Regulation of cytochrome P-450 CYPIA1 gene expression and proto-oncogene expression by growth factors in primary hepatocytes

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Received 12 June 1990

The effect of growth factors on the cytochrome P-450 (CYPIA1) gene expression was studied in primary mouse hepatocytes. Of the three growth factors used, i.e. epidermal growth factor (EGF), transforming growth factor α (TGFα) and insulin, only EGF or TGFα completely blocked CYPIA1 expression in the presence of the CYPIA1 inducer 3-methylcholanthrene (3-MC). This repression was not linked to cell cycle progression of the hepatocyte because insulin was active to induce 'early immediate genes' and DNA replication as well as EGF/TGFα but failed to suppress CYPIA1 expression. A specific EGF/TGFα receptor-mediated function may repress CYPIA1 gene expression and contribute to the acquisition of a xenobiotic drug resistance phenotype.

Hepatocyte; Growth factor; Cytochrome P-450 gene regulation

1. INTRODUCTION

Exposure of rats to carcinogens followed by tumor promoters induces macroscopic liver foci referred to as hyperplastic liver nodules [1,2]. These liver cell populations are regarded as preneoplastic and as possibly diploid progenitor cells of hepatocellular carcinoma showing significant changes in the proliferation pattern compared to normal hepatocytes [3,4]. Furthermore, the altered growth state is accompanied by rapid loss of nearly all cytochrome P-450 (CYP)-dependent activities leading to the xenobiotic drug resistance phenotype [5–7]. Until now, there are no data which could demonstrate a direct link between the state of proliferation of the hepatocyte and the mechanism of CYP repression. Recent results could show that TGFα acts as an autocrine growth factor in the regeneration step of hepatocytes after hepatectomy [8] and in hepatoma formation [9]. The process of liver regenerative growth in many aspects resembles the events in hyperplastic liver growth [10,11] suggesting that TGFα may play a crucial role also in CYP repression. In the present study we used an in vitro system of primary mouse hepatocytes which can be growth-stimulated by addition of growth factors such as EGF, TGFα and insulin. The expression of 'immediate early genes' like c-*fos*, c-*jun* or c-*myc* followed by DNA replication was found to be induced by EGF, TGFα or insulin to a similar extent. However, CYPIA1 gene expression was inhibited only by an EGF/TGFα receptor-mediated function.

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Abbreviations: EGF, epidermal growth factor; TGFα, transforming growth factor α; 3-MC, 3-methylcholanthrene; CYP, cytochrome P-450; EROD, 7-ethoxyresorufin-*O*-deethylase

2. MATERIALS AND METHODS

Adult C57BL6 mouse hepatocytes were isolated by collagenase perfusion and differential centrifugation [12]. Cells were cultured in serum-free MX-82 medium as described [13]. For Northern blot analysis of oncogene expression cells were washed and directly lysed in the culture dishes by 4 M guanidinium isothiocyanate/2-mercaptoethanol and total RNA was isolated by CsCl gradient centrifugation [14]. The c-*fos*-specific probe was a 1.1 kb *Pst*I fragment of pv-*fos*1 [15], the c-*myc*-specific probe a v-*myc* (MC29) 1.2 kb *Pst*I insert of pMC-Pst [16]. Northern blot analysis was described in detail elsewhere [17]. DNA probes were labeled with [^{32}P]dCTP by random oligonucleotide priming [18].

Cytochrome P-450 (CYPIA1) was purified from β-naphthoflavone-induced rat liver microsomes as described [19]. The antibody was produced in rabbits and purified by antigen-coupled Sepharose-4B (Pharmacia) using usual procedures. Hepatocyte microsomes were prepared by differential centrifugation [20]. Samples of 25 μg microsomal proteins were subjected to SDS-gel electrophoresis (SDS-PAGE) after denaturation by boiling according to Laemmli [21]. Proteins were transferred electrophoretically to nitrocellulose membranes (Amersham) by semi-dry blotting and incubated with anti-rat CYPIA1 antibody [19] in a 1:500 dilution. Detection was achieved with secondary anti-rabbit [^{125}I]Ig-F(ab)$_2$ (Amersham). In case of *FOS* analysis Western blots were carried out with an epitope-specific *FOS* anti-serum α-*fos*-454 [22] after immunoprecipitation of *FOS* from whole cell lysates with the same antibody followed by detection with Auro probe BL plus anti-rabbit IgG (Janssen) and silver enhancement. 7-Ethoxyresorufin-*O*-deethylase activity (EROD) was measured in microsomes [23], while CYP content was determined by CO difference spectroscopy [24]. [^{3}H]Thymidine incorporation into DNA was measured as described [13]. Insulin and rEGF was purchased from Boehringer (Mannheim, FRG), while TGFα, synthetic, was from Bachem (Basel, Switzerland).

