Interleukin-6, but not tumour necrosis factor- α , increases lipogenesis in rat hepatocyte primary cultures

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The Kupffer-cell products interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α) have been shown to stimulate hepatic lipogenesis *in vivo*. Studies were performed to define the direct effects of these cytokines on lipogenesis in primary-culture rat hepatocytes. Hepatocytes were cultured in the presence of IL-6 or TNF- α for periods of 24–72 h. IL-6 increased hepatocyte protein content per μ g of DNA. IL-6 also caused a dose- and time-dependent induction of hepatocyte capacity for incorporation of [2- 14 C]pyruvate into fatty acids (56% increase by 12.5 ng/ml IL-6 after 72 h of cytokine exposure). This increase in cellular lipogenic capacity was confirmed by using 3 H $_2$ O in-

corporation into fatty acids as tracer. TNF- α did not increase hepatocyte lipogenesis. In contrast with studies *in vivo*, neither IL-6 nor TNF- α had any acute (2 h of exposure) effects on rates of lipogenesis. Both IL-6 and TNF- α are known to increase macrophage prostaglandin synthesis acutely. The prostaglandin E agonist misoprostol free acid (0.1 μ M) acutely increased hepatocyte lipogenic rates by 14%. Thus, IL-6 can directly induce hepatocyte lipogenic capacity, and E-series prostaglandins can antagonize the acute inhibition of lipogenesis by glucagon. The observations provide further evidence for the role of Kupffer-cell products in the regulation of hepatocyte metabolism.

INTRODUCTION

Infectious and inflammatory diseases are associated with diverse changes in fuel homeostasis in man (Spitzer et al., 1988; Goldstein and Elwyn, 1989; Lowry, 1992). Glucose disposition and production may be altered, leading to hypo- or hyperglycaemia (Wolfe and Burke, 1978; Spitzer et al., 1988). Additionally, hepatic lipid metabolism is shunted away from ketogenesis and towards triacylglycerol synthesis, with hepatic lipogenesis also enhanced (Larsson-Berkstrom et al., 1990; Spitzer et al., 1988; Wolfe et al., 1985). The etiology of these metabolic changes is multifactorial, but changes in nutritional and hormonal status do not adequately explain the metabolic state of individuals under conditions of Gram-negative sepsis or chronic inflammation (Goldstein and Elwyn, 1989; Spitzer et al., 1988).

Kupffer cells are resident macrophages distributed along the hepatic sinusoid (Wardle, 1987). Kupffer cells generate a spectrum of bioactive molecules, including eicosanoids, cytokines and nitric oxide (NO), in response to macrophage stimuli (Decker, 1990). Hepatic endothelial cells may also contribute to bioactive-factor generation (Kuiper et al., 1988; Feder et al., 1993). These Kupffer- and endothelial-cell products can then function as paracrine factors to modulate hepatocyte metabolism and function. PGE $_2$ and NO have been shown to modulate hepatocyte glycogenolysis and the hepatocyte response to glucagon (Okumura et al., 1988; Brass and Garrity, 1990; Brass and Vetter, 1993). Similarly, interleukin-6 (IL-6) has been shown to be a major stimulus for the hepatic acute-phase response (Decker, 1990; Andus et al., 1991; Gaudie et al. 1992). Direct modulation of hepatocyte metabolic regulation by cytokines has been less well established.

Studies *in vivo* have demonstrated that the administration of tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1), interferon- γ or IL-6 can reproduce all or part of the altered hepatic lipid metabolism observed after lipopolysaccharide administration (Feingold et al., 1989; Grunfeld et al., 1990a, 1991a).

