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RESEARCH ARTICLE

Ordered transcriptional factor recruitment and epigenetic regulation of *tnf-α* in necrotizing acute pancreatitis

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Abstract The expression of the critical initiator cytokine TNF-α was strongly upregulated in vivo in acute necrotic pancreatitis (AP) in rodents and in vitro in TNF-α activated acinar AR42J cells. Upregulation of *tnf-α, inos, icam-1* and *il-6* occurred both in TNF-α receptor 1 and 2 knock-out mice, but not in TNF-α knock-out mice, in cerulein-induced acute pancreatitis. Chromatin immunoprecipitation analysis showed that transcriptional factors (ELK-1, SP1, NF-κB and EGR-1) and chromatin modification complexes (HDAC1, HDAC2, GCN5, PCAF and CBP) were recruited and/or released from the promoter in a strictly ordered mechanism. Activation of *tnf-α* gene was also accompanied by an ordered increased level of histone H3K9, H3K14 and H3K18-acetylation and H3K4 methylation, as well as H4K5 acetylation. A better knowledge of the molecular mechanisms that control *tnf-α* gene regulation will provide deeper understanding of the initiation and development of the inflammatory processes occurring in acute pancreatitis triggered by TNF-α cytokine.

Keywords Acute necrotic pancreatitis · Chromatin immunoprecipitation · ChIP · Epigenetic · Tumor necrosis factor alpha

Introduction

Acute pancreatitis (AP) is an initially localized inflammation of the pancreatic gland that may lead to local and systemic complications. Mortality due to AP is around 20–30% in the severe forms of the disease because of multiple organ failure [1–5].

The precise mechanisms by which etiological factors induce AP are not yet well known, but when initiated, common inflammatory pathways seem to be involved, with cytokines being their components of major importance [1, 6–8]. TNF-α is a key cytokine initiator of the inflammatory cascade in AP [2, 3, 9, 10], which induces the expression of pro-inflammatory genes such as *il-6* [11–13], *inos* or *icam-1* [14–16]. TNF-α interacts with two different surface receptors, TNF-α receptor 1 (TNFR1) and TNF-α receptor 2 (TNFR-2) [2, 17, 18]. In knock-out mice deficient in TNF-α receptors, the rate of mortality due to necrotizing AP decreased significantly, although there was no reduction in the severity of the pancreatic damage [19, 20].

TNF-α promoter contains elements critical for induction of the gene. It has been reported that a unique TNF-α enhancer complex containing Ets, Elk-1, Sp1, ATF-2-Jun,

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CBP and p300 is assembled on the TNF-α promoter in LPS-stimulated monocytes [21]. Nevertheless, the epigenetic regulation of *tnf*-α seems to be quite complex because the expression is cell-type specific [21, 22] and also stimulus specific [23, 24]. Thus, different sets of transcriptional factors are recruited in the *tnf*-α promoter upon ionophore stimulation (ATF-2/c-JUN and NFAT) and virus induction (ATF-2/c-JUN, NFAT and SP1) in fibroblasts or lymphocytes T and B [23].

At present, it is generally accepted that the precise regulation of gene expression by epigenetic mechanisms is required to upregulate cytokine expression in different experimental models. In this study, we provide new insights into the epigenetic mechanisms that control transcriptional activation of *tnf*-α in the initiation and development of AP.

Materials and methods

Experimental models of AP in animals

Young male Wistar rats and young male mice C57BL/6 were used in the experiments. Animals were cared for and handled in accordance with the European regulations (Council Directive 86/609/EEC), and the studies were approved by the Ethical Research Committee of the University of Valencia. Mice were either wild type, TNF-α receptor 1 knock-out (KO TNF-α R1), TNF-α receptor 2 knock-out (KO TNF-α R2) or TNF-α knock-out (KO TNF-α), and were kindly provided by Horst Bluethmann (Hoffmann-La Roche). Mice were treated with the cholecystokinin analogue cerulein to induce AP [25]. Cerulein was administered in seven injections at hourly intervals, with each injection containing 50 μg/kg body weight, and killed under anesthesia 1 h after the last injection of cerulein. AP was induced in rats by retrograde infusion of sodium taurocholate (3.5%; Sigma) into the biliopancreatic duct, as described by Aho et al. [26]. Rats were killed under anesthesia at 0, 0.5, 1, 3 and 6 h after the infusion of taurocholate or saline. Serum lipase activity was measured, and histological studies were performed to confirm the appropriate induction of pancreatitis.

AR42J pancreatic acinar cell line culture

The pancreatic acinar cell line AR42J (ATCC CRL 1492) was grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) containing 25 mmol/l glucose, 100 μg/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml fungizone, supplemented with 10% fetal bovine serum (FBS). To activate the *tnf*-α gene, AR42J cells were treated with 0–100 ng/ml of TNF-α (Sigma) for the indicated times.

