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Induction of rat alpha-1-acid glycoprotein by phenobarbital is independent of a general acute-phase response

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Abstract—Phenobarbital (PB) induces transcription of the α1-acid glycoprotein (AGP) gene, one of the major positive acute-phase proteins, the expression of which is controlled by a specific combination of glucocorticoids and cytokines. This raises questions as to the involvement of glucocorticoids and cytokine pathways in the PB-mediated effect on AGP gene expression. We found that the pattern of whole-serum proteins in PB-treated rats differed markedly from that observed during a typical acute inflammatory response (in turpentine-treated rats): levels of some positive acute-phase proteins (APP) increased slightly (α1-acid glycoprotein, haptoglobin, hemopexin and T-kininogen), while levels of α2 macroglobulin, the most sensitive marker of the acute-phase reaction, decreased. Among the negative APP, neither albumin nor prealbumin decreased while CBG increased. The cytokines involved in AGP gene regulation (mainly IL1, IL6 and TNFα) do not therefore seem to mediate the effect of PB on acute-phase protein expression. Glucocorticoid involvement is also ruled out by the observed enhancement of the effect of PB on AGP expression in adrenalectomized animals. Our results suggest that phenobarbital acts on AGP expression by a mechanism independent of the inflammatory pathway.

Key words: phenobarbital; inflammation; α1-acid glycoprotein; cytokines; glucocorticoids; acute phase protein

PB* exerts several effects on hepatocytes, including the induction of various specific forms of cytochrome P450-dependent monooxygenases [1–3]. PB acts at both the transcriptional and post-transcriptional levels [4–7]. In addition, repeated administration of PB leads to an increase in the serum level of AGP in a number of laboratory animals including dogs [8] and rats [9–11]. We recently reported that PB exerts its effects on rat AGP expression mainly at the transcriptional level [12]. AGP is a major APP in humans, rats and mice [13], and considerable progress has been made in recent years in understanding the regulation of APP gene expression. Expression of the AGP gene, like that of other genes encoding proteins involved in the acute-phase reaction, is controlled by a specific combination of the major regulatory mediators, *viz* glucocorticoids [14–16] and a cytokine network involving IL1, IL6 and TNFα [17–22]. None of these factors alone is able to elicit a full increase in the AGP serum level, and a suitable mixture of at least IL1, IL6 and glucocorticoids is required to provide stimulation roughly equivalent to that obtained after turpentine treatment [23–25].

Since PB, like cytokines and glucocorticoids, induces transcription of the AGP gene, the question arises as to whether these mediators could contribute to the effects of PB on AGP gene modulation. To investigate the mechanism responsible for the PB-induced increase in AGP, we compared the serum pattern of rat APP after PB and turpentine treatment; experiments were also conducted on ADX animals to distinguish the PB-induced response from that due to glucocorticoids.

Materials and Methods

Animals. Male Dark Agouti rats (CNRS, Orléans, France) weighing 200–250 g were housed at 21–24° under a 14 hr–10 hr light–dark cycle with laboratory chow (UAR) and tap water *ad lib*. Bilateral adrenalectomy was carried out under mild ether anesthesia and some animals were sham operated. ADX rats were subsequently maintained on 0.9% NaCl as drinking water to prevent serum hormonal and hemodynamic changes resulting from NaCl depletion. ADX animals were allowed to recover from surgery for 1 week and serum levels of corticosterone were checked before treatment.

Treatment. Lyophilized phenobarbital sodium (Gardenal®, Specia, France) was dissolved in apyrogenic sterile normal saline and injected subcutaneously in the interscapulum area daily for 7 days, as 75 mg/kg of PB in a volume of 10 mL/kg; control animals received the solvent alone.

Serum sampling. Blood samples were collected for protein analysis according to the following schedule: before the first injection of PB (or solvent alone); 24 hr after injections 2, 4 and 6; and 24 and 48 hr after the last injection. Some rats received a single subcutaneous injection of 1 mL of turpentine to induce a maximal inflammatory response, and blood samples were collected 48 hr later. Fifty microlitres of blood was taken by the retroorbital route by capillarity using an apyrogenic and non-inflammatory method. Blood was allowed to clot at room temperature, centrifuged and serum samples were stored at −20° until use.

Analytical procedures. The patterns of whole-serum proteins were determined by crossed immuno-electrophoresis with anti-whole rat serum antibodies and expressed as the relative changes in positive and negative APP concentrations [26]. AGP, α_2M, TK, Hpx, Hp, α1-PI, CBG, prealbumin and albumin were determined in serum samples by means of rocket immunoelectrophoresis using monospecific antisera. Results were expressed as variations relative to control values and tested for differences by Student's *t*-test; P values of ≤ 0.05 were considered significant.