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00145793/90/$3.50

Table I

Suppression of CYP activities by EGF or TGFα

Hepatocyte culture	CYP ± SD (nmol CYP/mg protein)	EROD ± SD (nmol EROD/min/mg protein)
0 h, control	0.83 ± 0.05	0.13 ± 0.01
3 days, control	0.17 ± 0.02	0.02 ± 0.01
3 days, 3-MC	0.46 ± 0.03	0.98 ± 0.02
3 days, 3-MC/EGF	0.18 ± 0.02	0.04 ± 0.01
3 days, 3-MC/TGFα	0.16 ± 0.02	0.03 ± 0.01

Hepatocytes were cultured with 3-MC or without (control) in the presence of EGF (10^{-8} M) or TGFα (10^{-8} M) for the times as indicated. CYP and EROD activities were obtained from 4 independent experiments.

3. RESULTS AND DISCUSSION

3.1. *Effects of growth factors on CYPIA1 expression*

In the present serum-free primary culture system, the content of total CYP decreased to 20% of that in freshly prepared cells within 3 days (Table I). However, in the presence of 3-methylcholanthrene (3-MC), a classical inducer of CYPIA1 [25,26], the CYP content decreased only to 50% of the original level, and a drastic increase in the ethoxyresorufin-*O*-deethylase activity (EROD), which is specific for CYPIA1 activity [23], was observed. This is due to the induction of CYPIA1 as confirmed by Western blot analysis (Fig. 1). Similar decrease of total CYP content and induction of CYPIA1 by 3-MC have been published using rat hepatocyte primary culture [27]. In our system CYPIA1 induction was not significantly affected in the presence of insulin (10^{-8} M). However, EGF and EGF in combination with insulin (Fig. 1) or TGFα alone (Table I) almost totally suppressed the induction of CYPIA1 by 3-MC. In regenerating liver [28], in preneoplastic liver lesions [28] and in hepatoma tissues of rodent and man [29,30] low levels of CYP-dependent activities were found. Furthermore, also the induction of CYP is suppressed in regenerating liver [31]. TGFα binds to the EGF receptor and may be directly involved in the control of liver size under regenerative growth [9]. Recent data suggest also a direct role of TGFα in the autocrine growth of hepatoma [8]. TGFα may, therefore, be involved in the suppression of CYPIA1 gene expression under these conditions.

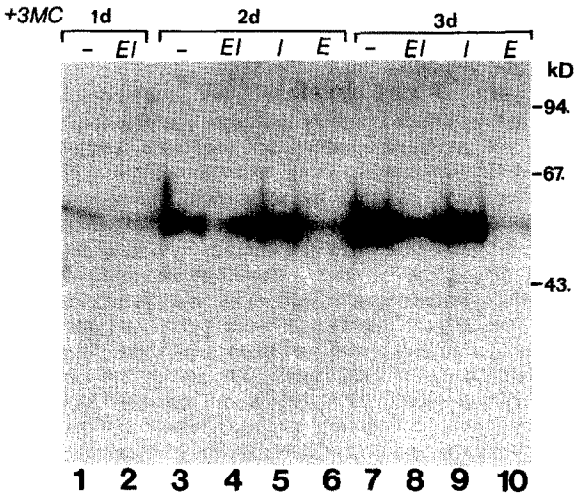

Fig. 1. Western blot analysis of CYPIA1 expression after 3-methylcholanthrene (3-MC) induction in vitro. Hepatocytes were treated with 3-MC (3 μM) for 1 day (lanes 1,2), 2 days (lanes 3–6) or 3 days (lanes 7–10) in the absence (−) or presence of 10^{-8} EGF (E), 10^{-6} M insulin (I) or EGF and insulin (EI). Western blot analysis was performed as described in section 2.

3.2. *Effects of growth factors on proto-oncogene expression and DNA replication in primary hepatocyte cultures*

Insulin at a concentration of 10^{-8} M leads to a transient expression of c-*fos* in primary mouse hepatocyte culture when cells were growth-arrested for 48 h. The highest accumulation of transcripts is observed around 10 min followed by a rapid decline (Fig. 2A). The c-*fos* expression precedes the c-*myc* expression which peaks around 1 h and declines to barely detectable levels at 2 h (Fig. 2B). EGF and insulin at 10^{-8} M are comparably effective in the accumulation of *FOS* protein. However, EGF elicited a nearly two times higher response which is further potentiated by the combination of EGF and insulin (Fig. 3A). Parallel to the induction of c-*fos* the

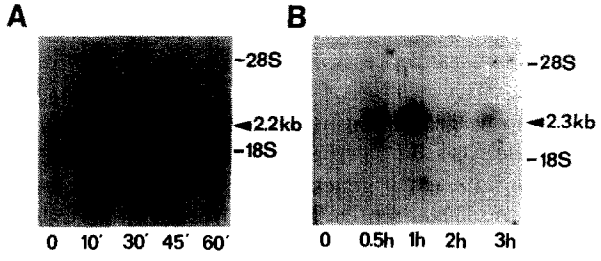

Fig. 2. Northern blot analysis of c-*fos* and c-*myc* transcripts in primary mouse hepatocytes after induction with insulin. Adult mouse hepatocytes were growth-arrested for 48 h in MX-82 medium. Insulin (10^{-8} M) was added and the cells were harvested at the times as indicated. 20 μg of total RNA were loaded per lane, separated on glyoxal agarose gels and subsequently blotted to nylon filters and hybridized with a v-*fos* specific probe (A) or with a v-*myc* specific probe (B).

