Biochem. J. (1993) 290, 637–640 (Printed in Great Britain)

RESEARCH COMMUNICATION

Phenobarbital induction of AP-1 binding activity mediates activation of glutathione S-transferase and quinone reductase gene expression

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Phenobarbital is an inducer of xenobiotic-metabolizing enzymes, such as cytochrome *P*-450, glutathione S-transferases (GSTs) and NAD(P)H:quinone reductase, as well as being a promoter of hepatocarcinogenesis. The molecular mechanisms regulating these biological activities are, however, unknown. In this paper we show that induction by phenobarbital of GST Ya and quinone reductase gene expression is mediated by regulatory elements, EpRE and ARE respectively, which are composed of two adjacent AP-1-like binding sites. EpRE was recently found to be activated by a Fos/Jun heterodimeric complex (AP-1). Here we show that phenobarbital induces an increase in AP-1 binding activity in nuclear extracts of cultured hepatoma cells. Furthermore, we observe that the induction of chloramphenicol acetyltransferase (CAT) activity from an EpRE Ya-cat gene construct and of AP-1 binding activity by phenobarbital is inhibited by the thiol compounds *N*-acetyl-L-cysteine and glutathione. These results suggest that the phenobarbital induction of AP-1 activity, leading to the AP-1-mediated transcriptional activation of the GST Ya and quinone reductase genes, may involve production of reactive oxygen species and an increase in intracellular oxidant levels, which is prevented by thiol compounds. In view of the involvement of AP-1 in the control of cell proliferation and transformation, the induction by phenobarbital of AP-1 binding activity observed here provides a possible molecular mechanism for the tumour-promoting activity of this drug.

INTRODUCTION

The induction of xenobiotic metabolism in mammalian liver by barbiturates is a well-established phenomenon. In rat liver, barbiturates induce specific cytochrome *P*-450 species [1,2], as well as the electrophile-metabolizing enzymes glutathione S-transferase (GST) [3,4] and NAD(P)H:quinone reductase [5]. The induction of these enzyme activities by phenobarbital was shown to involve the transcriptional activation of the expression of their respective genes, leading to a several-fold increase in mRNA levels [4–6]. In addition to being an inducer of gene expression, phenobarbital, like a number of other chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), phorbol esters and polychlorinated biphenyls, was reported to function in rodents as a hepatocarcinogenesis-promoting agent [7].

In an attempt to elucidate the mechanisms involved in the mammalian cell response to barbiturates, we have studied the induction of GST Ya subunit gene expression by phenobarbital. This chemical was found to increase GST Ya mRNA levels in rat liver [8] and in cultured hepatoma H4II cells [9] by 3–5-fold. Previous studies have shown that a single *cis*-regulatory element in the 5′-flanking region of the GST Ya gene is responsible for the induction of gene expression by a variety of xenobiotics such as planar aromatic (e.g. 3-methylcholanthrene, β-naphthoflavone, TCDD) and electrophilic (e.g. *trans*-4-phenyl-3-buten-2-one, t-butyl hydroquinone, dimethyl fumarate) compounds [10], as well as by phorbol 12-myristate 13-acetate (PMA) [11]. This DNA regulatory element, known as EpRE, was found to be composed of two adjacent AP-1-like binding sites and to be regulated by the binding of protein products of the c-*fos* and c-*jun* proto-oncogenes, forming the Fos/Jun heterodimeric (AP-1) complex [11]. Regulatory elements termed ARE, similar in structure to EpRE, have been described in rat GST Ya [12] and rat quinone reductase [13] genes, and were shown to confer inducibility by β-naphthoflavone and phenolic antioxidants.

In this paper we provide evidence that induction by phenobarbital of GST Ya and quinone reductase gene expression is mediated by the EpRE and ARE enhancers respectively. We also show that phenobarbital induces an increase in AP-1 binding activity in nuclear extracts of cells exposed to this drug. This induction, together with the involvement of the AP-1 transcription factor in cell proliferation, differentiation and transformation [14], may provide a molecular mechanism to account for the role of phenobarbital as tumour promoter in hepatocarcinogenesis.