Bioactive IL6 was measured in rat serum by using the IL6-dependent B9 hybridoma cell-line (27). B9 cells were cultured for 72 hr in the presence of serial dilutions of serum in 96-well plates. B9 cell proliferation was estimated

* Abbreviations: PB, phenobarbital; AGP, α1-acid glycoprotein; APP; acute-phase proteins; ADX, adrenalectomized; α_2M, α_2-macroglobulin; TK, T-kininogen; Hpx, hemopexin; Hp, haptoglobin; α1-PI, α1-proteinase inhibitor; CBG, corticosterone binding globulin; LIF, leukemia inhibitor factor; HGF, hepatocyte growth factor.

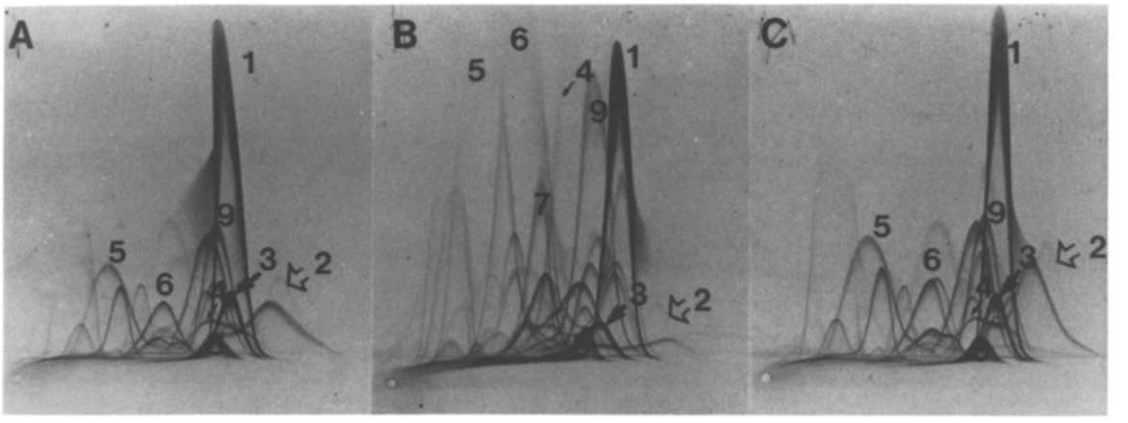

Fig. 1. Crossed immunoelectrophoresis of serum from control (A), turpentine-treated (B) and PB-treated (C) rats. (1) albumin; (2) corticosterone binding globulin (CBG); (3) α1-inhibitor III; (4) T-kininogen; (5) hemopexin; (6) haptoglobin; (7) α_2-microglobulin; (8) α_1-macroglobulin; (9) α1-proteinase inhibitor.

after 72 hr of incubation using the MTT colorimetric assay (28). HrIL6 was used as an internal standard in all assays.

Results

Quantification of serum proteins after PB or turpentine treatment of normal rats. Figure 1 shows the patterns of proteins in the sera of control animals 48 hr after seven injections of PB and 48 hr after a single injection of turpentine. Crossed immunoelectrophoresis of control (pattern A) and turpentine-treated (pattern B) animals analysed with polyspecific antibodies show very clear differences, distinguishing the inflammatory pattern from the "healthy" pattern; i.e. an increase in TK, α1-PI, Hp, α2M and Hpx, and a decrease in albumin, α1-I3 and CBG.

Pattern A (control) and pattern C (PB-treated) are similar, with no signs of inflammation (in particular, no marked increase in TK or decrease in α1-I3, albumin or CBG). More precise quantitation of positive and negative APP with specific antibodies showed that, 48 hr after 7 days of PB treatment, the serum concentrations of some APP (AGP, Hp, Hpx and TK) were elevated (Fig. 2), but to a lesser extent than in inflamed rats; the largest increase concerned AGP (4.3-fold). The serum level of α1-PI did not vary significantly after PB treatment, contrasting with the increase obtained after turpentine injection. Furthermore, α_2M, the most sensitive positive marker of the acute-phase reaction, increased sharply in inflamed rats but decreased slightly yet significantly in PB-treated rats. Among the negative APP, neither albumin nor prealbumin decreased significantly, and CBG [29], a protein down-regulated by glucocorticoids [30], increased. These results indicate that the ability of PB to modulate APP expression is limited to a few proteins and suggest that PB administration does not initiate a typical acute inflammatory response.

Determination of bioactive IL6 concentrations in serum after PB treatment of normal rats. Serum levels of bioactive IL6 were approx. 6 pg/mL before, during and after treatment with 0.9% NaCl and were not modified by PB.

