Korean J. Toxicol.
Vol. 9, No. 2, pp. 177-186, 1993

EFFECTS OF TUMOR NECROSIS FACTOR-ALPHA ON CYTOCHROME P-450-DEPENDENT DRUG METABOLISM IN PRIMARY MOUSE HEPATOCYTES CULTURES AND MOUSE HEPATOMA CELLS

Hyun Ho Jung, Hye Gwang Jeong[1], Michael Lee,
Byung Sun Yoo[2], and Kyu-Hwan Yang

Department of Life Science, Korea Advanced Institute of Science and Technology,
373-1 Kusong-Dong, Yusung-Gu, Taejon, 305-701, Korea
[1]Department of Environmental Science, Chosun University 375 Seoseok-dong,
Kwangju, 501-759, Korea
[2]Department of Biology, Kyonggi University, San 94-6 Yiui-Dong, Jangan-Gu, Suwon

(Received June 30, 1993)
(Accepted July 28, 1993)

ABSTRACT: *Previous results from several laboratories have demonstrated that tumor necrosis factor-alpha (TNFα) depressed cytochrome P-450 (P-450)-dependent drug metabolism in vivo. However, there is some debate whether the action of TNFα is mediated by its direct effects on hepatocytes, or is indirectly mediated through the release of other mediators like IL-1 from macrophages. In the present studies, we investigated the effects of TNFα on P-450-dependent drug metabolizing enzyme as measured by 7-ethoxyresorufin O-deethylase (EROD) activity. TNFα depressed the basal EROD activity in primary mouse hepatocyte cultures and in purified parenchymal cell cultures. The magnitude of suppression was related to the dose of TNFα. TNFα also reduced the levels of TCDD-preinduced EROD activity in Hepa-1 cells in dose-related fashion. TNFα and interferon-gamma (IFNγ) showed an additive effect in reduction of EROD activity. Microsomal level of P-450IA1 was reduced after treatment of TNFα in TCDD-preinduced Hepa-1 cells, and hepatic P-450 IA1 mRNA was decreased to a similar extent as P-450IA1. These results indicate that the reduction of P-450IA1 by TNFα was due to the decrease of its mRNA, through either transcriptional inactivation or mRNA destabilization. Although these results can not rule out a possibility that IL-1 release indirectly contributes to the actions of TNFα on P-450-dependent drug metabolism, it appears that suppressive effect of TNFα is due to a direct effect in the hepatocytes culture and Hepa-1 cells.*

Key Words: *Cytochrome P-450, tumor necrosis factor, mouse hepatocyte cultures, mouse hepatoma cells.*

INTRODUCTION

Tumor necrosis factor-alpha (TNFα) was initially described as a tumoricidal cytokine produced by activated macrophages (Carswell *et al.*, 1975). TNFα has been investigated mainly for its immunostimulating and antitumor activities (Oehler *et al.*, 1978; Keell and Cuetracaras, 1981; Shalaby *et al.*, 1985). Previous reports have shown that interferons (IFNs), TNFα and interleukin-1 (IL-1) depressed cytochrome P-450 (P-450) levels *in vivo* (Parkinson *et al.*, 1982; Ghezzi *et al.*, 1986a, 1986b; Bertani *et al.*, 1988 and 1989), and that IL-1 but not TNFα, depressed P-450 *in vitro* in cultured rat hepatocytes (Bertani *et al.*, 1988). The effect of TNFα *in vivo* was explained by postulating that it acts through increasing secretion of IL-1 by macrophages (Dinarello *et al.*, 1986; Bertani *et al.*, 1989).

Cytochrome P-450 is a group of heme-containing proteins that function as the terminal oxidase of the microsomal monooxidase system. The system detoxifies by catalyzing the oxidation of a wide variety of hydrophobic xenobiotics including drugs, pesticides, and environmental pollutants and of endogenous compounds such as fatty acids and steroids; on the other hand, some substrates, most notably the polyaromatic hydrocarbons become more toxic (or carcinogenic) as a consequence of P-450-dependent monooxygenase-catalyzed metabolism. In P-450 isozymes, P-450IA1 is one of the most widely studied. This isozyme is induced by polyhalogenated aromatic compound like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), as well as by a number of other chemicals; many of these xenobiotics are mutagenic, carcinogenic, or teratogenic in laboratory animals (Ioannides *et al.*, 1984). The gene for mouse P-450IA1 has been cloned and sequenced (Negishi *et al.*, 1981). Also monoclonal antibodies (mAbs) against P-450IA1 has become available recently (Park *et al.*, 1982). In this study we used mAb 1-7-1 recognizing rat polycyclic aromatic hydrocarbon-inducible P-450IA1 and P-450IA2.

