Impairment by interleukin 1 β and tumour necrosis factor α of the glucagon-induced increase in phosphoenolpyruvate carboxykinase gene expression and gluconeogenesis in cultured rat hepatocytes

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The influence of the inflammatory mediators interleukin 1 β (IL1 β) and tumour necrosis factor α (TNF α) on the glucagon-induced expression of phosphoenolpyruvate carboxykinase (PCK) and on glucose formation via gluconeogenesis was investigated in cultured rat hepatocytes. Gene expression was monitored by determination of mRNA levels and of enzyme activity. Glucose formation was estimated with newly synthesized radioactive glucose derived from a radiolabelled lactate precursor. Glucagon (0.1 or 1 nM) induced PCK mRNA transiently to a maximum 2 h after its application. In the presence of recombinant human (rh) IL1 β or rhTNF α the increase in PCK mRNA levels was totally inhibited at 0.1 nM glucagon, whereas at 1 nM glucagon the maximal increase was inhibited by only 25%. Glucagon (0.1 or 1 nM) induced PCK activity to a maximum after 4 h (4-fold and 6-fold over prestimulatory activity respectively). In the presence of rhIL1 β or rhTNF α the maximal

increase was inhibited by approx. 50%. Addition of rhIL1 β or rhTNF α 2 h after glucagon, at the maximal glucagon-induced PCK mRNA levels, accelerated the decay of PCK mRNA. Glucagon (0.1 or 1 nM) increased glucose formation from lactate by 1.3-fold and 1.7-fold respectively over unstimulated rates. In the presence of rhIL1 β or rhTNF α this increase in glucose formation was inhibited by 60–90%. At 0.1 nM, glucagon doubled the intracellular cAMP concentration. This increase was prevented by rhIL1 β or rhTNF α . At 1 nM, glucagon increased cAMP concentrations by 10-fold. In the presence of rhIL1 β or rhTNF α this increase was inhibited by 70%. From the results it is suggested that rhIL1 β and rhTNF α prevented glucagon-stimulated PCK gene expression and gluconeogenesis at least in part by inhibition of the glucagon-stimulated increase in cAMP concentrations.

INTRODUCTION

The switch from low to high expression of positive acute-phase proteins and from high to low expression of negative acute-phase proteins is characteristic of the acute-phase response in the liver. This is initiated by the synergistic action of the proinflammatory cytokines interleukin 1 β (IL1 β), tumour necrosis factor α (TNF α) and interleukin 6 (IL6), which are derived from keratinocytes, endothelial cells, fibroblasts or macrophages after stimulation during infection, neoplastic growth or tissue damage in the organism. In rat liver the synthesis and secretion of serum amyloid A, haptoglobin, haemopexin and α_1 -acid glycoprotein, among others, is stimulated by either IL1 β or TNF α alone or in conjunction with IL6. These proteins have been classified as type 1 acute-phase proteins. α_1 -Antitrypsin, α_1 -antichymotrypsin, α_1 -cysteine protease inhibitor, α_2 -macroglobulin and fibrinogen are induced by IL6 alone or by IL6-related cytokines such as leukaemia inhibitory factor or oncostatin M. These proteins have been classified as type 2 acute-phase proteins (reviewed in [1]).

In the organism the acute-phase reaction gives rise to a number of changes in carbohydrate metabolism comprising increased glucose uptake and utilization at the site of tissue lesion, inhibition of hepatic glycogen synthesis, glucose intolerance and insulin resistance (reviewed in [2]). During initial inflammation, blood glucose concentrations increase owing to the stimulation of glycogen breakdown in the liver. During prolonged sepsis, however, severe hypoglycaemia emerges after the depletion of glycogen stores (reviewed in [3]). During sepsis the expression of the key control enzyme of gluconeogenesis,

phosphoenolpyruvate carboxykinase (GTP) (PCK; EC 4.1.1.32), was inhibited, which was probably mediated by inflammatory cytokines [4]. In rat hepatocyte cultures the glucagon-dependent induction of PCK gene transcription was inhibited and PCK mRNA degradation was accelerated by recombinant human (rh) IL6 [5]. IL6 and TNF α were assumed to decrease PCK gene transcription in rat and mouse liver respectively [6,7].

