF stimulates fatty acid synthesis in the liver is not yet understood. In fat cells, TNF decreases lipid storage by decreasing levels of lipoprotein lipase and the lipogenic enzymes (6, 13). Preliminary data suggest that TNF can increase lipolysis in cultured fat cells (10). Each of these actions takes several hours and is mediated by changes in the levels of the relevant enzymes. In view of the slow turnover of lipogenic enzymes in the liver (14–17), it is unlikely that the rapid stimulation of hepatic lipogenesis seen after TNF administration (12) can be accounted for by increases in the levels of hepatic lipogenic enzymes.

Abbreviations: TNF, tumor necrosis factor alpha; BSA, bovine serum albumin; DTT, dithiothreitol.

The regulation of hepatic fatty acid synthesis is complex and has been studied in most detail with regard to changes in diet. When fasted rats are re-fed a fat-free diet, the rate of fatty acid synthesis increases rapidly (within minutes), as measured in vivo and in vitro by the incorporation of labeled substrate into lipid, and then increases progressively over several days (14, 15). However, the levels of the rate-limiting enzymes for fatty acid synthesis, acetyl-CoA carboxylase, and fatty acid synthetase, as conventionally measured under conditions of maximal activation, do not increase until several hours after the increase in lipogenesis (14–17). Instead, the rapid increases in hepatic fatty acid synthesis correlate with changes in the intracellular levels of the regulators of acetyl-CoA carboxylase activity (14, 15, 18). Hepatic levels of citrate, an activator of acetyl-CoA carboxylase, increase while levels of long chain fatty acyl CoA molecules, inhibitors of acetyl CoA carboxylase, acutely decrease with fat-free re-feeding (14, 15, 18–21). Paradoxically, one of the known actions of TNF in fat cells, i.e., stimulation of lipolysis, could potentially decrease lipogenesis in the liver. If TNF, by increasing lipolysis (8, 10), produced an increase in circulating free fatty acids in vivo and this in turn resulted in an increase in the intracellular levels of fatty acyl CoA, inhibition of hepatic acetyl-CoA carboxylase activity might occur.

Acetyl-CoA carboxylase activity is also regulated by phosphorylation and dephosphorylation (21, 22). Using partially purified rat liver acetyl-CoA carboxylase, Carlson and Kim (22) have shown that dephosphorylation reduces the $K_m$ for citrate activation from 2.4 to 0.2 mM and makes the enzyme less susceptible to inhibition by palmitoyl-CoA. The extrapolation of these findings to in vivo data is complex because there are multiple sites for phosphorylation on acetyl-CoA carboxylase (21), with opposing hormones such as insulin and epinephrine inducing phosphorylation at different sites (23–25). Recently a method has been developed for analyzing the phosphorylation state of acetyl-CoA carboxylase in situ by fast freezing, extracting in the presence of phosphatase and protease inhibitors, and assaying the ratio of the activity in the absence and presence of 2 mM citrate (26, 27). This "activity ratio" is inversely proportional to the degree of phosphorylation at the sites responsible for inactivating the enzyme. These studies indicate that nutritional status influences the activity ratio, and hence the phosphorylation state (27).

In this manuscript we delineate the mechanisms by which TNF stimulates hepatic lipogenesis by measuring the activities of lipogenic enzymes and their allosteric modulators in the livers of animals that have been treated with TNF.

## EXPERIMENTAL PROCEDURES

### Materials

Human TNF alpha with a specific activity of $5 \times 10^7$ units/mg produced by recombinant DNA techniques was kindly provided by Dr. H. Michael Shepard of Genentech, Inc., San Francisco, CA. Units are determined by the ability of TNF to induce cytolysis in an L-929 cell assay (28, 29). Male Sprague-Dawley rats were purchased from Simonsen Animal Vendors. BSA fraction V, acetyl-CoA, ATP, 2-mercaptoethanol, DTT, NADPH, malonyl-CoA, citrate, NADH, NAD, malate dehydrogenase, citrate lyase, α-ketoglutarate, α-ketoglutarate dehydrogenase, phenylmethylsulfonyl fluoride, benzamidine, N-tosyl-L-phenylalanine chloromethyl ketone, leupeptin, antipain, pepstatin, and 10% $BCl_3$/methanol were purchased from Sigma Chemical Company, St. Louis, MO. Sodium [$^{14}$C]bicarbonate was purchased from New England Nuclear, Boston, MA, [1-$^{14}$C]acetyl-CoA from Amersham Corporation, MP-Ready Solve scintillation fluid from Beckman Instruments, and Ultrafluor scintillation fluid from RPI, Inc.