Chromatin immunoprecipitation (ChIP) assay and RNApol ChIP

ChIP and RNApol ChIP procedures were performed as previously described [27, 28]. Briefly, rats were killed at the indicated times after taurocholate treatment, and the pancreases were excised and immersed for 10 min, or AR42J cultures were treated for 4 min, in 1% formaldehyde to crosslink the chromatin. After stopping the reaction by adding glycine to a final concentration of 0.125 M, the nuclei were obtained as described [27, 28]. Crosslinked chromatin was disrupted in a Vibra-Cell VCX-500 sonicator (Sonics and Materials) obtaining chromatin fragments of around 500 ± 300 bp. Aliquots of disrupted chromatin (equivalent to 50 μg DNA) were immunoprecipitated using 2 μg of antibodies against: NF-κB (sc-109), AP-1 (sc-1694), C/EBPβ (sc-150), SP1 (sc-59), ELK-1 (sc-355), ETS-2 (sc-351), EGR1 (sc-110), CBP (sc-369), PCAF (sc-8999), GCN5 (sc-6303), HDAC1 (sc-6298), HDAC2 (sc-6296), HDAC3 (sc-11417; from Santa Cruz), $H3K4me_2$ (ab-7766), $H3K4me_3$ (ab-8580), H3K9ac (ab-4441), H3K18ac (ab-1191), H4K5ac (ab-1758; from Abcam), $H3R17me_2$ (07-214), H3K14ac (07-353), H3K27ac (07-360), H4K16ac (07-329; from Upstate) for ChIP assay or RNA pol II (sc-899 from Santa Cruz) for RNApol ChIP. The primer sequences used for target gene PCR analysis of IP samples were: *tnf*-α (promoter): forward 5′-GGTGAGGACGGAGAGGAGATT-3′ and reverse 5′-TGGGAGTTAGTACCAGGGTGTTC-3′ (277 bp product); *tnf*-α (coding region, exon 4) forward 5′-CAGCCGATTTGCCATTTCAT-3′ and reverse 5′-TCCTTAGGGCAAGGGCTCTT-3′ (253 bp product); α-*actin* (promoter): forward 5′-AGGGACTCTAGTGCCCAACACC-3′ and reverse 5′-CCCACCTCCACCCTACCTGC-3′ (186 bp product); α-*actin* (coding region, exon 2): 5′-AGGATTCCTACGTGGGCGAC-3′ and 5′-TAGAGAGACAGCACCGCCTG-3' (280 bp product); β-*actin* (coding region, exon 2): forward 5′-AGAGCAAGAGAGGCA TCCTG-3′ and reverse 5′-GGGTCATCTTTTCACGGTTGG-3′ (245 bp product). PCR fragments were size-fractionated by 2% agarose gel electrophoresis, stained with ethidium bromide and analyzed with the FLA3000 electronic autoradiography system (Fujifilm) and ImageJ software (http://rsbweb.nih.gov/ij/).

RNA extraction and determination of mRNA steady-state levels

A small piece of the pancreas was excised and immediately immersed in 1 ml RNAlater solution (Ambion) to stabilize the RNA. Total RNA was isolated from pancreas and from AR42J cell cultures by the guanidinium thyocianate method [29]. The cDNA used as template for amplification in the PCR assay was constructed by reverse transcription

reaction using SuperScript II (Invitrogen) and random hexamers as primers [27]. Real-time PCR was performed using Syber Green PCR Master mix (Applied Biosystems) in an ABI GeneAmp 7000 Sequence Detection System. Each reaction was performed in triplicate, and the melting curves were constructed using Dissociation Curves Software (Applied Biosystems) to ensure that only a single product was amplified. The 18S *rRNA* was also analyzed as RT-PCR control. The following specific primers were used: *tnf-α*, forward 5′-CAGCCGATTTGCCATTTCAT-3′, reverse 5′-TCCTTAGGGCAAGGGCTCTT-3′, *β-actin*: forward 5′-TTCAACACCCCAGCCATGT-3′, reverse 5′-GTGGTACGACCAG AGGCATACA-3; *rRNA* 18S: forward 5′-AGTCCCTGCCCTTTGTACACA-3′, reverse 5′-GATCCGAGGGCCTCACTAAAC-3′. The threshold cycle (Ct) was determined, and the relative gene expression was expressed as follows: fold change $= 2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{target} - Ct_{housekeeping}$ and $\Delta(\Delta Ct) = \Delta Ct_{treated} - \Delta Ct_{control}$.

Immunohistochemistry

Pancreas specimens from control rats and rats at 3 h after induction of pancreatitis with taurocholate were fixed in formalin and embedded in paraffin. Immunohistochemistry was performed using the R.T.U. Vectastain Universal Elite ABC kit (Vector Labs, Burlingame, CA) following the manufacturer's instructions. Briefly, sections were deparaffinized and rehydrated by sequential immersion of slides in xylene followed by graded concentrations of ethanol, and finally with water. Inactivation of endogenous tissue peroxidases was performed by immersing the slides in a solution of 0.5% H_2O_2 in methanol for 30 min. Sections were incubated in 2.5% horse serum albumin to prevent non-specific binding of antibodies, and rabbit anti-rat-TNF-α antibody (AbD Serotec, Oxford, UK) was probed for 1 h. Afterwards, slides were incubated with biotinylated secondary antibody for 30 min and treated with pre-formed avidin and biotinylated horseradish peroxidase macromolecular complex (Vector Labs, Burlingame, CA) for 30 min. Sections were then washed with water, and peroxidase activity was developed using diaminobenzidine solution (Sigma) with 0.04% H_2O_2. Finally, tissue was counterstained with Mayer's hematoxylin solution (Sigma) for 4 min and mounted for light microscopic examination. Slides processed as above but without treatment with primary antibody against TNF-α were used as negative control of staining.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed in two steps. Firstly, a one-way ANOVA was carried out to find significance in the overall comparison of groups. Then, differences between individual groups were investigated by the Scheffé test. Differences were considered to be significant when $P < 0.05$.

Results

Expression of pro-inflammatory genes in TNF-α receptor 1 and 2 knock-out mice during AP

The expression of *tnf-α*, as well as inducible nitric oxide synthase (*inos*), intercellular adhesion molecule 1 (*icam-1*) and interleukin 6 (*il-6*), was measured in pancreas at 1 h after induction of AP by cerulein in wild-type mice and in TNF-α receptor 1 and 2 knock-out (KO TNF-α R1 and KO TNF-α R2) mice. Figure 1a shows that *tnf-α* upregulation occurred not only in wild-type mice, but also in mice devoid of either receptor 1 or 2, indicating that the presence of only one of these TNF-α receptors is sufficient to mediate TNF-α positive feed-back and consequently upregulation. Upregulation of *inos*, *icam-1* and *il-6* also occurred during AP in all three types of mice (wild type, KO TNF-α R1 and KO TNF-α R2) (Fig. 1a). Hence, either TNF-α receptor 1 alone or receptor 2 alone is sufficient to mediate the inflammatory cascade triggered by TNF-α.

