Repression of apoC-III gene expression by TNFα involves C/EBPδ/NF-IL6β via an IL-1 independent pathway

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Abstract Apolipoproteins A-II and C-III, which participate in the control of cholesterolemia and triglyceridemia, are negative acute phase proteins. Treatment of HepG2 cells with TNFα showed that apoA-II and apoC-III mRNA levels were decreased. Using transient transfection, we found that apoC-III gene expression is controlled at the transcriptional level. By competition and supershift experiments, we demonstrate that TNFα-induced complexes were related to C/EBPδ/NF-IL6β and p50 and that overexpression of C/EBPδ was able to reproduce the inhibitory effect of TNFα on the apoC-III promoter. RT-PCR failed to detect the IL-1 transcript in TNFα-treated HepG2 cells, suggesting that activation of C/EBPδ by TNFα is not related to the IL-1-signalling pathway.

Key words: Apolipoprotein; Transcription; Tumor necrosis factor α; C/EBPδ

1. Introduction

After trauma, infection, or inflammation, two classes of acute phase proteins (APP) may be identified: positive APPs, such as C-reactive protein and serum amyloid A (SAA) protein, whose plasma concentrations increase during inflammation [1], and negative ones, such as albumin and apolipoproteins (apo), whose levels decrease [2]. The acute phase response is mediated by cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor α (TNFα). These cytokines affect gene expression by acting at different levels, particularly by controlling the functional activity of several transcription factors. Among the transcription factors known to be activated by TNFα are the early response gene c-jun [3], the ubiquitous factor Sp1 [4], and the NF-κB transcription factor [5]. Whereas the induction of positive APPs has been well studied, little is known about the regulation of negative APPs. Previous findings have demonstrated that the down-regulation of negative acute phase genes involves post-transcriptional mechanisms [6] or a decrease in the expression of a positive transactivator [7]. Since it has been previously reported that apoC-III and apoA-II are decreased during infection and inflammation in humans and rats [8,9], we decided to investigate the regulation of the expression of these two genes by TNFα in HepG2, a human hepatoma cell line. We found that TNFα activates C/EBPδ/NF-IL6β and p50, and that C/EBPδ had a dominant negative effect on apoC-III promoter.

2. Materials and methods

2.1. Cell culture and transfections

HepG2 cells (4×10^5) were transfected by the calcium phosphate-DNA coprecipitation method [10] with a mixture of DNAs containing 7 μg of reporter chloramphenicol acetyltransferase (CAT) plasmid, apoC-III-890-CAT or −911/+29 A-II CAT [11,12], 2 μg of pCMVβGal plasmid, and 0.25–1 μg of expression vector and carrier plasmid added to keep the total amount of DNA constant at 10 μg. The C/EBPδ expression vector, generously provided by G. Ciliberto, was subcloned into the pCB6 vector [13]. RcCMV p50 was the kind gift of L. Schmitz. CAT assays were carried out in liquid phase, as previously described [14], and normalized for β-galactosidase activity. Human recombinant TNFα (R&D, Abingdon, UK) was added immediately after transfection.

2.2. RNA analysis

Total RNA was extracted with RNAzol (Bioprobe, Montreuil-sous-Bois, France). RNAs from untreated and TNFα-treated HepG2 cells were separated on formaldehyde containing 1.2% agarose gel. After transfer, the blots were hybridized with hapoC-III, hapoA-II, and 18S cDNA probes radiolabeled by the random priming method. Prehybridization, hybridization, and washing were carried out as described previously [15]. Specific hybridization signals were visualized by autoradiography and quantified by densitometry. Isolation of macrophages was carried out as previously described [16]. For detection of IL-1β mRNA by RT-PCR, 1 μg of total RNA was reverse-transcribed and amplified as previously described [16]. As a control of the reverse transcription reaction, amplification of β-actin cDNA was carried out on the same cDNA that was used to amplify the IL-1 transcript [17].