However, interpretation of these studies as reflecting hepatocyte-cytokine interactions are limited by the known changes in circulating hormone concentrations following cytokine administration (Evans et al., 1989), and the ability of each of the cytokines to modify Kupffer-cell production of other bioactive molecules (Peters et al., 1990b; Kawada et al., 1991). Studies *in vitro* have failed to demonstrate an acute (less than 2 h) effect of TNF- α or IL-1 on hepatocyte metabolism (Rofe et al., 1987), but 48 h of treatment with TNF- α has been shown to increase triacylglycerol production by a hepatoma cell line (Grunfeld et al., 1991b). Importantly, cytokines can stimulate macrophage prostaglandin production (Peters et al., 1990a; Ruhl et al., 1992), and E-series prostaglandins can acutely modulate hepatocyte metabolism (Brass and Garrity, 1990). To test the hypothesis that cytokines can act directly on hepatocytes to stimulate lipogenesis, fatty acid synthesis was studied in rat hepatocytes following chronic exposure to TNF- α or IL-6, and acute exposure to the cytokines or the prostaglandin E (PGE) analogue misoprostol free acid. The results demonstrate that chronic exposure to IL-6, but not TNF- α , resulted in an induction of hepatic lipogenesis, whereas misoprostol free acid, but neither cytokine, acutely stimulated fatty acid synthesis.

EXPERIMENTAL

Animal and cell isolations

Male fed Sprague–Dawley rats (380 $\pm$ 30 g, n = 14) were used in all experiments. Hepatocytes for primary culture were initially isolated by a modification of the method of Berry and Friend (1969) as previously detailed (Brass and Ruff, 1991). To permit use of the cells in primary culture experiments, additional modifications were made (Ben-Ze'ev et al., 1988), including the use of autoclaved or ethanol-washed glassware and instruments, and the passage of all solutions through a 0.2 μ m filter before use. All subsequent steps were done using sterile technique in a laminar-flow hood. Williams' Medium E (WME; Gibco, Grand

Abbreviations used: IL, interleukin; TNF- α , tumour necrosis factor- α ; PGE, prostaglandin E; WME, Williams' Medium E.

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Island, NY, U.S.A.) was used as the wash buffer. Hepatocytes for primary culture were separated from non-parenchymal cells by five 1 min centrifugations at 50 *g* in WME. Cell preparations ($n = 14$) used for culture had an average viability of $93 \pm 1\%$, based on Trypan Blue exclusion, had a wet weight of 17.0 ± 1.3 mg/ 10^6 cells, and contained 1.04 ± 0.10 mg of protein and 98 ± 11 μ g of DNA per 10^6 cells. Cells (1×10^6) were then plated in 35 mm-diameter plastic plates pre-coated with rat tail collagen (Sigma Chemical Co., St. Louis, MO, U.S.A.). The time of initial plating is referred to as 0 h of culture. The plating conditions included (total volume 1.5 ml) WME supplemented with 100 nM dexamethasone, 100 nM insulin, 50 units/ml penicillin, 50 μ g/ml streptomycin and 5% fetal-bovine serum, and the cells were maintained at 37°C under humidified air/CO₂ (19:1). After 4 h the medium was aspirated and replaced with identical medium, except that the insulin concentration was decreased to 10 nM and fetal-bovine serum was excluded, and the plates were maintained under these condition from 4 to 24 h of culture. At 24 h the medium was again changed and replaced by medium further supplemented as defined in individual experiments. In all cases, medium was subsequently changed at 24 h intervals during the course of culture. Plates were inspected at 24 h intervals by phase-contrast microscopy, and plates with poor cell adherence or contamination (yeast or bacterial) were discarded. Consistent with similar methods from other laboratories (Bonney et al., 1974; Bayad et al., 1991), this procedure yielded a plating efficiency of approx. 75%, and cell viability was maintained for a minimum of 96 h, based on lactate dehydrogenase leakage and metabolic integrity (results not shown).

Measurement of lipogenesis

Lipogenesis was quantified as incorporation of radioactivity from [2-¹⁴C]pyruvate or ³H₂O into hepatocyte fatty acids. At the culture time-point of interest, the medium was aspirated and replaced with 1.2 ml of WME supplemented with 50 units/ml penicillin and 50 μ g/ml streptomycin and allowed to equilibrate for 30 min at 37°C under humidified air/CO₂ (19:1). At the end of the preincubation period, 0.3 ml of substrate solution (5% defatted BSA, 50 mM pyruvate, 135 mM NaCl and either [2-¹⁴C]pyruvate (final sp. radioactivity approx. 30 d.p.m./nmol), or ³H₂O (final sp. radioactivity approx. 20 d.p.m./nmol) was added, as well as any compounds being evaluated as short-term regulators of lipogenesis. Plates were then incubated for 1 or 2 h at 37°C under humidified air/CO₂ (19:1).

