TNF-α downregulates the peroxisome proliferator activated receptor-α and the mRNAs encoding peroxisomal proteins in rat liver

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Abstract We have studied the effects of TNF-α on the mRNAs coding for the peroxisome proliferator activated receptor α (PPAR-α), and for catalase (Cat), acyl-CoA oxidase (AOX), multifunctional enzyme (PH), and β-actin in rat liver. Total RNA was isolated from livers of male SD-rats 16 h after administration of a single dose of 25 μg TNF-α and mRNAs were analyzed by a novel dot blot RNase protection assay. The mRNAs for PPAR-α and for Cat, AOX and PH were significantly reduced by TNF-treatment. In addition, the level of PPAR-α protein was also decreased after TNF. In contrast, the mRNA for β-actin was markedly increased implying that the effect of TNF on PPAR-α and the peroxisomal mRNAs is highly selective. This effect may have important implications in perturbation of the lipid metabolism induced by TNF-α.

Key words: Tumor necrosis factor α; Peroxisome; Peroxisome proliferator activated receptor

1. Introduction

Tumor necrosis factor α (TNF) is a potent cytokine with a wide range of biological activities [1–5]. One of its remarkable effects is the induction of severe hyperlipidemia by the stimulation of hepatic lipogenesis [6,7], the suppression of lipoprotein lipase and elevated rate of lipolysis in adipocytes [8,9] as well as by the liberation of fatty acids, among them arachidonic acid, from cell membranes by the activation of phospholipase A_2 [10,11]. Peroxisomes play a key role in lipid metabolism and especially in the degradation of polyunsaturated and very long-chain fatty acids by their β-oxidation system [12,13]. The genes encoding peroxisomal β-oxidation enzymes are regulated by nuclear receptors commonly referred to as peroxisome proliferator activated receptors (PPARs) which, after ligand activation bind to response elements in the promoter regions of peroxisomal β-oxidation genes thus acting as transcription factors [14–16].

In the rat liver, PPAR-α is highly expressed [17] and long chain fatty acids and arachidonic acid have been shown to serve as ligands activating PPAR-α in cultured cells [15,18]. Thus, the TNF mediated hyperlipidemia with increased release of activators of PPAR-α such as arachidonic acid should be expected to induce the transcription of peroxisomal β-oxidation enzymes in the liver. In a recent study however, we found that the activity of the peroxisomal β-oxidation enzyme acyl-CoA oxidase (AOX) was reduced and the corresponding protein levels of AOX and multifunctional enzyme (PH) were significantly *decreased* in rat liver 16 h after a single dose of TNF [19]. This observation raised the question whether TNF also affected the PPAR-α in rat liver. The present study was designed to answer this question by investigating the in vivo effects of TNF on the mRNAs encoding peroxisomal proteins and especially to assess the role of PPAR-α in the TNF-mediated downregulation of peroxisomal β-oxidation.

2. Material and methods

2.1. Animals

Male Spraque-Dawley rats (200 g) were kept under standardized conditions (22°C, 12 h 7.00 h/19.00 h light/dark cycle) and were provided with chow and tap water ad libitum. They were injected via the tail vein with 25 μg of human recombinant TNF in 0.5 ml 0.9% saline or saline alone in control animals. The TNF was generously provided by BASF/Knoll (Ludwigshafen, Germany). Sixteen hours after the injection the animals were sacrificed under ether anesthesia and the liver was removed and quickly frozen in liquid nitrogen.

2.2. Preparation of cRNA probes, RNA-isolation and dot blot RNase protection assay

cDNA fragments of rat hepatic AOX (2.0 kbp), PH (2.1 kbp), Cat (1.6 kbp), PPAR-α (1.6 kbp) and β-actin (0.68 kbp) were subcloned into the following restriction sites of either a pGEMZf (+), a pGEM-3zf or a pBSII SK vector AOX:*Eco*RV/*Eco*RI; PH:*Bam*HI/*Sph*I; Cat:*Hin*dIII/*Eco*RI; PPARα:*Bam*HI/*Xba*I. The cDNA fragment of PPAR-α was a generous gift of Dr. J.A. Gustafsson (Karolinska Institute, Huddinge, Sweden). The fragment of β-actin was a gift of Dr. H. Hermann (German Cancer Research Center, Heidelberg, Germany) which was subcloned in a pGEM3Z vector using the *Bgl*II restriction enzyme. The cDNAs were transcribed to corresponding cRNAs and were concomitantly digoxigenin-labeled by means of a commercial Dig-RNA-labeling kit (Boehringer Mannheim, Germany).

