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Adrenalectomy and Dexamethasone Treatment Alter the Patterns of Basal and Acute Phase Response-induced Expression of Acute Phase Protein Genes in Rat Liver

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Hormonal requirements for full hepatic expression of α_2-macroglobulin (α_2M), α_1-acid glycoprotein (AGP), haptoglobin (Hp) and γ-fibrinogen (Fb) were assessed at the level of mRNA. Prior to exposure to turpentine-induced inflammation, rats were either depleted of glucocorticoids by adrenalectomy or supplemented with an excess of dexamethasone. Adrenalectomy alone did not affect the basal level of acute phase protein (APP) expression except for α_2M mRNA, the level of which was enhanced. In contrast, dexamethasone treatment alone promoted full induction of α_2M, significant, but not maximal increase of AGP and Hp mRNAs and suppression of Fb. In adrenalectomized rats, acute phase (AP)-cytokines, released in response to inflammation, promoted full expression of Fb and Hp and increased the level of AGP mRNA whereas α_2M mRNA remained at the basal level. Inflammation in dexamethasone pretreated rats elicited changes which, in comparison to mRNA values for dexamethasone unpretreated inflamed rats, were seen as overexpression of α_2M, full expression of AGP and incomplete expression of Hp, whereas Fb mRNA remained at the basal level. These data suggest that glucocorticoids are the principal inducers of α_2M and AP-cytokines of Fb. For full induction of AGP, additive actions of glucocorticoids and AP-cytokines are required whereas expression of Hp is predominantly controlled by AP-cytokines. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

The acute phase response is initiated by activation of macrophages at the site of injury and subsequent release of a broad spectrum of mediators including the major acute phase (AP) cytokines, TNFα, IL-1 and IL-6. As redundant and multifunctional mediators, the AP-cytokines elicit a complex series of physiological responses, defined as the systemic reaction. This includes the AP-cytokine-mediated activation of the hypothalamic–pituitary–adrenal axis and the resulting secretion of the major counter-regulator of AP-cytokines, the glucocorticoids (GC). While suppressing the production and actions of AP-cytokines, the GC synergize with them in the induction of a hallmark process of the acute phase response, the transcriptional activation of the genes coding for a subset of plasma proteins, the acute phase reactants (APP) in the liver. The principal inducers of APP genes in human and rodent hepatoma cell systems have been identified as interleukin-6 (IL-6)-type and interleukin-1 (IL-1)-type cytokines whereas GC have been found to act primarily as factors enhancing the AP-cytokine-induced stimulation of APP genes [1]. Depending on the hormone requirement for maximal production in cultured rat liver cells, the APP species are classified into type I, requiring IL-1, IL-6 and dexamethasone, and type II, responding fully to IL-6 and dexamethasone. The prominent representatives of type I APP are α_1-acid glycoprotein (AGP) and

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haptoglobin (Hp) and of type II are α_2-macroglobulin (α_2M) and fibrinogen (Fb) [2]. In the past decade several APP genes have been cloned and the elements responsive to each of the AP-cytokines and GC exhaustively characterized in various human and rodent hepatoma cell culture systems [3].

In contrast to cell culture systems, hormone requirements for full expression of individual APP in the acute phase liver have not been determined as yet. Based on the findings that the level of AGP mRNA in the liver can be enhanced by inflammation of adrenalectomized rats lacking GC as well as by treatment of normal rats with dexamethasone, the mechanism regulating this and some other APP has been proposed to rely on two effector systems, GC and unknown stress-related factors [4]. The predicted factors have been identified as cytokines. Injection of rats with recombinant human rIL-6, was found, indeed, to promote a several-fold increase in mRNA levels of AGP, β-Fb, α_2M and cysteine proteinase inhibitor in the liver [2]. While the combined information from tissue culture cells and acute phase livers suggests that APP genes in both systems are up-regulated by synergistic actions of AP-cytokines and GC, the question of whether hormone requirements for full expression of individual APP in animals is similar to those in cultured cells cannot be answered unless assessed in the acute phase liver. However, experimental approaches to this problem are hampered by the finding that the functional relationship between GC and AP-cytokines outside as well as inside the liver depends on numerous factors. This is illustrated by the data showing that infusion of lipopolysaccharide (LPS) with the basal dose of GC stimulated production of IL-6 and TNF in a perfused rat liver preparation whereas the higher dose of GC acted suppressively [5]. Nevertheless, the efforts aimed at delineation between the contribution of GC and AP-cytokines to the expression of individual APP in the liver are gaining in importance not only for the sake of progression in fundamental science but also because these results may have direct implications for patients undergoing chronic glucocorticoid therapy.