### Animal procedures

Male Sprague-Dawley rats (200 g) were maintained on a reverse 12-hr light cycle (3 AM–3 PM dark, 3 PM–3 AM light). They were fed rat chow (Simonsen Animal Vendors) and water ad libitum until the start of the experiments. The animals were injected via the tail vein with 25 μg of TNF in 0.5 ml of 0.9% saline or saline alone. This dose is approximately one-quarter of that shown to produce tumor necrosis in vivo (30), and is the optimal dose for acutely stimulating hepatic lipogenesis in vivo (12). These conditions are identical to those used previously in this laboratory for studying lipogenesis in vivo (12). After TNF administration, animals are kept fasting to control for the anorectic effects of TNF (31) as diet influences fatty acid synthesis (14–21). Rats were killed either 90 min or 16 hr after injection to assess the mechanisms of the early and late response to TNF. Five rats were used for each condition with figures presenting data from one representative experiment of control and TNF-treated animals at a single time point. The text presents a summary of multiple repeats of the experiments. All assays were performed at concentrations that had been shown to be linear with respect to time. Data are presented as mean ± SEM. Significance was determined using the Student's $t$-test.

### Fatty acid synthetase activity

Fatty acid synthetase was measured by the method of Hsu, Butterworth, and Porter (32). Liver was homogenized

in a solution composed of 7 mM $KHCO_3$, 85 mM $K_2HPO_4$, 9 mM $KH_2PO_4$ (pH 8.0), 1 mM DTT, and BSA (1 mg/ml) using a Polytron. After filtering through gauze, the homogenate was sequentially centrifuged at 1,000 *g* for 30 min and 100,000 *g* for 45 min. The supernatant was used for the reaction. Fatty acid synthetase activity was assayed in 100 mM potassium phosphate buffer (pH 6.8), 5 mM 2-mercaptoethanol, 3 mM EDTA, 50 μM malonyl-CoA, 12.5 μM [1-$^{14}$C]acetyl-CoA (0.0216 μCi/ml), 0.3 mM NADPH. After incubation for 6 min at 37°C, the reaction was stopped by addition of trichloroacetic acid. An equal volume of absolute ethanol was added and the newly synthesized fatty acids were extracted four times with 5 ml of petroleum ether. The solvent was evaporated and the fatty acid radioactivity was counted in Ultrafluor scintillation fluid. One unit of enzyme activity is defined as that required to incorporate 1 μmol of [1-$^{14}$C]acetyl-CoA into fatty acid per min at 37°C.

### Acetyl-CoA carboxylase

The level of acetyl-CoA carboxylase was estimated by assaying its activity after maximal activation with citrate (33). In brief, liver was homogenized in two volumes of 50 mM Tris, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5, using a Polytron. The homogenate was sequentially centrifuged and re-extracted as previously described (33). The supernatant from a 105,000 *g* centrifugation was used. Acetyl-CoA carboxylase was maximally activated by incubation in 50 mM Tris, 20 mM citrate, 20 mM $MgCl_2$, 1 mM DTT, 0.5 mg/ml BSA, pH 7.5, for 30 min at 37°C. Acetyl-CoA carboxylase activity was then immediately assayed by dilution into 100 mM Tris (pH 7.5), 1 mM DTT, 0.2 mM acetyl-CoA, 20 mM $NaH^{14}CO_3$ (0.25 μCi/μmol), 5 mM ATP, 20 mM citrate, 20 mM $MgCl_2$, and BSA (0.5 mg/ml). After incubation for 5 min at 37°C the reaction was stopped by acidification and the samples were dried. The newly synthesized malonyl-CoA was assayed by dissolving the residue in water and counting in Ultrafluor scintillation fluid.