The regulation of PCK gene expression in the liver has been widely investigated. In the rat the enzyme is increased during the fasting period in the normal daily feeding rhythm, in starvation and in diabetes [8]. In cultured rat hepatocytes the PCK gene was activated by glucagon under the permissive action of glucocorticoids [9,10] and in rat hepatoma cells by cAMP or glucocorticoids alone [11,12]. Glucagon, via an increase in cAMP concentration, stimulated PCK gene transcription by way of activation of the transcription factor cAMP-response-element binding protein, which bound to the cAMP response element in the promotor of the PCK gene [13,14]. In cultured rat hepatocytes the activation of PCK gene expression by glucagon was counteracted by insulin by inhibition of glucagon-induced PCK gene transcription and the acceleration of degradation of mRNA for PCK [15,16].

Because during inflammation the co-operative actions of IL1 β , TNF α and IL6 are necessary for the induction of both type 1 and type 2 acute-phase proteins, it was the aim of this study to investigate whether in primary cultures of rat hepatocytes rhIL1 β and rhTNF α would also inhibit PCK gene expression, as had been shown recently for IL6 [5]. In addition it was intended to demonstrate whether glucose formation via gluconeogenesis was inhibited by rhIL1 β and rhTNF α as a potential consequence of

the inhibition by the cytokines of PCK activity. The results show that rhIL1 β and rhTNF α , like IL6, inhibited PCK gene expression by inhibition of the glucagon-induced increase in PCK mRNA levels and by acceleration of mRNA decay. Furthermore rhIL1 β and rhTNF α inhibited the glucagon-stimulated increase in PCK activity and in turn the glucagon-stimulated increase in gluconeogenesis. The inhibition of glucagon-increased PCK gene expression and gluconeogenesis could be due at least in part to the inhibition by rhIL1 β and rhTNF α of the glucagon-stimulated increase in cAMP.

MATERIALS AND METHODS

Animals and chemicals

Cells were prepared from male Wistar rats (200–300 g) kept with a normal daily feeding rhythm. Chemicals were of analytical grade and supplied by commercial sources. The digoxigenin RNA-labelling kit, the nucleic acid detection kit, fetal calf serum and collagenase A were from Boehringer (Mannheim, Germany). Dowex 1 $\times$ 8 (200–400 mesh) and insulin were purchased from Serva (Heidelberg, Germany), 3-isobutyl-1-methylxanthine, glucagon and dexamethasone were from Sigma (Deisenhofen, Germany). Culture medium M199 was supplied by Life Technologies (Eggenstein, Germany), culture dishes by Greiner (Nürtingen, Germany) and supported nitrocellulose BA-85 by Schleicher & Schüll (Dassel, Germany). rhTNF α and rhIL1 β were gifts from Dr. P. Scholz and Dr. P. Donner (Schering AG, Berlin, Germany). [U-¹⁴C]Lactate (5.74 GBq/mmol) was purchased from Amersham/Buchler (Braunschweig, Germany).

Experimental design

Hepatocytes were prepared by the collagenase perfusion technique and cultured in medium M199 for 48 h at 2.5 ml per dish (60 mm diameter) for determination of PCK mRNA levels, PCK activity and gluconeogenic rate, or at 1.5 ml per dish (35 mm diameter) for determination of cAMP levels at a density of 7.5×10^4 cells/cm². During the first 4 h of culture the medium contained 4% (v/v) fetal calf serum, 100 nM dexamethasone and 0.5 nM insulin, which was included as a growth factor to improve hepatocyte viability. Serum was omitted during the subsequent 44 h of culture. Experiments were started with two initial washes with insulin-free but dexamethasone-containing medium and culture was continued in fresh medium supplemented with glucagon and cytokines at the indicated concentrations. When the decay of glucagon-induced PCK mRNA was to be determined, the cytokines were added to the cells 2 h after glucagon (at the maximal glucagon-induced PCK mRNA levels). Cells were harvested at the indicated time points and either total RNA was prepared for Northern blot analyses or cells were homogenized and enzyme activity was determined in post-mitochondrial supernatants essentially as described [9].