Expression of pro-inflammatory genes in TNF-α knock-out mice during AP

The upregulation of *inos* and *icam-1* that occurred in wild-type mice with AP was abrogated in TNF-α KO mice (Fig. 1b). However, the induction of *il-6* expression still occurred in TNF-α KO mice with AP, although to a lesser extent, probably because additional cellular signals and pro-inflammatory cytokine Il-1β may be involved in the activation of the gene during AP initiation. Indeed, *il-6* expression increased almost 16 times in wild-type mice with AP, whereas it increased up to 8 times in the KO mice with AP (Fig. 1b).

Analysis of *tnf-α* gene expression in AP

AP induced by taurocholate in rats was used in order to study the regulation of *tnf-α* expression and particularly the recruitment of transcription factors to *tnf-α* promoter. This experimental model of AP is well established in rats and provides enough material to carry out the chromatin immunoprecipitation assays and compare the results of the immunoprecipitation assays within the same animal. The expression of the *tnf-α* gene in AP induced by taurocholate was analyzed by RNApol ChIP assay as well as by semi-

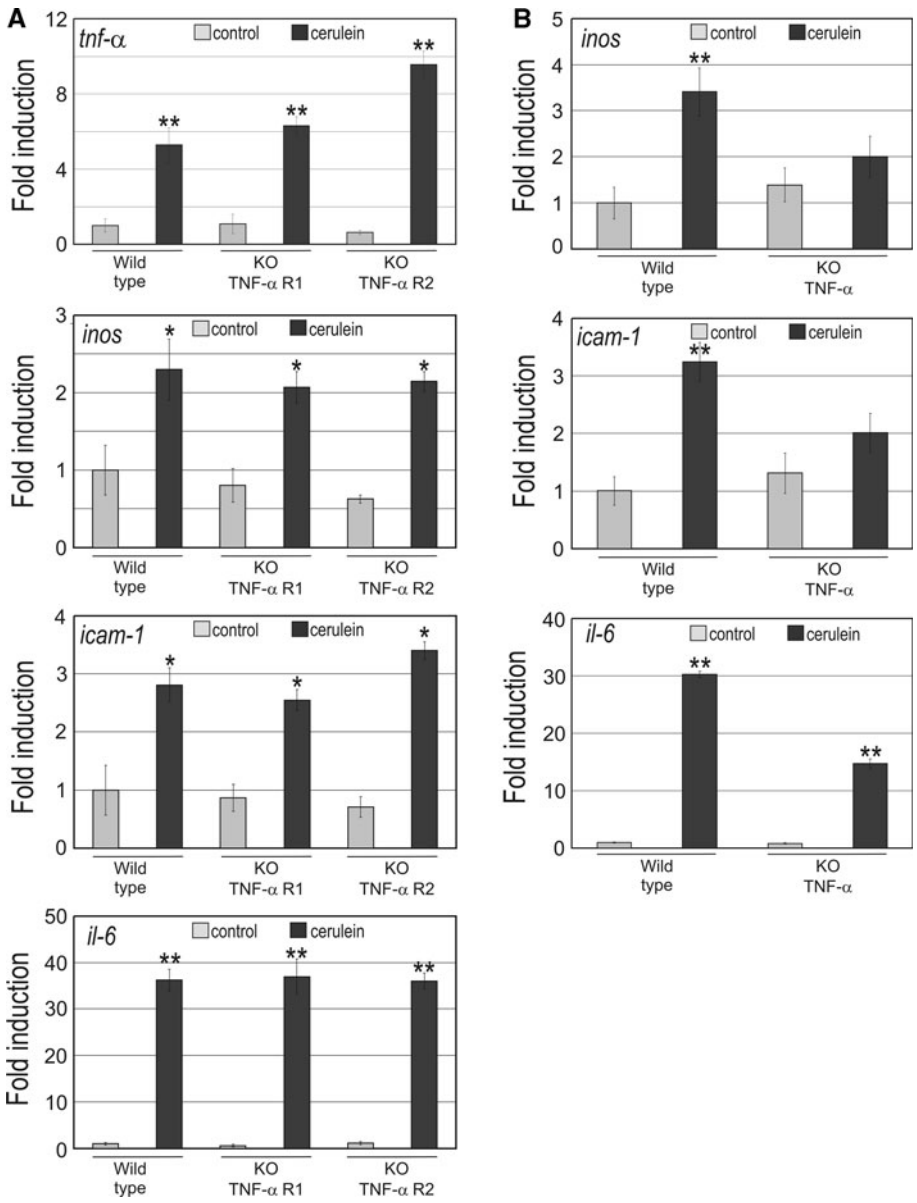
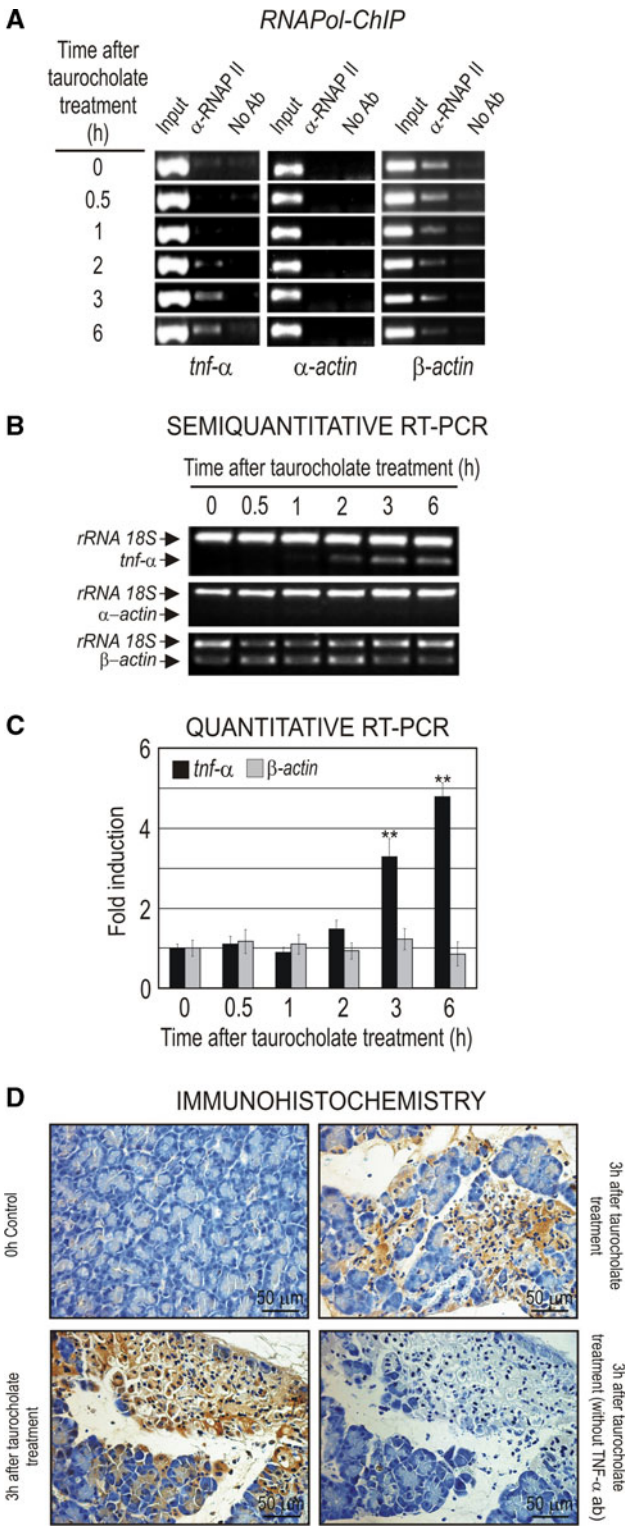