The phosphorylation state of acetyl-CoA carboxylase was estimated by assaying the ratio of activity at 0 and 2 mM citrate using the method of Jamil and Madsen (26, 27). A lobe of liver was frozen in situ with a Wollenberger clamp cooled in liquid $N_2$. The liver was powdered while frozen, then rapidly homogenized in 1.5 volumes of 50 mM Tris (pH 7.5), 2 mM EDTA, 1 mM EGTA, 100 mM NaF, 1 mM DTT, BSA (1 mg/ml), 0.39 mM PMSF, 1 mM benzamidine, 0.01 mM TPCK, leupeptin (0.4 μg/ml), antipain (0.4 μg/ml), and pepstatin (0.4 μg/ml) to inhibit dephosphorylation and proteolytic activation. The homogenate was centrifuged at 27,000 *g* for 20 min at 0°C. The supernatant was added to a reaction mixture including 50 mM Tris (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, BSA (0.75 mg/ml), 20 mM $NaH\ ^{14}CO_3$ (26,000 cpm/μmol), 4 mM ATP, and 0.5 mM acetyl-CoA in the absence or presence of 2 mM citrate in a final volume of 0.9 ml. After incubation for 4 min at 37°C, the reaction was stopped by acidification and the samples were dried. The residue was dissolved in water and counted in MP-Ready-Solv scintillation fluid. One unit of enzyme activity is defined as that required to form 1 μmol of malonyl-CoA per min at 37°C.

### Citrate levels

A lobe of liver was frozen in situ using a Wollenberger clamp cooled in liquid nitrogen. Liver was powdered with a mortar and pestle while still frozen. Citrate was then extracted in 3.5 volumes of 8% $HClO_4$ in 40% ethanol as described by Williamson and Corkey (34). The homogenate was centrifuged at 25,000 *g* and the pellet was re-extracted. Both supernatants were pooled and centrifuged again at 25,000 *g* for 10 min. The supernatant was adjusted to pH 6 by addition of 3 M $K_2CO_3$ containing 0.5 M triethanolamine base. Citrate was measured by incubating the extract in 50 mM triethanolamine, 10 mM $MgSO_4$, 5 mM EDTA, pH 7.4, buffer supplemented with 10 μg/ml NADH and 25 μg/ml malate dehydrogenase (34). Absorbance was recorded at 340 nm for 5 min until stabilized, after which citrate lyase (50 μg/ml) was added to start the reaction. The change in absorbance for each sample was measured every 30 sec for 10 min using a Beckman DU-50 spectrophotometer with kinetics modules, and compared to a standard curve of citrate.

### Plasma free fatty acid levels

Blood for free fatty acids was placed in iced, heparinized glass tubes within 30 sec of sampling, centrifuged at 1,000 *g* at 4°C, and plasma was separated. The free fatty acid content of plasma was determined by a modification of the extraction method of Ko and Royer (35), as previously described (36). After acidification and extraction of the plasma with heptane–isopropyl alcohol containing pentadecanoic acid as an internal standard, the heptane was evaporated and 0.05 ml of 10% $BCl_3$/methanol was added to convert the fatty acids to their respective methyl esters. After the reaction had proceeded for 10 min, two layers were present: an oily film adhering to the walls of the reaction vial and the $BCl_3$/methanol layer. The latter solution, after transfer to another vial, was evaporated with nitrogen. Methylene chloride (0.025 ml) was added to the vial and a sample was injected into a gas chromatograph [10' × ¼" ID glass column containing 10% SP-2330 (Supelco, Inc., Bellefonte, PA)] with an FID detector and an oven temperature of 190°C. The amounts of the individual fatty acids were then determined by comparison with the pentadecanoic acid internal stan-

dard and integrated with a 5880 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA). Eight analyses of one sample gave a coefficient of variation of 1.9% in our laboratory. The values for individual fatty acids were summed and expressed as nmol total free fatty acids per ml of plasma.

### Fatty acyl-CoA levels

Livers were frozen in situ using a Wollenberger clamp cooled in liquid nitrogen; the sample was powered with a mortar and pestle, then extracted with 9 volumes of 5% $HClO_4$ containing 2 mM EDTA (37). The homogenate was centrifuged for 10 min at 1000 *g*. The pellet containing fatty acyl-CoA was suspended in 2 ml $H_2O$ and 0.8 ml of 0.85 N KOH containing 20 mM 2-mercaptoethanol and incubated for 20 min at room temperature to cleave fatty acid from coenzyme. The extract was acidified with 60% $HClO_4$, incubated for 5 min at 4°C, and then centrifuged for 10 min at 1000 *g*. The supernatant containing the released coezyme A was adjusted to pH 6.0–6.5 by addition of 0.2 ml saturated $KH_2PO_4$ followed by titration with 10 N KOH. The sample was then centrifuged to remove potassium perchlorate. The released coenzyme A was assayed fluormetrically (38). Samples were added to 100 mM potassium phosphate buffer (pH 7.0), 100 $\mu$M NAD, 100 $\mu$M $\alpha$-ketoglutarate, 2 mM cysteine, 2 mM $MgCl_2$, and 1 mM EDTA. Fluorescence (excitation 345 nm, emission 455 nm) was measured before and after addition of 6.0 units of $\alpha$-ketoglutarate dehydrogenase and compared to a standard curve of coenzyme A.