In present study, we investigated the effects of TNFα on the P-450-dependent drug metabolizing enzyme activity (EROD) in primary mouse hepatocyte cultures and in mouse hepatoma cell line, Hepa-1 cells. The level of P-450IA1 isozyme and mRNA were also measured using Western blot and dot blot analysis.

MATERIALS AND METHODS

Chemicals

Chemicals and other cell culture materials were obtained from the following sources: 7-ethoxyresorufin and resorufin (Pierce Chemical Co., Rockford, IL); 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Chemsyn Science Lab., Lenexa, KS); recombinant mouse Interferon gamma (IFNγ) and recombinant mouse tumor necrosis factor alpha (TNFα) (Genzyme Corp., Boston, MA); Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden); Collagenase type I (Sigma Chemical Co., St. Loius, MO); Hank's Balanced Salt Solution (HBSS), Waymouth's MB752/1 medium, alpha Mi-

nimum Essential Medium (α-MEM) (lacking ribonucleotides and deoxyribonucleosides), and fetal calf serum (FCS) (Gibco Laboratories, Chargin Falls, OH); All other chemicals were of the highest commercial grade available.

Isolation, Purification, and Culture of Primary Mouse Hepatocytes

Mouse hepatocytes were isolated using a collagenase perfusion method as described previously (Klaunig *et al.*, 1977) with minor modifications. The hepatocyte suspension was purified by centrifugation over Percoll (density=1.08 g/m*l* and an osmolarity=315 mOsm) as described (Pertoft and Laurent, 1982; Smedsrfd and Pertoft, 1985). Modified Waymouth's medium, supplemented with testosterone, thyroxine, hydrocortisone, estradiol, glucagon, insulin, linoleic acid and oleic acid as described (Decad *et al.*, 1979), was used as the culture medium. Hepatocyte suspension was adjusted to 5.0×10^5 cells/m*l*, and 2 m*l* was pipetted into individual wells of 6-well plates precoated with collagen. The cultures were incubated at 37℃ in a humidified 95% air and 5% CO_2 incubator. The medium was changed 4 hr after the initial plating to remove unattached cells and cellular debris, TCDD and TNFα were added directly. The medium, TCDD and TNFα were renewed every 24 hr. TCDD was dissolved in DMSO and the final concentration of DMSO in culture was 0.1 %.

Hepa-1 Cell Culture and EROD Induction by TCDD

Hepa 1c1c7 cells, a subclone of mouse hepatoma Hepa-1 cells (Bernhard *et al.*, 1973), were maintained in α-MEM supplemented with 10% FCS and antibiotic-antimycotic mixture (100 units of penicillin, 100 μg of streptomycin and 0.25 μg of amphotericin B per m*l*). 2 m*l* of hepatocyte suspension (1×10^6 cells/m*l*) was plated in each well of 6-well plates. EROD in Hepa-1 cells was preinduced by treatment of TCDD (10 nM) for 24 hr. After incubation, the culture was washed twice with complete α-MEM and EROD-preinduced Hepa-1 cells were incubated for an additional 24 hr with TNFα at 37℃ in 95% air and 5% CO_2.

EROD Assay

After culture, hepatocytes and Hepa-1 cells were harvested with a rubber policeman, centrifuged and were resuspended in 0.1 M potassium phosphate buffer, pH 7.4. Cells were then sonicated. EROD activity was assayed by the method of Burke *et al.* (1985). The protein concentration was determined according to Bradford (1976) using bovine serum albumin as the standard.

Preparation of Microsome and Immunoblot Analysis of P-450IA1 in Hepa-1 Cells

Microsomal proteins of Hepa-1 cells were isolated using differential centrifugation, separated by 7.5% sodium dodecylsulfate polyacrylamide electrophoresis, and transferred to nitrocellulose as described (Laemmli, 1970; Towin *et al.*, 1979). For P-450IA1 detection, the filter was first blocked with 3% (w/v) low fat milk in TBS (50 mM Tris-200 mM NaCl, pH 7.4) for 1 hr at room temperature. The filter was subsequently incubated with mAb 1-7-1 at 1 : 2000 dilution in TBS, and alkaline phosphatase conjugated antimouse IgG. Immunoreactive protein was visualized