MATERIALS AND METHODS

Cell culture, transfection and chloramphenicol acetyltransferase (CAT) assays

HepG2 hepatoma cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum as described [9]. Transfections were carried out by incubation of cells for 6 h with calcium phosphate-precipitated plasmid DNAs (5 μg of test plasmid, 5 μg of RSV*gal* internal control plasmid and 10 μg of pGEM-1 carrier DNA plasmid per 10 cm dish). After transfection, the cells were glycerol-shocked for 1 min, supplemented with fresh medium containing 10% fetal calf serum and, after a 12 h recovery at 37 °C, exposed to xenobiotic inducers. Protein extracts were prepared 16–24 h after induction. CAT activities were measured as described [9] and were calculated after normalizing each assay relative to the internal control of β-galactosidase activity expressed from the cotransfected RSV*gal* vector. All

Abbreviations used: CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; NAC, *N*-acetylcysteine; PMA, phorbol 12-myristate 13-acetate; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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CAT activity data are derived from three separate transfection experiments.

Oligonucleotides and plasmid constructs

Synthetic oligonucleotides containing the 41 bp EpRE region between nucleotides −754 and −714 of the GST Ya gene [10], the ARE xenobiotic-inducible element of the rat quinone reductase gene [13] (TCTAGAGTCACAGTGACTTGGCA), the EpRE motif mutant 5′ gatcGAGACAAAGCA and the AP-1 consensus sequence 5′ gatcATGACTCGCA (where AP-1-like sequences are underlined and lower-case letters represent cloning linkers) were prepared by O. Goldberg (Weizmann Institute). To serve as probes for AP-1 binding activity in electrophoretic mobility shift assays, the double-stranded AP-1 consensus sequence, cloned into the *Bam*HI site of pGEM-1 (Promega) and excised by *Xba*I and *Eco*RI, was labelled at the 5′ protruding ends by [α-^{32}P]dATP and Klenow DNA polymerase. The EpRE Ya-cat and ARE Ya-cat plasmids were constructed by ligation of the EpRE or ARE oligonucleotides respectively into the −187 site of the GST Ya gene minimal promoter driving the expression of the CAT gene [10], and were transfected for transient expression into HepG2 cells.

Electrophoretic mobility shift assays

HepG2 cells were grown on 100 mm plates and, after 6 h of exposure to phenobarbital, were used to prepare nuclear extracts [15]. Mobility shift assays were performed as described previously [11] using 10 μg of nuclear extract.

RESULTS

Phenobarbital-inducible elements of GST Ya and quinone reductase genes

Since earlier work has demonstrated that the inducible expression of the GST Ya [10] and quinone reductase [13] genes by a variety of planar aromatic and electrophilic compounds is mediated by regulatory sites with similar structures, i.e. EpRE and ARE respectively (Figure 1), it was of interest to determine whether the same elements are also responsible for induction by barbiturates. The plasmid constructs EpRE Ya-cat and ARE Ya-cat, containing the EpRE or ARE enhancer linked to the minimal Ya promoter (nt −187) driving CAT expression [10], were transfected into HepG2 cells. After exposure of the transfected cells to phenobarbital, extracts were prepared and CAT activities measured. The transfection experiments included the RSV*gal* plasmid as an endogenous control, and all CAT activities were normalized against β-galactosidase activity. Figure 2 shows that phenobarbital activates the GST Ya gene promoter via the EpRE enhancer, increasing by 4–5-fold the expression of CAT activity. Similarly, the quinone reductase ARE enhancer promotes a 3–4-fold increase in Ya-cat activity by phenobarbital.

EpRE (GST Ya) 5′-AA**TGACATTGC**TAATGG**TGACAAAGC**AA-3′

ARE (quinone reductase) 5′-GT**CTAGAGTCACAGTGACTTGGC**AA-3′

Figure 1 Structures of the regulatory elements EpRE and ARE

EpRE is derived from mouse GST Ya [10] and ARE is derived from the rat quinone reductase [13] gene. Bold letters indicate the AP-1-like sequences, and arrows show their orientation.