## RESULTS

### Fatty acid synthetase activities

We first measured the activity of fatty acid synthetase in livers from rats that had been treated with TNF for 90 min or 16 hr prior to study. The data in **Fig. 1** are expressed in units of fatty acid synthetase activity per liver because the activity in the whole liver determines total fatty acid synthesis and, indirectly, secretion into the circulation. These values can be more directly compared to total lipogenesis in vivo. In addition, we have shown that liver size increases 16 hr after TNF treatment (12). Fatty acid synthetase activity in the liver was the same in controls and animals that had been treated with TNF for 90 min prior to study (Fig. 1, left panel), the time at which the increase in de novo hepatic fatty acid synthesis peaks as measured by incorporation of $^3H_2O$ into lipid in vivo (12). Also, no difference in the activity of fatty acid synthetase was seen when activity was expressed per mg protein in the supernatant of the liver homogenate (control: 1.11 ± 0.09 mU/mg, TNF: 1.00 ± 0.05 mU/mg).

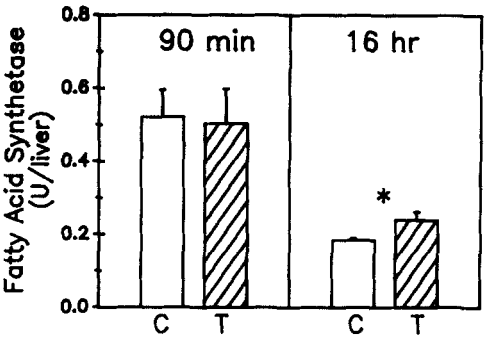


**Fig. 1.** Effect of TNF on fatty acid synthetase. Rats were injected with saline (C) or 25 $\mu$g TNF (T) intravenously, then kept fasting. At the indicated times, livers were excised, homogenized, and assayed for fatty acid synthetase activity as described in Experimental Procedures (32). Values are the means ± SEM for five rats for each condition; *, $P < 0.05$.

The total activity of fatty acid synthetase was 30% higher in the livers of animals that had been treated with TNF 16 hr earlier compared to controls (Fig. 1, right panel). In three similar experiments the activity of fatty acid synthetase per liver in animals treated with TNF was 33 ± 2% higher than that found in control animals. However, when expressed per mg protein, there was no significant increase in fatty acid synthetase activity (control: 0.68 ± 0.02 mU/mg, TNF: 0.75 ± 0.05 mU/mg), indicating that fatty acid synthetase activity was elevated in proportion to the increase in liver size and total liver protein that is seen 16 hr after TNF treatment (12).

### Acetyl-CoA carboxylase activity

The level of acetyl-CoA carboxylase in liver is estimated by measuring acetyl-CoA carboxylase activity after maximal stimulation by citrate (33). We first measured acetyl-CoA carboxylase after maximal activation using 20 mM citrate. As seen with fatty acid synthetase, livers from animals that had been exposed to TNF in vivo 90 min prior to study showed no significant increase in maximally activated acetyl CoA carboxylase activity (**Fig. 2,** left panel). Likewise, no increase was seen when acetyl-CoA carboxylase activity was expressed per mg protein in the supernatant of the liver homogenate (control: 19.2 ± 1.1 mU/mg, TNF: 21.0 ± 1.6 mU/mg).