Radiochemical determination of glucose formation from lactate

Determinations were performed as described [17]. Briefly, after 48 h of culture, cells were washed twice as described above, except that the medium was supplemented with 2 mM lactate. Experiments were initiated by adding fresh medium containing 1 μ l (0.0074 MBq) of [U-¹⁴C]lactate per ml. After a 30 min equilibration period, zero-time samples (100 μ l) were taken. After 2 and 4 h of incubation, 100 μ l samples were again taken for determination of newly synthesized radioactive glucose. During this period product formation was linear with time. The 100 μ l aliquots were applied to a 0.5 cm $\times$ 1 cm resin of Dowex

(formate form) and glucose was eluted with 2 ml of water. For determination of specific radioactivity, lactate in the zero-time samples was eluted from the resin with 2 ml of 0.5 M sodium formate.

Tools and assays

Northern blots for determination of PCK mRNA levels were performed as described [9,15]. Total RNA was isolated from two pooled culture dishes: 15 μ g was separated on denaturing agarose gels, transferred to nitrocellulose membranes and PCK mRNA detected by the use of digoxigenin-labelled anti-sense cRNA probes. These were generated by the use of T3 RNA polymerase from the *Hind*III-linearized plasmid pBS-PCK, which contained a *Pst*I 1.2 kb cDNA fragment coding for rat PCK [18].

PCK activity was determined by quantification of the product phosphoenolpyruvate after conversion with pyruvate kinase and lactate dehydrogenase [19].

cAMP was determined by the use of the ¹²⁵I-labelled cAMP radioimmunoassay system supplied by Amersham-Buchler (Braunschweig, Germany). After 48 h of culture, cells were washed three times with medium A [20 mM Hepes (pH 7.4)/120 mM NaCl/1.2 mM MgSO₄/1.2 mM KH₂PO₄/2.5 mM CaCl₂/5 mM glucose/2 mM lactate] and culture was continued in 1 ml of medium A. Hormones and cytokines were added after a 10 min equilibration period at the indicated concentrations and incubation was continued for 3 min. The medium was sucked off and the cells were then frozen in liquid nitrogen and thawed again in 1 ml of 10 mM HCl containing 1 mM 3-isobutyl-1-methylxanthine to inhibit endogenous phosphodiesterase activity. Cells were disrupted by incubation for 1 h at 4 °C and the resulting homogenate was centrifuged for 10 min at 6000 g. A 20 μ l portion of the supernatant was applied to the cAMP assay system.

Statistical analysis was performed by use of Student's *t* test for unpaired values, including the number of different cell cultures as indicated in the figure legends. Values in the presence of glucagon and rhIL1 β or rhTNF α were compared with values in the presence of glucagon alone; significant inhibition by the cytokines was defined by *P* values as indicated in the figure legends.

RESULTS

Concentration dependence of the glucagon/cytokine antagonism in PCK mRNA increase

IL1 β and TNF α , co-operatively with IL6, cause the acute-phase reaction in the liver. As shown in a previous study, IL6 completely inhibited the induction of PCK mRNA by 0.1 nM glucagon [5]. To investigate whether IL1 β or TNF α was also able to inhibit glucagon-induced PCK gene expression, cultured rat hepatocytes were treated for 2 h with 0.1 or 1 nM glucagon in the presence of increasing concentrations of rhIL1 β (Figure 1B) or rhTNF α (Figure 1A). Physiological concentrations of glucagon (0.1 nM) increased PCK mRNA levels to approx. 60% of the increase reached with 1 nM glucagon. Whereas with an inducing concentration of glucagon of 0.1 nM the cytokines at 100 ng/ml prevented the increase in glucagon-induced PCK mRNA levels, with an inducing concentration of glucagon of 1 nM they failed to inhibit effectively, even at high (100 ng/ml) concentrations. With an inducing concentration of glucagon of 0.1 nM, the half-maximal inhibitory concentration of either cytokine was 5 ng/ml. Similar cytokine concentrations had been determined in the plasma of patients suffering from primary septic shock [20]. Thus the inhibition by rhIL1 β and rhTNF α of the glucagon-induced PCK mRNA increase is operative at physiological