In this study the influence of GC on the expression of selected APP was investigated in rats which were either depleted of GC by bilateral adrenalectomy ensuring a more than 90% inhibition of GC production [6], or brought into the state of hypercorticism by injection of dexamethasone prior to exposure to turpentine-induced inflammation. It was expected that the abundant release of AP-cytokines in the turpentine-treated rats lacking GC would stimulate expression of APP species that are under the predominant control of AP-cytokines, whereas impaired production of AP-cytokines in the inflamed rats supplemented with an excess of dexamethasone would cause preferential expression of GC-inducible APP genes in the liver. Differences between these two patterns of APP expression should enable delineation of the contribution of GC and AP-cytokines to the full expression of each of the examined APP. The results showed that demands for GC decreased and for AP-cytokines increased in the following sequence: α_2M, AGP, Hp and Fb. Thus one prominent member of class II APP, α_2M, was found to be fully inducible by dexamethasone and another one, Fb, by AP-cytokines.

EXPERIMENTAL

Animals

The experiments were performed on adult male albino Wistar rats of weight 250–300 g. Prior to exposure to inflammation one group of rats was submitted to a bilateral adrenalectomy, the second to dexamethasone injection, whereas the third one was left untreated.

The acute phase reaction of rats was promoted by administration of turpentine oil which is one of the most widely used locally inflammatory substance [7]. The rats received s.c. injection of turpentine oil in the abdominal region (1 μl/g of body wt) and were killed 18 h later. 30 min before treatment with turpentine the group assigned for hormone pretreatment received i.p. 250 μl of 1% (v/v) ethanol in saline containing 1 mg of dexamethasone. Treated animals had *ad libitum* access to water and a standard laboratory diet throughout. Bilateral adrenalectomy was performed under anesthesia and the rats were provided with 0.9% NaCl as drinking water. 4 days after surgery the adrenalectomized rats were exposed to turpentine-induced inflammation.

The control rats were submitted to the same pretreatment as those exposed to inflammation but received 0.9% NaCl (1 μl/g of body wt) instead of turpentine.

Plasmids

Plasmids encoding the following rat acute proteins were used (the insert size in base pairs at the restriction-endonuclease *Pst*I site of pBR322 is given in parentheses): pIRL21, α_1-acid glycoprotein (AGP) (85)); pIRL25, haptoglobin (Hp) (1300); pIRL20, γ-fibrinogen (Fb) (1650) and α_2-macroglobulin (α_2M) (650) were donated by H. Baumann of the Roswell Park Memorial Institute, Buffalo, NY, U.S.A., plasmid pTAT-3SP encoding rat tyrosine aminotransferase (TAT) (850) was donated by R. Miesfeld of the University of Arizona, Tucson, AZ, U.S.A. The inserts were radioactively labelled with [α-^{32}P]dCTP using an Amersham nick translation kit (Amersham International, Buckinghamshire, U.K.) according to the manufacturer's instructions to an s.a. of 5–7 $\times$ 10^6 dpm/μl DNA. The radioactivity used for the

hybridization was not lower than 3×10^6 dpm/ml of hybridization buffer.

Isolation and quantitative analyses of rat liver mRNA

Total liver RNA from treated and control rats was isolated by a procedure based on the extraction of the RNA with guanidine hydrochloride [8]. For quantification of mRNAs for AGP, α_2M, Hp, Fb and TAT, the RNA samples were submitted to dot-blot. Before transfer to nitrocellulose, the isolated RNA was denatured for 10 min at 65°C in 50% formamide, 2.2 M formaldehyde and 10 mM sodium phosphate and tested for the presence of degradation products by electrophoretic analysis of the sample [9]. For dot-blot analyses 10, 5 and 2.5 μg RNA aliquots were directly blotted on to nitrocellulose filters. The filters were prehybridized in 5 × Denhardt solution, 6 × SSPE, 0.5% SDS, 50% formamide and 100 μg/ml denatured salmon sperm DNA for 2–4 h at 42°C [10]. The hybridization with [α-^{32}P]dCTP nick translated plasmid DNAs was carried out in the same buffer for 24 h at 42°C. The filters were extensively washed at 37 and 65°C in 0.1 × SSPE, 0.5% SDS. The blots were scanned with a PhosphorImager (Molecular Dynamics, U.S.A.) and quantitated using the ImageQuant program. The changes in mRNA concentration were expressed as a percentage of the control value (100%).