AGP and CBG serum levels during PB treatment in sham-operated and ADX animals. Figure 3 shows that the PB-induced increase in AGP serum levels was potentiated in ADX rats. Basal serum levels of AGP (mean 160 mg/L) were reduced by 30% relative to normal controls (220 mg/L), suggesting that endogenous glucocorticoids are required to maintain basal expression of the AGP gene. In the control rats (ADX and non-ADX) receiving 0.9% NaCl, only slight variations were observed. In contrast, AGP increased throughout PB treatment; the increase was moderate and reached 600 mg/L in sham-operated rats (three-fold increase), whereas it was rapid and reached 1200 mg/L (7-fold increase) in ADX rats.

These data clearly show that PB can act independently of glucocorticoids on AGP gene expression. Furthermore, it appears that endogenous glucocorticoids partially attenuate sensitivity to PB induction *in vivo*.

Discussion

During the acute inflammatory process elicited by turpentine in the rat, the production and plasma concentration of a number of proteins increased (AGP, α_2M, Hp, Hpx, TK, etc.), whereas that of others is decreased (albumin, CBG, prealbumin, α1I3, etc.) [31–32]. Irrespective of the cause of inflammation, expression of the genes encoding these proteins changes; this expression is mainly under the control of a network of cytokines (IL1, IL6, TNFα and HSF III/LIF) [17–22, 33, 34] and glucocorticoids [14–16].

In previous studies we found that the increased serum levels of AGP after PB treatment are paralleled by an increase in AGP mRNA [12]. Although a post-transcriptional mechanism cannot be ruled out, nuclear run-on transcription results suggested that PB acted mainly at the transcriptional level. IL1, IL6 and glucocorticoids also exert their inductive effects on the AGP gene at this level [35–37], raising the possibility that the action of PB is mediated by inflammatory glucocorticoid and/or cytokine pathways.

Our results clearly indicate that the plasma protein pattern obtained after PB treatment differs strongly from that observed during a typical acute inflammatory response. The serum concentrations of some positive APP (AGP, Hpx, Hp and TK) increased less strongly after PB treatment than after turpentine challenge. PB failed to influence any of the other positive APP (αPI and α_2M, despite a 225-fold increase in the latter after turpentine treatment) or negative APP (albumin, prealbumin and α1I3). CBG, a negative APP [26, 29], increased during PB treatment. The absence of any effect on α_2M or negative APP, together with the opposite response of CBG to PB and turpentine, clearly indicate that PB does not induce a typical acute inflammatory response.

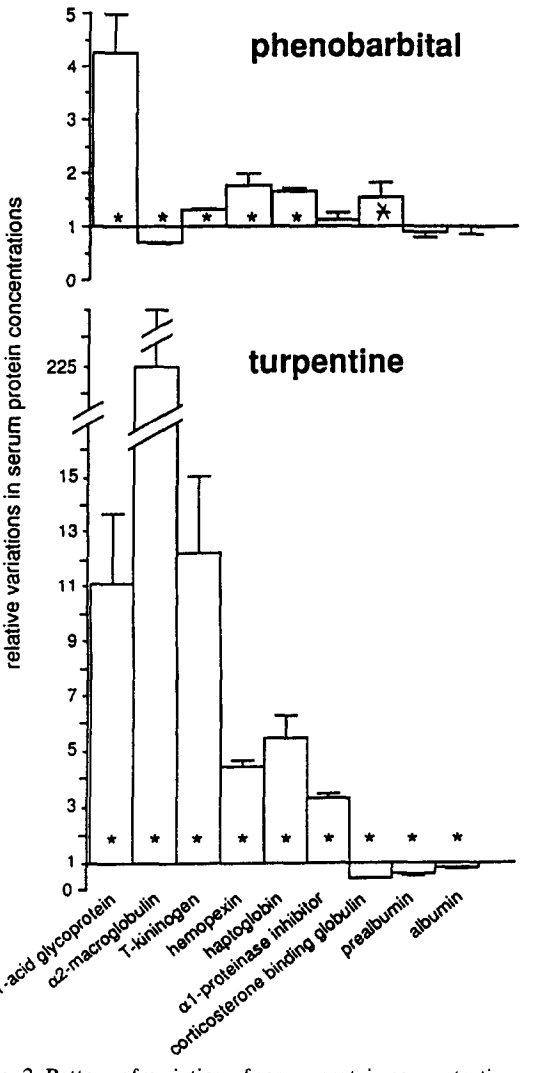

Fig. 2. Pattern of variation of serum protein concentrations 48 hr after seven injections of phenobarbital or a single injection of turpentine. Serum samples were collected 48 hr after the last injection of phenobarbital or turpentine. Control rats received 0.9% NaCl. Results are means ± SD of three rats per group and are expressed as variations relative to the controls.