2.3. Nuclear extract preparation and in vitro DNA binding assays

Nuclear proteins were extracted as previously described [18], except that 50 mM NaF was added in all buffers. Electrophoretic mobility shift assays (EMSA) were carried out as previously described [19] with oligonucleotides CIIID: 5′-CTCAGTCTCCTAGGGATTTCCCAA-CTCTCCCGCCC [11] and AlbD: 5′-TGGTATGATTTTGTAAT-GGGGTAGGGA [20]. In supershift experiments, nuclear extracts were pre-incubated for 4 h at 4°C with 1 μl of the following antibodies: anti-C/EBPα, anti-C/EBPδ, anti-c-rel (Santa Cruz), anti-C/EBPβ (gift of S.-C. Lee), anti-p65, and anti-p50 (kindly provided by A. Israël) prior to the binding reaction.

3. Results

Fig. 1A shows that TNFα treatment of HepG2 cells resulted in a decrease in apoC-III and apoA-II mRNA accumulation. We observed a twofold decrease in apoC-III and apoA-II mRNA after 24 h as well as 8 h (data not shown) of incubation with 10 ng of TNFα. In order to determine whether this inhibition was exerted at the transcriptional level, HepG2 cells were transiently transfected with apoC-III-890-CAT or −911/+29 A-II CAT plasmids and stimulated by TNFα. The region between −890 and +24 of the apoC-III gene and the region between −911 and +29 of the apoA-II gene contain all the regulatory elements required for tissue-specific expression and nutritional and hormonal control

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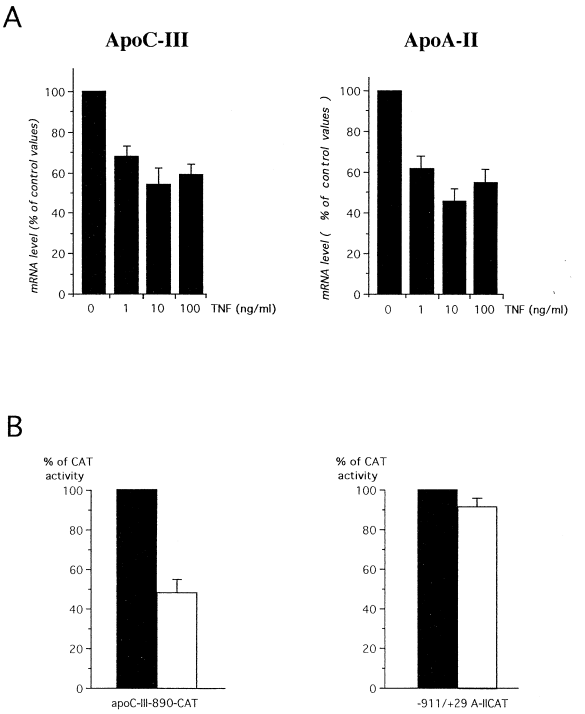

Fig. 1. Inhibition of apoC-III and apoA-II gene expression by TNFα. A: 30 μg of total RNA from HepG2 cells treated with 1, 10, or 100 ng/ml TNFα for 24 h was analyzed by Northern blotting and hybridized with apoC-III and apoA-II cDNA probes. After densitometric quantification and normalization with the 18S probe, apoC-III and apoA-II mRNA levels were expressed as a percentage of normalized values measured in untreated cells. B: HepG2 cells were transfected with apoC-III-890-CAT or −911/+29 A-II CAT promoter constructs and treated with (white column) or without (black column) 10 ng of TNFα for 36 h. Results are expressed as the mean values ± S.D. of the normalized chloramphenicol acetyltransferase activity of three independent experiments.

[11,21,22]. As shown in Fig. 1B, 24 h of TNFα treatment induced a twofold inhibition of apoC-III promoter activity, which suggests that TNFα inhibits apoC-III gene expression at the transcriptional level (if not also post-transcriptionally). We did not observe any significant change in CAT gene expression under the control of the −911/+29 apoA-II promoter. This could indicate either that the −911/+29 promoter region does not comprise the TNFα responsive element controlling apoA-II transcription or that TNFα inhibits apoA-II gene expression at the post-transcriptional level. We could not assess the effect of TNFα on apolipoprotein mRNA stability, since actinomycin D (5 ng/ml) dramatically increases TNFα cytotoxicity, as has been previously reported [23].

