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## **Tristetraprolin regulates the expression of the human inducible nitric oxide synthase gene**

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**Running title:** TTP regulates human iNOS expression

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**Abbreviations:** 3'-UTR, 3'-untranslated region; AUF1, AU-rich element RNA-binding protein 1; ARE, AU-rich element; cds, coding sequence; CM, cytokine mixture; ELAV, embryonic lethal abnormal vision; GM-CSF, granulocyte/macrophage colony stimulating factor; hnRNP, heteronuclear ribonucleoprotein; IFN- $\gamma$ , interferon- $\gamma$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; IP, immunoprecipitation; KSRP, KH-type splicing regulatory protein; p38 MAPK, p38 mitogene activated protein kinase; MAPKAPK-2/MK2, mitogen-activated protein kinase-activated protein kinase 2; NF- $\kappa$ B, nuclear factor  $\kappa$ B; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NO-synthase; qRT-PCR, quantitative real time reverse transcription polymerase chain reaction; RPA; RNase protection assay; shRNA, short hairpin RNA; siRNA, small interfering RNA; STAT-1 $\alpha$ , signal transducer and activator of transcription-1 $\alpha$ ;

TNF- $\alpha$ , tumor necrosis factor- $\alpha$  □ □ □ □, tristetraprolin; WB, Western Blot

## Abstract

The expression of human inducible NO synthase (iNOS) is regulated both by transcriptional and post-transcriptional mechanisms. Stabilization of mRNAs often depends on activation of p38 mitogene activated protein kinase (p38 MAPK). In human DLD-1 cells, inhibition of p38 MAPK by compound SB203580 or by overexpression of a dominant negative p38 MAPK $\alpha$  protein resulted in a reduction of human iNOS mRNA and protein expression, whereas human iNOS promoter activity was not affected. An important RNA binding protein regulated by the p38 MAPK pathway and involved in the regulation of the stability of several mRNAs is tristetraprolin. RNase protection, qRT-PCR and Western blot experiments showed that cytokines used to induce iNOS expression in DLD-1 cells also enhanced tristetraprolin expression. SB203580-incubation reduced cytokine-mediated enhancement of tristetraprolin expression. Consistently, overexpression or down-regulation of tristetraprolin in stably transfected DLD-1- or A549/8 cells resulted in enhanced or reduced iNOS expression by modulating iNOS-mRNA stability, respectively. In UV-crosslinking experiments, recombinant tristetraprolin did not interact with the human iNOS mRNA. However, co-immunoprecipitation experiments showed interaction of tristetraprolin with the KH-type splicing regulatory protein (KSRP) which is known to recruit AU-rich element containing mRNAs to the exosome for degradation. This tristetraprolin-KSRP interaction was enhanced by cytokines and reduced by SB203580-treatment. We conclude that tristetraprolin positively regulates human iNOS expression by enhancing the stability of human iNOS mRNA. Since tristetraprolin does not directly bind to the human iNOS mRNA but interacts with KSRP, tristetraprolin is likely to stabilize iNOS mRNA by capturing the KSRP-exosome complex.

## Introduction

Regulation of human inducible nitric oxide synthase (iNOS) expression takes place at a large part by post-transcriptional mechanisms modulating mRNA stability (Kleinert et al., 2004; Rodriguez-Pascual et al., 2000).

AU-rich elements (AREs) are critical cis-acting elements in the 3'-untranslated regions (3'-UTRs) of many cytokine, transcription factor and proto-oncogene mRNAs and are targets for trans-acting proteins regulating mRNA stability and translation (Bevilacqua et al., 2003). The human iNOS mRNA contains five of such AREs in the 3'-UTR. In transfection experiments with human A549 or DLD-1 cells, the 3'-UTR of the human iNOS mRNA destabilized the mRNA of a heterologous reporter gene (Rodriguez-Pascual et al., 2000).

A number of ARE binding proteins have been identified that can interact with AU- and U-rich regions. These include AUF-1 (Zhang et al., 1993), the ELAV protein family members (most important HuR, (Brennan and Steitz, 2001)), the KH-type splicing regulatory protein (KSRP, (Chen et al., 2001)), and tristetraprolin (Carballo et al., 1998)).

HuR is known to stabilize several inducible ARE-containing mRNAs (Brennan and Steitz, 2001). Gel retardation experiments showed high affinity interaction of HuR with the human iNOS 3'-UTR (Rodriguez-Pascual et al., 2000). Consequently, enhancing or reducing HuR expression in human DLD-1 cells resulted in an upregulation or downregulation of cytokine-induced iNOS expression, respectively (Rodriguez-Pascual et al., 2000).

In contrast to HuR tristetraprolin, a zinc finger protein, has been shown to destabilize the mRNAs of several immediate-early genes such as c-fos, IL-3, IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF (Blackshear, 2002). Tristetraprolin<sup>-/-</sup> mice develop a severe inflammatory phenotype due to an increased expression of pro-inflammatory cytokines like TNF- $\alpha$  and GM-CSF. This enhanced cytokine expression is a consequence of the loss of the negative regulation at the post-transcriptional level (Carballo et al., 1998).

In line with this data, Chen et al. showed tristetraprolin-dependent ARE-mRNA degradation activity of the human exosome (a complex of exonucleases that catalyzes the 3'  $\rightarrow$  5' decay

pathway of mRNAs (Chen et al., 2001). By interaction with different ARE-binding proteins like KSRP and tristetraprolin the exosome is recruited to these mRNAs to degrade them subsequently (Chen et al., 2001).

Analyses of the mechanisms of stabilization of ARE containing mRNAs revealed the critical involvement of the p38 mitogene activated protein kinase (p38 MAPK) (Kracht and Saklatvala, 2002). Pharmacological inhibition of p38 MAPK $\alpha$  and  $\beta$  (by SB203580) or inhibition of these enzymes by overexpression of dominant negative isoforms resulted in enhanced degradation of different ARE-containing mRNAs like the cyclooxygenase-2 (COX-2) mRNA (Ridley et al., 1998) and the TNF- $\alpha$  mRNA (Mahtani et al., 2001). Interestingly, in rat primary mesangial cells, p38 MAPK $\alpha$  and  $\beta$  regulate iNOS expression in opposite directions (Lui et al., 2004).

The exact pathway by which p38 MAPK stabilizes ARE-containing mRNAs is not known. p38 MAPK was found either directly or via the mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MAPKAPK-2/MK2) to control the expression and posttranslational modification of tristetraprolin (Carballo et al., 2001; Tchen et al., 2004), suggesting that at least in part, the p38 MAPK-mediated stabilization of ARE-mRNAs may work by modulating tristetraprolin expression/activity.

In the current study, we first analyzed the effect of p38 MAPK inhibition on iNOS expression. Our experiments did not show any influence of the p38 MAPK inhibitor SB203580 on cytokine-induced human iNOS promoter activity, but inhibition of cytokine-induced iNOS mRNA- and protein expression. Overexpression of a dominant negative isoform of murine p38 MAPK $\alpha$  also decreased cytokine-induced iNOS expression. To further elucidate the molecular mechanism of p38 MAPK mediated iNOS mRNA regulation we analyzed the involvement of tristetraprolin in this post-transcriptional regulation. The data show that the cytokines inducing human iNOS expression also enhanced tristetraprolin expression. In addition, SB203580- incubation reduced the cytokine-mediated enhancement of tristetraprolin expression. Overexpression of tristetraprolin resulted in a marked increase in

iNOS expression, which was based on mRNA stabilization. UV-crosslink experiments failed to show any interaction of tristetraprolin with the human iNOS mRNA. However, co-immunoprecipitation experiments showed cytokine-enhanced interactions of tristetraprolin with KSRP, which is known to be essential for the degradation of ARE-containing mRNAs by the exosome. Therefore, tristetraprolin appears to be involved indirectly in regulation of human iNOS expression by interaction with KSRP. By capturing the KSRP-exosome complex tristetraprolin seems to dislodge this complex from the iNOS mRNA. This results in enhanced mRNA stability and thereby enhanced iNOS expression.

## Material and Methods

### *Reagents*

Trypsin-, glutamine-, and pyruvate-solutions, PMSF, leupeptin, aprotinin, agarose, IPTG, polyvinylpyrrolidone, tRNA, BSA, actinomycin D, protein A-agarose beads, monoclonal anti- $\beta$ -tubulin- and monoclonal anti-FLAG antibodies, horseradish-peroxidase-coupled anti-rabbit and anti-mouse IgG were purchased from Sigma, Deisenhofen, Germany. Monoclonal anti-iNOS antibodies were obtained from R&D systems, Wiesbaden, Germany. Isotopes were obtained from NEN/Dupont, Köln, Germany. Restriction enzymes, Taq polymerase, Klenow DNA polymerase, dNTPs, oligo-dT primer, Ficoll 400, pGEX-2T and glutathione-agarose affinity beads were purchased from Amersham-Pharmacia, Freiburg, Germany. The monoclonal anti-HA antibodies were obtained from New England Biolabs, Heidelberg, Germany. RNase A, RNase T1, DNase I, T3 and T7 RNA polymerase, FuGene and complete EDTA-free protease inhibitor cocktail tablets were obtained from Roche Diagnostics, Mannheim, Germany. The QuantiTect Probe RT-PCR Kit was from Qiagen, Hilden, Germany. All oligonucleotides and dual labeled probes were from MWG Biotech, Ebersberg, Germany. Human interferon- $\gamma$  (IFN- $\gamma$ ) interleukin-1 $\beta$  (IL1- $\beta$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were obtained from Strathmann, Hannover, Germany. FCS, DMEM and RPMI were purchased from PAN-Systems, Nürnberg, Germany. G 418 and SB203580 were purchased from Calbiochem, Bad Soden, Germany. Zeocin, pZeoSV2(-) and pCDNA3 were purchased from Invitrogen, Groningen, The Netherlands. pCR-Script was from Stratagene, Heidelberg, Germany and psiRNA-hH1-GFPzeo was obtained from InvivoGen, San Diego, USA. The Bradford reagent mix for determination of protein concentration was obtained from BioRad, Munich, Germany. The Dual-Luciferase Reporter Assay System and Passive Lysis Buffer were purchased from Promega, Heidelberg, Germany. The polyclonal anti-tristetraprolin antibody was a kind gift of Dr. William Rigby (Department of Medicine, Dartmouth Medical School, Lebanon, USA), the polyclonal anti-KSRP antibody was a kind gift of Dr. Ching-Yi Chen (Department of Biochemistry & Molecular Genetics, University of Alabama,



Birmingham, USA) and the monoclonal anti-KSRP antibody was a kind gift of Dr. Douglas L. Black (Howard Hughes Medical Institute at UCLA, Los Angeles, USA).

### ***Cell culture, cytokine treatment and RNA isolation***

Human alveolar epithelial A549/8 cells and human colon carcinoma DLD-1 cells were grown in DMEM with 2 mM L-glutamine, penicillin and streptomycin and 5 or 10 % heat-inactivated fetal bovine serum, respectively. Eighteen hours before cytokine activation, cells were washed with PBS and incubated with DMEM containing 2 mM L-glutamine in the absence of serum and phenol red. iNOS expression in DLD-1 cells was induced with a cytokine mixture (CM) containing IFN- $\gamma$  (100 U/ml), IL-1 $\beta$  (50 U/ml) and TNF- $\alpha$  (10 ng/ml) for the corresponding time periods depending on the experiment. In some experiments cells were treated with SB203580 at various concentrations 1 h before and during cytokine incubation. Afterwards, supernatant of the cells (300  $\mu$ l) was used to measure NO $_2^-$  by the Griess reaction or the Sievers Nitric Oxide Analyzer (ADIInstruments, Spechbach, Germany), and cells were processed for RNA isolation by guanidinium thiocyanate/phenol/chloroform extraction as described (Rodriguez-Pascual et al., 2000) or for protein extraction as described below.

### ***RNase protection analyses***

To obtain a human tristetraprolin cDNA fragment a PCR reaction with the primers hTTP-5P1 (5'-CCGGATCCCCGGGCCACCCTCCTGTGC-3') and hTTP-3P1 (5'-GGAAGCTTGAG-AAGGCAGAGGGTGACAG-3') was performed. The PCR fragments were digested with *Hind*III and *Bam*HI and cloned into pCR-Script to generate pCR-hTTP. DNA sequences of the clones were determined using the dideoxy chain termination method with a sequencing kit from Pharmacia.