Total RNA was extracted from liver tissue by means of guanidinium thiocyanate/phenol chloroform using the Roti-Quick-Kit (Carl Roth, Karlsruhe, Germany). RNA was quantified by spectrophotometry at 260/280 nm and its integrity was assessed by denaturing 1% agarose gel electrophoresis (ratio of ribosomal 28S vs 18S). For the dot blot RNase protection assay developed recently in our laboratory [20] 1 μl = 1 μg each of total RNA was dotted on a nylon membrane ('Qiabrane', Qiagen, Hilden, Germany) and crosslinked by means of an UV Stratalinker (Stratagene). Blots were prehybridized at 68°C for 2 h and hybridized overnight at the same temperature with digoxigenin-labeled cRNA probes adjusted to a concentration of 100 ng/ml. After hybridization, membranes were incubated for 10 min at room temperature with ribonuclease A (1 μg/ml) followed by washing and incubation in blocking buffer (Blocking reagent, Boehringer, Mannheim Germany). Dig-labeled RNA hybrids were detected by chemiluminescence using alkaline phosphatase-labeled anti Dig Fab-fragments and a chemiluminescence substrate (CDP-Star, Boehringer Mannheim, Germany). The signals obtained were quantified by densitometry (Quantimet 500+, Leica, Germany). For statistical analysis Student's *t*-test was used.

2.3. Immunoblotting

Liver was homogenized in a buffer containing 10 mM Tris-HCl, 20 mM sodium molybdate, 1.5 mM EDTA and 0.6 M KCl, pH 8. For immunoblotting equal amounts of protein from liver homogenate were subjected to SDS-PAGE and after electrotransfer onto nitrocellulose the sheets were incubated overnight with a monospecific anti-

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body to PPAR-α. The antibody was a generous gift of Dr. J.A. Gustafsson, Karolinska Institute, Huddinge, Sweden. After repeated washing, a peroxidase-conjugated goat anti-rabbit antibody (1:10000, Sigma, München, Germany) was added for 1 h at room temperature, and the immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham International, Amersham, England).

3. Results and discussion

The animals tolerated the injection of TNF well and did not develop any signs of toxicity.

The mRNAs for the peroxisomal β-oxidation enzymes AOX and PH were significantly decreased 16 h after TNF-treatment (Fig. 1). This is in line with our previously reported results of immunocytochemistry and western blot analysis which had revealed significant reduction of the corresponding enzyme proteins in TNF-treated animals [19]. Concomitantly, the mRNA for PPAR-α (Fig. 1) as well as the corresponding PPAR-α-protein (Fig. 2) were significantly reduced after TNF-treatment. Most recently, another member of the PPAR-family, the PPAR-γ has been reported to be downregulated by TNF in isolated adipocytes [21]. In contrast to PPAR-α, however, the PPAR-γ is not expressed in the liver [17] and its main function is supposed to be the regulation of adipocyte gene expression and differentiation [22].

The TNF-mediated reduction of PPAR-α found in the present study most probably contributes to the decrease of peroxisomal β-oxidation after TNF-treatment. Another mechanism for TNF-induced downregulation of peroxisomal β-oxidation could be related to its interaction with c-jun. TNF stimulates the expression of c-jun [23] and c-jun and PPAR-α mutually inhibit each other's transactivation functions [24]. Thus, the two effects of TNF, the direct reduction of PPAR-α level and the induction of c-jun expression which would inhibit the PPAR-α function may have an additive effect in the diminuition of peroxisomal β-oxidation. Since the peroxisomal β-oxidation plays an important role for degradation of very long chain and polyunsaturated fatty acids [12,13] the *diminuition* of the peroxisomal β-oxidation after TNF-treatment could contribute to the TNF-mediated hyperlipidemia. The biological importance of the hyperlipidemia after TNF-treatment may be related to the protective effect of lipoproteins against endotoxin toxicity and viruses as suggested by Harris et al [25]. Accordingly, the downregulation of PPAR-α could play an important role in this response to TNF. Moreover, the downregulation of peroxisomal β-oxidation which is involved in degradation of prostaglandins [26] and leukotriens [27] could contribute to the intensification of inflammation-inducing effects of TNF. Indeed, PPAR-α most recently has been discussed to play a central role in inflammation control [28].

In addition to the mRNAs of PPAR-α and the peroxisomal β-oxidation enzymes, the mRNA for catalase was also significantly decreased 16 h after TNF-treatment (Fig. 1). This result does not correlate with the enzyme activity and protein level of catalase which were only slightly but insignificantly decreased at this time after TNF-treatment [19]. This discrepancy suggests that catalase may have a longer turn-over rate than AOX and PH. Indeed, in livers of rats treated for 5 days with TNF, catalase activity was reported to be significantly decreased [29].