Figure 1 Concentration dependence of the glucagon/cytokine antagonism towards the increase in PCK mRNA

Rat hepatocytes were cultured for 48 h and PCK mRNA was induced for 2 h as described in the Materials and methods section with either 0.1 or 1 nM glucagon (Ggn) in the presence of increasing rhTNF α (A) or rhIL1 β (B). Controls were treated with increasing cytokine concentrations in the absence of glucagon (0 Ggn). Total RNA was isolated from two pooled culture dishes for Northern blot analyses, and PCK mRNA was detected with digoxigenin-labelled cRNA probes; amounts were quantified by means of videodensitometry. In each single experiment the highest value of PCK mRNA was set arbitrarily at 1, to which the other values are relative. Values are means $\pm$ S.E.M. for seven different cell cultures. Statistics: Student's *t* test for unpaired values; differences between values in the presence of 0.1 or 1 nM glucagon and values in the absence of glucagon are significant at the $P \leq 0.01$ level (points labelled a). Differences between values in the presence of cytokines and values in the absence of cytokines are significant at the $P \leq 0.05$ (b) and $P \leq 0.01$ (c) levels.

Figure 2 Induction by 0.1 nM glucagon of PCK mRNA levels and enzyme activity and inhibition by rhIL1 β and rhTNF α

Rat hepatocytes were cultured for 48 h and PCK mRNA levels (left panel) and enzyme activity (right panel) induced with 0.1 nM glucagon (Ggn) in the absence (■) or presence of 50 ng/ml rhTNF α (TNF; ○) or rhIL1 β (IL1; △) as described in the Materials and methods section. At the indicated time points cells were harvested for the preparation of total RNA for Northern blot analyses or the determination of enzyme activity. PCK mRNA was detected with digoxigenin-labelled cRNA probes; amounts were quantified by means of videodensitometry. In each single experiment the highest value of PCK mRNA levels reached in the presence of glucagon was set arbitrarily at 1, to which the other values were made relative. Results are expressed as means $\pm$ SEM for three to six different cell cultures. Statistics: Student's *t* test for unpaired values, which compared values in the absence of cytokines with values in the presence of either rhIL1 β or rhTNF α ; differences are significant at the $P \leq 0.005$ level (a).

concentrations of both hormone and cytokine. In all subsequent experiments rhIL1 β and rhTNF α were applied at 50 ng/ml to achieve full inhibitory efficiency.

Time course of the glucagon-induced increase in PCK mRNA level and enzyme activity and antagonism by rhIL1 β and rhTNF α

The time course of the inhibition of the glucagon-induced increase in PCK mRNA level and enzyme activity by rhIL1 β and rhTNF α

Figure 3 Induction by 1 nM glucagon of PCK mRNA levels and enzyme activity and inhibition by rhIL1 β and rhTNF α

Rat hepatocytes were cultured for 48 h and PCK mRNA (left panel) and enzyme activity (right panel) induced with 1 nM glucagon (Ggn) in the absence (■) or presence of 50 ng/ml rhTNF α (TNF; ○) or rhIL1 β (IL1; △) as described in the Materials and methods section. At the indicated time points cells were harvested for preparation of total RNA for Northern blot analyses or determination of enzyme activity. PCK mRNA was detected with digoxigenin-labelled cRNA probes; amounts were quantified by means of videodensitometry. In each single experiment the highest value of PCK mRNA levels reached in the presence of glucagon was set arbitrarily to 1, to which the other values are relative. Results are expressed as means $\pm$ S.E.M. for three to six different cell cultures. Statistics: Student's *t* test for unpaired values, which compared values in the absence of cytokines with values in the presence of either rhIL1 β or rhTNF α ; differences are significant at the $P \leq 0.005$ (a), $P \leq 0.025$ (b) or $P \leq 0.05$ (c) level.