For statistical analysis the mean ± SD ± SE are calculated from the data based on five to seven independent measurements. Statistical significance of differences was estimated by Fisher *t*-test. When below the 5% level ($p < 0.05$) the difference was considered to be statistically significant.

RESULTS

It has been shown previously that synthesis of APP in the liver of inflamed rats is switched on after a 4 h lag period when the increase in the plasma GC content has already reached its peak. Therefore, progressive elevation in the plasma APP concentration over 4–24 h proceeds in parallel with a decrease in the plasma GC level [11]. In the present studies, changes in the levels of mRNAs for two class I (AGP and Hp) and two class II (α_2M and Fb) APP, as well as for a GC inducible gene, TAT, were assessed 18 h following turpentine injection when the increase in expression of APP is approaching the maximum. Figure 1 shows that at this time interval the concentration of Hp mRNA was 3-fold, of α_2M and Fb four to 5-fold and of AGP 10-fold higher in the acute phase than in the control liver, whereas the level of TAT remained unchanged. As judged from the significant fall in the basal level of TAT mRNA in the liver of adrenalectomized rats (Fig. 2), the production of GC at the fourth post-adrenalectomy day was effectively inhibited. Such an implication is in accordance with data showing that 10 days following removal of adrenal glands the plasma corticosterone concentration in rats was reduced to 5% of the basal value [6]. The adrenalectomy itself also elicited a small but statistically significant increase in the level of α_2M mRNA, the reason of which is unclear at present, whereas expression of the other APP remained virtually unaltered. Following inflammation of adrenalectomized rats, the levels of Fb and Hp mRNAs were maximally enhanced, that of TAT mRNA significantly reduced ($p < 0.001$), whereas the magnitude of increase in AGP mRNA approached only half of the value obtained for nonadrenalectomized rats exposed to turpentine-induced inflammation (Fig. 2). However, expression of the major APP, α_2M, remained at the basal level. The presence of GC hormones was thus implicated to be a prerequisite for induction of the α_2M gene and full expression of AGP in the acute phase liver whereas AP-cytokines released in rats lacking GC can act as inducers promoting maximal expression of Hp and Fb.

Figure 3 shows that treatment of normal rats with dexamethasone resulted in the expected enhancement in the level of TAT mRNA. Moreover, exposure of dexamethasone treated rats to turpentine exerted no additional influence on the expression of this gene. Dexamethasone alone elicited a selective increase in the mRNA levels of α_2M, AGP and Hp whereas the

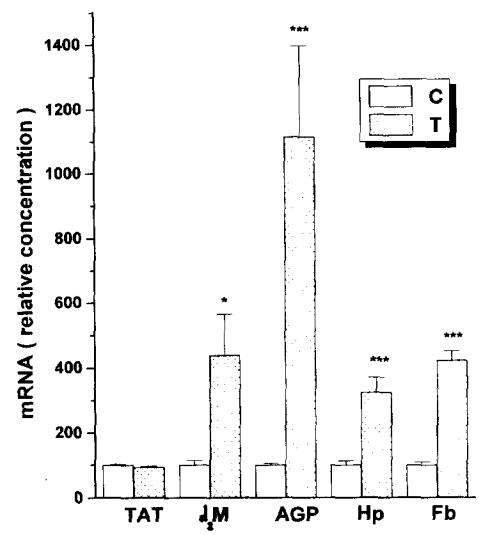

Fig. 1. Inflammation-induced changes in mRNA levels of TAT and individual APP. The total RNA was isolated from the livers of control (saline administered) rats (C) and turpentine treated rats (T) 18 h following treatment. The relative concentration of mRNAs for TAT, α_2M, AGP, Hp and Fb was determined by hybridization of the isolated RNA with the corresponding radioactively labeled cDNA probes and quantification of the retained radioactivity. The inflammation-induced changes in mRNA concentration were expressed as percentages of the corresponding control values (100%). The values are mean ± SE for 5–7 separate determinations, done in triplicate. Statistical significance of differences: *$p < 0.05$, ***$p < 0.001$.