To generate radiolabeled human iNOS,  $\beta$ -actin, luciferase, and tristetraprolin antisense probes for RNase protection assays, 0.5  $\mu$ g of the linearized plasmids pCR-NOS II-human, pCR-

$\beta$ -actin-human, pXcm-GAPDH-human (Witteck et al., 2003), pCR-luc-pGl2 (Rodriguez-Pascual et al., 2000), and pCR-hTTP were *in vitro* transcribed using T3 or T7 RNA polymerase and  $\alpha$ -<sup>32</sup>P-UTP. To quantify human iNOS, luciferase and tristetraprolin mRNA levels, RNase protection experiments were performed as described (Rodriguez-Pascual et al., 2000). In all experiments,  $\beta$ -actin- or GAPDH mRNA was also determined for normalization purposes. Densitometric analyses were performed using a Phospho-Imager (BioRad, Munich, Germany). The protected fragments of iNOS, luciferase, tristetraprolin,  $\beta$ -actin and GAPDH were 386 nt, 230 nt, 174 nt, 109 nt and 108 nt, respectively.

### ***Quantitative reverse transcription/polymerase chain reaction (qRT-PCR)***

One-Step RT-PCR was performed with the QuantiTect RT PCR Kit (Qiagen, Hilden, Germany) in 25  $\mu$ l reactions in a 96-well spectrofluorometric thermal cycler (iCycler, BioRad, München, Germany). RNA was isolated as described above. For real-time qRT-PCR (RT-reaction: 50 °C 30 min, 95°C 15 min; PCR-reaction: 94°C 15 s, 60°C 60 s, 40 cycles), the oligonucleotides listed below served as sense and antisense primers and Taqman hybridization probes. Taqman hybridization probes were double labeled with 6-carboxyfluorescein (FAM) as reporter fluorophore and carboxytetramethyl-rhodamine (TAMRA) as quencher. All primers and dual-labeled probes (5'-FAM, 3'-TAMRA) were from MWG-Biotech, Ebersberg, Germany. Fluorescence was monitored at each 60°C step.

#### mRNA

iNOS	sense	TGCAGACACGTGCGTTACTCC
	antisense	GGTAGCCAGCATAGCGGATG
	hybridization probe	TGGCAAGCACGACTTCCGGGTG
GAPDH	sense	CCCATGTTTCGTCATGGGTGT
	antisense	TGGTCATGAGTCCTTCCACGATA
	hybridization probe	CTGCACCACCAACTGCTTAGCACCC

tristetraproli n	sense	TTCGCCCACTGCAACCTC
	antisense	CGCCCACTCTCTGAGAAGGTC
	hybridization probe	CCCCTCGCGCTACAAGACTGAGCTATG
luciferase	sense	AAAAAGTTGCGCGGAGGAG
	antisense	TTTTTCTTGCGTCGAGTTTTCC
	hybridization probe	TGTGTTTGTGGACGAAGTACCGAAAGGTCTTAC

Each experimental reaction was performed in triplicate. All primer/probes sets had efficiencies of 100% ( $\pm 10\%$ ).

To calculate the relative expression of iNOS-, luciferase- or tristetraprolin mRNA in DLD-1- or A549/8 cells the  $2^{(-\Delta\Delta C(T))}$  method (Livak and Schmittgen, 2001) was used. According to this method the C(T) values for iNOS-, luciferase- or tristetraprolin mRNA expression in each sample were normalized to the C(T) values of GAPDH mRNA in the same sample. Then the values of untreated cell samples were set 100 % and the percentage of iNOS-, luciferase- or tristetraprolin expression was calculated.

### ***Actinomycin D experiments***

To analyze the effects of experimental interventions on the iNOS mRNA stability, cells were incubated as indicated and iNOS expression was induced by cytokines for 6h. Then, 10  $\mu$ M actinomycin D was added and RNAs were prepared 0 to 18h thereafter. Relative iNOS- and GAPDH mRNA amounts were determined by qRT-PCR and iNOS mRNA was normalized to GAPDH mRNA. The relative amount of iNOS mRNA at 0h actinomycin was set 100 %. Curve fittings of the resulting actinomycin D time curves were performed by non-linear regression using Graphpad Prism 3.0 (GraphPad Software, San Diego, U.S.A.).

### ***Analysis of human iNOS promoter activity in stably and transiently transfected cells***

In order to investigate the effect of inhibition of the p38 mitogene activated protein kinase (p38 MAPK) on cytokine-induced iNOS promoter activity and iNOS mRNA expression, pools of stably transfected DLD-1 cells containing a 16 kb fragment of the human iNOS promoter (see GenBank AC005697 for the sequence of the 5'-flanking sequence of the human iNOS gene) cloned in front of a luciferase reporter gene, were incubated for 18h with DMEM without FCS and without phenol red. Before cytokine activation the cells were pretreated for 1h with the p38 MAPK inhibitor SB203580 at the concentrations indicated. Then cells were incubated with the cytokine mixture for the time periods indicated and RNAs were isolated as described above. To analyze luciferase activity, the cells were lysed in 1x Passive Lysis Buffer and luciferase activity was determined using the Dual-Luciferase Reporter Assay System.

DLD-1 cells were transiently transfected by lipofection with FuGene (Roche) according to the manufacturer's recommendations. 1.5  $\mu\text{g}$  of the plasmid pNOS2(16)Luc (containing a 16 kb fragment of the human iNOS promoter) were combined with 0.5  $\mu\text{g}$  of the renilla reporter gene plasmid pRL-SV40 for normalization of transfection efficiency. After overnight incubation, cells were incubated for 5h with or without CM. Then the cells were lysed in 1x Passive Lysis Buffer provided by the Dual-Luciferase-Reporter-Assay-System, and firefly and renilla luciferase activities were determined in 40  $\mu\text{l}$  and 20  $\mu\text{l}$  of the extracts, respectively. The light units of the firefly luciferase were normalized by those of renilla luciferase after subtraction of extract background.

### ***Western blot experiments***

To study the expression of iNOS protein, total cell protein was fractionated into nuclear and cytoplasmic extracts as described (Greenberg and Ziff, 1984). For iNOS Western blots, 10-50  $\mu\text{g}$  of cytoplasmic proteins were separated on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes by semi-dry electroblotting. All further steps were performed as described (Rodriguez-Pascual et al., 2000). For the detection of human iNOS, an anti-iNOS-

antibody was used. Immune complexes were detected by using anti-mouse horseradish peroxidase-conjugated immunoglobulin. The immunoreactive proteins on the blots were visualized by the enhanced chemiluminescence detection system (ECL, Amersham).

To study the expression of human tristetraprolin (endogenous or HA-tagged) and the transfected dominant negative isoform of p38 MAPK $\alpha$  (FLAG-tagged) cytoplasmic extracts (10-50  $\mu$ g protein) were separated on 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes by semi-dry electroblotting. All further steps were performed as described (Rodriguez-Pascual et al., 2000). For the detection of endogenous tristetraprolin a polyclonal anti-tristetraprolin-antibody (Brooks et al., 2002) was used. For detection of HA-tagged tristetraprolin and dominant negative FLAG-tagged p38 MAPK $\alpha$  protein monoclonal anti-HA-antibodies (HA-TAG 262K, NEB) and anti-FLAG-antibodies (Anti-FLAG M2, Sigma) were used, respectively. For detection of KSRP a polyclonal anti-KSRP-antibody (Gherzi et al., 2004) or a monoclonal anti-KSRP-antibody (Hall et al., 2004) was used. Immune complexes were detected by using anti-mouse or anti-rabbit horseradish peroxidase-conjugated immunoglobulin. The immunoreactive proteins on the blots were visualized by the enhanced chemiluminescence detection system (ECL, Amersham).

### **Immunoprecipitation**

For immunoprecipitation, cell extracts were digested for 30 min at 30 °C with RNase A (40  $\mu$ g) and RNase T1 (100000 U). Then these extracts were preincubated with protein A-agarose beads (Sigma, Deisenhofen, Germany) in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 10 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM sodium pyrophosphate, 50 mM disodium glycerol phosphate, 10 mM ascorbic acid, 2 mM EDTA, 10 % glycerol, 1 % NP40, 1/50 v/v complete EDTA-free protease inhibitor cocktail) for 1 h at 4 °C. These precleared extracts were incubated with polyclonal or monoclonal antibodies or a rabbit pre-immune serum (negative control) overnight at 4 °C in RIPA buffer. Subsequently protein-antibody complexes were captured by incubating with protein A-agarose beads for 5 h at 4 °C in RIPA

buffer. Beads were washed three times with RIPA buffer and co-immunoprecipitated proteins were analyzed by Western Blotting.

### ***Purification of GST-TTP protein***

To generate pGEX-HA-hTTP, a bacterial expression plasmid coding for a GST-HA-tristetraprolin fusion protein (GST-TTP), the plasmid pCMV-hTTP-Flag (Lai et al., 1999), kindly provided by Dr. Blackshear) was digested with *Hind*III. The fragment was purified by gel electrophoresis and cloned into pCR-Script (Stratagene) to generate pCR-HA-hTTP. Then pCR-HA-hTTP was digested with *Cla* I and *Eco*RV and the ends were blunt ended by treatment with the Klenow enzyme. The fragment was purified by gel electrophoresis and cloned into pGEX-2T (Amersham-Pharmacia) digested with *Sma*I. DNA sequences of the clones were determined using the dideoxy chain termination method with a sequencing kit from Pharmacia. Purified GST-TTP fusion protein was prepared using the plasmid pGEX-HA-hTTP as described (Rodriguez-Pascual et al., 2000). The yield of the purification procedure was determined by comparison to a BSA standard on Coomassie blue-stained SDS-PAGE. The electrophoresis revealed a 63-kDa band corresponding to the fusion protein. The same procedure was used to purify GST protein from *Escherichia coli* cultures transformed with the plasmid pGEX-2T.

### ***UV-crosslinking experiments***

cDNAs encoding for subfragments of human iNOS 3'-UTR have been described previously (Rodriguez-Pascual et al., 2000). To obtain the 5'-UTR and cds of the human iNOS mRNA and the 3'-UTR of the human c-fos mRNA PCR reactions were performed using the primers N2-5UTR-5P1 (5'-CCAAGCTTATAACTTT-GTAGCGAGTCG-3') and N2-5UTR-3P1 (5'-CCAAGCTTCTCTATGGCTTTACAAAGC-3') or N2-cds-5P1 (5'-CCAGATCTCGAGATGGCCTGTCCTTG-3') or N2-cds3-P1 (5'-CCGCGGCCGCTCAGAGCGCTGACATCTCCA-3') and hum-fos-AUFL-5P1 (5'-CATGCATTGTTGAGGTGGTC-3') and hum-fos-AUFL-

3P1 (5'-CTTGGAACAATAAGCAAACAATG-3'), respectively. The PCR fragments were restricted with *HindIII* or *XhoI* and *NotI*, respectively or treated with T4 polynucleotide kinase, and cloned in pCR-Script to generate pCR-iNOS-5'-UTR, pCR-iNOS-cds and pCR-hum-fos-AUFL. To generate radiolabeled iNOS 5'-UTR, cds or 3'-UTR or human c-fos AUFL sense probes for RNA binding experiments, 0.5-1  $\mu\text{g}$  of DNA (linearized plasmids or PCR fragments) were *in vitro* transcribed as described above. Radiolabeled transcripts were analyzed by urea-denaturing electrophoresis in order to estimate the yield and the specific activity. Incorporated radioactivity in transcripts was usually higher than 80 % and the specific activity ranged from 0.2-0.5  $\mu\text{Ci}/\text{pmol}$ .