For longer term studies, animals were treated with saline or TNF and then fasted for 16 hr before being studied. As expected with fasting, maximally activated acetyl-CoA carboxylase in livers from control rats decreased (Fig. 2, right). However, the amount of maximally stimulated acetyl-CoA carboxylase activity was significantly higher ($P < 0.001$) in the animals that had been treated with TNF 16 hr previously compared to controls (Fig. 2, right). The activity of acetyl-CoA carboxylase was also higher when expressed per mg protein (control: 8.08 ± 0.45 mU/mg, TNF: 9.70 ± 0.48 mU/mg, $P < 0.05$). In several similar experiments, total acetyl-

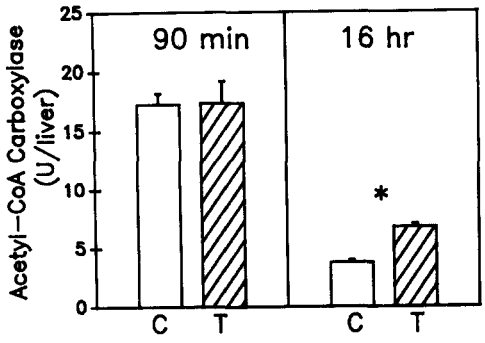


**Fig. 2.** Effect of TNF on acetyl-CoA carboxylase. Experimental design is the same as that in Fig. 1. At the indicated time, livers were excised, homogenized, and assayed for acetyl-CoA carboxylase activity under conditions of maximal citrate activation by the method of Inoue and Lowenstein (33) as described in Experimental Procedures; C, control; T, TNF; *, $P < 0.001$.

CoA carboxylase activity expressed per liver from animals that had been treated with TNF 16 hr earlier was 58% higher that found in controls, while activity expressed per mg protein increased by 38%.

We then determined whether activation of acetyl-CoA carboxylase by dephosphorylation could account for the acute stimulation of fatty acid synthesis by measuring the ratio of enzyme activity at 0 and 2 mM citrate, according to the method of Jamil and Madsen (26, 27). Their assay has several differences from the standard method of Inoue and Lowenstein (33) used above. First, the liver is rapidly frozen and extracted. Second, the homogenization buffer contains both NaF to inhibit dephosphorylation and protease inhibitors to inhibit proteolytic activation. Finally, the supernatant from a 27,000 *g* centrifugation is used directly in the reaction mixture. As can be seen in **Table 1,** the ratio of acetyl-CoA carboxylase activity at 0 and 2 mM citrate did not significantly change in liver extracts from animals treated with TNF 90 min before study. (An increase in the activity ratio would be consistent with activation.) Likewise, no significant change in the activity ratio was seen in the livers from animals that had been treated 16 hr earlier with TNF.

The functional phosphorylation state, calculated based on the nomogram published by Jamil and Madsen (26, 27), is also presented in Table 1. This method calculates the amount of phosphorylation only in the two critical sites for regulating enzyme activity and usually does not exceed 1.0. (A decrease in phosphorylation would be consistent with activation.) Similar to their observations (27), we calculated that the enzyme in fed animals is relatively dephosphorylated compared to control animals that were fasted for 16 hr (Table 1). However, we calculated no significant change in the functional phosphorylation state of acetyl-CoA carboxylase from livers of animals at either 90 min or 16 hr after treatment with TNF compared to that seen with control animals (Table 1).

### Hepatic levels of citrate

Because extensive previous data have indicated that the acute increase in hepatic fatty acid synthesis correlates best with changes in the level of modulators of acetyl-CoA carboxylase activity, rather than in levels of the enzyme itself (14, 15, 18), we measured the two key allosteric regulators of acetyl-CoA carboxylase in liver after exposure to TNF in vivo. The hepatic concentration of citrate was higher ($P < 0.05$) in liver 90 min after administration of TNF (**Fig. 3**). In three similar experiments TNF increased hepatic citrate concentrations by an average of 51% ($P < 0.001$).

As expected, the hepatic intracellular levels of citrate decreased after 16 hr in the fasted state in both control and TNF-treated animals (Fig. 3). However, citrate levels remained relatively elevated ($P < 0.05$) in animals that had been treated with TNF 16 hr prior to measurement when compared to control animals (Fig. 3). In three similar experiments citrate levels were 49% higher in animals that had been treated with TNF.