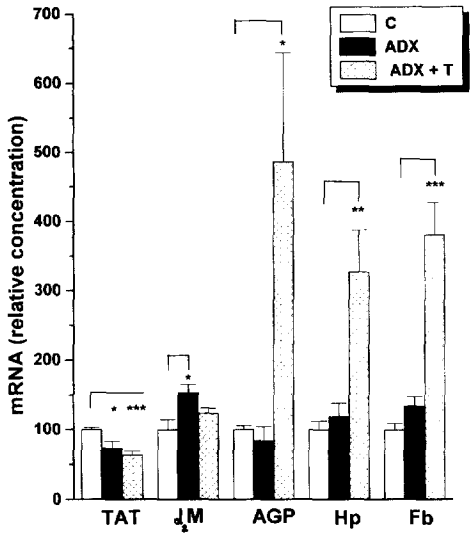

Fig. 2. The effects of adrenalectomy on the levels of TAT and APP mRNAs in the liver of rats untreated and treated with turpentine. The total RNA was isolated 4 days after bilateral adrenalectomy from the liver of rats which 18 h prior to sacrifice were injected with saline (ADX) or turpentine (ADX + T). For quantification of TAT, α_2M, AGP, Hp and Fb mRNAs the isolated RNA was hybridized with nick translated cDNA probes encoding each of the examined proteins and the amount of hybridized mRNA estimated by measurement of the radioactivity of the signals. The estimates are expressed as percentages of the corresponding control values (100%). The values are mean ± SE for 5–7 separate determinations, done in triplicates. Statistical significances of differences: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

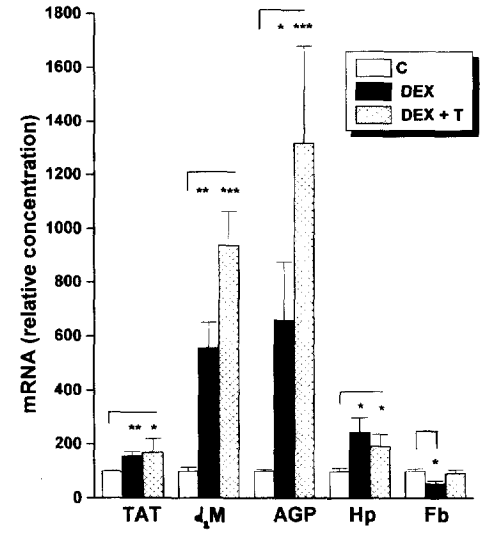

Fig. 3. The effects of dexamethasone administration on the level of TAT and APP mRNAs in uninduced liver and the liver of rats exposed to inflammation. Thirty min following administration of 1 mg of dexamethasone rats were injected either with saline (DEX) or turpentine (DEX + T) and killed 18 h later. The total RNA was isolated from the liver and radioactivity of the complex obtained by hybridization of RNA aliquots with radioactively labeled cDNA probes for TAT and APP quantified. The values are mean ± SE for 5–7 separate experiments expressed as percentages of deviation from the control level (100%). Statistical significance of differences: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

concentration of Fb mRNA was reduced to about 50% of the basal value. When expressed as a percent of the corresponding value for unpretreated rats exposed to inflammation (Fig. 1) the level of α_2M mRNA in the liver of normal dexamethasone administered rats was approximately 20% higher, and of Hp, AGP and Fb 30%, twice and 8-fold lower, respectively (Figs 1 and 3). In response to inflammation the concentration of α_2M mRNA in the liver of dexamethasone pretreated rats increased to double the level of that observed for normal rats exposed either to inflammation or treatment with dexamethasone. This, together with the data showing that inflammatory stimuli/AP-cytokines produced in turpentine-treated rats lacking GC failed to stimulate expression of α_2M (Fig. 2), strongly suggests that GC hormones are the principal inducer of α_2M. Eighteen h following injection of turpentine the amount of AGP mRNA in dexamethasone pretreated rats was doubled and approached the level observed for unpretreated rats exposed to turpentine-induced inflammation, (Figs 3 and 1). By promoting half of the maximal AGP expression when acting alone, dexamethasone and inflammatory stimuli/AP-cytokines appear to be equally potent inducers of this gene, yet incapable of eliciting its full expression unless acting together. The effect of inflammation on the expression of Hp in the liver of dexamethasone pretreated rats was seen as a slight impairment rather than enhancement of the dexamethasone-induced increase in the level of Hp mRNA. Thus, the value remained 40% below that observed in the acute phase liver of unpretreated rats. In response to inflammatory stimuli, the basal level of Fb in the liver of dexamethasone pretreated rats was restored.