For UV-crosslinking experiments radiolabeled iNOS-5'-UTR-, -cds- or -3'-UTR RNA and human c-fos3'-UTR RNA was incubated with 0.6  $\mu\text{g}$  purified GST fusion protein in a volume of 25  $\mu\text{l}$  in binding buffer (10 mM Hepes pH 7.6, 3 mM  $\text{MgCl}_2$ , 5 mM EDTA, 2 mM DTT, 5% Glycerol, 0.5% NP-40, 3 mg/ml Heparin, and 0.5 mg/ml yeast tRNA) supplemented with 40 mM KCl and RNasin (0.3 U/ml, final) for 20 min at 37 °C. Then the probes were irradiated with UV-C light (125 mJ) for 30 min on ice. RNA not protected by protein binding was digested by addition of RNases T1 (20 U/assay) and RNase A (20  $\mu\text{g}/\text{assay}$ ) for 30 min at 30 °C. The reaction was stopped by addition of 6  $\mu\text{l}$  5X Laemmli loading buffer (312.5 mM Tris-HCl pH 6.8, 5 mM EDTA, 15 % SDS, 50 % Glycerin, 0.015 % bromphenolblue, 40 mM DTT). After denaturation at 80 °C for 10 min the samples were separated in 12 % SDS polyacrylamide gels, the gels were dried and exposed to X-ray films.

***Establishment of cell lines expressing a sense or antisense tristetraprolin mRNA, a dominant negative p38 MAPK $\alpha$  vector-derived small interfering RNA for downregulation of tristetraprolin expression.***

The plasmids pZeo-hTTP-HA-sense or -antisense were generated by cloning a 1000 bp *HindIII* fragment from pCMV-hTTP-flag (Lai et al., 1999) containing the cDNA sequence of a HA-tagged tristetraprolin protein in both orientations into pZeoSV2(-) (Invitrogen). DNA

sequences of the clones were determined using the dideoxy chain termination method with a sequencing kit from Pharmacia. To generate DLD-1 cells overexpressing a sense or antisense HA-tagged tristetraprolin mRNA, cells were transfected with 5  $\mu$ g of pZeo-hTTP-HA-sense or -antisense by lipofection with FuGene according to the manufacturer's recommendations. Stable transfectants were selected with Zeocin (200  $\mu$ g/ml). As a control, DLD-1 cells stably transfected with the pZeoSV2(-) vector were generated as well (Zeo). Pooled population of cells and single clones were characterized for the expression of sense tristetraprolin-HA mRNA by Western Blots using a monoclonal anti-HA-antibody and by PCR analyses. To test for antisense tristetraprolin-HA RNA expression RNase protection experiments using a radiolabeled sense tristetraprolin-HA probe were performed.

To generate DLD-1 cells overexpressing a dominant negative murine FLAG-tagged p38 MAPK $\alpha$  cells were transfected with 4,5  $\mu$ g of pCDNA3 (Invitrogen) and 0.5  $\mu$ g pCS3-FLAG-p38AGF (Winzen et al., 1999) by lipofection with FuGene according to the manufacturer's recommendations. Stable transfectants were selected with G418 (1 mg/ml). As a control, DLD-1 cells stably transfected with the pCDNA3 vector were generated as well (pCDNA3). Pooled populations of cells were characterized for p38AGF-FLAG expression by Western blots using a monoclonal anti-FLAG-antibody and by RT-PCR analyses.

The plasmid psiRNA-hH1-GFPzeo-TTP was generated by cloning a double stranded oligonucleotide

(5'-  
ACCTCACAAGACTGAGCTATGTCGGATCAAGAGTCCGACATAGC-

TCAGTCTTGTTTTTTG-3'; sequence of the siRNA repeats directed against the human tristetraprolin mRNA underlined) into the Bbs I sites of psiRNA-hH1-GFPzeo (Invivogen).

The DNA sequence of the construct was determined using the dideoxy chain termination method with a sequencing kit from Pharmacia. To generate A549/8 cells stably expressing shRNAs directed against the human tristetraprolin mRNA, cells were transfected with 5  $\mu$ g of



psiRNA-hH1-GFPzeo-TTP by lipofection with FuGene according to the manufacturer's recommendations. Stable transfectants were selected with Zeocin (200  $\mu\text{g/ml}$ ). As the psiRNA-hH1-GFPzeo vector codes for a GFP-zeocin resistance fusion protein the zeocin-resistant cell pools were also selected for GFP expression by fluorescence activated cell sorting. As a control, A549/8 cells stably transfected with the empty psiRNA-hH1-GFPzeo vector were generated as well.

### ***Statistics***

Data represent means  $\pm$  SEM. Statistical differences were determined by factorial analysis of variance followed by Fisher's protected least-significant-difference (PLSD) test for comparison of multiple means.

## Results

### *Inhibition of p38 mitogene activated protein kinase (p38 MAPK) by compound SB203580 or by overexpression of a dominant negative p38 MAPK markedly reduces iNOS mRNA and protein expression, but has no effect on human iNOS promoter activity.*

In order to test the effect of SB203580-mediated inhibition of p38 MAPK on human iNOS promoter activity and iNOS mRNA expression, we used DLD-1 cells stably transfected with pNOS2(16)Luc, a construct containing a 16 kb human iNOS promoter fragment (see GenBank AC005697 for the sequence of the 5'-flanking region of the human iNOS gene, (Kleinert et al., 2004)) cloned in front of luciferase. These cells were preincubated with SB203580 (0 to 10  $\mu$ M) for 1h. Then iNOS expression was induced by a cytokine mixture (CM; 100 U/ml IFN- $\gamma$ , 10 ng/ml TNF- $\alpha$  and 50 U/ml IL-1 $\beta$ ). After 2 to 48h, cells were lysed and RNA was purified. To analyze the iNOS- and luciferase mRNA expression, quantitative RNase protection experiments were performed using antisense probes for human iNOS- and luciferase mRNA. For normalization, expression of  $\beta$ -actin mRNA was determined in the same reaction. qRT-PCR experiments were performed as well using the primer/probes described in Material and Methods. For normalization in qRT-PCR experiments the expression of GAPDH mRNA was determined in a parallel reaction using the same RNA sample. As shown in **figure 1**, no iNOS mRNA (panel A and C) or protein (panel D) expression was detected in unstimulated DLD-1 cells. However, in accordance with previous results (Rodriguez-Pascual et al., 2000) basal levels of luciferase mRNA were detected (**figure 1**, panels B and C). Basal luciferase activity was measured in extracts of these cells (data not shown) either. Incubation of the cells with CM resulted in marked induction (>50 fold) of iNOS mRNA expression (**figure 1**, panels A and C). In contrast, luciferase mRNA expression was only induced about 5 fold (**figure 1**, panels B and C). CM-incubation also resulted in a nearly 5 fold enhancement of luciferase activity in extracts of these cells (data not shown). Inhibition of p38 MAPK by SB203580 resulted in marked reduction of the

human iNOS mRNA (**figure 1**, panels **A** and **C**), protein expression (**figure 1**, panel **D**) and iNOS-mediated NO production (**figure 1**, panel **E**). In contrast, SB203580 did not influence basal or cytokine-induced luciferase mRNA expression (**figure 1**, panels **B** and **C**).

There are 4 different isoforms of p38 MAPK (see Introduction), but only two (p38 MAPK $\alpha$  and  $\beta$ ) are inhibited by SB203580. Since SB203580 inhibited CM-induced iNOS expression in DLD-1 cells and Lui et al. described opposite effects of p38 MAPK $\alpha$  (stabilizing) or  $\beta$  (destabilizing) on iNOS expression in rat mesangial cells (Lui et al., 2004) we also tested the effect of inhibition of p38 MAPK $\alpha$  by overexpression of a dominant negative p38 MAPK $\alpha$  isoform. We stably transfected DLD-1 cells with pCS3-FLAG-p38AGF (Winzen et al., 1999) leading to overexpression of a dominant negative murine p38 MAPK $\alpha$  protein in these cells. As shown in **figure 1** (panel **F**) stable overexpression of this dominant negative p38 MAPK $\alpha$  protein resulted in marked inhibition of cytokine-induced iNOS expression in human DLD-1 cells.

Therefore, these data indicate that human iNOS expression depends both on transcriptional and post-transcriptional mechanisms. Furthermore, activation of the p38 MAPK pathway seems to be critically involved in the post-transcriptional regulation of human iNOS expression.

***Cytokines enhance expression of tristetraprolin and inducible nitric oxide synthase (iNOS) in human DLD-1 cells.***

Tristetraprolin has been described as a destabilizing protein for ARE-containing mRNAs (Blackshear, 2002). Recent evidence also suggests that tristetraprolin is involved in the regulation of mRNA stability of ARE containing mRNAs in response to p38 MAPK (Mahtani et al., 2001; Stoecklin et al., 2004). Therefore, we analyzed the role of tristetraprolin in human iNOS expression. First, we tested the effect of cytokine incubation on tristetraprolin expression in DLD-1 cells. As shown in **figure 2**, cytokine-incubation of DLD-1 cells resulted in enhanced tristetraprolin mRNA (**figure 2**, panels **A** and **B**) and protein expression

(**figure 2**, panel **C**). Interestingly, the time curve of the enhancement of tristetraprolin expression paralleled the time curve of iNOS induction (Rodriguez-Pascual et al., 2000).

***Inhibition of p38 MAPK by SB203580 reduces cytokine-induced tristetraprolin expression.***

It has been reported that tristetraprolin expression is regulated by the p38 MAPK pathway (Tchen et al., 2004). Therefore, we analyzed the influence of SB203580-mediated inhibition of p38 MAPK on the cytokine-stimulated tristetraprolin expression. As shown in **figure 3**, SB203580 reduced cytokine-induced tristetraprolin expression.

***Biological role of tristetraprolin: Overexpression of sense or antisense tristetraprolin mRNA or downregulation of tristetraprolin expression by small interfering RNAs (siRNAs).***

In order to determine whether tristetraprolin plays a role in the regulation of iNOS gene expression in intact cells, we generated stably transfected DLD-1 cells which constitutively express sense tristetraprolin mRNA (**pZeo-hTTPs**) or antisense tristetraprolin mRNA (**pZeo-hTTPas**) under the control of the early SV40 promoter (pZeoSV2 expression plasmid). Pooled cell populations were analyzed for stable integration of the DNA and tristetraprolin expression. As a control, DLD-1 cells stably transfected with the (empty) pZeoSV2 vector were generated as well (**pZeo**). As shown in **figure 4** (panel **A**), Western blot experiments revealed enhanced tristetraprolin protein expression in **pZeo-hTTPs** cells, whereas a decrease of tristetraprolin protein expression was seen in **pZeo-hTTPas** cells. Overexpression of sense tristetraprolin-cDNA resulted in enhanced cytokine-induced iNOS mRNA expression (**figure 4**, panels **B** and **C**). This result was unexpected, because tristetraprolin is generally believed to be an ARE-binding protein leading to mRNA degradation. However, consistent with the results above, reduction of tristetraprolin expression by an antisense approach impaired cytokine-induced iNOS mRNA expression (**figure 4**, panels **D** and **E**). Also cytokine-induced iNOS-dependent NO production was enhanced by tristetraprolin overexpression in DLD-1-

pZeo-hTTPs cells and reduced by tristetraprolin downregulation in DLD-1-pZeo-hTTPas cells (**figure 4, panel F**).

To support this unexpected result of tristetraprolin-mediated enhancement of iNOS expression we aimed to downregulate tristetraprolin expression in a different cell line using small interfering RNAs (siRNAs). Therefore, we generated A549/8 cells stably transfected with psiRNA-hH1-GFPzeo-TTP. In these cells siRNAs containing a hairpin structure (shRNAs) were generated, which were directed against the human tristetraprolin mRNA. As shown in **figure 5** (panels A and B) tristetraprolin expression was reduced in A549/8-psiRNA-hH1-GFPzeo-TTP cells (**siTTP**) on RNA- and protein level compared to A549/8 cell stably transfected with the empty vector psiRNA-hH1-GFPzeo (**GFP**). As shown above for the DLD-1-pZeo-hTTPas cells, siRNA-mediated downregulation of tristetraprolin expression resulted in a marked reduction of cytokine-induced iNOS mRNA expression also in A549/8 cells (see **figure 5** panel C).

Therefore, tristetraprolin seems to be positively involved in the regulation of cytokine-induced iNOS expression.