was monitored with 0.1 nM (Figure 2) and 1 nM (Figure 3) inducing concentrations of glucagon. Because it was not possible to study the many different conditions with a single cell preparation, values in Figures 2 and 3 were obtained in separate series of experiments. For the determination of mRNA levels, in each single experiment the highest value reached in the presence of glucagon was set equal to 1, to which the other values were made relative. The relative increases in PCK mRNA levels reached with 0.1 and 1 nM glucagon can be taken from Figure 1. Glucagon at 0.1 nM (Figure 2) or 1 nM (Figure 3) increased PCK mRNA levels maximally after 2 h. After the maximal increase, PCK mRNA levels remained elevated for another 2 h and started to decline again during the subsequent culture period. In the presence of either rhIL1 β or rhTNF α the maximal increase in PCK mRNA levels at 2 h was almost totally abolished at an inducing concentration of glucagon of 0.1 nM (Figure 2) and was inhibited by 25% at 1 nM glucagon. Prestimulatory levels were reached again after a further 2 h (Figure 3).

Glucagon at 0.1 nM (Figure 2) and 1 nM (Figure 3) increased PCK activity maximally after 4 h to approx. 4-fold and 6-fold respectively over prestimulatory activity. It remained elevated during the experimental time course for at least a further 2 h. In the presence of either rhIL1 β or rhTNF α the glucagon-induced increase in PCK activity was inhibited by approx. 50% at 0.1 nM glucagon (Figure 2) or 1 nM glucagon (Figure 3).

In controls (in the absence of glucagon) a slight increase with time in PCK mRNA levels was observed. This increase was totally prevented in the presence of rhIL1 β or rhTNF α (results not shown).

Acceleration by rhIL1 β and rhTNF α of the decay of glucagon-induced PCK mRNA

IL6 antagonized the induction of PCK gene expression by glucagon in part by the acceleration of mRNA decay [5]. To investigate whether also rhIL1 β and rhTNF α accelerated the decay of glucagon-induced PCK mRNA, the cytokines were

Figure 4 Acceleration of glucagon-induced PCK mRNA decay by rhIL1 β and rhTNF α

Rat hepatocytes were cultured for 48 h and PCK mRNA was induced with 0.1 nM glucagon (Ggn; ■) as described in the Materials and methods section. rhTNF α (TNF; ○) or rhIL1 β (IL1; △) at a concentration of 50 ng/ml were added 2 h after glucagon. At the indicated time points cells were harvested for preparation of total RNA for Northern blot analyses. PCK mRNA was detected with digoxigenin-labelled cRNA probes; amounts were quantified by means of videodensitometry. In each single experiment the highest value of PCK mRNA reached in the presence of glucagon was set arbitrarily at 1, to which the other values are relative. Results are means $\pm$ S.E.M. for three different cell cultures. Statistics: Student's *t* test for unpaired values, which compared values in the absence of cytokines with values in the presence of either rhIL1 β or rhTNF α ; differences are significant at the $P \leq 0.005$ level (a). The arrows indicate the time of the addition of glucagon and the time of the addition of rhIL1 β or rhTNF α .

applied 2 h after glucagon, at the maximal glucagon-induced PCK mRNA levels (Figure 4). In a previous study it was demonstrated by the use of nuclear run-on assays that glucagon stimulated PCK gene transcription transiently, with a maximum value after 30 min. After a further 90 min, transcription had again declined almost to the prestimulatory basal levels [15]. Therefore it was reasonable to monitor the decay of PCK mRNA 2 h after glucagon by estimation of the remaining mRNA in the absence of a transcriptional inhibitor; it was even necessary, because the use of transcriptional inhibitors prevented the degradation of glucagon-induced PCK mRNA, probably by preventing the synthesis of an as yet unknown degradation factor [15,21]. If the cytokines were added 2 h after glucagon, the decay of glucagon-induced PCK mRNA was nearly doubled, independently of whether PCK mRNA had been previously induced with 0.1 nM (Figure 4) or 1 nM glucagon (results not shown).