Fig. 1 Expression of tumor necrosis factor alpha (*tnf-α*), inducible nitric oxide synthase (*inos*), intercellular adhesion molecule 1 (*icam-1*) and interleukin 6 (*il-6*) in pancreas from **a** wild-type mice and TNF-α receptor 1 or 2 knock-out mice, and **b** TNF-α knock-out mice with cerulein-induced AP. Steady-state mRNA levels of *tnf-α, inos, icam-1* and *il-6* were measured by quantitative RT-PCR in pancreas from mice treated with saline (control) or cerulein as indicated in Methods. The *error bars* correspond with the standard deviation of 4–5 independent RT-PCR measurements. The statistical significance is indicated as follows: $^{**}P < 0.01$ or $^{*}P < 0.05$ vs. control or time 0

quantitative and quantitative RT-PCR (Fig. 2). We have previously described the RNApol ChIP assay as an experimental approach to asses the actual transcription rate [27]. The results indicate that elongating RNApol II was already present in the *tnf-α* coding region at 2 h of AP induction and thereafter (Fig. 2a), and produces the increase of the steady-state mRNA level at 2–3 h after AP induction, a level that stayed high at least until 6 h post-treatment (Fig. 2b, c). The upregulation of *tnf-α* expression in pancreas in the early stage of AP seems to be primarily ascribed to acinar cells [28–30]. Accordingly, the immunohistochemical analysis showed that pancreatic acinar cells produce TNF-α at 3 h after induction of AP with taurocholate (Fig. 2d).

Ordered transcriptional factor recruitment to *tnf-α* promoter in AP

In order to explore the epigenetic regulation of *tnf-α* in AP, we carried out a kinetic analysis of the recruitment of transcriptional factors to the gene promoter by ChIP assay. First, we analyzed the putative binding sites for transcriptional factors in the promoter by using MatInspector Software (Genomatrix) (Fig. 3a). The analysis showed a complex scenario in which up to nine transcriptional factors might theoretically bind the promoter. To know if they actually bind in vivo, we performed a ChIP assay using the corresponding antibodies (see "Materials and methods"). Figure 3b, c shows that EGR-1, ELK-1, SP1 and NF-κB



◄ **Fig. 2** The *tnf-α* gene expression in taurocholate-induced AP in rats. **a** RNApol ChIP analysis. α- and *β-Actin* genes were used as negative and positive control, respectively, of the RNApol ChIP assay. **b** Semiquantitative RT-PCR and **c** quantitative RT-PCR analysis of the *tnf-α* mRNA level. The *rRNA* 18S was used as an internal control, and α- and *β-actin* genes were used as negative and positive control, respectively, of the RT-PCR analysis. In the histogram, the quantitative RT-PCR values obtained for *tnf-α* or *β-actin* analysis were normalized against those obtained for *rRNA* 18S analysis, giving an arbitrary value of 1 to the non-treated sample. The *error bars* correspond with the standard deviation of at least three independent RT-PCR measurements. The statistical significance is indicated as follows: $^{**}P < 0.01$ or $^{*}P < 0.05$ vs. control or time 0. **d** Immunohistochemical detection of TNF-α production by acinar cells in rat pancreas 3 h after AP induction. Immunostaining negative control with tissue treated in absence of primary TNF-α antibody is also shown

were bound to the promoter when the gene was activated in AP.

We further analyzed if an ordered recruitment of these factors could occur during *tnf-α* activation after AP induction. The factor EGR1 was bound to the promoter at 1 h after AP induction, followed by SP1 and NFκB (2 h) and ELK-1 (3 h) (Fig. 3b, c). The recruitment of the other putative transcriptional factors, C/EBP*β* (Fig. 3b, c), ATF-2, AP-1, ETS-1 or SRF, was not detected in our experiments (results not shown).