We focused our attention on the transcriptional repression of apoC-III. Previous studies have shown that the element D (between −160 and −142) of the apoC-III promoter could bind NF-κB [24] and C/EBP [25]. Therefore we compared the EMSA patterns of nuclear extracts of cells treated with TNFα for 4 h with those of untreated cells. Gel retardation assays that were carried out with untreated cell nuclear extracts formed two broad complexes, CIIID1 and CIIID2 (Fig. 2A). These complexes were related to members of the C/EBP family, since the AlbD oligonucleotide, a high-affinity binding site for C/EBP [20], competed with them. We demonstrated by supershift experiments that CIIID1 and CIIID2 were formed by heteroduplexes containing C/EBPα and C/EBPβ [26]. In addition to CIIID1 and CIIID2, nuclear extracts from TNFα-treated cells revealed a third, faster-migrating complex, CIIID3, also displaced by AlbD, and two other binding complexes, designated a and b, which were not displaced by an excess of the AlbD domain (Fig. 2A). CIIID3 consisted only of C/EBPδ/NF-IL6β and none of the other factors tested, e.g. C/EBPα, C/EBPβ (Fig. 2B), p65, p50, and c-rel (data not shown). On the other hand, the a complex was supershifted by anti-p50 and partially by anti-p65 but not by anti-c-rel (Fig. 2C), nor by anti-C/EBPα, anti-C/EBPβ or anti-C/EBPδ (data not shown), suggesting that a complex could be a p50/p65 heterodimer. In contrast, the b complex was only supershifted by anti-p50 and not by any other antibodies against members of the NF-κB family (Fig. 2C) or the C/EBP family (data not shown), suggesting that the b complex is made of p50 subunits. These results provide evidence that TNFα activates binding of three different transcriptional factors that belong to distinct families.

The activation of p50 and C/EBPδ in HepG2 cell nuclei after TNFα induction prompted us to evaluate the functional role of these factors on the apoC-III promoter. As shown in Fig. 3, C/EBPδ inhibited apoC-III transcription, reproducing the inhibitory effect of TNFα. In contrast, p50 induced positive transactivation of the apoC-III promoter. In order to assess the resulting transactivation effect of C/EBPδ and p50 on apoC-III transcription, we carried out cotransfection experiments. As shown in Fig. 3A, addition of C/EBPδ reversed positive transactivation by p50. On the other hand, the repressive effect of C/EBPδ was not affected by transfection of increasing amounts of p50 (Fig. 3B). The above results suggest a dominant negative role of C/EBPδ on p50 in the control of apoC-III gene transcription during TNFα incubation.

Several studies have shown that IL-1 can activate C/EBPδ [27,28]. Furthermore, it has been demonstrated that hepatocytes and hepatoma cell lines are able to synthesize IL-1 [29,30]. Therefore, one could hypothesize that TNFα induces IL-1 secretion in HepG2 cells, which in turn could activate C/EBPδ. In order to detect IL-1 mRNA, we carried out RT-PCR, using IL-1β primers [16]. As shown in Fig. 4, IL-1β cDNA (391 bp) was detected in macrophages (lane 1) but not in TNFα-treated or untreated HepG2 cells (lanes 2 and 3). This suggests that IL-1β was not synthesized by HepG2 cells exposed to TNFα before activation of C/EBPδ/NF-IL6β and that this activation proceeded via an independent IL-1 pathway.

4. Discussion

The acute phase of the inflammatory response is characterized by wide-ranging physiological changes, particularly by an altered pattern of protein synthesis in the liver characterized by increased or decreased plasma levels of APPs. These physiological responses are initiated and coordinated by various inflammatory mediators, including cytokines, such as IL-1, IL-6, and TNFα. The main effect of cytokines in APP expression is due to modifications in gene transcription. However, post-transcriptional events may also contribute to altered plasma APP. Until now, only a few transcription factors, such as c-jun, Sp1, and NF-κB, have been reported to be functionally activated by TNFα. Our findings provide evidence, for the first time, that NF-IL6β, the human homolog of C/EBPδ, is activated by TNFα, a pro-inflammatory cyto-