Glucagon-stimulated increase in glucose formation from lactate, and antagonism by rhIL1 β and rhTNF α

PCK catalyses the control step in gluconeogenesis from pyruvate to phosphoenolpyruvate. Because the glucagon-stimulated induction of PCK mRNA level and enzyme activity was attenuated by rhIL1 β and rhTNF α , it was assumed that glucagon-stimulated gluconeogenesis might also be inhibited by the cytokines. Glucose formation via gluconeogenesis was estimated by determination of newly synthesized radioactive glucose, starting from radioactive lactate as a precursor (Table 1). The basal non-stimulated gluconeogenic rate ($\pm$ S.E.M.) (2.05 ± 0.18 μ mol/h per g wet wt.; $n = 10$) was essentially unaltered by either cytokine (results not shown). With 0.1 nM glucagon, the gluconeogenic rate was not stimulated over basal rates, whereas 1 and 10 nM glucagon increased gluconeogenic rates significantly by 1.3-fold (2.68 ± 0.16 μ mol/h per g wet wt.; $n = 10$) and 1.7-fold (3.38 ± 0.32 μ mol/h per g wet wt.; $n = 10$) respectively. In the

Table 1 Glucagon-stimulated glucose formation from lactate, and antagonism by rhIL1 β and rhTNF α

Rat hepatocytes were cultured for 48 h. Then cells were treated for 4 h with 0.1, 1 or 10 nM glucagon in the absence or presence of 50 ng/ml rhIL1 β or rhTNF α . The gluconeogenic rate was calculated from newly synthesized glucose derived from radiolabelled lactate as described in the Materials and methods section. Radioactive products were determined in 100 μ l samples taken from the culture medium after the 4 h incubation period. Values are expressed as the increase over non-stimulated gluconeogenic rate, which was 2.05 ± 0.18 μ mol/h per g wet wt. ($n = 10$). Results are means $\pm$ S.E.M. for five to ten different cell cultures, each run in duplicate. Statistics: Student's *t* test for unpaired values. Values in the presence of cytokines are different from values in the absence of cytokines at the * $P \leq 0.0025$, ** $P \leq 0.01$ or *** $P \leq 0.025$ level. Abbreviation: n.d., not detectable.

[Glucagon] (nM) ...	Gluconeogenic rate (μ mol glucose/h per g wet wt.)		
	0.1	1	10
Without addition	n.d.	0.63 ± 0.09	1.33 ± 0.27
+ IL1 β	n.d.	$0.05 \pm 0.03^*$	$0.43 \pm 0.11^{**}$
+ TNF α	n.d.	$0.22 \pm 0.07^{**}$	$0.50 \pm 0.12^{***}$

Table 2 Glucagon-stimulated increase in cAMP, and antagonism by rhIL1 β , rhTNF α and insulin

Rat hepatocytes were cultured for 48 h. Then cells were stimulated for 3 min with 0.1 or 1 nM glucagon in the absence or presence of 50 ng/ml rhIL1 β or rhTNF α , or 1 nM insulin, in buffer A as described in the Materials and methods section. Cells were quickly frozen and the resulting homogenates used for radioimmunological determination of cAMP. Values are expressed as the increase over non-stimulated zero-time concentrations of cAMP, which were 218 ± 26 fmol/mg wet wt. Results are means $\pm$ S.E.M. for five to ten and two different cell cultures, each run in duplicate in the series with 0.1 nM glucagon and with 1 nM glucagon respectively. Statistics: Student's *t* test for unpaired values; * differences between values in the presence of cytokines or insulin and values in the absence of rhIL1 β or rhTNF α or insulin are significant at the $P \leq 0.005$ level. Abbreviation: n.d., not detectable.