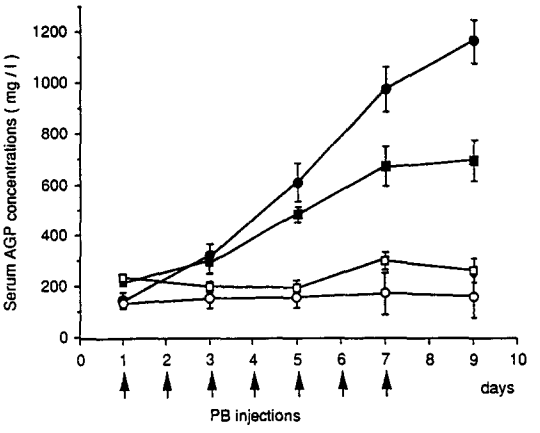

Fig. 3. Effect of adrenalectomy on the PB-induced increase in AGP serum levels. Adrenalectomized rats (circles) and control rats (squares) received 0.9% NaCl (○, □) or PB 75 mg/kg/day for 7 days (●, ■) and aliquots of blood were taken at day 1, 3, 5, 7 and 9 for serum protein determination. Results (mg/L) are means ± SD (three animals) for PB-treated and NaCl-treated control rats. Values for NaCl-treated ADX rats are means ± range (two animals).

APP have been divided into two major classes depending on the cytokines involved in their induction [24]. There are also remarkable differences in the hormonal regulation of a given specific APP within each class [24]. AGP, which belongs to class I, can be induced in the apparent absence of glucocorticoids, although α_2M (representative of class II) cannot. The significant increase in the serum concentration of the class I APP (AGP, Hp and Hpx) suggest the participation of IL1 and/or TNFα. In contrast, the fact that the serum concentrations of the class II proteins α_2M and α1PI, neither of which are modified by PB treatment, are strictly regulated by IL6, argues against the involvement of this cytokine in the PB effect. However, if PB acts on macrophage-monocytes and stimulates IL1 production, the production of IL6 by the same cells should also be stimulated.

Another argument against the involvement of cytokines in PB-mediated effects is the well-known induction of CytP450IIB, whereas IL1 [38, 39] and IL6 [40] have been reported to depress expression of these genes. Moreover, the possibility that PB induction of the CytP450IIB gene could be minimized by weak concomitant cytokine release is not supported by our observation that PB treatment did not influence serum levels of bioactive IL6.

Results obtained in ADX animals showed the need for endogenous glucocorticoids to maintain a basal regular level of circulating AGP. Unexpectedly however, the effect of PB on AGP expression was enhanced in ADX animals, ruling out the involvement of glucocorticoids in PB action. Similar results have previously been reported, showing that glucocorticoids also negatively modulate the effect of PB on CytP450IIB gene expression [41]. These data are also reminiscent of those reported by Yiangou *et al.* [42], who found that heavy metals such as Hg strongly induced the mouse AGP gene in the absence of glucocorticoids. As suggested by these authors, this may be due to the structure of the AGP gene promoter.

During the last 5 years numerous studies have been conducted to evidence nucleotidic consensus sequences in the AGP gene promoter as well as transcriptional factors, both of which are involved in transcriptional regulation of the AGP gene. While distal regulatory elements responsive to IL1, IL6, TNFα, LIF and phorbol esters have been located far upstream (nucleotides −5300 to −5150), [35] a single glucocorticoid responsive element (GRE) has been evidenced downstream (−120 to −105) of the AGP gene promoter [36, 43]. Moreover, it is now known that GRE alone is not sufficient to activate transcription of the AGP gene and that other sequences in the immediate vicinity are required. These include the binding sites for ANF2 (C/EBP, IL-6DBP) and ANF1 located in the close downstream region (−105 to −65) [44]. Recently, using gel shift assays, we identified a 10 bp sequence located very close to GRE and potentialy involved in AGP induction by PB (submitted for publication). This 10 bp consensus sequence has previously been described in the promoter of the CytP450IIB1-2 gene, and transfection assays have shown that it is involved in gene regulation [45]. The induction of the AGP gene by PB could thus be mediated by the interaction of specific transacting factors with the 10 bp

consensus sequence in the AGP promoter region. Owing to the proximity of the GRE consensus sequence, the presence of glucocorticoids might thus prevent or reduce simultaneous binding of these PB-transacting factors, as evoked for AGP induction by heavy metals [42].

Our results suggest that PB could act on AGP gene expression by a mechanism independent of the inflammatory pathway (i.e. glucocorticoid- and cytokine-dependent). The direct effects of PB have been described on other proteins not involved in the metabolism of foreign compounds. This includes genes involved in the tumor-promoting effects of phenobarbital, such as HGF [46], TGF-β1 [47], proto-oncogenes *Ki-ras*, *fos* and *myc* [48], and the ferrochelatase gene [49]. The biological significance of the induction of an acute-phase protein gene (AGP) by PB is not clear, but comparison of the molecular mechanisms involved in the induction of the AGP gene with that of other PB-inducible genes may provide further insight into the complex array of regulatory pathways that govern the PB-inducible expression of various genes [50].

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