At the end of the incubation, the medium was aspirated and the cells were rapidly washed with and harvested in cold PBS. A portion of the cells (0.4 ml) was mixed with 1.0 ml of 0.3 M NaOH in 90% methanol. This mixture was then saponified for 2 h at 65°C, and acidified with 12 M HCl (36 μ l/ml of sample); the lipids were extracted twice into an equal volume of iso-octane and the organic phases pooled. The iso-octane was then evaporated under air. The residue was resuspended in 600 μ l of iso-octane/ethyl acetate (3:1, v/v) for further fractionation. Unesterified fatty acids were isolated by mini-column chromatography on Silica Gel 60 (EM Science, Gibbstown, NJ, U.S.A.) based on the method of Ingalls et al. (1993). A slurry (2 ml) of iso-octane/Silica Gel 60 (1:1, v/v) was loaded on to a Pasteur pipette mini-column and washed with 2×1.0 ml of iso-octane/ethyl acetate (3:1, v/v). The hepatocyte extract was then loaded on to the column and washed with 2×1.0 ml of iso-octane/ethyl acetate (3:1, v/v).

Unesterified fatty acids were then eluted with 5 ml of iso-octane/ethyl acetate/acetic acid (75:25:2, by vol.), and the eluent

was collected directly into glass scintillation vials. Preliminary studies using radiolabelled standards demonstrated greater than 90% recovery of palmitic acid in the collected fractions, with no contamination from water or pyruvate (experimental blanks were routinely less than 100 d.p.m. per sample, and were specifically quantified for each set of studies), or from cholesterol (results not shown). Radioactivity in the fatty acid fractions was quantified by addition of BioSafe II Scintillation Cocktail (Research Products International, Mt. Prospect, IL, U.S.A.) and scintillation counting in a Beckman LS 5000 CE instrument with quench correction.

Other assays

Protein content was determined by the method of Bradford (1976) with BSA as standard. DNA content was quantified by the method of Labarca and Paigen (1980), by using a bisbenzimidazole dye concentration of 10 ng/ml and a Turner 110 filter fluorimeter.

Materials

TNF- α (human recombinant) and IL-6 (human recombinant) were purchased from Promega Co. (Madison, WI, U.S.A.). ³H₂O and [2-¹⁴C]pyruvate were purchased from American Radio-labelled Chemicals (St. Louis, MO, U.S.A.). Collagenase (Type II) from Worthington Biochemicals (Fairchild, NJ, U.S.A.) was used in hepatocyte isolations. Insulin was purchased from Sigma. Glucagon was a gift from Lilly Laboratories (Indianapolis, IN, U.S.A.) and misoprostol free acid was a gift from G. D. Searle (Skokie, IL, U.S.A.). All other chemicals were of reagent grade.

Data analysis

Data are presented as means $\pm$ S.E.M., with n referring to the numbers of experiments using distinct hepatocyte preparations. For each hepatocyte preparation, observations were routinely made on duplicate culture plates with the average value used as the best-estimate value for that experiment. Comparisons were made by using Student's paired t test based on simultaneously conducted studies (same hepatocyte preparation differing only in the variable of interest), with $P < 0.05$ considered significant.

RESULTS

The effects of IL-6 and TNF- α on lipogenesis were evaluated in primary rat hepatocyte cultures. Treatment of hepatocytes with cytokines was initiated at 24 h of culture, and lipogenesis was assessed after 72 h of culture (48 h of cytokine exposure) in the absence of hormones or cytokines with 10 mM pyruvate as substrate to approximate to non-substrate-limited lipogenic rates (Agius and Alberti, 1985; Carmona and Freedland, 1989). Insulin (10 nM) was included throughout the 48 h of cytokine exposure. This insulin concentration was selected as a mid-range in the insulin-lipogenic expression dose-response curve (6.7 ± 2.0 , 8.2 ± 1.8 and 11.6 ± 2.1 nmol of pyruvate/h per mg of protein for 1.0, 10.0 and 100 nM insulin respectively; $n = 4$). Additionally, under these conditions, lipogenic capacity could be decreased by inclusion of 250 nM glucagon or increased by changing to media with a higher insulin concentration (results not shown). IL-6 (1.25 ng/ml) increased incorporation of [2-¹⁴C]pyruvate into hepatocyte fatty acids per mg of cellular protein by 14% (Table 1). However, 12.5 ng/ml IL-6 was without significant effect on the rate of lipogenesis per mg of cellular protein. IL-6 is known to induce hepatocyte protein synthesis (Andus et al., 1991), and