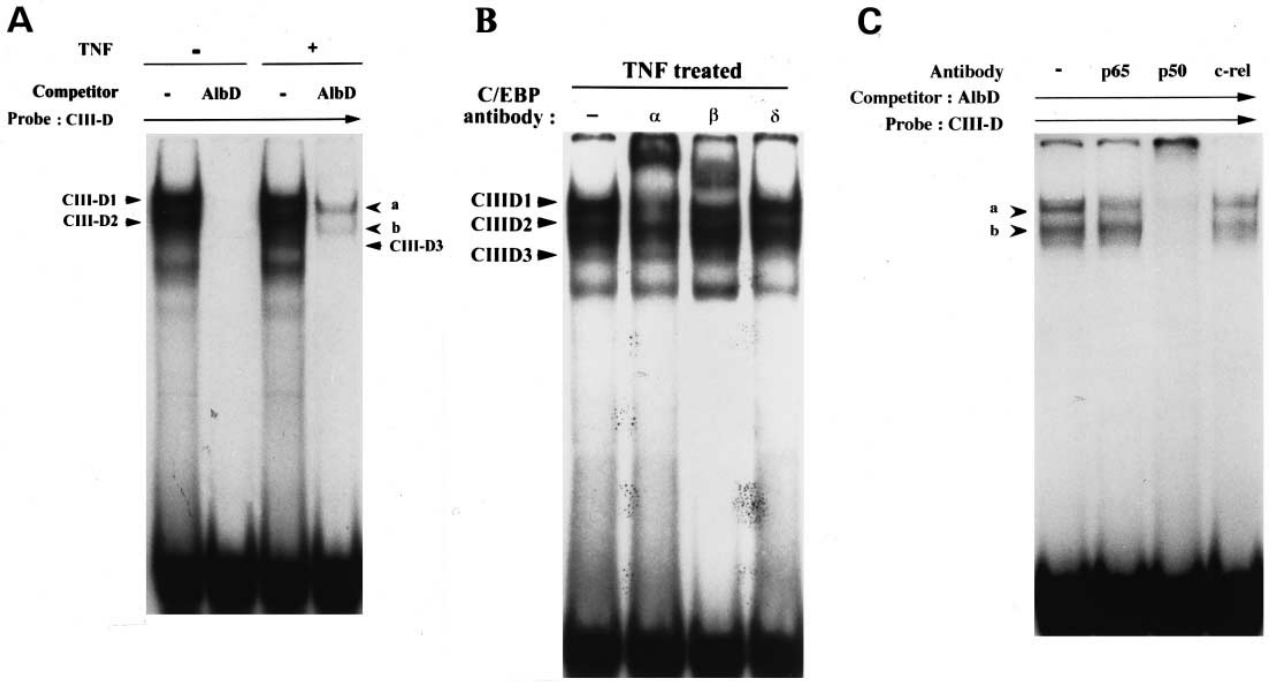

Fig. 2. TNFα treatment activates DNA binding of C/EBPδ and p50. Nuclear extracts prepared from cells treated for 4 h with TNFα, as well as those of untreated cells, were analyzed by means of EMSA, using the double-stranded CIIID oligonucleotide, in the presence or absence of a 100-fold molar excess of cold competitor AlbD (panel A) in the presence of anti-C/EBPα, anti-C/EBPβ or anti-C/EBPδ antibodies (panel B) or in the presence of a 100-fold molar excess of cold AlbD competitor and antibodies raised against members of the NF-κB family (panel C).

kine, in the HepG2 human hepatoma cell line. We and others have reported that, in addition to IL-6, C/EBPδ is activated by IL-1β, inducing an increase in mRNA and protein [26,27]. Since it is possible that C/EBPδ was activated by the IL-1 secretion of hepatoma cells induced by TNFα, we decided to look for IL-1β mRNA following TNFα treatment. After 4 h of incubation with TNFα, the mRNA of IL-1β in HepG2 cells was not detected, whereas C/EBPδ/NF-IL6β binding was activated. Therefore, in addition to IL-6 and IL-1, we found that TNFα is able to activate C/EBPδ by its own pathway, which attributes a central role to this transcriptional factor in the regulation of the acute phase of inflammation.

Decreased expression of transactivator [7] and post-transcriptional control [6] have been implicated in the regulation of negative APP expression. In the present study we further assess our recent observation that in the context of apoC-III

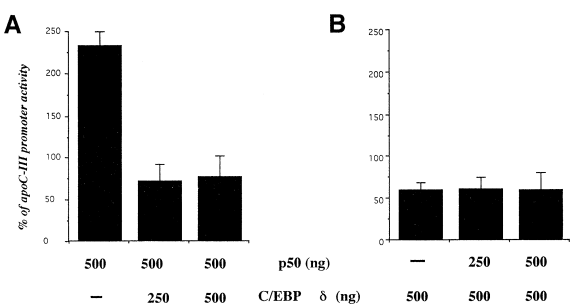

Fig. 3. Negative dominant effect of C/EBPδ on apoC-III transcription. HepG2 cells were co-transfected with apoC-III-890-CAT and various quantities of C/EBPδ and p50 expression vectors. CAT values measured in the cotransfection experiments are expressed as the percentage of apoC-III promoter activity alone.