***Overexpression or down-regulation of tristetraprolin modulates human iNOS mRNA stability, but does not change human iNOS promoter activity.***

To test whether the effects of enhancement or reduction of tristetraprolin expression on iNOS expression resulted from tristetraprolin-mediated changes in the stability of the human iNOS mRNA, we performed actinomycin D experiments. DLD-1-pZeo, -pZeo-TTP-sense and -pZeo-TTP-antisense cells were incubated with CM for 6 h. Then actinomycin D (10  $\mu$ g/ml) was added to stop transcription and RNA was isolated after 0, 2, 4, 6 and 18 h. Expression of iNOS mRNA in comparison to GAPDH was determined by qRT-PCR. As shown in **figure 6** (panel A), compared to pZeo cells ( $t_{1/2} = 3.95 \pm 0.24$  h), overexpression of tristetraprolin resulted in a nearly twofold enhancement of human iNOS mRNA stability ( $t_{1/2} = 6.76 \pm 0.64$

h). Accordingly, downregulation of tristetraprolin expression reduced iNOS mRNA stability ( $t_{1/2} = 3.12 \pm 0.16$  h).

Several RNA-binding proteins have been described to bind to DNA and regulate promoter activity, too (Donev et al., 2002; Fiset and Chabot, 2001; He et al., 2000; Katahira et al., 2001). Therefore we analyzed whether enhancement or reduction of tristetraprolin expression changes human iNOS promoter activity. We transiently transfected pNOS2(16)Luc and pRL-SV40 (a construct containing renilla luciferase, for normalization of transfection efficiency) into DLD-1-pZeo, DLD-1-pZeo-hTTPs, and DLD-1-pZeo-hTTPas cells. After transfection the cells were incubated in the presence or absence of the cytokine-mixture and firefly and renilla luciferase activity were analyzed in extracts of these cells. As shown in **figure 6** (panel **B**), cytokine incubation resulted in similar enhancement (about 3 fold) of iNOS promoter activity in all three cell lines. Therefore, the effect of tristetraprolin on iNOS expression is unlikely to involve regulation of human iNOS promoter activity.

In summary, the enhancing effect of tristetraprolin overexpression on human iNOS mRNA expression results from enhanced mRNA stability.

### ***Tristetraprolin does not interact with the human iNOS mRNA***

In order to analyze the interaction of tristetraprolin with the human iNOS mRNA 3'-UTR, we purified recombinant GST-TTP protein for UV-crosslinking experiments. Purified GST-TTP and GST protein (as negative control) was incubated with labeled transcripts comprising the human iNOS 3'-UTR sequence and tristetraprolin/RNA complex formation was assayed by UV-crosslinking. As shown in **figure 7** (panel **A**), we detected no complex formation between recombinant GST-TTP-protein and the whole 3'-UTR transcript. In contrast, as shown before by RNA gelshift experiments (Rodriguez-Pascual et al., 2000) GST-HuR specifically binds to the human iNOS 3'-UTR sequence. Then we analyzed the interaction of GST-TTP with the human iNOS 5'-UTR and the human iNOS coding sequence (cds). GST-TTP did not interact with one of these iNOS mRNA fragments either (see **figure 7**, panel **B**). However, using a

human c-fos 3'-UTR RNA, a known target for tristetraprolin binding, instead of human iNOS mRNA fragments, resulted in a specific tristetraprolin/RNA complex formation (see **figure 7**, panel C). These experiments demonstrated that tristetraprolin does not bind to any region of the human iNOS mRNA.

***Tristetraprolin interacts with the KH-type splicing regulatory protein (KSRP) in human DLD-1 cells***

The experiments above showed post-transcriptional regulation of cytokine-induced iNOS expression by tristetraprolin without binding of tristetraprolin to the iNOS mRNA. Therefore, it seems very likely that tristetraprolin interacts with other proteins important for the stability of iNOS mRNA. KSRP has been shown to be an essential component of the degradation machinery of mRNAs containing AREs (Chen et al., 2001; Gherzi et al., 2004). Therefore, we tested the interaction of tristetraprolin and KSRP by co-immunoprecipitation experiments. Cellular extracts from CM- or untreated DLD-1 cells were incubated with polyclonal anti-tristetraprolin antibodies or with a rabbit pre-immune serum as negative control. The immunoprecipitated material was analyzed by Western Blotting, using a monoclonal anti-KSRP antibody. As shown in **figure 8** (panel A), tristetraprolin displayed a marked protein-protein interaction with KSRP. This interaction was enhanced by cytokine-incubation. To analyze the effect of SB203580 on this tristetraprolin-KSRP interaction cellular extracts from CM- or untreated DLD-1 cells pretreated with or without 10  $\mu$ M were incubated with monoclonal anti-KSRP antibodies or with a rabbit pre-immune serum as negative control. As shown in **figure 8** (panels B and C) also immunoprecipitation with monoclonal anti-KSRP antibodies and Western Blotting of the precipitates with polyclonal anti-tristetraprolin antibodies revealed the same tristetraprolin-KSRP protein-protein interaction. As shown above this interaction was enhanced by cytokine treatment. SB203580 treatment reduced this cytokine enhancement.

## Discussion

Gene expression is controlled both by transcriptional and post-transcriptional mechanisms. A central part of the post-transcriptional modulation of gene expression is mediated by regulation of mRNA stability. The stability may vary considerably from one mRNA to another and can be modulated by various extracellular stimuli (Wilusz et al., 2001). A tight control of mRNA stability enables cells to fine-tune the expression of genes, permits rapid changes in the levels of mRNAs and provides a mechanism for prompt termination of protein production. The rate of mRNA decay is determined by cis-acting sequences within the mRNA which are recognized by trans-acting factors. Dysregulation of the expression of RNA-binding proteins or mutation of important cis-acting binding sequences and thereby dysregulation of mRNA stability has been associated with different diseases like chronic inflammatory diseases (Kontoyiannis et al., 1999), cancer (Gouble et al., 2002) and Alzheimer's disease (Hollams et al., 2002).

The best-characterized cis-acting sequences responsible for mRNA decay in mammalian cells are the AU-rich elements (AREs) present within the 3'-untranslated regions (3'-UTRs) of short-lived mRNAs from cytokine, proto-oncogene and growth-factor genes, whose expression has to be regulated exactly (Bakheet et al., 2001). These AREs are involved in deadenylation and subsequent degradation of mRNAs (Shyu et al., 1991) and have also been described to stimulate 5'-decapping (Gao et al., 2001). More than 15 proteins are known that bind to AREs (Hollams et al., 2002). Only few of them have been shown clearly to regulate mRNA stability. These include the AU-rich element RNA-binding protein 1 (AUF1) (Zhang et al., 1993), the embryonic lethal abnormal vision (ELAV) proteins (also named Hu proteins), especially HuR (Brennan and Steitz, 2001), the KH-type splicing regulatory protein (KSRP, (Chen et al., 2001)) and the tristetraprolin family of zinc-finger RNA binding proteins (Lai et al., 1999).

One important signal transduction pathway involved in the regulation of ARE-mRNA degradation is the p38 mitogene activated protein kinase (p38 MAPK) pathway (Kracht and



Saklatvala, 2002). Inhibition of p38 MAPK either by pharmacological inhibitors or overexpression of dominant negative p38 MAPK isoforms resulted in enhanced degradation of different ARE-containing mRNAs like the cyclooxygenase-2 (COX-2) mRNA (Ridley et al., 1998), the IL-1 $\beta$  mRNA (Kracht and Saklatvala, 2002), the TNF- $\alpha$  mRNA (Mahtani et al., 2001) and the iNOS mRNA (Lui et al., 2004).

In order to analyze the effect of inhibition of p38 MAPK on the human iNOS promoter activity and iNOS mRNA and protein expression in parallel, we used DLD-1 cells stably transfected with pNOS2(16)Luc (containing a 16 kb fragment of the human iNOS promoter, see GenBank AC005697 for the sequence of the 5'-flanking region of the human iNOS gene). RNase protection experiments and quantitative real time RT-PCR (see figure 1, panels **A**, **B**, **C** and **E**), Western blots (figure 1, panel, **D**), nitrite measurements (figure 1, panel, **E**) and luciferase activity determination (data not shown) revealed that inhibition of p38 MAPK by SB203580 or a dominant negative p38 MAPK $\alpha$  isoform reduced iNOS mRNA and protein expression but not luciferase mRNA- or protein expression. This effect of p38 MAPK inhibition on iNOS expression has been shown in human osteoarthritic chondrocytes (Poljakovic et al., 2003) and human kidney epithelial cells (Martel-Pelletier et al., 1999). In transient transfection experiments using fragments of the human or rat iNOS promoter reduction of LPS and/or cytokine-induced promoter activity by SB203580-mediated inhibition of the p38 MAPK pathway has been described (Bhat et al., 2002; Kristof et al., 2001). However, in our experiments the activity of the stably transfected human 16 kb iNOS promoter was not effected by p38 MAPK inhibition. Therefore, in DLD-1 cells p38 MAPK seems to regulate human iNOS expression at the post-transcriptional level.

The exact mechanism of p38 MAPK-mediated stabilization of ARE-containing mRNAs is not known. p38 MAPK was found to control the expression and post-translational modification of tristetraprolin either directly (Carballo et al., 2001) or via its substrate MAPKAPK-2/MK2 (Tchen et al., 2004), suggesting that at least in part the p38 MAPK-mediated stabilization of ARE-mRNAs may work by modulating tristetraprolin expression/activity.

In an attempt to further characterize the involvement of tristetraprolin in human iNOS expression, we analyzed the effect of cytokine-incubation on expression of tristetraprolin in DLD-1 cells. As shown in **figure 2**, CM incubation enhanced tristetraprolin mRNA (**figure 2**, panels **A** and **B**) and protein (**figure 2**, panel **C**) expression. The time curve of tristetraprolin induction was similar to the time curve of human iNOS induction in these cells (Rodriguez-Pascual et al., 2000). As cytokine-induced expression of iNOS is reduced by SB203580-treatment, we then analyzed whether this treatment also influences tristetraprolin expression. As shown in figure 3, preincubation with 10  $\mu$ M SB203580 decreased tristetraprolin expression. A similar effect of p38 MAPK inhibition had been described in murine RAW macrophages (Tchen et al., 2004)

As tristetraprolin appeared to be a good candidate for an ARE-binding protein modulating human iNOS expression, we stably transfected human DLD-1 cells with an eucaryotic expression vector coding for a HA-tagged tristetraprolin protein. To downregulate endogenous tristetraprolin expression, cells containing the tristetraprolin-HA cDNA in an opposite orientation were generated as well. Pools of clones were analyzed for cytokine-induced iNOS expression. Unexpectedly, as shown in **figure 4**, overexpression of HA-tagged tristetraprolin protein (**pZeo-hTTPs**) resulted in enhanced cytokine-induced iNOS mRNA expression (**figure 4**, panels **B** and **C**) and NO production (**figure 4**, panel **F**) compared to control cells stably transfected with the expression vector (**pZeo**). Accordingly, transfection of the antisense construct (**pZeo-hTTPas**) resulted in reduced iNOS mRNA (**figure 4**, panels **D** and **E**) and NO production (**figure 4**, panel **F**).

As the stabilizing effect of tristetraprolin on iNOS expression in DLD-1 cells was highly unexpected, we decided to expand this analysis using another cell line and a different method to modulate tristetraprolin expression. We generated A549/8 cells (**siTTP**) stably transfected with a vector (psiRNA-hH1-GFPzeo-TTP) coding for siRNAs directed against the human tristetraprolin mRNA. As control, A549/8 cell pools transfected with the empty vector (**GFP**) were generated. By using the siRNA technique we were able to downregulate tristetraprolin

mRNA and protein expression (**figure 5**, panels **A** and **B**) in A549/8 cells. As shown in DLD-1 cells, also in A549/8 cells downregulation of tristetraprolin expression resulted in reduction of cytokine-induced iNOS mRNA expression (see **figure 5** panel **C**). Therefore, in contrast to its described destabilizing activity (Blackshear, 2002), tristetraprolin seems to be positively involved in cytokine-induced iNOS expression in human epithelial cells.

To analyze the effect of the modulation of tristetraprolin expression on human iNOS mRNA stability, actinomycin D experiments were performed with the three different stably transfected DLD-1 cell pools (pZeo, pZeo-hTTPs and pZeo-hTTPas). These experiments (see **figure 6**, panel **A**) showed that tristetraprolin overexpression (pZeo-hTTPs cells) enhanced, whereas tristetraprolin downregulation (pZeo-hTTPas cells) decreased iNOS mRNA stability compared to cells transfected with the expression vector (pZeo cells). Therefore, tristetraprolin-mediated modulation of human iNOS expression is likely to result from post-transcriptional effects of tristetraprolin.