The effects of an altered composition and/or amount of APP inducers on the level of expression of individual APP in rats which prior to turpentine treatment were depleted of GC or supplemented with dexamethasone are summarized in Fig. 4. The data are expressed as a percent of the value obtained for control rats exposed to turpentine-induced inflammation, assuming that GC and AP-cytokines produced under conditions of undisturbed hypothalamic–pituitary–adrenal axis promote full (100%) expression of each of the estimated APP. When stimulated by AP-cytokines produced in rats lacking GC (ADX + T), expression of α_2M and AGP was reduced to 28 and 43% of the maximal value (C + T), respectively, whereas Hp and Fb were fully expressed. The rats supplemented with dexamethasone responded to inflammation (DEX + T) by a further increase in α_2M mRNA to a level double that of the control rats exposed to inflammation (C + T) or treatment with dexamethasone (Fig. 3). In contrast to the impaired expression of AGP in inflamed rats

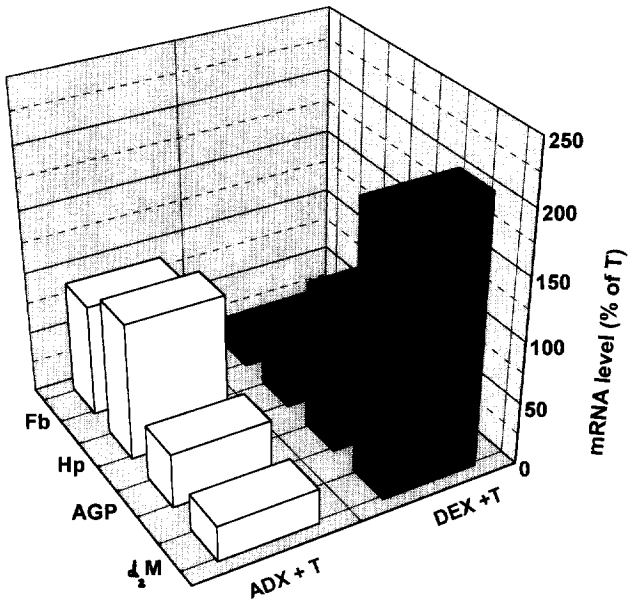

Fig. 4. Inflammation-induced changes in mRNA levels of α_2M, AGP, Hp and Fb in adrenalectomized (ADX + T) and dexamethasone (DEX + T) pretreated rats expressed as the percentage of values for control rats exposed to inflammation.

lacking GC (ADX + T) those supplemented with dexamethasone responded to inflammation (DEX + T) by full expression of this APP. However, under these conditions the levels of Hp and Fb expression were 40 and 80% below those observed for control rats exposed to inflammation (C + T), respectively. Thus, it might be concluded that hormonal demands for maximal production of APP are specific for each of the estimated APP.

DISCUSSION

Hormonal requirements for the complete expression of α_2M, AGP, Hp and Fb in the liver of rats exposed to turpentine-induced inflammation were assessed by measurement of their mRNA levels under conditions of undisturbed functioning of the hypothalamic–pituitary–adrenal axis and when the composition and/or amount of APP inducers were altered by removal of the adrenal glands or injection of dexamethasone. Since a regulatory feedback circuit exists between AP-cytokines and GC hormones in animals and humans [12–14] and both types of mediator act as APP inducers [15], it was reasonable to assume that rats lacking GC would respond to inflammation by enhanced production of AP-cytokines and consequent activation of AP-cytokine inducible APP genes. However, APP species inducible by GC would be preferentially expressed in rats supplemented with an excess of the AP-cytokines suppressor, dexamethasone, before exposure to inflammation. The results showed that Fb and Hp were fully, and AGP incompletely, induced following inflammation in adenalectomized rats, whereas α_2M remained uninduced (Fig. 2). Inflammation in rats supplemented with an excess of dexamethasone elicited an overproduction of α_2M mRNA, incomplete expression of AGP and Hp and basal production of Fb (Fig. 3).