with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

Isolation of Total Cellular RNA and Dot Blot Analysis in Hepa-1 Cells

Total cellular RNAs in the Hepa-1 cells were isolated using strong denaturant guanidinium thiocyanate method as described elsewhere (Sambrook *et al.*, 1989) For slot hybridization analysis, isolated RNAs were serially diluted, denatured (65° C, 5 min in 2.0 M formaldehyde, 50% (v/v) formamide), transferred on a nitrocellulose filter through a filtration manifold, hybridized, and autoradiographed as described (Giachelli and Omiecinski, 1987). The oligonucleotide probe for the hybridization of P-450IA1 mRNA was synthesized chemically using the Pharmacia DNA synthesizer as described (Giachelli and Omiecinski, 1987). The sequence is 5'-d (TCTGGTGAGCATCCAGGACA)-3' and this oligonucleotide is complementary to region 1650-1669 in the P450IA1 cDNA sequence. The probe was 5'-end labeled with [γ-^{32}P]ATP.

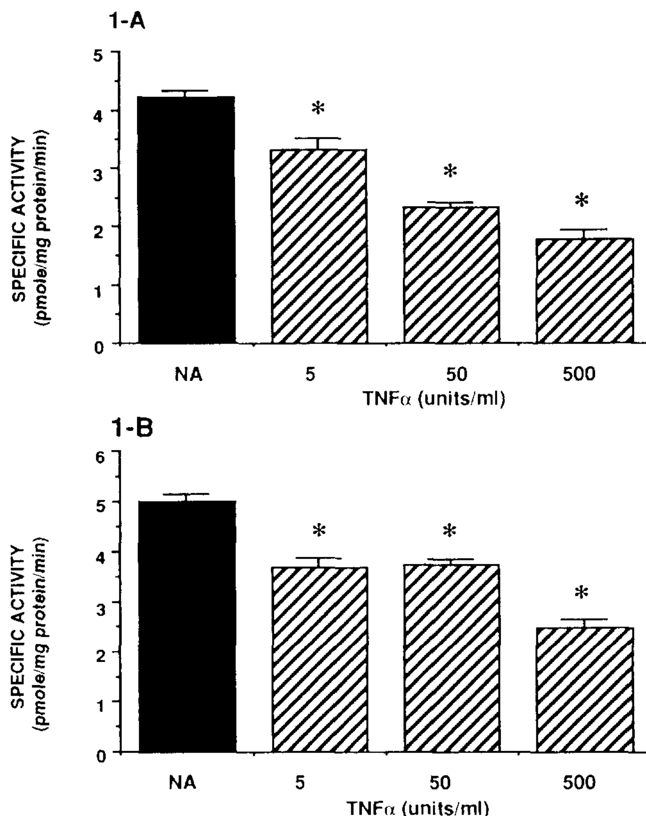

Figure 1. Effect of TNFα on the 7-ethoxyresorufin O-deethylase (EROD) activity in primary mouse hepatocyte cultures (1-A) and in purified primary mouse hepatocyte cultures (1-B). After culture for 48 hr. cells were harvested and measured EROD activity. Each bar represents the mean± S.E. of three replicate cultures. * Significantly different from the NA (naive) as determined by Dunnett's 't' test at $P \leq 0.01$.

Statistics

All cultures were done in triplicate and experiments were repeated three times. For results of all studies, a Dunnett's t test was performed in a one-way analysis of variance of the means showed treatment effects.

RESULTS

Figure 1 shows the effect of TNFα on the basal EROD activity in primary mouse hepatocyte cultures and purified parenchymal hepatocyte cultures. Isolated hepatocytes were cultured for 48 hr in the presence of TNFα. In primary hepatocytes, EROD activity was depressed in dose-dependent fashion by treatment of TNFα: 5, 50, and 500 units/m*l* of TNFα reduced the EROD activity to 78.7, 55.3, and 42.5% of the naive (NA) value, respectively (Figure 1-A). Also 5, 50, and 500 units/m*l* of TNFα reduced the EROD activity to 73.6, 74.6, and 49.7% of NA in the purified parenchymal hepatocyte cultures, respectively (Figure 1-B).

Pretreatment of TCDD (10 nM) for 24 hr markedly increased EROD activity in Hepa-1 cells. After preinduction, TNFα was added and the cells were cultured for an additional 24 hr. TCDD-preinduced EROD activity was reduced in dose-dependent fashion by the treatment of TNFα: 5, 50, and 500 units/m*l* of TNFα reduced preinduced EROD activity to 93.7, 78.9, and 52.2% of the TCDD value,