Some RNA-binding proteins like AUF1 or hnRNP A1 have been described to also display DNA-binding activity and to regulate the promoter activity of different genes (Fuentes-Panana et al., 2000; Shen and Masters, 2001). To exclude the possibility that the tristetraprolin-mediated effects on human iNOS expression also resulted from regulation of human iNOS promoter activity by tristetraprolin, we transiently transfected the plasmid pNOS2(16)Luc into the three different cell pools (pZeo, pZeo-hTTPs and pZeo-hTTPas) and determined luciferase activity after cytokine-induction. As shown in **figure 6** (panel **B**) modulation of tristetraprolin expression in DLD-1 cells did not result in changes of cytokine-induced human iNOS promoter activity.

The 3'-UTR of the human iNOS contains five AREs, two of which had been shown to interact with the mRNA stabilizing factor HuR (Rodriguez-Pascual et al., 2000). Therefore, we analyzed the exact binding site of tristetraprolin in the human iNOS 3'-UTR sequences. Surprisingly, as shown in **figure 7** (panels **A** and **B**), UV-crosslinking experiments indicated that recombinant GST-TTP protein did not interact with any region of the human iNOS

mRNA (3'-UTR, 5'-UTR, cds). However, the same GST-TTP protein was able to bind to the c-fos-3'-UTR, a known target of tristetraprolin (**figure 7**, panel **C**) and to the human TNF- $\alpha$  3'-UTR (data not shown). Therefore, tristetraprolin does not seem to interact with the human iNOS mRNA.

The results shown above indicate an indirect stimulatory effect of tristetraprolin on human iNOS expression. Lai et al. also reported that tristetraprolin mutants without RNA binding activity enhanced the stability of ARE containing mRNAs like the TNF- $\alpha$  transcript (Lai et al., 2002). This unexpected effect of mutant tristetraprolin seems to be based on an interaction of these proteins with enzymes stimulating the degradation of ARE-containing mRNAs (Lai et al., 2002). Supporting this hypothesis, Chen et al. showed tristetraprolin- and KSRP-dependent ARE-mRNA degradation activity of the human exosome (Chen et al., 2001). The purified exosome, a complex of exonucleases catalyzing the 3'  $\rightarrow$  5' decay pathway of mRNAs, was not able to bind to and degrade ARE-containing mRNAs. However, ARE-binding proteins such as KSRP and tristetraprolin recruited the exosome to these mRNAs and enabled degradation (Chen et al., 2001; Gherzi et al., 2004). Therefore, we analyzed the interaction of tristetraprolin with KSRP in human DLD-1 cells. As shown in **figure 8** (panels **A** and **B**), tristetraprolin displayed a protein-protein interaction with KSRP, which was enhanced by cytokine treatment. As shown in **figure 8**, panel **B**) the cytokine enhancement of the tristetraprolin-KSRP interaction was reduced by SB203580-treatment.

KSRP markedly destabilized human iNOS mRNA and overexpression of KSRP downregulated human iNOS expression (Linker et al., 2005). Therefore, without binding of tristetraprolin to the human iNOS mRNA the enhanced expression of tristetraprolin after cytokine treatment and the cytokine-enhanced interaction of tristetraprolin with KSRP seems to result in a dislodgement of the KSRP-exosome complex from the human iNOS mRNA. Thereby enhanced tristetraprolin expression in cytokine-treated cells seems to stabilize iNOS mRNA and thus enhances iNOS expression. SB203580-mediated inhibition of the p38 MAPK

pathway reduced the enhancement of tristetraprolin expression by cytokines and thereby destabilized iNOS mRNA.

In conclusion, our data support the following mechanism: Cytokines required for iNOS induction also enhance tristetraprolin expression, most likely by p38 MAPK activation. The elevated tristetraprolin protein in turn reduces the recruitment of exosomes to the human iNOS mRNA by enhanced interaction with KSRP. The reduced ability of the exosome to bind to and to degrade the human iNOS mRNA results in an enhanced iNOS mRNA stability. This leads to a higher iNOS protein expression and an increase of iNOS-dependent NO production.

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## Footnotes

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## Figure legends

### **Figure 1. Effect of compound SB203580-mediated inhibition of p38 MAPK on human iNOS mRNA and protein expression and human 16 kb iNOS promoter activity**

DLD-1 cells stably transfected with pNOS2(16)Luc, a construct containing a 16 kb fragment of the human iNOS promoter in front of a firefly luciferase reporter gene, were preincubated for 18h in medium without FCS and phenol red. Then the cells were pretreated for 1h with SB203580 (**SB**, 0 to 10  $\mu$ M). Thereafter, cells were incubated with or without cytokine mixture (**CM**, 100 U/ml IFN- $\gamma$ , 10 ng/ml TNF- $\alpha$  and 50 U/ml IL-1 $\beta$ ) in presence or absence of SB203580 at the concentrations indicated. Total RNA and cytoplasmic proteins were prepared after 6h and 24h, respectively.

**Panel A:** Representative RNase protection experiment to detect iNOS mRNA expression in the stably transfected DLD-1 cells. Experiments were performed using antisense RNA probes for human iNOS- and  $\beta$ -actin mRNA (used for normalization). The positions of the protected **iNOS-** and  **$\beta$ -actin** fragments are indicated.

**Panel B:** Representative RNase protection experiment to detect luciferase mRNA expression in the stably transfected DLD-1 cells. Experiments were performed using antisense RNA probes for luciferase- and human  $\beta$ -actin mRNA (used for normalization). The positions of the protected luciferase (**Luc**) - and  **$\beta$ -actin** fragments are indicated.

**Panel C:** Summary of densitometric analyses of nine different gels and 4 qRT-PCR analyses using RNA isolated from stably transfected DLD-1 cells. Data (means  $\pm$  SEM) represent relative **iNOS-** and luciferase (**Luc**) mRNA levels (\*\* $p < 0.001$ ; ns = not significant vs iNOS- or luciferase mRNA levels in cells not treated with SB203580, respectively).

**Panel D:** Western blots using monoclonal anti-iNOS-antibodies or monoclonal anti-tubulin-antibodies for the detection of iNOS- and  $\beta$ -tubulin expression in DLD-1 cells. Cytoplasmic extracts from DLD-1 cells incubated with (**CM**) or without cytokines in the presence (+) or absence (-) of 10  $\mu$ M SB203580 were used. The presence of **iNOS-** and  $\beta$ -tubulin protein is

indicated. As a positive control bacterial expressed human iNOS protein (**rP**) was loaded on the gel. This blot is representative of three other blots showing similar results.

**Panel E:** Analysis of the iNOS-mediated NO production in DLD-1 cells treated with or without 10  $\mu$ M SB203580 by measurement of nitrite in supernatants of the cells. Cells were preincubated for 18h in serum-free medium and then pretreated with or without 10  $\mu$ M SB205830 (**SB**) for 1h. Afterwards cells were treated with or without a cytokine-mixture (**CM**) for another 48h. Columns (means  $\pm$  SEM, n = 10) represent the relative nitrite levels determined (100% corresponds to 407 pmol/ml/48h; \*\*\* p < 0.001 versus CM-treated DLD-1 cells).

**Panel F:** DLD-1 cells stably transfected with pCS3-FLAG-p38(AGF) (Winzen et al., 1999), a construct coding for a dominant negative murine p38 MAPK $\alpha$  isoform (**p38DN**), or the empty plasmid **pCDNA3** (Invitrogen), were preincubated for 18h in medium without FCS and phenol red. Then cells were incubated with or without cytokine mixture (**CM**). Total RNA was prepared after 6h. iNOS mRNA and GAPDH mRNA concentrations were determined by qRT-PCR and iNOS mRNA was normalized to GAPDH mRNA. A summary of 6 qRT-PCR analyses is shown. Data (means  $\pm$  SEM) represent relative iNOS mRNA levels (\*\*\* p < 0.001; ns = not significant vs CM-treated stably pCDNA3-transfected DLD-1 cells).

## **Figure 2. Cytokine mixture (CM) enhances tristetraprolin expression DLD-1 cells**

DLD-1 cells were preincubated for 18h in medium without FCS and phenol red. Then cells were incubated with or without **CM** for the time periods indicated and total RNA or cytoplasmic proteins were isolated.

**Panel A:** Representative RNase protection experiment detecting tristetraprolin-, iNOS-, and GAPDH mRNA expression in DLD-1 cells. Experiments were performed using antisense RNA probes for human tristetraprolin-, iNOS- and G $\square$  $\square$ P $\square$  mRNA (used for normalization). The positions of the protected tristetraprolin (**TTP**-), iNOS- and G $\square$  $\square$ P $\square$  fragments are indicated. (**M**: molecular weight standard,  $\Phi$ X174-restricted with *Hinf* I; **T**: tRNA control).

**Panel B:** Summary of densitometric analyses of eight different gels similar to panel A and 5 different qRT-PCR analyses. Columns (means  $\pm$  SEM) represent relative tristetraprolin mRNA levels in CM-treated cells compared to control cells (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; ns = not significant vs control cells).

**Panel C:** Western blot using specific anti-tristetraprolin- and anti- $\beta$ -tubulin antibodies and cytoplasmic extracts from DLD-1 cells. This blot is representative of three other blots showing similar results.

**Figure 3. SB203580-treatment reduces cytokine-mediated enhancement of tristetraprolin expression in DLD-1 cells**

DLD-1 cells were preincubated for 18h in medium without FCS and phenol red. Then cells were pretreated for 1h with 10  $\mu$ M SB203580. Afterwards cells were incubated with or without a cytokine mixture (CM) for 6h and total RNA or cytoplasmic proteins were isolated.

**Panel A:** Summary of densitometric analyses of 7 different qRT-PCR analyses. Columns (means  $\pm$  SEM) represent relative tristetraprolin mRNA levels (\*\*\*)  $p < 0.001$ ; ns = not significant vs CM-treated cells).

**Panel B:** Western blot using specific anti-tristetraprolin- and anti- $\beta$ -tubulin antibodies and cytoplasmic extracts from DLD-1 cells. This blot is representative of three other blots showing similar results.

**Figure 4. Overexpression of tristetraprolin enhances and downregulation of tristetraprolin expression reduces cytokine-induced iNOS expression.**

DLD-1 cells were stably transfected with eucaryotic expression vectors containing a tristetraprolin-HA cDNA either in sense (pZeo-hTTPs) or antisense (pZeo-hTTPas) orientation. Pools of cells were tested for tristetraprolin expression or for cytokine-induced iNOS mRNA in comparison to DLD-1 cells stably transfected with the empty expression vector pZeoSV2 (pZeo). For analysis of iNOS expression the cells were preincubated for 18h

in medium without FCS and phenol red before being exposed to cytokines (**CM**) for the time periods indicated.

**Panel A:** Analysis of tristetraprolin protein expression in cytoplasmic extracts from untreated DLD-1 wildtype, -pZeo, -pZeo-hTTPs and -pZeo-hTTPas cells by Western Blot using polyclonal anti-tristetraprolin- and monoclonal  $\beta$ -tubulin antibodies. This blot is representative of three other blots showing similar results.

Densitometric analyses of these blots revealed the following relative levels of TTP protein expression:

	Mean	SEM
pZeo	100.00	0.00
pZeo-hTTPs	171.00	6.93
pZeo-hTTPas	65.89	5.82

**Panel B:** Representative RNase protection experiment detecting iNOS and GAPDH mRNA expression in **pZeo-hTTPs-** and **pZeo** cells. Experiments were performed using antisense RNA probes for human iNOS- and G $\square\square$ P $\square$  mRNA (for normalization). The positions of the protected **iNOS-** and **G $\square\square$ P $\square$**  fragments are indicated. (**N:** human iNOS probe; **T:** tRNA control; **M:** molecular weight standard,  $\Phi$ X174-restricted with *Hinf* I; **co:** RNA from cells incubated in medium without FCS only).

**Panel C:** Summary of densitometric analyses of seven different gels and four qRT-PCR analyses using RNA isolated from stably transfected DLD-1-**pZeo-** or **-pZeo-hTTPs** cells. Data (means  $\pm$  SEM) represent relative **iNOS** mRNA levels (\*  $p < 0.05$ ; \*\*\*  $p < 0.001$ ; ns = not significant vs pZeo cells incubated for 4h with CM).