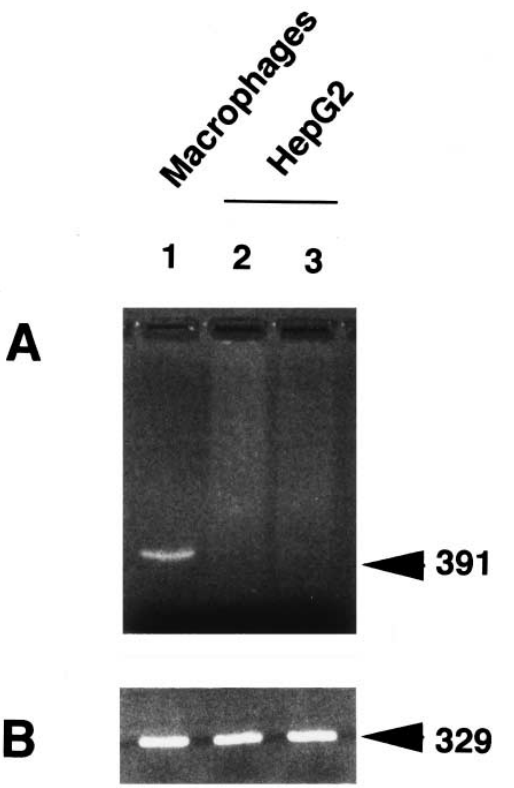

Fig. 4. Absence of IL-1β mRNA in TNFα-treated HepG2 cells. Ethidium bromide-stained agarose gel of PCR experiments. 1 μg of RNA from cells treated with TNFα for 4 h (lane 3) and from untreated cells (lanes 1 and 2) was reverse-transcribed and amplified by PCR, using IL-1β primers (A) or β-actin primers (B).

promoter, C/EBPδ acts as a repressor of transcription [26]. In that study we showed that this inhibition is not mediated by the D and C domains, which are able to bind C/EBP, but rather by the I domain, located in the enhancer region, where it cannot bind [26]. We suggest that C/EBPδ, but not C/EBPα, disrupts the transcriptional complex by a protein-protein interaction. Moreover, in the present study, we demonstrate that TNFα, in addition to C/EBPδ, activates binding of p50 in HepG2 cells which has a positive transactivation effect on the apoC-III promoter. p50 belongs to a family which contains the Rel domain, required for DNA binding, as well as to form homodimers and heterodimers with other Rel family proteins [31]. A previous study has reported that p50 can interact physically and functionally with members of the C/EBP family [32]. Cotransfection of C/EBPδ with p50 revealed that the inhibitory effect of C/EBPδ predominates over the positive effect of p50 on the apoC-III promoter. The physiological relevance of the activation of p50 on the expression of the apoC-III gene remains to be elucidated.

Since its discovery, C/EBPδ/NF-IL6β has been implicated in the induction of several positive APPs [33,34]. The binding and transactivating capacities of C/EBPδ have recently been reported to depend on tyrosine and serine/threonine phosphorylation [26,35]. Our results agree with these findings, since the omission of sodium fluoride, which is a phosphatase inhibitor, in the nuclear extract buffer markedly decreases the binding activity of C/EBPδ (data not shown). Several recent reports suggest that protein kinases are activated playing a critical role in the TNFα signalling pathway (review in [36]), raising the possibility of the direct activation of C/EBPδ by the signal-transducing protein kinase cascade triggered by TNFα. These results have led us to suggest that C/EBPδ could play a pivotal role in the acute phase reaction by exercising transcriptional control over positive and negative APPs. The activation of C/EBPδ could possibly disrupt or enhance the actions of transcriptional complexes by interfering with transcription factors, leading to an increase or a decrease in transcription. The mechanism involved in this regulation may be a protein-protein interaction which could be modulated by the phosphorylation status of C/EBPδ. Therefore, C/EBPδ is capable of inducing a high level of transcription of positive APPs and at the same time of repressing apoC-III gene expression.

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