Induction of AP-1 binding activity by phenobarbital

To test whether induction of GST Ya and quinone reductase gene expression is mediated by an increase in AP-1 binding activity, hepatoma HepG2 cells were exposed to phenobarbital for 6 h, and nuclear extracts were prepared and analysed by electrophoresis mobility shift for AP-1 binding activity using an AP-1 binding site oligonucleotide probe. Figure 3(a) shows that, compared with the nuclear extract from uninduced cells, exposure to phenobarbital resulted in a marked increase in AP-1 binding activity. The competition experiment shown in Figure 3(b)

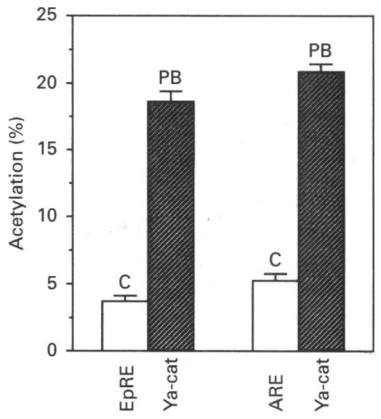

Figure 2 Phenobarbital-inducible elements of the GST Ya and quinone reductase genes

DNA (5 μg) from the EpRE Ya-cat or ARE Ya-cat plasmid constructs (containing the EpRE or ARE chemical-inducible enhancer sequences of the mouse GST Ya or rat quinone reductase gene respectively), 5 μg of RSV*gal* internal control and 10 μg of pGEM-1 DNA carrier were cotransfected in HepG2 cells. After transfection, the cells were untreated (C) or exposed for 6 h to 2 mM phenobarbital (PB), then washed and incubated for 18 h in fresh medium. Extracts were prepared and assayed for β-galactosidase and CAT activities; all CAT activities were normalized to β-galactosidase.

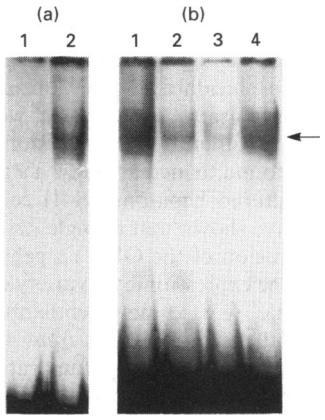

Figure 3 Induction of AP-1 binding activity by phenobarbital

(**a**) HepG2 cells were exposed for 6 h to 2 mM phenobarbital, nuclear extracts were prepared and electrophoretic mobility shift assays were carried out as described [11] using 10 μg protein samples and 2×10^4 c.p.m. of ^{32}P-labelled AP-1 oligonucleotide probe. (**b**) Specificity of the induced AP-1 binding activity. Nuclear extracts (10 μg) from 2 mM phenobarbital-treated HepG2 cells were incubated with 2×10^4 c.p.m. of ^{32}P-labelled AP-1 oligonucleotide probe in the absence (lane 1) or presence of the following unlabelled oligonucleotides: 100 × molar excess of AP-1 (lane 2) or EpRE (lane 3), or a 200 × molar excess of the T → A mutant of the EpRE motif (AGACAAAGC) (lane 4). The arrow indicates the AP-1 complex.

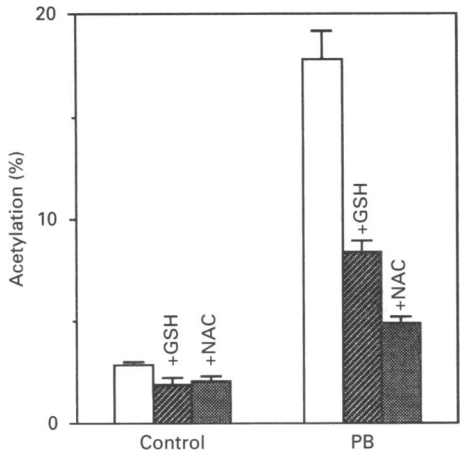

Figure 4 GSH and NAC inhibit activation of EpRE Ya-cat gene expression by phenobarbital

EpRE Ya-cat plasmid DNA (5 μg), 5 μg of RSV*gal* internal control and 10 μg of pGEM-1 DNA carrier were cotransfected in HepG2 cells. The cells were exposed for 60 min to 10 mM GSH or 30 mM NAC, followed by 6 h treatment with 2 mM phenobarbital (PB) as indicated. Cells were then washed and incubated with fresh medium for 18 h. CAT activities were measured and normalized relative to internal β-galactosidase activity. All CAT activity data are derived from three separate transfections.