**Panel D:** Representative RNase protection experiment detecting iNOS and GAPDH mRNA expression in **pZeo-hTTPas-** and **pZeo** cells. Experiments were performed using antisense RNA probes for human iNOS- and G $\square\square$ P $\square$  mRNA (used for normalization). The positions of the protected **iNOS-** and **G $\square\square$ P $\square$**  fragments are indicated. (**N:** human iNOS probe; **T:** tRNA control; **M:** molecular weight standard,  $\Phi$ X174-restricted with *Hinf* I; **co:** RNA from cells incubated in medium without FCS only).

**Panel E:** Densitometric analyses of six different gels and three qRT-PCR analyses using RNA isolated from stably transfected DLD-1-**pZeo-** or **-pZeo-hTTPas** cells. Data (means  $\pm$  SEM) represent relative **iNOS** mRNA levels (\*\* $p < 0.001$ ; ns = not significant vs pZeo cells incubated for 4h with CM).

**Panel F:** Analysis of iNOS-mediated NO production in **pZeo**, **pZeo-hTTPs** and **pZeo-hTTPas** cells by measurement of nitrite in supernatants of the cells. Cells were preincubated for 18h in serum-free medium and then treated with a cytokine-mixture for another 48h. Columns (means  $\pm$  SEM,  $n = 12$ ) represent the relative nitrite levels determined (100% corresponds to 407 pmol/ml/48h; \*\*\*  $p < 0.001$  versus CM-treated DLD-1-pZeo cells).

**Figure 5. Downregulation of tristetraprolin expression by siRNA reduces cytokine induced iNOS mRNA expression.**

A549/8 cells were stably transfected with psiRNA-hH1-GFPzeo-TTP. In these cells siRNAs containing a hairpin structure (shRNAs) were generated, which were directed against the human tristetraprolin mRNA. Stable transfectants were selected for zeocin resistance and GFP expression. As a control, A549/8 cells stably transfected with the empty psiRNA-hH1-GFPzeo vector were generated as well. The cells were preincubated for 18h in medium without FCS and phenol red before being exposed to cytokines (**CM**) for 6h. Then total RNA or protein extracts were prepared.

**Panel A:** Summary of four qRT-PCR analyses using RNA isolated from stably transfected A549/8-psiRNA-hH1-GFPzeo-TTP (**siTTP**) or -psiRNA-hH1-GFPzeo (**GFP**) cells. Data (means  $\pm$  SEM) represent relative tristetraprolin mRNA levels (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; vs psiRNA-hH1-GFPzeo cells incubated for 6h with CM).

**Panel B:** Analysis of tristetraprolin and  $\beta$ -tubulin protein expression in A549/8-psiRNA-hH1-GFPzeo-TTP (**siTTP**) or -psiRNA-hH1-GFPzeo (**GFP**) cells by Western Blot using polyclonal anti-tristetraprolin- and monoclonal  $\beta$ -tubulin antibodies and cytoplasmic extracts

from stably transfected A549/8 cells. The position of  $\beta$ -tubulin and tristetraprolin is indicated. This blot is representative of three other blots showing similar results.

**Panel C:** Statistic analysis of five qRT-PCR analyses using RNA isolated from stably transfected A549/8-psiRNA-hH1-GFPzeo-TTP (**siTTP**) or -psiRNA-hH1-GFPzeo (**GFP**) cells. Data (means  $\pm$  SEM) represent relative iNOS mRNA levels (\*\*\*)  $p < 0.001$  vs psiRNA-hH1-GFPzeo cells incubated for 6h with CM).

### **Figure 6. Effect of tristetraprolin expression on human iNOS mRNA stability and iNOS promoter activity**

**Panel A:** DLD-1-pZeo control cells (**pZeo**), DLD-1-pZeo-hTTPs cells (**pZeo-hTTPs**) or DLD-1-pZeo-hTTPas cells (**pZeo-hTTPas**) were incubated with a cytokine mixture for 6h. Then 10  $\mu$ M actinomycin D was added and RNAs were prepared after 0 to 18 h. iNOS mRNA and GAPDH mRNA concentrations were determined by qRT-PCR and iNOS mRNA was normalized to GAPDH mRNA.

A summary of 6 qRT-PCR analyses is shown. Data (means  $\pm$  SEM) represent relative iNOS mRNA levels (\*  $p < 0.05$ ; \*\*  $p < 0,01$ ; ns = not significant vs 0h actinomycin D). Curve fitting was performed using Graphpad Prism for Macintosh.

**Panel B:** DLD-1-pZeo control cells (**pZeo, black columns**), DLD-1-pZeo-hTTPs cells (**pZeo-hTTPs, hatched columns**) or DLD-1-pZeo-hTTPas cells (**pZeo-hTTPas, gray columns**) were transfected with a luciferase reporter gene under the control of the 16 kb human iNOS promoter. After transfection, cells were incubated with or without a mixture of cytokines (**CM**) for 8h. Columns (mean  $\pm$  SEM) represent the cytokine-induced activation of the iNOS promoter expressed as a percentage of the corresponding basal values determined in the absence of cytokines (\*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; ns = not significant vs control cells).

### **Figure 7. Recombinant tristetraprolin protein does not bind to the human iNOS mRNA**

Several  $^{32}\text{P}$ -radiolabeled RNA transcripts were incubated with 0.6  $\mu\text{g}$  of purified bovine serum albumin (**BSA**), glutathione S-transferase (**GST**), GST-HuR- (**HuR**) or GST-TTP (**TTP**) fusion protein. The radiolabeled RNAs were synthesized from plasmids containing the human iNOS **3'-UTR**, iNOS **5'-UTR** or iNOS coding sequence (**cds**). As a control radiolabeled **c-fos-3'-UTR** RNA was also included. The positions of RNA/protein complexes are indicated.

### **Figure 8. Tristetraprolin interacts with KSRP**

DLD-1 cells were preincubated for 18h in medium without FCS and phenol red. Then the cells were incubated with (**CM**) or without (**Co**) a cytokine mixture for 4h. Some cells were pretreated with SB203580 (10  $\mu\text{M}$ ; **SB**) for 1h. Extracts were prepared and RNase A/T1 digested as described in Material and Methods.

**Panel A:** RNase pretreated cell lysates were subjected to immunoprecipitation using polyclonal anti-tristetraprolin antibodies (**IP:  $\alpha$ -TTP**) or a pre-immune serum (**IP: Pre□□**). Co-precipitation of KSRP was analyzed by performing Western Blots using monoclonal anti-KSRP antibodies (**WB:  $\alpha$ -KSRP**). As control KSRP expression was analyzed in the extract from untreated DLD-1 cells (5% of the extract input, **In**). The presence of the **KSRP** protein is indicated. One representative out of three co-immunoprecipitation analyses is shown.

**Panel B:** RNase pretreated cell lysates from cells preincubated for 1h with or without SB203580 (**SB**) were subjected to immunoprecipitation using monoclonal anti-KSRP antibodies (**IP:  $\alpha$ -KSRP**) or a pre-immune serum (**IP: Pre□□**). Co-precipitation of tristetraprolin was analyzed by performing Western Blots using polyclonal anti-tristetraprolin antibodies (**WB:  $\alpha$ -TTP**). The presence of the tristetraprolin protein (**TTP**) and of the immunoglobulin heavy chain (**hc**) is indicated. One representative out of three co-immunoprecipitation analyses is shown.

**Panel C:** To normalize for precipitated KSRP protein of the experiment shown in panel **B** the amount of KSRP protein was analyzed performing Western Blots using monoclonal anti-

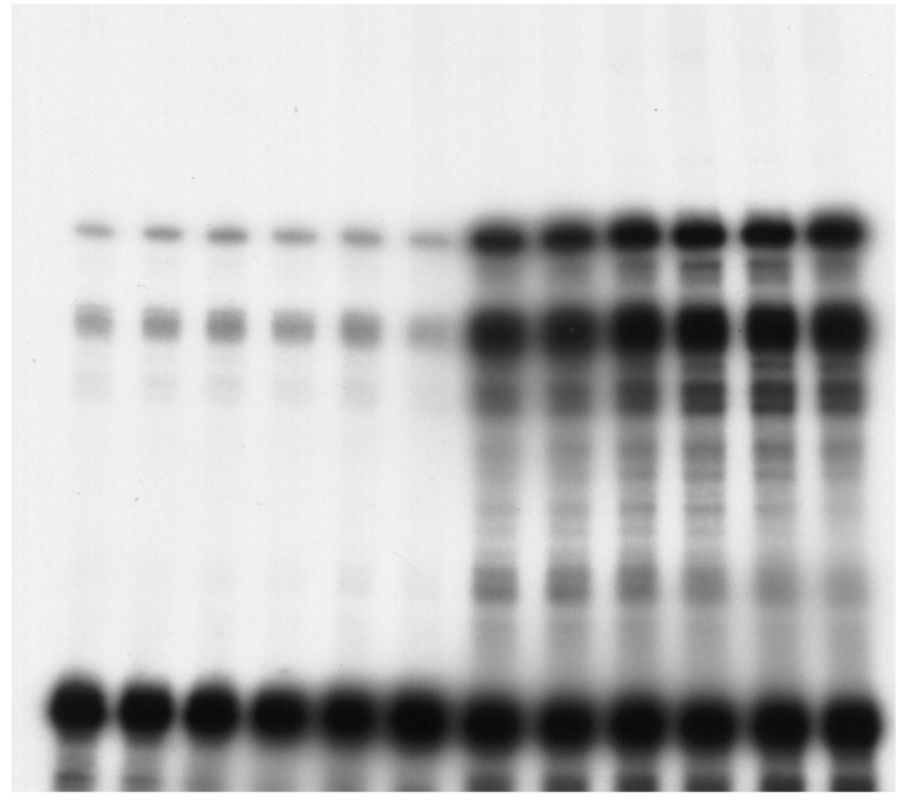


KSRP antibodies (**WB:  $\alpha$ -KSRP**). As control KSRP expression was analyzed in the extract from untreated DLD-1 cells (5% of the extract input, **In**). The presence of the **KSRP** protein and of the immunoglobulin heavy chain (**hc**) is indicated. One representative out of three co-immunoprecipitation analyses is shown.