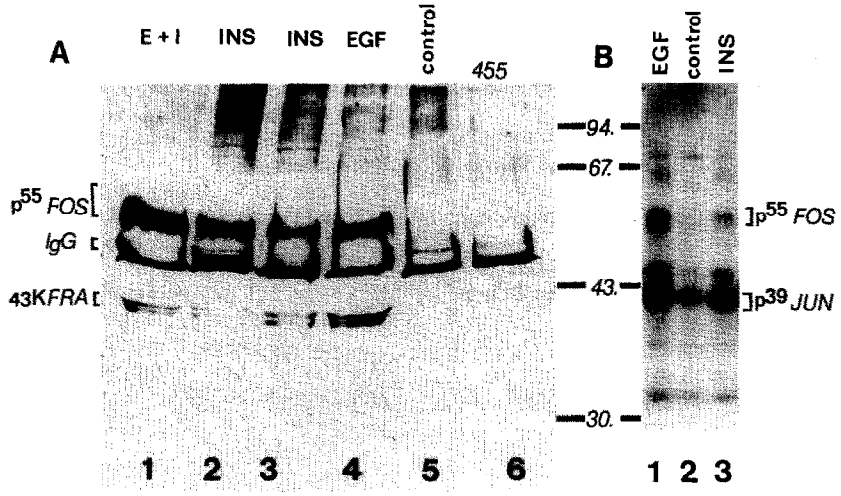

Fig. 3. Western blot analysis of *FOS* and *JUN*/P[39] expression in primary mouse hepatocytes after induction by different growth factors. Adult mouse hepatocytes were growth-arrested in arginine-free MX-82 medium for 72 h and then stimulated for 1 h with the growth factors as indicated. (A) Cells were lysed, c-*fos* protein (*FOS*) was immunoprecipitated with α-*fos* 454 antibody and immunocomplexes bound to protein A Sepharose 4B (Pharmacia). Western blot analysis was carried out using a 1:1000 dilution of α-*fos* 454 and Auro Probe BL plus (Janssen) anti-rabbit IgG for detection followed by silver enhancement. Cells were stimulated with EGF (10^{-8} M, lane 4), insulin (INS) (10^{-6} M, lane 2; 10^{-8} M, lane 3) or EGF and insulin (10^{-8} M, each respectively, lane 1). Unstimulated control (c) is shown in lane 5, while EGF (10^{-8} M) stimulated cells immunoprecipitated with rabbit preimmunoserum 455 is shown in lane 6. Molecular mass markers are shown in kDa. (B) After 1 h growth factor stimulation with EGF (10^{-8} M) or insulin (10^{-8} M) cells were pulse-labeled for 15 min with [^{35}S]methionine (300 μCi/10 cm dish/5×10^6 cells). After the labelling period, the cells were lysed in RIPA buffer and cleared supernatants were incubated with α-*fos* 454 antibody. Immunoprecipitates were analyzed using 12% SDS-polyacrylamide gels followed by autoradiography.

fos-related gene product *FRA*-1 at 43 kDa is expressed [30]. Similar effects are seen in the induction of *JUN*/P[39]-protein which coprecipitates with *FOS* in the immuno-analysis (Fig. 3B). Reinitiation of DNA synthesis after a 48 h growth arrest of the hepatocyte in response to growth factors (10^{-8} M) was determined by [^{3}H]thymidine incorporation into DNA and expressed in cpm $\times 10^3/2.5 \times 10^5$ cells ± SD (no addition: 12.8 ± 2.1; EGF: 59.8 ± 3.8; insulin: 36.5 ± 4.5; EGF/insulin: 68.3 ± 2.5).

Previous studies failed to show that insulin can induce c-*fos* expression in primary rat hepatocytes [11]. It is well established that insulin is one important liver-specific growth factor and necessary during the process of liver regeneration [31]. Our data indicate that EGF and insulin are equally potent hepatotrophic mitogens in mouse hepatocytes in primary culture and can simulate mechanisms of liver regeneration in vivo [31]. These growth factors activate 'early response' genes like c-*fos*, c-*jun* and c-*myc* which possibly act cooperatively in the competence phase of the hepatocytes as demonstrated in vivo during liver regeneration [9].

Although EGF/TGFα and insulin are equally potent in the activation of proto-oncogenes which is followed by mitogenic response, only EGF/TGFα can suppress the induction of CYPIA1 gene expression in the present hepatocyte culture system. The induction of hepatocyte DNA replication and proto-oncogene expression in vitro is therefore not generally accompanied by an altered CYPIA1 gene expression, and the growth regulatory pathways of the hepatocytes after activation of 'early response' genes are not directly linked to the repression of CYPIA1 gene expression. The growth factors EGF and TGFα via the EGF receptor may control the CYPIA1 mediated drug resistance phenotype of hepatocytes during regenerative growth and tumor development.

Acknowledgements: The authors are indepted to Dr D. Müller, Institut für Tumorforschung, Marburg, for providing the anti-*Fos*-specific α-*fos*-454 antibody and the c-*fos*- and c-*myc*-specific cDNA probes.

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