Analysis of *tnf-α* gene expression in TNF-α activated AR42J cells

To know the precise order of recruitment of the transcriptional factors to the promoter during *tnf-α* activation, we used the pancreatic acinar cell line AR42J. The use of cell lines to elucidate the epigenetic mechanism of gene expression provides a more accurate time-dependent profile of the transcriptional factors, chromatin modification complex recruitment and histone post-translational modifications needed for gene activation than AP-induced models. Moreover, the use of cell lines also avoids the response variability to the stimulus of animal models.

RNApol ChIP showed that RNA polymerase was present in the coding region of *tnf-α* at 10–20 min, increased significantly at 30 min and stayed bound up to 180 min after treatment of AR42J cells with TNF-α (50 ng/ml) (Fig. 4a). The presence of the elongating RNA polymerase in the coding region of the gene produced *tnf-α* mRNA accumulation, which is noticeable between 30 or 60 min and 180 min after TNF-α treatment (Fig. 4b, c). The apparent delay in the increase of mRNA level, in comparison with RNApol ChIP data, could be attributed to the accumulation of mRNA since the latter methodology is able to measure steady-state levels of mRNA and not the actual transcriptional rate as RNApol ChIP does.

Ordered transcriptional factor recruitment to *tnf-α* promoter in TNF-α activated AR42J cells

Figure 5 shows that the recruitment of transcriptional factors to *tnf-α* promoter upon TNF-α activation followed a

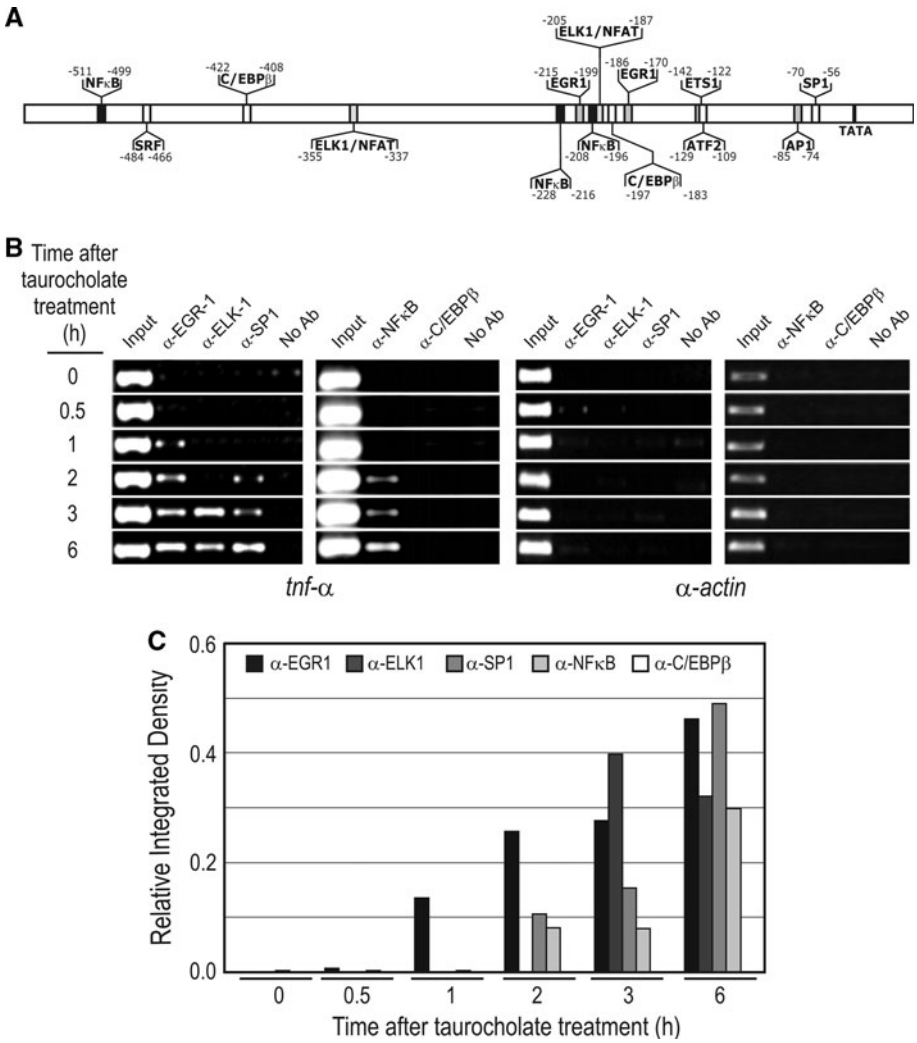


Fig. 3 ChIP assay of *tnf*-α promoter occupation in taurocholate-induced AP in rats. **a** Putative binding sites for transcriptional factors in the *tnf*-α promoter analyzed by MatInspector Software. **b** The immunoprecipitated samples with indicated antibodies were analyzed by PCR using primers of the *tnf*-α promoter region. **c** ImageJ analysis of PCR signals. α-*Actin* gene has also been included as a negative control of the ChIP experiment

strict order as occurred with AP models. ELK-1 and SP1 were bound earlier, at 10 to 20 min after TNF-α treatment, NF-κB and EGR-1 were bound later (30 min), and finally C/EBPβ was bound at 60 min. It is noteworthy that when the gene was fully active, from 90 to 180 min (Fig. 5), all the transcriptional factors were released from the promoter with the exception of SP1.