[Glucagon] (nM) ...	cAMP Concentration (fmol/mg wet wt.)		
	Without	0.1	1
Without addition	55 ± 17	224 ± 19	2100
+ IL1 β	n.d.	$78 \pm 8^*$	631
+ TNF α	n.d.	$80 \pm 5^*$	657
+ insulin	n.d.	$69 \pm 6^*$	n.d.

presence of rhIL1 β or rhTNF α the glucagon-stimulated increase in gluconeogenic rate was inhibited by 60–90 %.

Glucagon-stimulated increase in cAMP levels, and antagonism by rhIL1 β and rhTNF α

rhIL1 β and rhTNF α inhibited the glucagon-induced increase in PCK mRNA, enzyme activity and gluconeogenesis. Because glucagon acts via the elevation of intracellular cAMP, the cytokines might attenuate the glucagon-stimulated increase in cAMP levels. Cultured rat hepatocytes were stimulated for 3 min with 0.1 or 1 nM glucagon in the absence or presence of cytokines and, as a control, in the presence of insulin. Subsequently cAMP levels were determined by means of a radioimmunoassay. In zero-time controls cAMP concentrations were 218 ± 26 fmol/mg

wet wt. Glucagon at 0.1 or 1 nM increased cAMP levels to 442 ± 38 or 2318 fmol/mg wet wt. respectively. Relative to this increase, in controls that were incubated in the absence of glucagon, a slight increase in cAMP levels was observed (Table 2). In the presence of rhIL1 β or rhTNF α , 0.1 nM glucagon failed to increase cAMP levels over basal, unstimulated levels. The increase in cAMP levels obtained with 1 nM glucagon was attenuated by 70 % in the presence of the cytokines but it was still clearly higher than the increase obtained with 0.1 nM glucagon in the absence of cytokines.

Because insulin is known to attenuate the glucagon-stimulated increase in cAMP, cAMP levels were determined in hepatocytes that had been treated with glucagon and insulin. As expected, insulin inhibited the glucagon-induced increase in cAMP levels.

DISCUSSION

The results presented demonstrate that rhIL1 β and TNF α inhibited the glucagon-induced increase in PCK mRNA levels and enzyme activity and the glucagon-stimulated gluconeogenesis in cultured rat hepatocytes. Furthermore the increase in cAMP levels brought about by glucagon was attenuated by the cytokines.

Inhibition of PCK gene expression by rhIL1 β and rhTNF α

The activation of PCK gene expression by glucagon is mediated by the cAMP-activated transcription factor cAMP-response-element binding protein [22,23]. In cultured rat hepatocytes the cytokines prevented the glucagon-stimulated increase in cAMP at physiological (0.1 nM) glucagon concentrations (Table 2). This might have led consecutively to the inhibition by rhIL1 β and rhTNF α of the glucagon-stimulated increase in PCK mRNA levels and enzyme activity (Figure 2). In cultured rat hepatocytes glucagon increased cAMP concentrations transiently, with a maximum after 5 min [24]. Therefore at first glance it might be surprising that the short transient increase in cAMP caused the maximal increase in PCK mRNA levels only 2 h after glucagon (Figures 2 and 3). Yet it had been shown that glucagon stimulated PCK gene transcription maximally after only 30 min [15], which accounted for the subsequent maximal increase in PCK mRNA levels after 2 h.

At 1 nM glucagon concentrations the cytokines attenuated the glucagon-stimulated increase in cAMP by 70 % (Table 2). However, cAMP levels were still high enough to induce the PCK gene maximally. This might account for the weak inhibition by rhIL1 β and rhTNF α of the maximal glucagon-induced increase in PCK mRNA levels at 2 h (Figure 3). Nevertheless a substantial attenuation by the cytokines of the glucagon-induced increase in PCK mRNA levels, enzyme activity and gluconeogenesis was observed at 4–6 h (Figure 3 and Table 1). This indicates that rhIL1 β and rhTNF α could inhibit glucagon-induced PCK gene expression post-transcriptionally, e.g. by acceleration of the degradation of PCK mRNA (see below).