Surprisingly, the mRNA for β-actin was more than 100% increased after TNF-treatment (Fig. 1). Since the biochemical data do not provide any information to the cellular origin of β-actin mRNA, in situ hybridization experiments are now in progress to elucidate this. In rat liver besides the hepatocytes, the perisinusoidal fat-storing cells (Ito-cells, lipocytes) may be

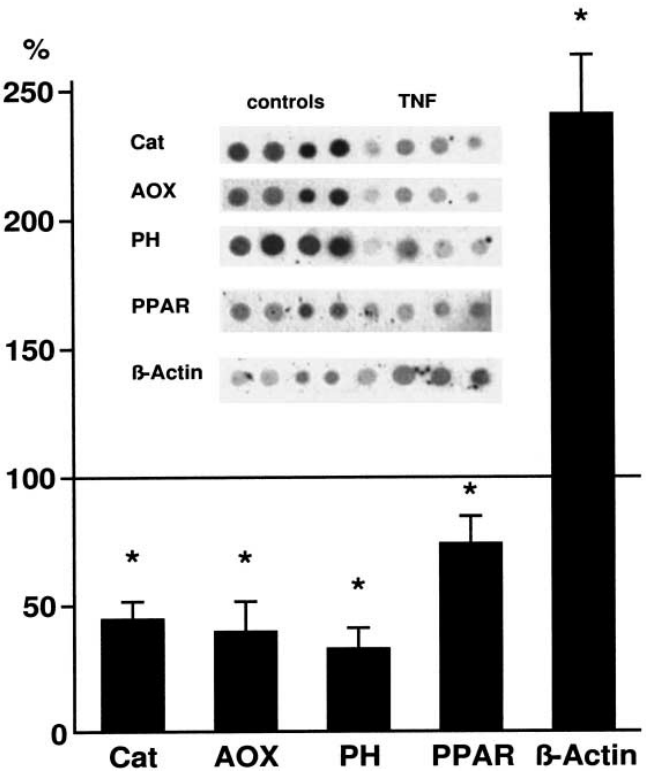

Fig. 1. Dot blot RNase protection assays for mRNAs of catalase (Cat), acyl-CoA oxidase (AOX), multifunctional enzyme (PH), peroxisome proliferator activated receptor-α (PPAR) and β-actin. The diagram below shows the results of the densitometric analysis. Controls were set to 100%, $n=4$ animals per group. The bars represent means with S.E.M. *Significant differences to controls ($P<0.05$).

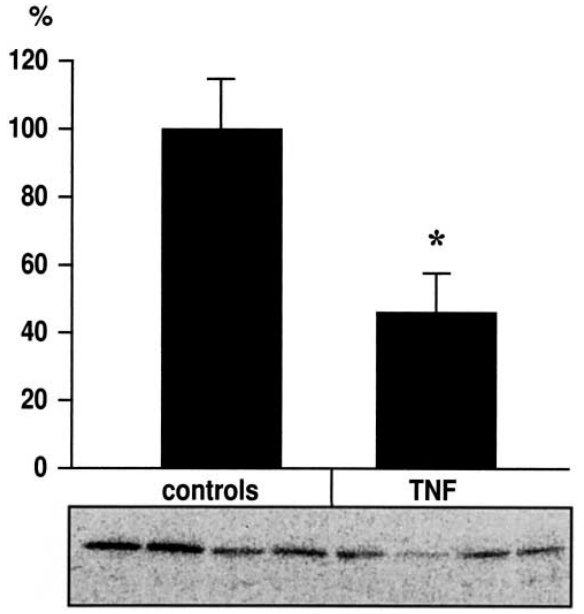

Fig. 2. Immunoblot of total liver homogenate from 4 controls and 4 TNF-treated animals incubated with an antibody to PPAR-α followed by visualization with the ECL technique. Controls were set to 100%. The diagram shows the results of the densitometric analysis. The bars represent means with S.E.M. *Significant difference to controls ($P < 0.05$).

promising candidates since they have been reported to transform to myofibroblasts after TNF-treatment [30].

The mRNA of β-actin which is considered as a stable constitutive protein, has been widely used as an intrinsic control for normalization of different mRNA levels. Accordingly, TNF treatment did not alter β-actin mRNA in fibroblasts [31], adenocarcinoma cell lines [32,33] or adipocytes [21]. However, our current results of *increased* β-actin mRNA in rat liver in combination with a recent report of *decreased* β-actin mRNA in microvascular endothelial cells after exposure to TNF [34] cast doubt on the value of β-actin as a reliable reference for normalization of other mRNAs under experimental conditions.

The mechanisms by which TNF downregulates the mRNAs for PPAR-α and catalase remains unknown. In the last years, several novel proteins have been identified that interact with members of the TNF-receptor superfamily to initiate intracellular signal transduction events [5,23,35,36]. Hitherto, however no direct link has been established between TNF-action and the regulation of peroxisomal genes. In contrast to TNF, a variety of peroxisome proliferating agents such as perflourodecanioc acid and WY 14,643 induce the mRNA of PPAR-α in rat liver and hepatoma cell lines [37]. The knowledge of various factors upregulating the mRNA of PPAR-α in combination with the downregulation of PPAR mRNA by TNF as revealed here, may be helpful for elucidation of the exact mechanisms involved in the regulation of PPAR-α.

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