Ordered histone modification pattern in *tnf*-α upregulation in TNF-α activated AR42J cells

The activation of a specific gene needs a precise pattern of histone modifications, in several cases following an ordered mechanism, to allow the gene to reach a fully active transcriptional state. To establish whether this ordered sequence of epigenetic marks occurs during *tnf*-α activation, we performed a time-dependent ChIP assay of histone acetyltransferase (HAT) and histone deacetylase (HDAC) complex recruitment, as well as site-specific modification of histones, in AR42J cells treated with TNF-α (Fig. 6). Figure 6a, b shows that, in unstimulated cells, HDAC1 and HDAC2 are bound to *tnf*-α promoter, maintaining a repressed state of the gene, and that they were released 10 min after TNF-α treatment, when RNA polymerase started to be detectable in the coding region of the gene (Fig. 5). In coincidence with HDAC release, GCN5-containing and PCAF-containing histone acetyltranferase complexes are recruited to the promoter. However, the binding of GCN5 complex displays a transient pattern since 60 min after *tnf*-α induction the complex is released from the promoter. On the contrary, the PCAF complex remains permanently bound to the promoter during *tnf*-α activation. The release of GCN5-containing complex was coincident with the recruitment of the HAT CREB-binding protein (CBP) 60 min after TNF-α treatment.

The recruitment of histone modification complexes is accompanied by an ordered site-specific modification of

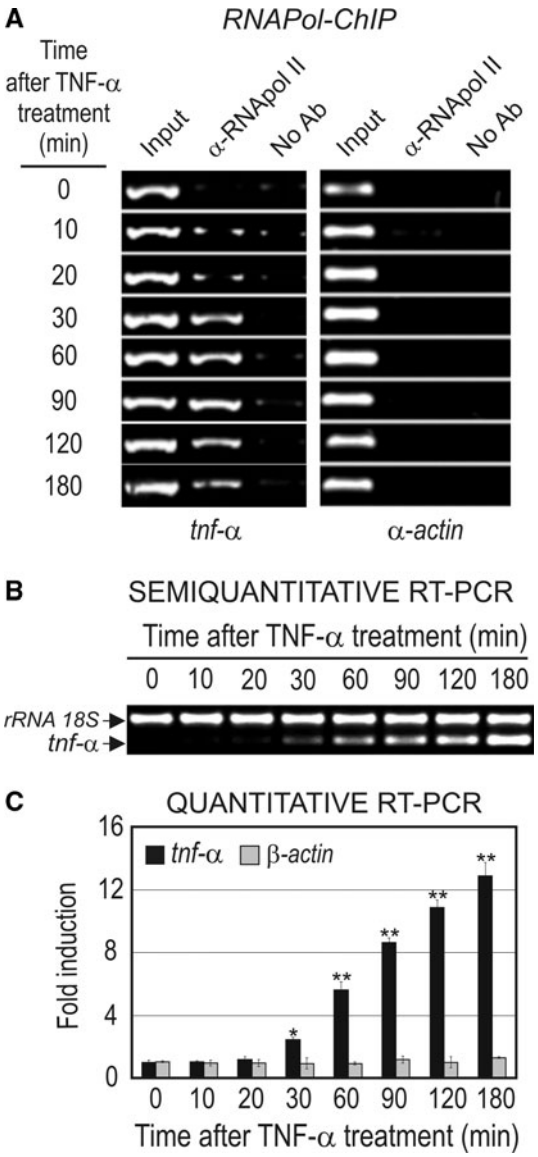


Fig. 4 The *tnf-α* gene expression in TNF-α-activated AR42J cells. **a** RNApol ChIP analysis. **b** Semiquantitative RT-PCR and **c** quantitative RT-PCR analysis of the *tnf-α* mRNA level. *rRNA* 18S was used as an internal control and *β-actin* as negative control of the RT-PCR analysis and *α-actin* as negative control of ChIP assay. The statistical significance is indicated as follows: $^{**}P < 0.01$ or $^{*}P < 0.05$ vs. control or time 0

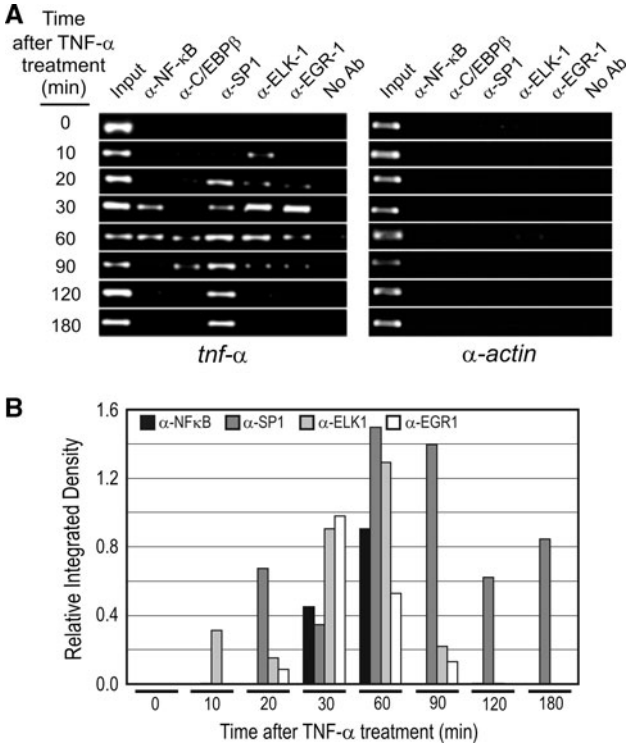


Fig. 5 **a** ChIP assay o*f tnf-α* promoter occupation in TNF-α-activated AR42J cells. **b** ImageJ analysis of PCR signals. The *α-actin* gene has been included as negative control of the ChIP experiment

the histones in *tnf-α* promoter (Fig. 6c–e). In the repressed state, there is a basal level of histone acetylation in lysine 14 and 18 of the histone H3 (Fig. 6c, d; H3K14ac and H3K18ac), modifications that markedly increase when the gene is fully activated. The significant increase in the acetylation level also occurs in lysine 9 of the histone H3 (Fig. 6c–d; H3K9ac). It is worth nothing that there is a transient increase, from 30 to 60 min of induction, in the acetylation level of lysine 5 in histone H4 (Fig. 6c, d; H4K5ac), which could be produced by GCN5 activity recruited at that time to the promoter of the gene. Finally, the methylation of the lysine K4 seems also to be necessary for *tnf-α* activation since this residue was di-methylated at 20 min of induction, and tri-methylated at 90 min of induction, when the gene became fully activated.