IL1 β and TNF α , together with IL6, cause a common set of biological processes, which induce in a concerted fashion the host defence reaction comprising fever, stimulation of hepatic acute-phase protein synthesis and augmentation of lymphocyte protective functions. The pleiotropic efficacy of the cytokines is due to the presence of high-affinity receptors present on the cell surface of a great variety of target cells.

IL1 β and TNF α bind to monomeric receptors that transduce the signal into the cell [25]. Intracellular events comprise the activation of neutral sphingomyelinase and phospholipase C. The activation of sphingomyelinase liberates ceramide, which leads to the stimulation of ceramide-activated protein kinase and

to downstream activation of MAP kinase and cytoplasmic phospholipase A2. The activation of phospholipase C generates diacylglycerol, a prominent activator of protein kinase C. Evidence exists that activation of protein kinase C in turn activates the transcription factor NF κ B, which is a prominent mediator of IL1 β - and TNF α -stimulated gene expression (reviewed in [26,27]). At present no reports are available that describe the effects of IL1 β or TNF α on changes in cellular cAMP levels in hepatocytes. However, it has been reported that conditioned medium from activated splenocytes impaired the isoproterenol-stimulated increase in cAMP levels in rat cardiac myocytes [28].

Post-transcriptional regulation of PCK gene expression by rhIL1 β and rhTNF α

Very few reports are available on the post-transcriptional regulation of mRNA stability by cytokines. TNF α stabilized IL1 mRNA by a protein kinase C-dependent mechanism in human fibroblasts [29] and glucose transporter (GLUT-1) mRNA in 3T3-L1 preadipocytes [30]. Transferrin receptor mRNA was stabilized by IL2 in B6 T-cells [31]. In Hep3B cells the repression by TGF β of gene expression of negative acute-phase genes was due at least in part to destabilization of mRNA [32]. TNF α shortened the half-life of the mRNA for nitric oxide synthase in endothelial cells [33]. However, nothing is known about the mechanism by which cytokines could regulate mRNA turnover. In cultured rat hepatocytes, glucagon stabilized PCK mRNA by binding a stabilizing protein in the 3' untranslated region of the mRNA; this was antagonized by insulin [16]. Because the cytokines, like insulin, inhibited glucagon-induced PCK gene expression by the acceleration of mRNA degradation, it is possible that cytokine-induced changes in protein–mRNA interactions caused the destabilization of PCK mRNA.

Inhibition by rhIL1 β and rhTNF α of glucagon-stimulated gluconeogenesis in cultured rat hepatocytes

Induction of PCK activity by physiological (0.1 or 1 nM) glucagon concentrations was prevented by rhIL1 β and rhTNF α (Figures 2 and 3). Gluconeogenesis was stimulated only at 1 and 10 nM glucagon concentrations (Table 1), which was again prevented by rhIL1 β and rhTNF α . Therefore because the glucagon/cytokine antagonism was effective at one order of magnitude higher glucagon concentrations in the regulation of gluconeogenesis than in the regulation of PCK activity, the cytokines must affect another still unknown regulatory step in gluconeogenesis, which is different from the regulation of substrate flux through PCK.

Although the inhibition of PCK gene expression by endotoxin or cytokines seems well documented [4–7], only few reports show a correlation with a concomitant decrease in gluconeogenesis [34,35].

Hypoglycaemia is one complication in the development of septic shock. Because glucose utilization in the periphery is strongly enhanced during sepsis, it is feasible to suppose that the reduced capacity of the liver to supply glucose might be a major cause of hypoglycaemia. The results presented here show that in the presence of cytokines, hepatocytes were still able to produce glucose at a basal rate, although their response to glucagon was impaired. This had also been shown previously in the septic-rat model [4]. Because insulin resistance also emerges during prolonged sepsis, the general failure of regulation by glucoregulatory hormones seems to lead to the impairment of glucose homeostasis in septic shock.

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