Differently from adrenalectomy, dexamethasone treatment alone altered the pattern of APP expression in uninduced liver wherein mRNAs of α_2M attained the level equal to and those of AGP and Hp 30–40% below, the corresponding values obtained for the acute phase liver of unpretreated rats. In contrast to them, Fb mRNA was 50% lower relative to the basal level. (Figs 3 and 1). This feature differed from that observed for cultured H-35 rat liver cells where dexamethasone alone was an ineffective inducer of the four examined APP [15] and, to a lesser extent, from that obtained for primary hepatocytes where α_2M and AGP [15, 16] but not Hp and Fb [15] were inducible by dexamethasone alone. Inflammation in dexamethasone pretreated rats, which was expected to inhibit or strongly restrict the production of AP-cytokines, did elicit a further increase of α_2M and AGP but not of Hp mRNAs, while Fb mRNA increased to the basal level (Fig. 3). The possibility that the applied dose of dexamethasone failed to inhibit/restrict the production of AP-cytokines disagrees with the observed uninduced state of Fb, as well as with the reported data showing that endotoxin, when infused together with a stress-related dose of GC in the isolated rat liver, acted suppressively on the release of AP-cytokines [5]. However, the possibility that GC hormones are the sole inducer/regulator of α_2M is inconsistent with the data, showing that it was not possible to restore the level of endotoxin-induced increase in α_2M mRNA in rats which were exposed to endotoxin following adrenalectomy and implantation with chronic release pellet containing 25 mg of dexamethasone [6]. Thus, it appears likely that the potency of GC to transactivate α_2M is influenced, apart from AP-cytokines, by some extrahepatic factors affected by both adrenalectomy and inflammation. This implies complex structural/functional characteristics of the GC regulatory unit in the α_2M gene which at the present time is known to contain an IL-6 RE in the promoter region [17–19] and a conserved consensus sequence for a potential GC receptor binding site in the promoter-proximal 5′-flanking region [20].

Apart from confirming the previously reported capability of dexamethasone to induce expression of AGP in the liver of normal rats [4], our results demonstrated that the magnitude of this increase was approximately equal for dexamethasone pretreated rats (Fig. 3) and for adrenalectomized rats exposed to inflammation (Fig. 2) and also that each of the two values was approximately half of that observed for unpretreated rats exposed to inflammation (Fig. 1). This suggests an additive action of GC and AP-cyto-

kines which is in accord with the observed mode of GC and AP-cytokine action in primary hepatocytes [15]. Dexamethasone pretreated rats responded to turpentine treatment by a further increase of AGP mRNA to a level reaching, but not exceeding, that observed in the acute phase liver of unpretreated rats (Fig. 3). This could be related to the structural characteristic of the AGP gene, which apart from cytokine responsive elements [21], contains a complex GC regulatory unit [22–24]. Based on the functional properties of this unit [25], the rat α_1-AGP gene has been classified as a delayed primary response gene regulated by GC through a mechanism involving both the GREs found in genes that respond rapidly to GC and the delayed GREs having sequence characteristic for secondary response genes whose induction requires ongoing protein synthesis [26]. It might be speculated that in the liver of normal rats dexamethasone activates the mechanism involving GREs which respond rapidly to GC whereas the mechanism activated in the state of inflammation involves both types of GREs.

Hp has been defined as class I APP whose full expression in H-35 cells is achieved by additive action of IL-1 and IL-6 and in primary hepatocytes by a combined action of IL-1, IL-6 and dexamethasone [15]. The finding that adrenalectomized rats responded to inflammation by a maximal increase of Hp mRNA suggested that principal inducers of Hp in the rat liver could be AP-cytokines also (Fig. 2). Dexamethasone treatment of normal rats was found to promote a significant increase in Hp mRNA, the magnitude of which remained unchanged following inflammation (Fig. 3). Thus dexamethasone acted as a potent inducer of α_2M and a modest inducer of AGP and Hp in uninduced liver whereas its inducer potency following inflammation became twice as high for α_2M and AGP but unchanged for Hp. Whether the latter can be related with the observed colocalization of GRE function in the Hp gene with the IL-6 responsive element [27], is unclear at the present time.

Gamma fibrinogen, which contains three class II IL-6 responsive elements in the promoter region [28], responded fully to inflammation of adrenalectomized (Fig. 2) but not of dexamethasone pretreated rats (Fig. 3), as if either the amount/composition or the inducer potency of AP-cytokines were impaired in the presence of an excess of dexamethasone. Dexamethasone treatment itself suppressed expression of Fb in uninduced liver so that the effect of inflammation was seen as restoration of the basal level of Fb expression. These data suggest that AP-cytokines are likely to be the sole inducer of Fb in rats, whereas for full expression of Fb in cultured rat cells the presence of both IL-6 and dexamethasone are required [15, 29, 30].

Taken together, our results reveal that the four examined APP can be distinguished by the level and the ratio of GC hormones and AP-cytokines that they require for maximal production. Processes regulating the rate of synthesis of these two types of mediator can be considered, therefore, to be a key part of the mechanisms regulating expression of APP in the liver.

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