Table 1 Effect of IL-6 on lipogenesis rate

Rat hepatocytes were placed in primary culture as described in the text. From 24 to 72 h of culture, plates contained IL-6 at the concentrations indicated. After 72 h in culture, lipogenesis was measured with [2-¹⁴C]pyruvate as tracer. Cells were harvested for quantification of lipogenesis rates, protein content and DNA content. Control rates of lipogenesis were 18.1 ± 3.3 nmol of pyruvate/h per mg of protein ($n = 11$) or 1.19 ± 0.18 nmol of pyruvate/h per μg of DNA ($n = 11$), with rates in the table normalized to control = 100 to permit assessment of IL-6 effects despite variability between preparations in the absolute control rates of lipogenesis. Values are means $\pm$ S.E.M. with n in parentheses; * $P < 0.05$ versus control.

IL-6 (ng/ml)	Rate of lipogenesis per mg of protein (% of control)	Protein (mg of protein/ μg of DNA)	Rate of lipogenesis per μg of DNA (% of control)
0	100	0.084 ± 0.019 (12)	100
0.0125	110 ± 6 (4)	0.067 ± 0.015 (4)	121 ± 9 (4)
0.125	109 ± 9 (6)	0.064 ± 0.013 (6)	110 ± 8 (6)
0.50	126 ± 27 (5)	0.078 ± 0.015 (5)	115 ± 16 (5)
1.25	114 ± 6 (7)*	0.073 ± 0.012 (8)	118 ± 10 (7)*
12.5	113 ± 14 (9)	0.090 ± 0.011 (11)*	122 ± 11 (9)*

Figure 1 Time dependence of IL-6 stimulation of lipogenesis

Beginning at 24 h of culture, plates were maintained in the absence or presence of 12.5 ng/ml IL-6. At times indicated, rates of lipogenesis per μg of DNA were quantified by using [2-¹⁴C]pyruvate (●) or ³H₂O (○) as tracer, and normalized to rates from control plates = 100% (control rates 1.17 ± 0.19 and 2.01 ± 0.13 nmol of H₂O/h per μg of DNA at 72 and 96 h respectively; see the text for absolute lipogenesis rates with [2-¹⁴C]pyruvate). Values are means $\pm$ S.E.M. with n in parentheses; * $P < 0.05$ versus absence of IL-6.

12.5 ng/ml IL-6 increased protein content per μg of DNA in the rat primary culture hepatocyte system (Table 1). Therefore, to approximate better to lipogenic capacity per hepatocyte, rates of lipogenesis were also expressed per μg of DNA (Table 1). IL-6 treatment for 48 h was associated with a dose-dependent increase in lipogenic capacity per μg of DNA, with a 22% induction observed with 12.5 ng/ml IL-6. In contrast, TNF- α at concentrations up to 100 ng/ml did not increase rates of lipogenesis, but 100 ng/ml caused a 30% decrease (1.7 ± 0.4 , 1.5 ± 0.1 and 1.1 ± 0.3 nmol of pyruvate/h per mg of DNA for control, 10 and 100 ng/ml TNF- α respectively).

The induction of lipogenesis by IL-6 was time-dependent (Figure 1), with a 56% increase in [2-¹⁴C]pyruvate incorporation into fatty acids after 96 h of culture (72 h of treatment with 12.5 ng/ml IL-6). This increase in IL-6 efficacy was observed during a time period when expression of lipogenic capacity is stable under control culture conditions [0.86 ± 0.21 ($n = 6$), 1.19 ± 0.18 ($n = 11$) and 1.15 ± 0.34 ($n = 7$) nmol of pyruvate/h per μg of DNA at 48, 72 and 96 h of culture respectively].

