

Regulation of cytochrome P-450 CYP1A1 gene expression and proto-oncogene expression by growth factors in primary hepatocytes

Martin Höhne¹, Volker Becker-Rabbenstein¹, Georg F. Kahl¹ and Hisaaki Taniguchi²

¹*Institute of Pharmacology and Toxicology, University of Göttingen, D3400 Göttingen, FRG and* ²*Institute of Experimental Pathology, German Cancer Research Center, D6900 Heidelberg, FRG*

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The effect of growth factors on the cytochrome P-450 (CYP1A1) gene expression was studied in primary mouse hepatocytes. Of the three growth factors used, i.e. epidermal growth factor (EGF), transforming growth factor α (TGF α) and insulin, only EGF or TGF α completely blocked CYP1A1 expression in the presence of the CYP1A1 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/TGF α but failed to suppress CYP1A1 expression. A specific EGF/TGF α receptor-mediated function may repress CYP1A1 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 TGF α 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 TGF α 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, TGF α 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, TGF α or insulin to a similar extent. However, CYP1A1 gene expression was inhibited only by an EGF/TGF α receptor-mediated function.

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* [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 [³²P]dCTP by random oligonucleotide priming [18].

Cytochrome P-450 (CYP1A1) 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 μ g 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 CYP1A1 antibody [19] in a 1:500 dilution. Detection was achieved with secondary anti-rabbit [¹²⁵I]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]. [³H]Thymidine incorporation into DNA was measured as described [13]. Insulin and rEGF was purchased from Boehringer (Mannheim, FRG), while TGF α , synthetic, was from Bachem (Basel, Switzerland).

Correspondence address: M. Höhne, Institute of Pharmacology and Toxicology, Robert-Koch-Strasse 40, D-3400 Göttingen, FRG

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

Table I
Suppression of CYP activities by EGF or TGF α

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

Hepatocytes were cultured with 3-MC or without (control) in the presence of EGF (10^{-8} M) or TGF α (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 CYP1A1 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 CYP1A1 [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 CYP1A1 activity [23], was observed. This is due to the induction of CYP1A1 as confirmed by Western blot analysis (Fig. 1). Similar decrease of total CYP content and induction of CYP1A1 by 3-MC have been published using rat hepatocyte primary culture [27]. In our system CYP1A1 induction was not significantly affected in the presence of insulin (10^{-8} M). However, EGF and EGF in combination with insulin (Fig. 1) or TGF α alone (Table I) almost totally suppressed the induction of CYP1A1 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]. TGF α 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 TGF α in the autocrine growth of hepatoma [8]. TGF α may, therefore, be involved in the suppression of CYP1A1 gene expression under these conditions.

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

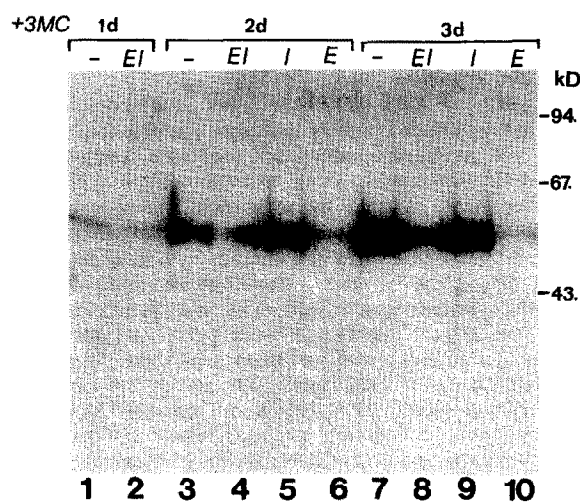


Fig. 1. Western blot analysis of CYP1A1 expression after 3-methylcholanthrene (3-MC) induction in vitro. Hepatocytes were treated with 3-MC (3 μ M) for 1 day (lanes 1,2), 2 days (lanes 3-6) or 3 days (lanes 7-10) in the absence (-) or presence of 10^{-8} EGF (E), 10^{-6} M insulin (I) or EGF and insulin (EI). Western blot analysis was performed as described in section 2.

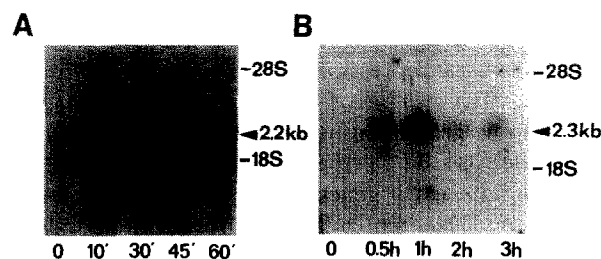


Fig. 2. Northern blot analysis of *c-fos* and *c-myc* transcripts in primary mouse hepatocytes after induction with insulin. Adult mouse hepatocytes were growth-arrested for 48 h in MX-82 medium. Insulin (10^{-8} M) was added and the cells were harvested at the times as indicated. 20 μ g of total RNA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

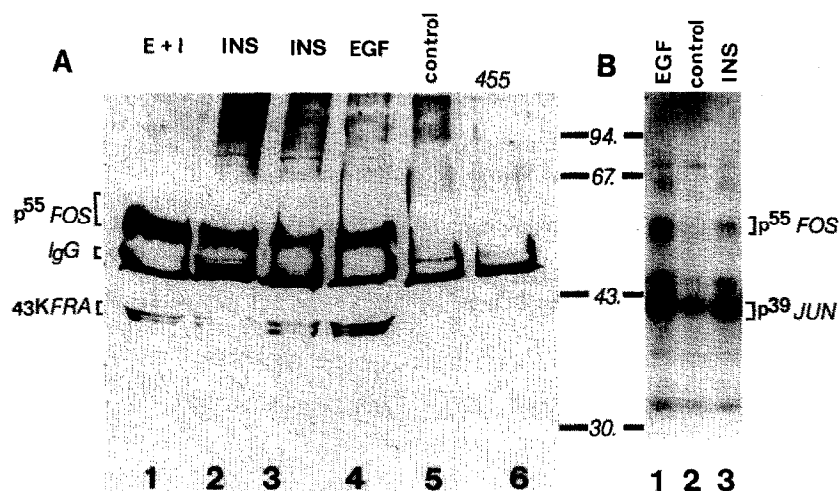


Fig. 3. Western blot analysis of *FOS* and *JUN/P³⁹* expression in primary mouse hepatocytes after induction by different growth factors. Adult mouse hepatocytes were growth-arrested in arginine-free MX-82 medium for 72 h and then stimulated for 1 h with the growth factors as indicated. (A) Cells were 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 μ Ci/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/P³⁹*-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 $\text{cpm} \times 10^3 / 2.5 \times 10^5 \text{ cells} \pm \text{SD}$ (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/TGF α and insulin are equally potent in the activation of proto-oncogenes which is followed by mitogenic response, only EGF/TGF α can suppress the induction of *CYP1A1* 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 *CYP1A1* gene expression, and the growth

regulatory pathways of the hepatocytes after activation of 'early response' genes are not directly linked to the repression of *CYP1A1* gene expression. The growth factors EGF and TGF α via the EGF receptor may control the *CYP1A1* mediated drug resistance phenotype of hepatocytes during regenerative growth and tumor development.

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