TABLE 1. Activity ratio and calculated phosphorylation state for acetyl-CoA carboxylase

| Treatment[a] | Time | Activity Ratio[b] (0/2.0 mM citrate) | Phosphorylation State[c] (mol P/mol enzyme) |
|---|---|---|---|
| Control | 90 min | 0.43 ± 0.01 | 0.11 ± 0.02 |
| | | N.S. | |
| TNF | 90 min | 0.43 ± 0.03 | 0.11 ± 0.04 |
| Control | 16 hr | 0.26 ± 0.01 | 0.38 ± 0.02 |
| | | N.S. | |
| TNF | 16 hr | 0.28 ± 0.004 | 0.34 ± 0.01 |

[a]N = five rats for each condition; value, mean ± SEM; N.S., not significant.
[b]The ratio of the activity of acetyl-CoA carboxylase measured in the absence of citrate to the activity measured in the presence of 2.0 mM citrate was determined by the method of Jamil and Madsen (26, 27) as described under Experimental Procedures.
[c]The phosphorylation state was calculated as described by Jamil and Madsen (26).

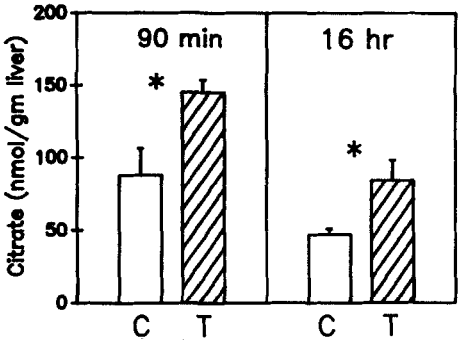


**Fig. 3.** Effect of TNF on hepatic citrate levels. Experimental design is the same as that in Fig. 1. At the indicated times, livers were quick-frozen and extracted, and citrate levels were assayed as described in Experimental Procedures (34); C, control; T, TNF; *, $P < 0.05$.

### Free fatty acids and fatty acyl-CoA

Because TNF and other cytokines appear to increase lipolysis in fat cells (8, 10), it was of interest to determine whether TNF increased circulating free fatty acid levels. As can be seen in **Fig. 4,** circulating free fatty acid levels were significantly higher (53%) in the plasma of rats 90 min after treatment with TNF.

After fasting for 16 hr, the plasma levels of free fatty acids increased as expected (Fig. 4). Although after a 16-hr treatment with TNF the absolute quantitative increase in free fatty acids was similar to that observed after 90 min, the increase was not significant in the face of the high levels seen in fasted controls (Fig. 4, right). There was no difference in the chain length or saturation of fatty acids circulating in the plasma from control or TNF-treated animals at 90 min or 16 hr (data not shown).

Fatty acyl-CoA molecules are allosteric inhibitors of acetyl-CoA carboxylase. If the increase in plasma free fatty acids resulted in a similar increase in hepatic fatty acyl-CoA, this could result in a decrease in acetyl-CoA carboxylase activity which might offset the effects of citrate. We found no significant change in the concentration of fatty acyl-CoA in livers from animals 90 min after treatment with TNF compared to livers from control animals (**Fig. 5**). In three similar experiments fatty acyl-CoA levels in livers of TNF-treated animals averaged 90% of controls. As expected, fatty acyl-CoA levels rose in control rats after 16 hr of fasting. However, the levels of fatty acyl-CoA were lower ($P < 0.005$) in the livers from TNF-treated animals. In three similar experiments fatty acyl-CoA levels in livers from TNF-treated animals averaged 65% of that in control animals.

## DISCUSSION

Previously we reported that administration of TNF to intact animals stimulates the de novo synthesis of fatty acids in the liver as well as increasing serum triglyceride levels (12). TNF treatment produced a rapid rise (by 90 min after administration) in serum triglycerides which remained significantly elevated for 17 hr. In parallel, de novo synthesis of fatty acid in the liver, as measured by the incorporation of $^3H_2O$ into lipid in vivo, was increased by 60% when measured 1–2 hr after TNF administration. Although hepatic lipogenesis decreased in both control and TNF-treated animals over the next 17 hr, lipogenesis in TNF-treated animals remained elevated compared to control animals at all times. The effects of TNF persist for over 17 hr despite the fact that TNF is rapidly cleared from the circulation with a half-life of 6–11 min and is degraded (39–41). (The increase in hepatic lipogenesis could also be demonstrated in vitro by measuring acetate incorporation into lipid in liver slices from rats treated with TNF in vivo (12).) In addition, we found in vivo that newly synthesized fatty acid, presumably of liver origin, rapidly appeared in serum (12).