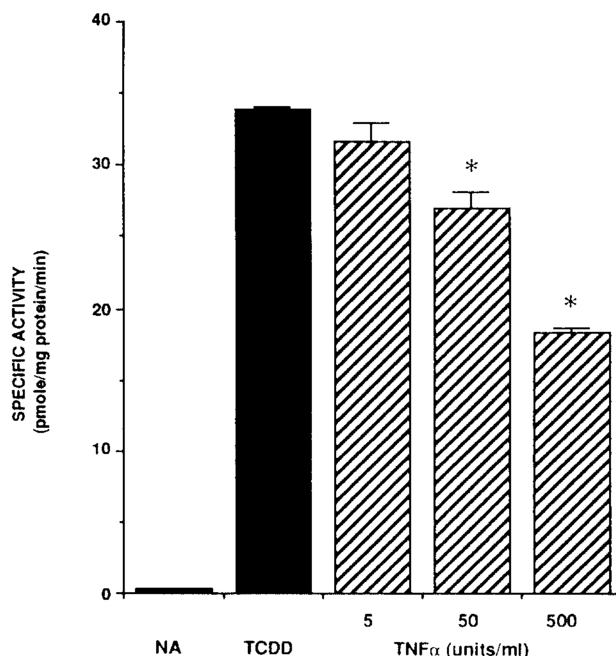

Figure 2. Effect of TNFα on the TCDD-preinduced 7-ethoxyresorufin O-deethylase (EROD) activity in Hepa-1 cells. Hepa-1 cells were preincubated with TCDD (10 nM) for 24 hr and then cultures were washed out twice with fresh medium. TNFα was then added and cultured for additional 24 hr. After culture, cells were harvested and measured EROD activity. Each bar represents the mean± S.E. of three replicate cultures. * Significantly different from the TCDD-preinduced (TCDD) as determined by Dunnett's t' test at P± 0.01.

Table I. Additive effects of TNFα and IFNγ on the 7-ethoxyresorufin O-deethylase (EROD) activity in primary mouse hepatocyte cultures and in Hepa-1 cells

Treatment	EROD (pmole/mg protein/min) Hepatocytes[1)]	Hepa-1 cells[2)]
Naive (NA)	5.61± 0.07[a)]	29.04± 1.03
TNFα (500 units/ml)	4.05± 0.44*	21.36± 0.80*
IFNγ (500 units/ml)	2.97± 0.43*	23.31± 1.51*
TNFα+IFNγ	2.04± 0.25*	16.85± 0.94*

[1)]Mouse hepatocytes were isolated and cultured with TNFα and/or IFNγ. After culture for 48 hr, cells were harvested and measured EROD activity.

[2)]EROD activity in Hepa-1 cells was preinduced by treatment of TCDD (10 nM) for 24 hr, and washed twice with α-MEM and then TNFα or/and IFNγ were treated. After culture for additional 24 hr, cells were harvested and measured EROD activity.

[a)]Each value represents the mean specific activity ± S.E. derived from three replicate cultures per treatment group.

*; Significantly different from naive as determined by Dunnett's 't' test at $P<0.01$.

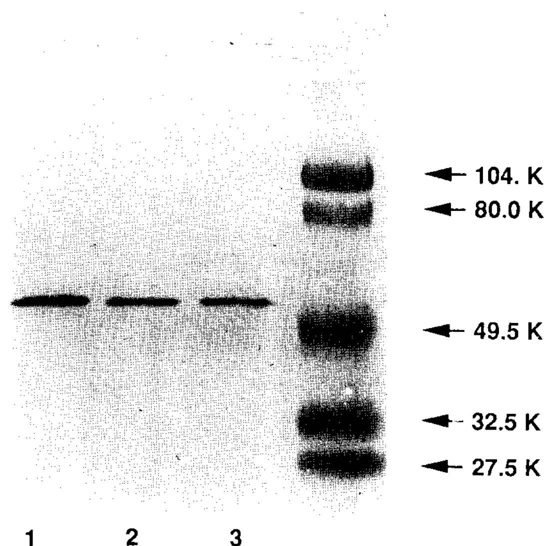

Figure 3. Western immunoblot of P-450IA1 in Hepa-1 cells. Hepatic microsomal protein samples were resolved by electrophoresis in a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose. The blots were visualized using antibodies raised against P-450IA1 as described in Materials and Methods. Hepa-1 cells were preincubated with TCDD (10 nM) for 24 hr and then cultures were washed out twice with fresh medium. TNFα was added and cultured for an additional 24 hr. Lane 1: TCDD-preinduced; 2: TNFα (50 units/ml); 3: TNFα (500 units/ml).

respectively (Figure 2).

Simultaneous administration of TNFα and IFNγ caused an additive reduction of EROD activity in primary hepatocyte cultures and TCDD-preinduced EROD activity in Hepa-1 cells (Table 1).