As [2-¹⁴C]pyruvate incorporation into fatty acids may be modified by changes in the specific radioactivity of the cytosolic citrate pool independently of any changes in rates of lipogenesis, the effects of IL-6 were also assessed by using ³H₂O as a tracer to measure lipogenic rates (Figure 1). IL-6 stimulated ³H incorporation into hepatocyte fatty acids, with a time-dependent response similar to that observed with [2-¹⁴C]pyruvate as the radiolabel (Figure 1). Additionally, comparisons of the mean absolute incorporation rates for ³H₂O and [2-¹⁴C]pyruvate for control conditions after 72 h of culture yield a ³H/¹⁴C ratio of 0.98. This ratio is in good agreement with the predicted relationship of 0.87 nmol of ³H₂O/nmol of acetate incorporated into fatty acids from synthesis *de novo* (Jungas, 1968), and further validates the current estimates of lipogenic rates.

Studies *in vivo* with lipopolysaccharide and TNF- α indicate a rapid (less than 2 h) stimulation of lipogenesis (Grunfeld et al., 1988) distinct from the long-term induction observed *in vivo* and demonstrated for IL-6 above. Neither 10 ng/ml TNF- α nor 12.5 ng/ml IL-6 had any acute effect on the rate of lipogenesis in the primary culture hepatocyte system (Table 2). Glucagon, an inhibitor of lipogenesis (Lane et al., 1979; Beynen et al., 1979), was included in these incubations to provide a lipogenic rate that could be stimulated. Thus, insulin (100 nM) induced an acute 34% increase in lipogenic rate. Studies using the primary hepatocyte culture system or freshly isolated hepatocytes demonstrated no acute effects of IL-6 or TNF- α on lipogenic rates in the absence of glucagon (results not shown).

Table 2 Short-term regulation of lipogenesis

Hepatocytes were maintained in primary culture for 48 h as detailed in the text. Lipogenesis from ³H₂O was assessed during 2 h incubations in the presence of 250 nM glucagon and other additions as detailed below. Rates of lipogenesis were calculated as nmol of H₂O/h per mg of protein, and rates in the presence of IL-6 or TNF- α were normalized to simultaneously incubated plates in the absence of cytokines set equal to 100 (control rate = 20.6 ± 5.1 nmol of H₂O/h per mg of protein; $n = 8$). Values are means $\pm$ S.E.M.; * $P < 0.05$ versus control.

Conditions	n	Rate of lipogenesis (%)
Control	4	100
+ 12.5 ng/ml IL-6	3	96 ± 12
+ 10 ng/ml TNF- α	2	96 ± 13
+ 100 nM Insulin	2	134 ± 2
+ 0.1 μM Misoprostol free acid	4	$114 \pm 6^*$
+ 0.3 μM Misoprostol free acid	6	$106 \pm 2^*$
+ 1.0 μM Misoprostol free acid	6	99 ± 7

The acute stimulation of lipogenesis *in vivo* by IL-6 and TNF- α may be due to an indirect effect of the cytokines. Misoprostol free acid (0.1 μ M), a PGE agonist, acutely stimulated the rate of lipogenesis in the hepatocyte system (Table 2). Increasing the misoprostol concentration to 1 μ M resulted in loss of the compound's stimulatory effect on lipogenesis.

DISCUSSION

Cytokines are associated with diverse changes in metabolic regulation *in vivo* (Evans et al., 1989; Grunfeld and Palladino, 1990; Andus et al., 1991). The interrelationships between expression of specific cytokines and the paracrine action of those compounds make assessment of specific cellular actions of individual cytokines difficult based on observations *in vivo* alone. IL-6 has a major effect on hepatic homeostasis that is integral to man's response to inflammatory or infectious stimuli (Andus et al., 1988, 1991; Gaudie et al., 1992). The present studies demonstrate that IL-6 can also directly influence hepatic metabolism by increasing lipogenesis.