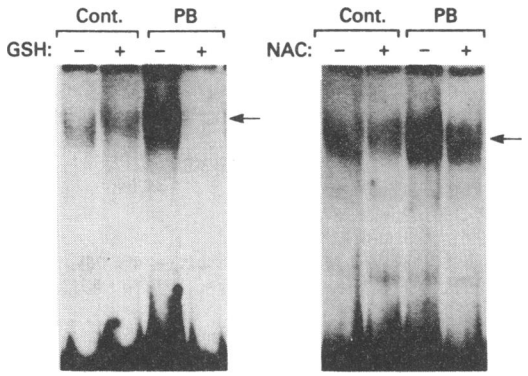

Figure 5 GSH and NAC inhibit induction of AP-1 binding activity by phenobarbital

HepG2 cells were untreated (Cont.) or treated for 6 h with 2 mM phenobarbital (PB) after 60 min of exposure to 10 mM GSH or 30 mM NAC as indicated. Gel shift assays were carried out using 10 μg nuclear extract samples and 2×10^4 c.p.m. of ^{32}P-labelled AP-1 oligonucleotide probe. The arrow indicates the AP-1 complex.

indicates that the phenobarbital-induced AP-1 binding activity is specific for the AP-1 as well as for the EpRE binding sites. Thus lanes 2 and 3 show that both the AP-1 and EpRE oligonucleotides compete with the binding of the AP-1 complex to the ^{32}P-labelled AP-1 oligonucleotide probe. There is no competition, however (lane 4), by an oligonucleotide containing a T → A mutation at position 1 of the EpRE motif (AGACAAAGC), which has no basal or inducible activity [11].

GSH and *N*-acetylcysteine (NAC) inhibit induction of EpRE Ya-cat and AP-1 binding activity by phenobarbital

The effect of thiol compounds on the induction of the EpRE Ya-cat gene and AP-1 binding activity by phenobarbital was studied. HepG2 cells were transfected with the EpRE Ya-cat construct and exposed for 60 min to NAC or GSH prior to induction by phenobarbital. Figure 4 shows that the induction of CAT activity from the EpRE Ya-cat construct by phenobarbital is inhibited by pretreatment of cells with GSH or NAC.

To test whether the inhibition of EpRE Ya-cat induction by thiol compounds is due to a block in the induction of AP-1 binding activity, nuclear extracts were prepared from HepG2 cells treated with phenobarbital in the absence or presence of GSH or NAC. AP-1 binding activities were then compared by electrophoresis mobility shift assays. Figure 5 indicates that the induction of AP-1 binding activity by phenobarbital was blocked in the presence of GSH or NAC.

DISCUSSION

The present study provides evidence for a molecular mechanism by which barbiturates could activate the expression of the electrophile-metabolizing enzymes GSTs and NAD(P)H: quinone reductase, and promote hepatocarcinogenesis. We show that the phenobarbital-inducible expression of the GST Ya subunit and quinone reductase genes is mediated by the same regulatory elements (composed of the two adjacent AP-1-like binding sites [11]), i.e. EpRE and ARE, that were previously found to be responsive to induction by a variety of chemical agents such as planar aromatic hydrocarbons, diphenols, PMA and electrophilic compounds [10,13].

Previous studies have demonstrated that the EpRE is activated by Fos/Jun heterodimeric complex binding [11], and a correlation between the induction of GST Ya gene expression and an increase in AP-1 binding activity by various chemicals was recently observed (S. Bergelson, R. Pinkus and V. Daniel, unpublished work). The present results indicate that exposure of cells to phenobarbital induces a 3–5-fold increase in AP-1 binding activity compared with untreated cells.