Discussion

The degree of pancreatic injury in acute pancreatitis correlates directly with TNF-α levels, and this pro-inflammatory cytokine plays a major role in the initiation of AP [33–35]. The use of mice devoid of TNF-α or its receptors allowed us to elucidate the contribution of this primary cytokine to the inflammatory cascade and the receptor(s) involved. So far, the inflammatory effects of TNF-α have been ascribed to its type I receptor [36]. Accordingly, the lack of TFN-α R1 signaling diminished induction of pro-inflammatory cytokines after brain injury [37]. Contrarily, our results show that the upregulation of pro-inflammatory mediators (*tnf-α, inos, icam-1* and *il-6*) in the pancreas during AP also occurs in KO TNF-α R1 mice, and hence it is not exclusively mediated by TNF-α receptor 1. Both TNF-α receptors 1 and 2 seem to be responsible for the pancreatic inflammatory cascade in the initial course of AP, as *inos*, *icam-1*, *il-6* and *tnf-α* mRNA levels were

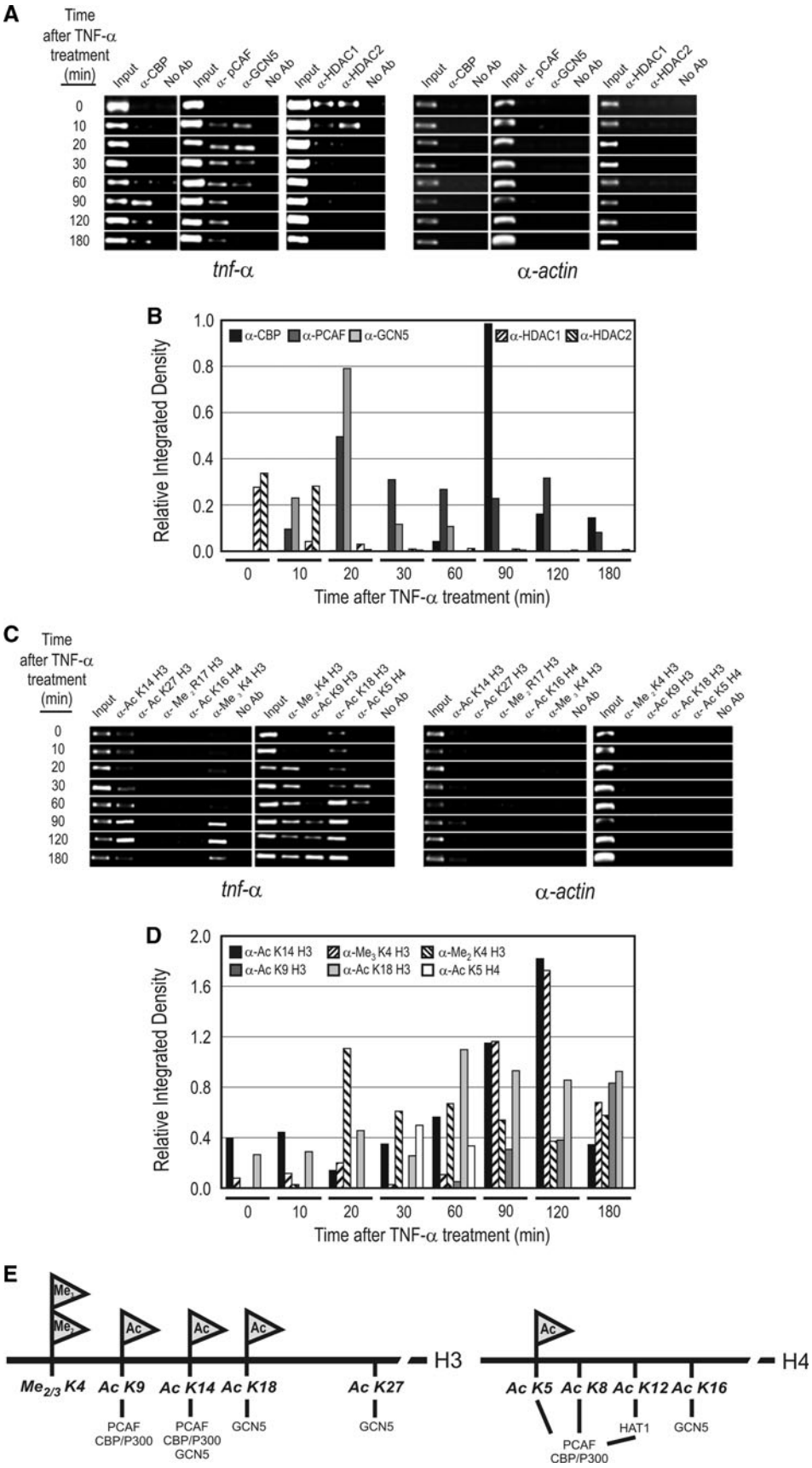


Fig. 6 ChIP assay of chromatin-modifying complex occupation (**a**, **b**) and site-specific histone modifications (**c**–**e**) in *tnf-α* promoter in TNF-α-activated AR42J cells. The *α-actin* gene has been included as negative control for the ChIP experiment

similar in TNF-α R1 and TNF-α R2 KO mice. Consequently, the positive feedback responsible for *tnf-α* induction in the pancreas may be mediated either by receptor 1 or 2. In any case, *tnf-α* upregulation is critical for the pancreatic inflammatory network since TNF-α KO mice did not exhibit any significant increase in *inos* and *icam-1* mRNAs levels, and only a half increase in *il-6* mRNA. The results suggest that upregulation of *il-6* seems to be mediated not only by TNF-α, but probably also by the other initiation cytokine IL-1*β*.