In vivo, administration of either IL-6 or TNF- α results in induction of hepatic lipogenesis (Adi et al., 1992; Grunfeld et al., 1990b; Feingold et al., 1989). This induction is associated with increased activity of the key lipogenic enzymes acetyl-CoA carboxylase and fatty acid synthetase (Grunfeld et al., 1988, 1990a). In the primary culture hepatocyte system, IL-6, but not TNF- α , induced cellular lipogenic capacity over a 48 h period. The time course of this induction *in vitro*, and its quantification following removal of the cytokines from the incubation (see the Experimental section), are consistent with induction of enzyme biosynthesis. TNF- α can induce Kupffer-cell IL-6 production (Decker, 1990; Ulich et al., 1991), and many of the metabolic effects of TNF- α can be blocked by anti-IL-6 antibodies (Starnes et al., 1990). Although direct effects of TNF- α on liver have been difficult to demonstrate (Rofe et al., 1987), IL-6 is able to increase hepatocyte protein synthesis and to enhance selectively the expression of proteins involved in the acute-phase response (Andus et al., 1988, 1991; Decker, 1990). Induction of hepatocyte lipogenesis by IL-6 is a direct action of the cytokine, presumably mediated through the hepatocyte IL-6 receptor (Pietzko et al., 1993). As the portal circulation may contain bacteria and other potential Kupffer-cell stimulants (Jacob et al., 1977; van Deventer et al., 1988), it is possible that Kupffer-cell IL-6 expression contributes to physiological, as well as pathophysiological, regulation of hepatic lipogenesis and plasma triacylglycerol concentrations.

In vivo, TNF- α and lipopolysaccharide also cause an acute stimulation of hepatic lipogenesis independent of increased enzyme expression (Grunfeld et al., 1990a). Short-term regulation of lipogenesis occurs primarily at the level of acetyl-CoA carboxylase secondary to phosphorylation/dephosphorylation in response to hormones, as well as allosteric regulation by citrate and long-chain acyl-CoAs (Lane et al., 1979). As TNF- α and IL-6 had no short-term effects on hepatocyte lipogenesis (Table 2), consistent with other acute metabolic studies (Rofe et al., 1987; Vaartjes et al., 1990), it is probable that the short-term actions of the cytokines are mediated by other bioactive molecules. The lack of acute effects of TNF- α on rates of lipogenesis were confirmed by using freshly isolated rat hepatocytes in suspension incubated under varied hormonal conditions, including the absence and presence of glucagon (results not shown).

E-series prostaglandins antagonize the effects of glucagon by inhibiting hormone-stimulated hepatocyte cyclic AMP production (Brass and Garrity, 1985; Brass et al., 1987; Garrity et

al., 1989), and thus were predicted to increase acutely glucagon-inhibited rates of lipogenesis. TNF- α and IL-6 are both known to increase macrophage prostaglandin production (Peters et al., 1990a; Ruhl et al., 1992). The PGE analogue misoprostol free acid, a PGE agonist, increased hepatocyte lipogenesis in the presence of 250 nM glucagon by 14% (Table 2). Similar data were obtained with the PGE agonist 16,16-dimethyl-PGE₂ (results not shown). PGE can stimulate hepatic adenylate cyclase at high concentrations (Garrity et al., 1983). This activation of adenylate cyclase through G_s would antagonize the G_i-mediated inhibition of adenylate cyclase observed at lower PGE concentrations (Garrity et al., 1989), and may be responsible for the loss of misoprostol efficacy observed. The regulation of hepatic fatty acid oxidation is interrelated with lipogenesis, as malonyl-CoA, the product of the acetyl-CoA carboxylase reaction, is an inhibitor of mitochondrial fatty acid oxidation (McGarry and Foster, 1980). PGE inhibits glucagon-stimulated fatty acid oxidation (Brass et al., 1987), consistent with activation of acetyl-CoA carboxylase and PGE stimulation of lipogenesis. NO, a Kupffer-cell product (Decker, 1990), antagonizes glucagon action in hepatocytes (Brass and Vetter, 1993) and would thus be predicted to stimulate lipogenesis rates. Although IL-6, PGE and NO all work by distinct mechanisms, the action of each of these compounds will be to increase hepatic rates of lipogenesis.

Bioactive molecules generated by Kupffer cells and hepatic endothelial cells in response to varied stimuli have the capacity to contribute to the regulation of hepatic metabolism. Altered long-term expression of hepatic metabolic enzymes by IL-6 may be critical in the transition to the chronic inflammatory state, and would thus act in concert with other key bioactive molecules.

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