Luc

$\beta$ -actin

**B**



SB ( $\mu$ M) 0 .1 .3 1 3 10 0 .1 .3 1 3 10

Co

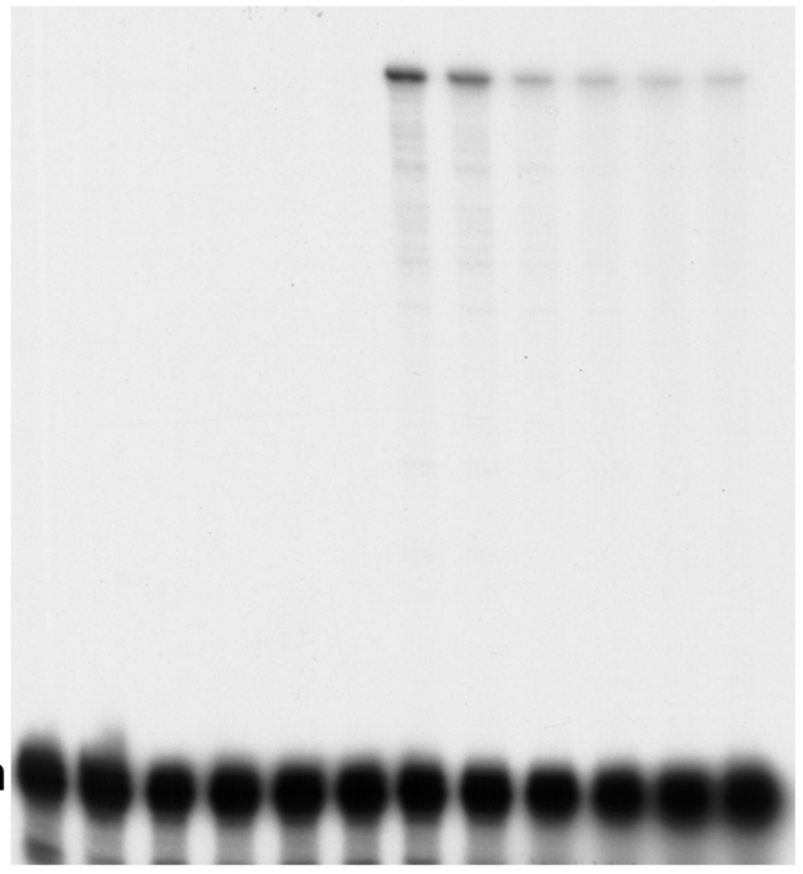
CM

**Fig. 1**

**A**

iNOS

$\beta$ -actin



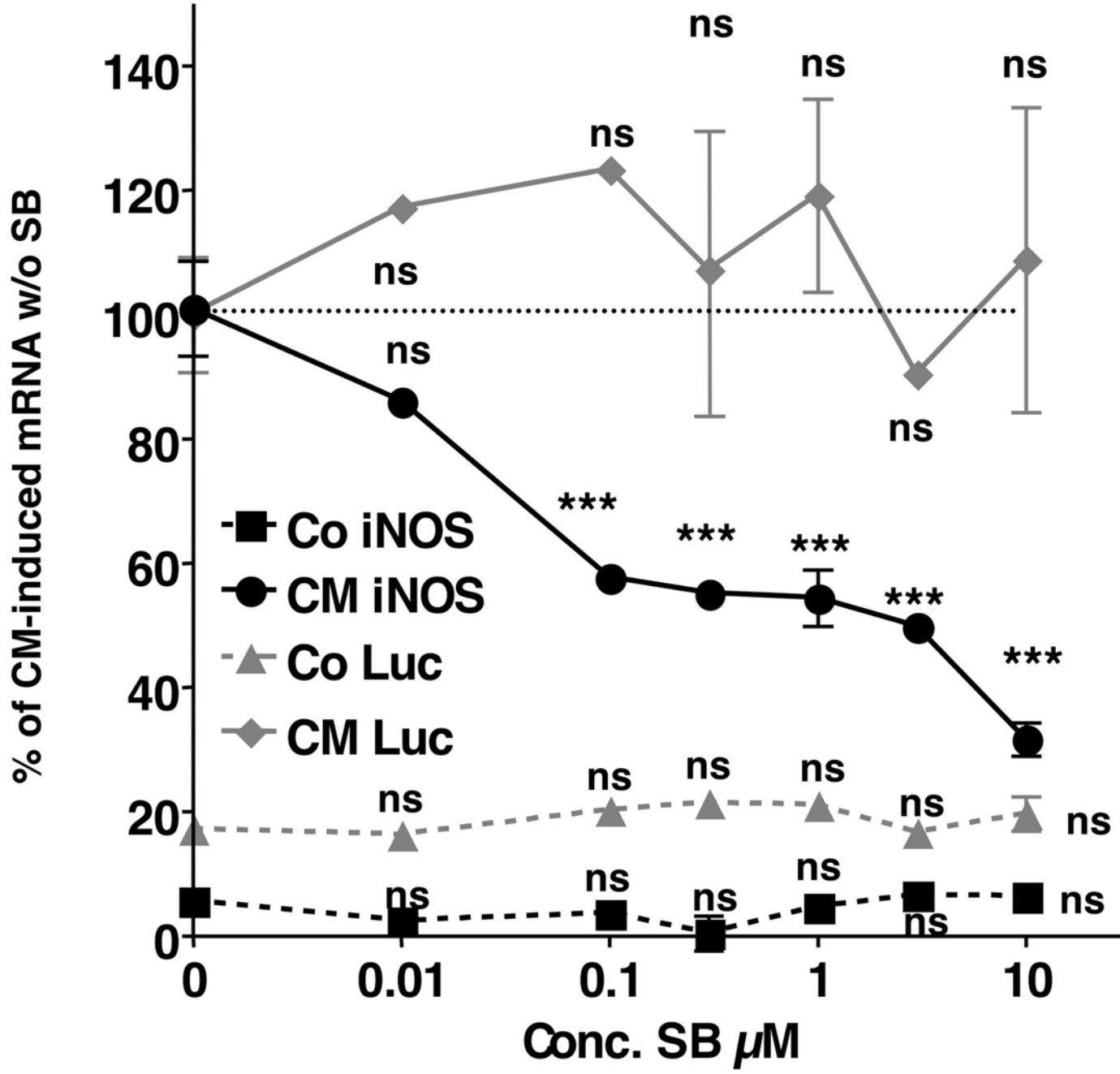
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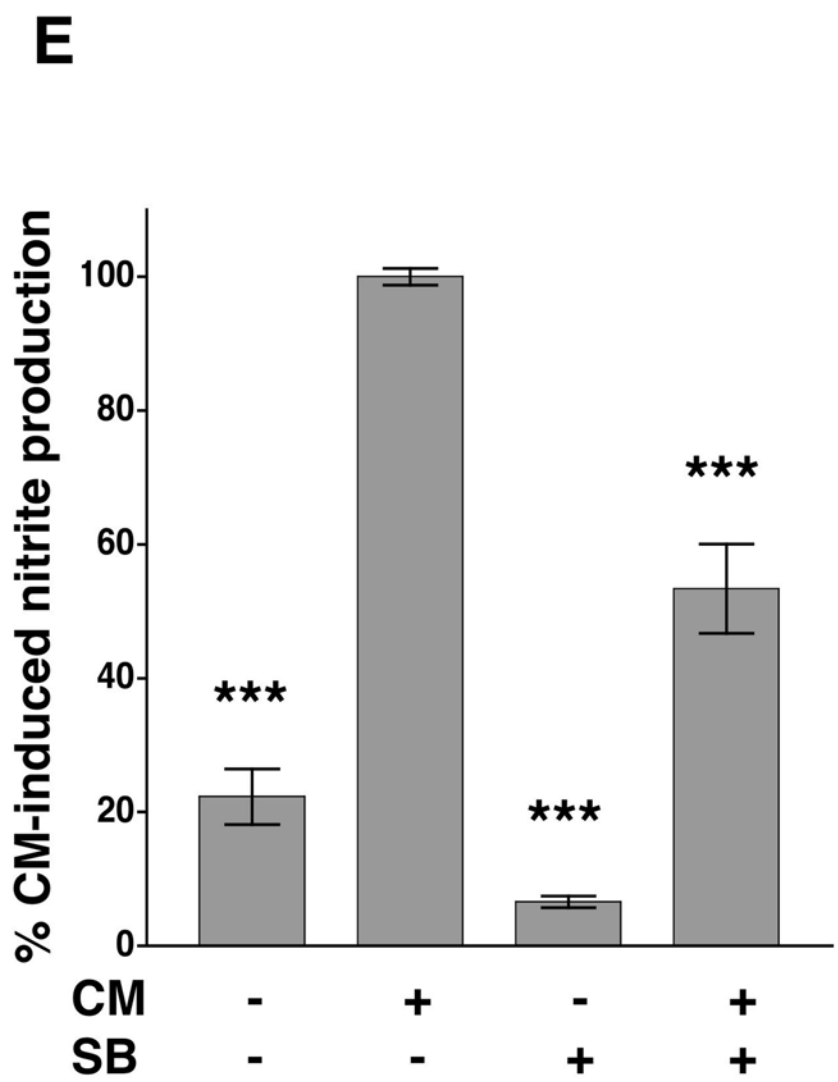
Co

CM

Fig. 1

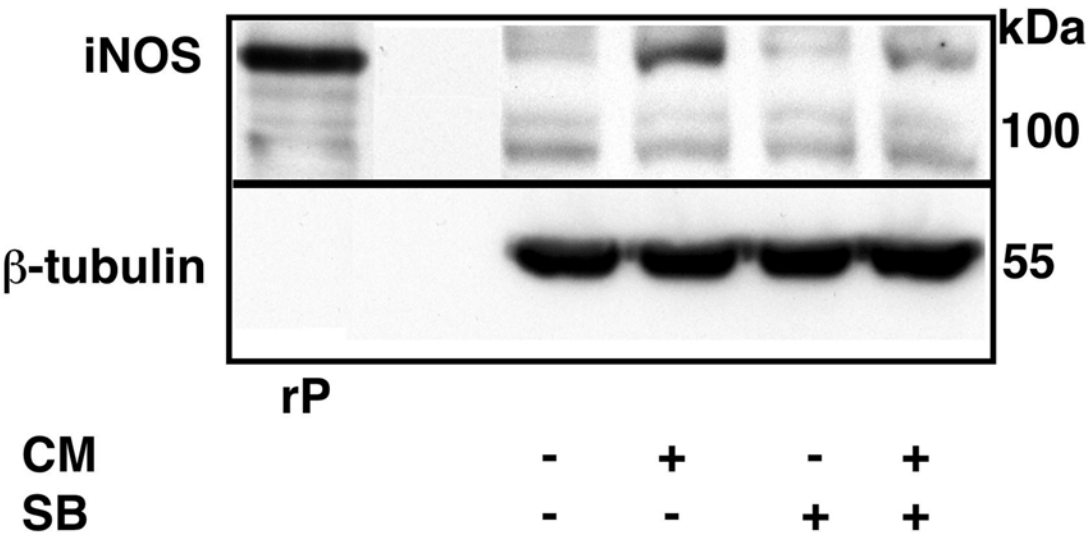
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**Fig. 1**