In this report we have delineated several mechanisms by which TNF may increase hepatic fatty acid synthesis. The actions of TNF are strikingly similar to the mechanisms by which hepatic fatty acid synthesis is increased when fasted animals are re-fed a fat-free diet (14–18). Changes in the levels of rate-limiting enzymes of fatty acid synthesis as conventionally measured (under conditions of maximal stimulation) can only be detected many hours after treatment with TNF. Fatty acid synthetase activity was 33% higher in the livers of animals that had been treated with TNF 16 hr previously when compared to control animals. When the levels of acetyl-CoA carboxylase are estimated by measuring its activity after maximal activation by citrate, total activity of acetyl-CoA carboxylase was 58% higher in livers of animals that had been treated with TNF 16 hr earlier compared to control animals. This correlates well with the 68% increase in total hepatic fatty acid synthesis that occurs in vivo (12).

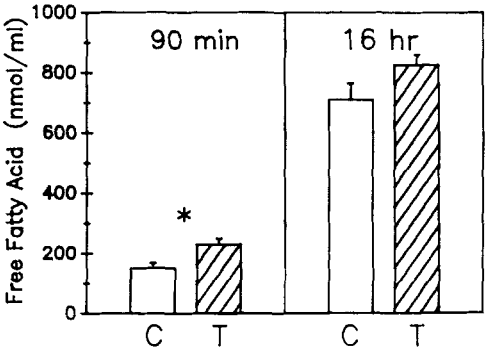


**Fig. 4.** Effect of TNF on plasma free fatty acid levels. The experimental design is the same as that in Fig. 1. At the indicated time, blood was drawn into heparinized tubes and the plasma was assayed for free fatty acids as described in Experimental Procedures; C, control; T, TNF; *, $P < 0.02$.

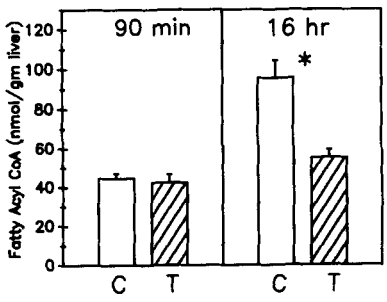


**Fig. 5.** Effect of TNF on hepatic fatty acyl CoA levels. Experimental design is the same as that in Fig. 1. At the indicated time, livers were quick-frozen and extracted. Fatty acyl CoA levels were assayed as described in Experimental Procedures (38); C, control; T, TNF; *, $P < 0.005$.

When acetyl-CoA carboxylase activity was expressed per mg protein, activity was 38% higher in TNF-treated animals; this correlates well with the 35% increase seen when hepatic de novo fatty acid synthesis was expressed per gram liver. Further study will be required to determine whether the long term effects of TNF on acetyl-CoA carboxylase are mediated by changes in the synthesis or degradation of the enzyme.

In contrast, there was no increase in either fatty acid synthetase or maximal acetyl-CoA carboxylase activity when measured 90 min after the administration of TNF. Again, this is similar to studies of dietary manipulation where the increase in lipogenesis precedes the rise in enzyme levels (14–18). Instead, early changes in lipogenesis induced by refeeding were due to allosteric regulation or covalent modification of acetyl-CoA carboxylase. The acute regulation of acetyl-CoA carboxylase activity is felt to be mediated by a variety of intracellular regulators, including citrate, which activates the enzyme, and fatty acyl-CoA molecules, which inhibit the enzyme (14, 15, 18–21). The active form of acetyl-CoA carboxylase is a filamentous polymer. Citrate promotes the polymerization of acetyl-CoA carboxylase whereas fatty acyl-CoA molecules cause its disaggregation (14, 18, 19, 21).

We found that TNF administration led to an acute rise in the hepatic intracellular concentrations of citrate by 51% after 90 min of exposure. This correlates well with the 61% increase in de novo fatty acid synthesis per gram liver seen in vivo after similar exposure to TNF (12). It should be noted that the concentrations of citrate in both control and TNF-treated animals are below the $K_m$ for activation of acetyl-CoA carboxylase (0.2 to 2.4 mM citrate). Therefore, small changes in citrate levels would be able to activate the enzyme, although these calculations (14, 15, 26, 27, 34) do not take into account intracellular water space or the distribution of citrate between cytosol and mitochondria.