The AP-1 complex seems to play a general role in signal transduction from the membrane to the nucleus. There is ample evidence that the rapid and transient induction of AP-1 activity by a variety of extracellular stimulatory agents (e.g. growth factors, hormones, neurotransmitters, u.v. irradiation) enables the transcription of AP-1-dependent genes and plays an important role in cell proliferation, differentiation and neoplastic transformation [14,16,17]. The fundamental role of AP-1 in cell proliferation is indicated by the increases in AP-1 activity and AP-1-dependent gene expression during normal cell proliferation and upon induction of neoplastic transformation by viral oncogenes, carcinogens and tumour promoters. The present data, indicating that phenobarbital induces an increase in AP-1 activity, may explain previous observations [7] on the role of phenobarbital in promoting hepatocarcinogenesis.

The finding that phenobarbital is one of the many chemical agents of diverse structure that activate GST Ya and quinone reductase gene expression through the induction of Fos/Jun heterodimeric (AP-1) complex binding to enhancers containing AP-1-like sequences (see Figure 1) raises the question of the transduction signal serving as the ultimate inducer of AP-1 activity. There is convincing evidence which implicates cellular pro-oxidant states or increased concentrations of active oxygen, organic peroxides and radicals in promoting neoplastic transformation [18,19]. Many tumour promoters and carcinogens have been demonstrated to induce conditions of oxidative stress that can be prevented by the enzymes of cellular antioxidant defence (superoxide dismutase, GSH-dependent peroxidase, catalase), low-molecular-mass thiol scavenger molecules (GSH, NAC) and antioxidants [18]. An involvement of active oxygen in

the induction of AP-1 activity and GST Ya gene expression by phenobarbital is supported by the observed inhibitory effect of the thiol compounds GSH and NAC (Figures 4 and 5). GSH is known to play a major role in maintaining the intracellular redox potential by removing reactive oxygen species by direct reaction, scavenging or via the GSH peroxidase/GSH system [20], and NAC acts by raising the intracellular levels of cysteine (and hence of GSH) and by scavenging oxygen radicals [21]. Any increase in intracellular oxidant levels would result in a consumption of reduced GSH and low thiol levels. The present findings suggest that phenobarbital induction of AP-1 activity, leading to the AP-1-mediated transcriptional activation of the GST Ya and quinone reductase genes, occurs at low thiol levels created by the generation of intracellular oxidants, since this induction can be prevented by increasing the intracellular thiol levels. The mechanisms by which phenobarbital may induce the formation of active oxygen are unknown, although a possible perturbation of the mitochondrial and microsomal cytochrome *P*-450 electron-transport chain has been suggested [18].

In vitro studies have shown that the DNA-binding activity of the Fos/Jun heterodimer is regulated by the redox of a cysteine residue in the DNA-binding domains of the two proteins [22] and have identified a nuclear protein that, by reducing this cysteine, modulates the DNA-binding activity of these proteins [23]. These observations would imply that the AP-1 complex requires a reducing environment for DNA binding and activation of gene expression. The present results, however, indicate that phenobarbital induction of AP-1 binding activity and AP-1-mediated transcriptional activation of the GST Ya gene involves an increase in intracellular oxidant levels. In the absence of direct information about the mechanism by which phenobarbital induces AP-1 binding activity, one can only speculate that these apparently contradictory aspects of the redox regulation of gene expression may be due to the different levels, i.e. Fos/Jun expression or function, at which this regulation takes place.

The results presented here clearly establish that phenobarbital induces increased levels of AP-1 binding activity and activates the phase II xenobiotic-metabolizing enzyme GST Ya and quinone reductase genes via regulatory elements containing AP-1-like binding sites. We assume that the phenobarbital-inducible cytochrome *P*-450 genes (*P-450b* and *P-450e*) may be regulated by a similar mechanism. The regulatory sequences of these cytochrome *P*-450 genes, however, require further characterization in order to determine an involvement of the AP-1 transcription factor in their expression. Several molecular mechanisms have been proposed to account for the transcriptional activation of *P*-450 genes by phenobarbital (for a review, see [24]). Future studies on the induction of AP-1 activity by phenobarbital should clarify the mechanism by which this chemical agent increases intracellular oxidant levels and the molecular events involved in the redox activation of the AP-1 transcription factor.

This work was supported in part by grants from the Basic Research Foundation of the Israel Academy of Sciences and Humanities and the Leo and Julia Forscheimer Center for Molecular Genetics.

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Received 16 December 1992/12 January 1993; accepted 14 January 1993