We have also examined the epigenetic mechanisms involved in *tnf-α* gene activation in vivo in taurocholate-induced AP, as well as in vitro in TNF-α activated acinar AR42J cells. This cell line was selected since acinar cells behave as inflammatory cells and are considered to be a primary source of TNF-α in experimental AP [28–30; Fig. 2d], even before leukocytes are able to increase the cytokine production and propagate the inflammatory cascade [36]. Consequently, the upregulation of pro-inflammatory cytokines in the initiation of AP should be mainly ascribed to acinar cells. In addition, the in vitro experiments on acinar AR42J cells show similar kinetics to AP regarding the gene expression of pro-inflammatory cytokines.

It is well known that TNF-α, IL-1*β* or IL-6 is critical for the amplification of the inflammatory signal in AP (see reviews in [1–3]). We found that *tnf-α* was induced when AR42J cells were incubated with TNF-α, but not with IL-1*β* or IL6 (results not shown). Therefore, we used TNF-α-activated acinar AR42J cells to study the epigenetic mechanism of *tnf-α* gene expression. When we analyzed the transcriptional factor recruitment during AP or in TNF-α-activated acinar AR42J cells, only ELK-1, SP1, NF-*κ*B, EGR-1 and C/EBP*β* of the at least nine putative factors that can be bound to the promoter were implicated in *tnf-α* induction. Consequently, the transcriptional factors involved in TNF-α upregulation were essentially the same in vivo in AP models and in vitro in acinar AR42J cells. The fact that these transcriptional factors are required for *tnf-α* gene activation seems to be confirmed when AP is induced in the presence of pentoxifylline, since the anti-inflammatory compound reduced markedly the recruitment of the transcriptional factors NF-*κ*B, SP1 and ELK-1, but not EGR1, to *tnf-α* promoter and inhibits gene expression (material accessible on line, Fig. S1). Pentoxifylline has been studied as a potential modulator of the immunological response in AP because it inhibits TNF-α production [38, 39] and diminishes the leukocyte infiltrate and edema after taurocholate- or cerulein-induced pancreatitis in rats [39, 40].

Our study goes a step forward by showing that *tnf-α* expression in vivo and in vitro follows an ordered transcriptional factor recruitment mechanism (Figs. 3 and 5). The factors ELK-1 and SP1 were bound earlier, NF-*κ*B and EGR-1 were bound later, and finally C/EBP*β*, at least in the AR42J cell line, was bound to *tnf-α* promoter. The recruitment of the transcriptional factors to *tnf-α* promoter in our experiments was, at least in part, distinct from those that have been found in other cell lines under different stimuli. Tsai et al. [21] have described the presence of an enhanceosome, composed by ATF2/c-JUN, ETS1/2, ELK-1, EGR1 and SP1, assembled in *tnf-α* promoter in the LPS-stimulated monocytic cell line. The differences in recruitment of factors to *tnf-α* promoter may be due to the gene responding by a cell-specific and/or stimulus-specific mechanism to different pro-inflammatory circumstances as occurs for instance in LPL, viral and chronic or acute pancreatitis inductions.

The strictly ordered mechanism for *tnf-α* transcriptional activation is also extended to chromatin modification complex recruitment and histone post-translational modifications (Fig. 6). Several authors have also reported that the transcription factors bound to the *tnf-α* promoter (ELK-1, SP1, NF-*κ*B, EGR-1 and C/EBP*β*) physically interact with PCAF, GCN5 and/or CBP/p300 in an inducible manner and, in many cases, its presence in the promoter is a prerequisite for HAT recruitment and activation of target genes [41–46]. In many genes, individual binding sites have only weak affinity for the specific factor, but cooperative binding of adjacent proteins can generate cooperative functional responses allowing the recruitment of coactivators of transcription, for instance CBP/P300, and activating the RNApol II complex (see reviews in [47–49]). The specificity in gene transcription is then achieved by the assembly of these complexes, transcriptional factors and chromatin modifying and remodeling complexes within particular promoters, as seems to occur in *tnf-α*. The complexes then induce conformational changes in the chromatin structure and epigenetic setting that integrate cellular and environmental signaling and determine the expression of specific pro-inflammatory genes (see reviews in [48, 50–52]).

In our studies we showed that *tnf-α* promoter is able to integrate the signals occurring during AP through an ordered recruitment of transcriptional factors. The binding and release of these factors may serve as a specific and transitory mark in the promoter for the recruitment of chromatin modifier complexes HAT and HDAC that may produce the district specific histone modification pattern needed to activate the gene (Fig. 6). In this context, Engdahl et al. [53] showed that THP-1 monocytes or PMA-differentiated cells treated with LPS increase histone H3 and H4 acetylation at the TNF-α promoter and that prostaglandin metabolite 15d-PGJ2 is able to block LPS-induced TNF-α expression by decreasing the acetylation level. They also showed that

inhibition of HDAC with TSA, or overexpression of CBP, overcomes 15d-PGJ2-mediated repression of the TNF-α promoter. In the same THP-1 cell line, the treatment with a high concentration of glucose activates *tnf*-α and *cox2* genes, not only by recruitment to promoter of NF-κB, CBP/p300 and PCAF, as well as histone acetylation, but also by increasing the transcriptional activity of NF-κB p65 over NF-κB-regulated inflammatory genes [54].

These studies and ours demonstrate the importance of epigenetic regulation in the control of TNF-α expression. These findings may have relevance for inflammatory disorders in which TNF-α is overproduced, as occurs in AP. Further experiments are needed to elucidate if the strict ordered epigenetic mechanism for *tnf*-α expression is involved in TNF-α-induced remodeling of the chromatin of *tnf*-α promoter during AP.

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