Because of the suggestion that TNF increases lipolysis in cultured fat cells (8, 10), it might be anticipated that circulating free fatty acid levels would rise and possibly induce an increase in intracellular fatty acyl-CoA molecules which might counteract the effect of citrate. While we did demonstrate that TNF induces an acute increase in circulating free fatty acids, the hepatic intracellular levels of fatty acyl-CoA were not higher in animals 90 min after TNF treatment. After 17 hr, during which time the rats were kept fasting, circulating free fatty acids rose in both control and TNF-treated animals. Although there was a corresponding increase in hepatic fatty acyl-CoA levels in control animals, fatty acyl-CoA levels did not rise in TNF-treated animals. The lower levels of fatty acyl-CoA after 17 hr in the TNF-treated animals could contribute to the increased synthesis of fatty acid by decreasing feedback inhibition of acetyl-CoA carboxylase. Thus, at both early and late time points, hepatic fatty acyl-CoA levels are relatively lower than what might be anticipated from the plasma free fatty acid levels in TNF-treated animals. While it is not possible to precisely determine metabolic fluxes by measuring static levels, these data are consistent with an increased utilization of free fatty acid in TNF-treated animals. Thus, re-esterification of fatty acid most likely contributes to the hypertriglyceridemia seen with TNF treatment; this is not surprising since there is a 2.2-fold acute increase in triglycerides 90 min after TNF administration and only a 61% increase in de novo synthesis of fatty acid.

Another potential mechanism for regulation of acetyl-CoA carboxylase is a change in its phosphorylation state, with dephosphorylation activating the enzyme. However, we did not find a change in the functional phosphorylation state of acetyl-CoA carboxylase after administration of TNF as detected by the ratio of its activities at 0 and 2 mM citrate. Thus, dephosphorylation of acetyl-CoA carboxylase does not appear to play a role in the increase in fatty acid synthesis induced by TNF.

The data on the acute effects of TNF should be contrasted with those on regulation of lipogenesis by glucagon which acutely decreases fatty acid synthesis. Glucagon treatment of hepatocytes produces a rapid decrease in intracellular levels of citrate, the allosteric activator of acetyl-CoA carboxylase (42). At the same time, glucagon treatment results in phosphorylation of acetyl-CoA carboxylase (43, 44), which inhibits the enzyme by increasing the $K_m$ for citrate (21, 22). In contrast, at early time points when TNF increases fatty acid synthesis, TNF induces an increase in the hepatic concentrations of citrate, an allosteric activator of acetyl-CoA carboxylase, while inducing no change in the phosphorylation state, as assessed by measuring the activity ratio of the enzyme. In this light, it is of interest that glucagon levels have been reported to be increased after TNF administration (45) and that TNF is able to stimulate hepatic fatty acid synthesis in insulin-deficient streptozotocin-induced diabetic rats, under conditions where circulating insulin levels do

not rise (C. Grunfeld et al., unpublished results). Thus, the effects of TNF cannot simply be explained by changes in the levels of these counter-regulatory hormones.

Our data (this paper and ref. 12) and that of others (46, 47) imply a central role for the liver in mediating certain systemic effects of TNF. Several laboratories have demonstrated that TNF stimulates the production of acute phase proteins by cultured liver cells, while the synthesis of other hepatic proteins is simultaneously decreased (46, 47). We have demonstrated that TNF increases hepatic lipogenesis in vivo (12) and now demonstrate that TNF could produce these changes by acutely increasing levels of citrate, an intracellular regulator of acetyl-CoA carboxylase, and in the long term by increasing the levels of lipogenic enzymes. When labeled TNF is injected in vivo, a significant percentage localizes to the liver (39) and preliminary studies indicate the presence of TNF receptors on mouse liver membranes (48). Whether all of the effects of TNF on hepatic function seen in vivo result from the direct action of TNF on hepatocytes remains to be demonstrated. Other cytokines, including interleukin-1 and the interferons, have been reported to have the same catabolic effects on fat cells as TNF (10, 49–51). It is not yet known whether other cytokines stimulate hepatic lipogenesis in vivo or whether the effects of TNF on lipogenesis are mediated by induction of another cytokine or messenger (52, 53).

In summary, we present evidence for multiple mechanisms by which TNF stimulates lipogenesis in vivo. A rapid increase in hepatic citrate concentration occurs early after TNF administration. Subsequently, the levels of acetyl-CoA carboxylase and fatty acid synthetase are also increased compared to controls, while fatty acyl-CoA levels decrease. Further work will be required to determine whether TNF increases the synthesis or decreases the degradation of acetyl-CoA carboxylase under these conditions and how TNF increases citrate levels.

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