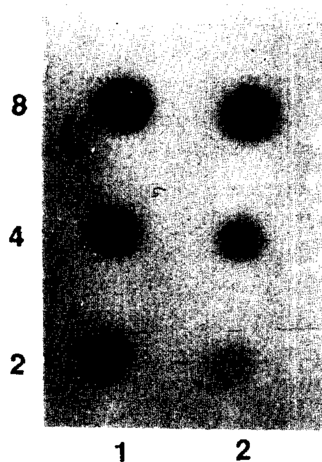

Figure. 4 Autoradiogram of dot blot analysis for P-450IA1 mRNA in Hepa-1 cells. Hepa-1 cells were preincubated with TCDD (10 nM) for 24 hr and then cultures were washed out twice with fresh medium. TNFα was then added and cultured for an additional 24 hr. The amount of total cellular RNAs (mg) loaded onto the each dot are shown on the left side. After baking the nitrocellulose filter, the [^{32}P]ATP-5'-end labeled probe was hybridized and the filter was exposed to film. Lane 1: TCDD-preinduced; 2: TNFα (500 units/m*l*).

In Figure 3, the level of microsomal P-450IA1 apoprotein was measured using western immunoblotting technique. P-450IA1 in Hepa-1 cells was preinduced with TCDD (10 nM) for 24 hr and then treated with TNFα (50 and 500 units/m*l*) for additional 24 hr. P-450IA1 decreased to about 40% of TCDD-preinduced control level by treatment of TNFα.

To investigate the mechanism of suppression of P-450IA1 by TNFα, a dot blot analysis using oligonucleotide probe was performed with the total cellular RNAs isolated from Hepa-1 cells. Cells were preincubated with TCDD (10 nM) for 24 hr and then TNFα (500 units/m*l*) was treated for additional 24 hr. As shown in Figure 4, the amount of P-450IA1 mRNA was significantly reduced in TNFα treated group compared with TCDD-preinduced control, indicating that the reduction of P-450IA1 by TNFα was due to the decrease of mRNA.

DISCUSSION

Considering the facts that TNFα is a potent macrophage stimulators, that macrophage-like cells (Kupffer cells) could be included in primary mouse hepatocytes, and that macrophage-derived products, most notably IL-1, have been reported to markedly depress liver drug metabolism (Ghezzi *et al.*, 1986a, 1986b), it was important to confirm that the depression of drug metabolizing enzyme by TNFα was due to a direct effect on mouse hepatocytes. In present study, TNFα depressed EROD activity in mouse liver parenchymal cells, which were percoll-through purified from crude hepatocytes. In mouse hepatoma cell line, Hepa-1 cells which are only parenchymal cells, the basal (constitute) enzyme activity is very low or

undetectable. So we preinduced the EROD activity by treatment of TCDD (10 nM) for 24 hr. TNFα showed suppressive effect on the preinduced enzyme activity. These results tentatively suggest that suppressive effect of TNFα on microsomal enzyme activity is a direct one and independent from IL-1 action.

TNFα and IFNγ have been shown to act synergistic fashion in some cases. A strong synergistic interaction between these two cytokines has also been found when their cytotoxic or tumoricidal activity was measured on cervical, breast and ovarian carcinoma cell lines (Brouckert *et al.*, 1986). The effect of TNFα and IFNγ on the EROD activity in this study was found to be additive.

The results shown in this study elucidate that preinduced microsomal level of P-450IA1 was reduced after treatment of TNFα in Hepa-1 cells, and hepatic P-450IA1 mRNA was also decreased to a similar extent as P-450IA1 apoprotein. It appeared that the decreased apoprotein levels was a consequence of reduced transcription and/or altered post-transcriptional processes by a mechanism involving altered mRNA regulation.

EROD was reported as a selective indicator enzyme of P-450IA1 (Burke *et al.*, 1985) which has been shown to catalyze the activation of polycyclic aromatic hydrocarbons to the ultimate carcinogenic metabolites (Conney, 1982). Induction of P-450IA1 has also been associated with both the carcinogenecity and the potential toxicity of inducing agents (Gelboin, 1980; Poland and Glover, 1982; Ioannides *et al.*, 1984). Considering that many chemicals are metabolized by P-450-dependent monooxygenase system, depression of P-450 might be result either in diminished ability to eliminate a drug (therefore leading to increased toxicity) or in reduced efficiency of drugs that require P-450-dependent metabolic activation (e.g. cyclophosphamide). The results of this study may be helpful to investigate clinically relevant interrelation between TNFα and other drugs.

ACKNOWLEDGEMENTS

This Work was supported by a Korea Science and Engineering Foundation Research Grant.

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