**D**



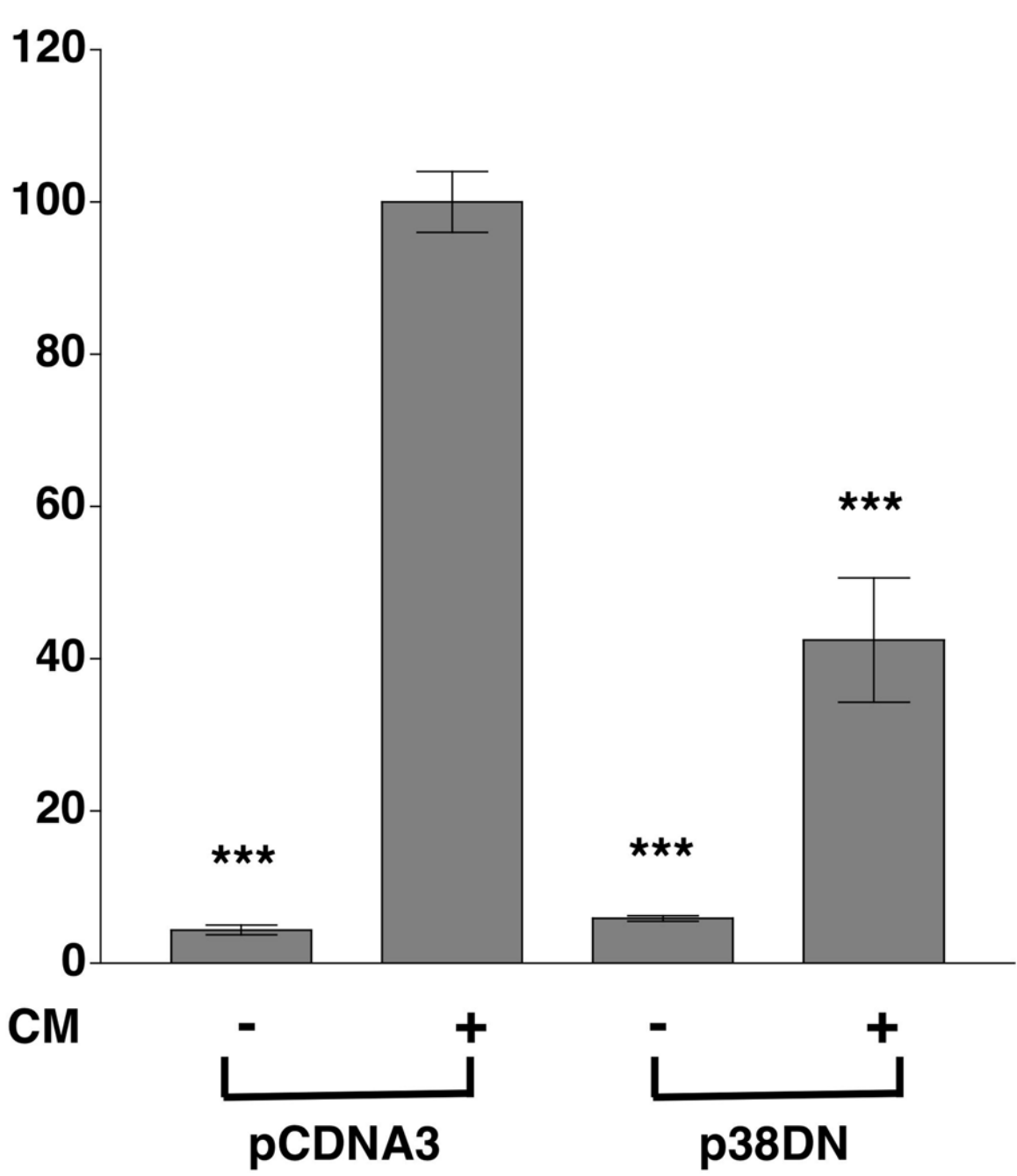


Fig. 1

F

Fig. 2

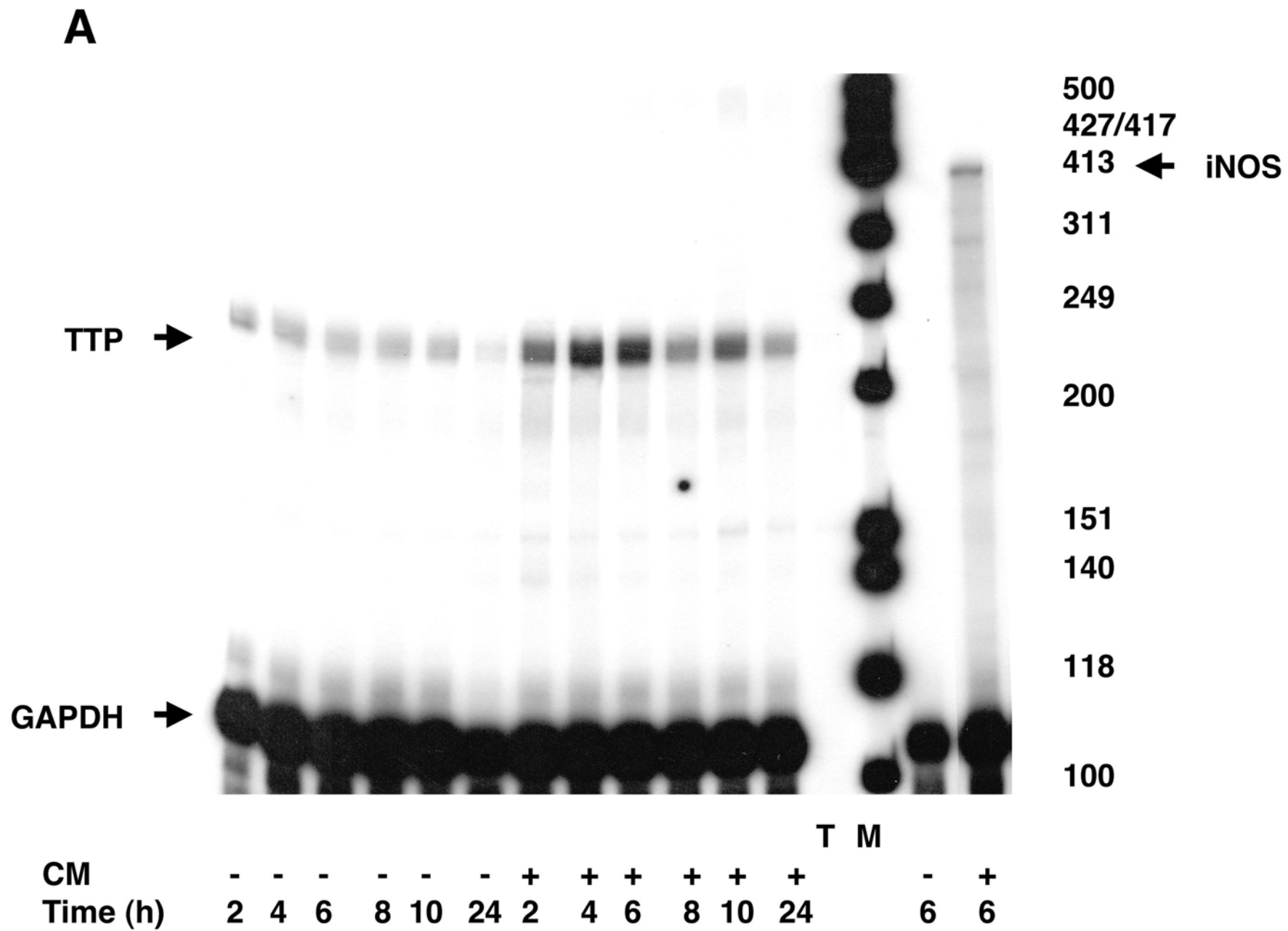


Fig. 2

**B**

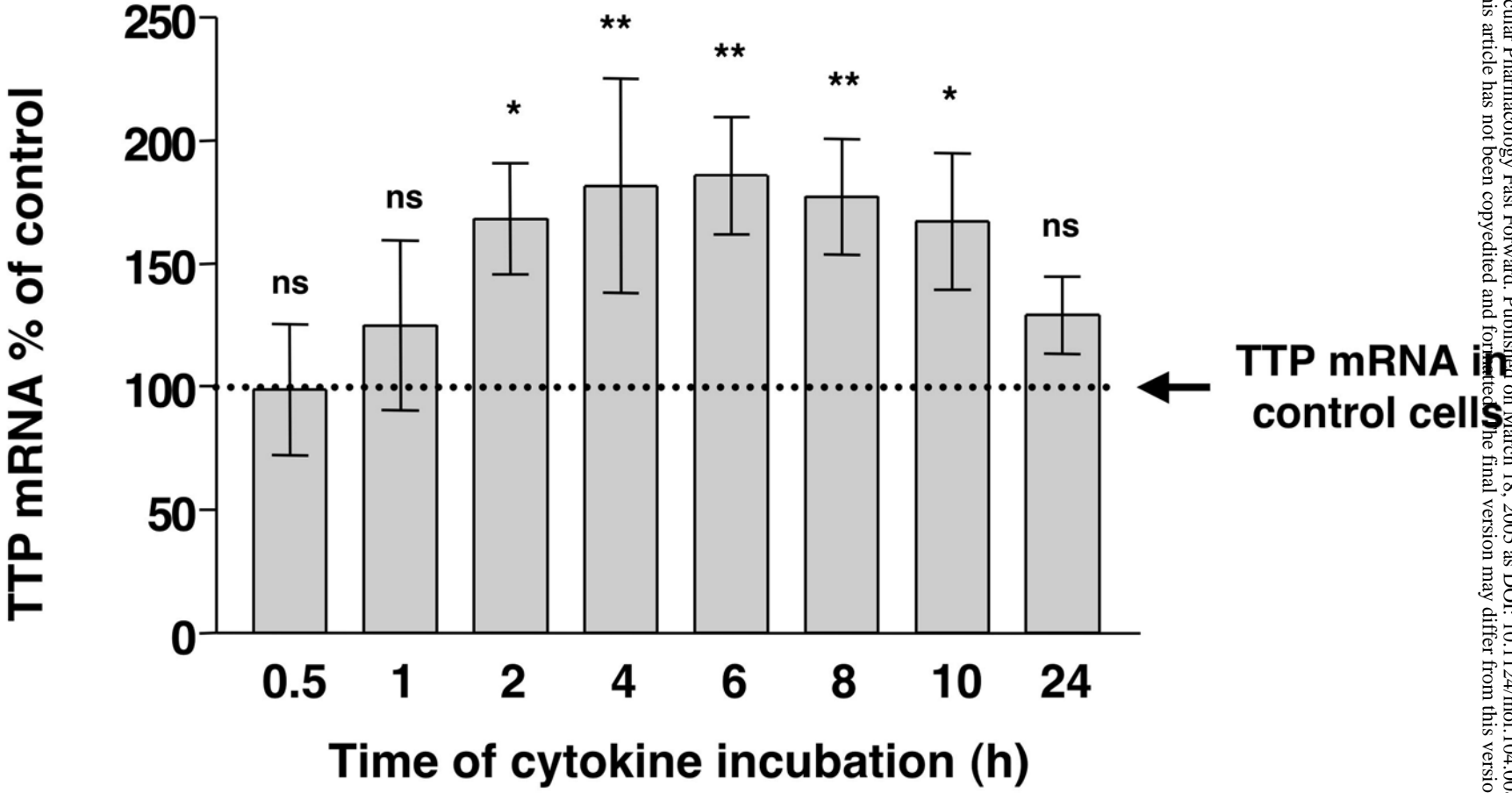


Fig. 2

C

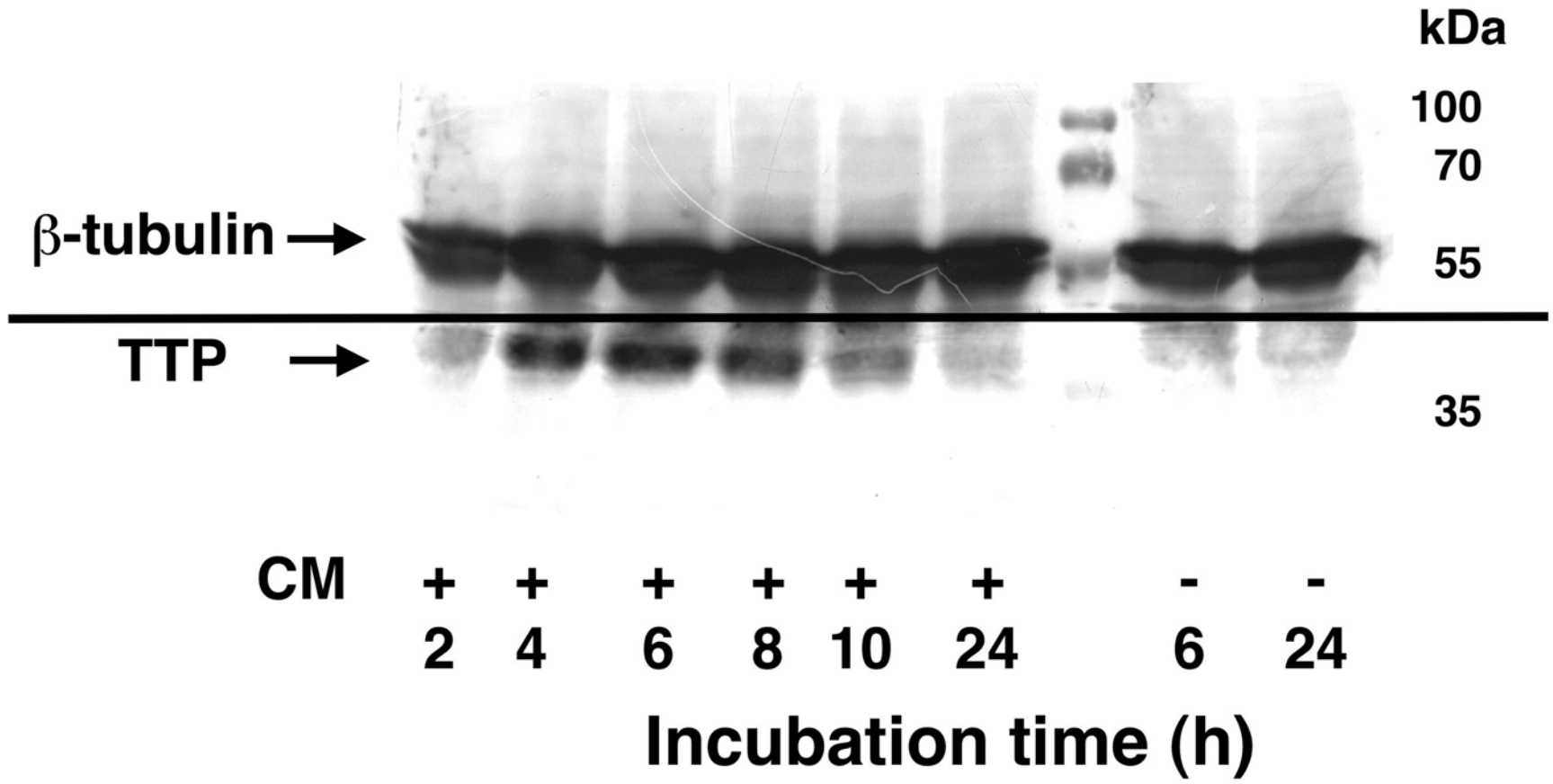
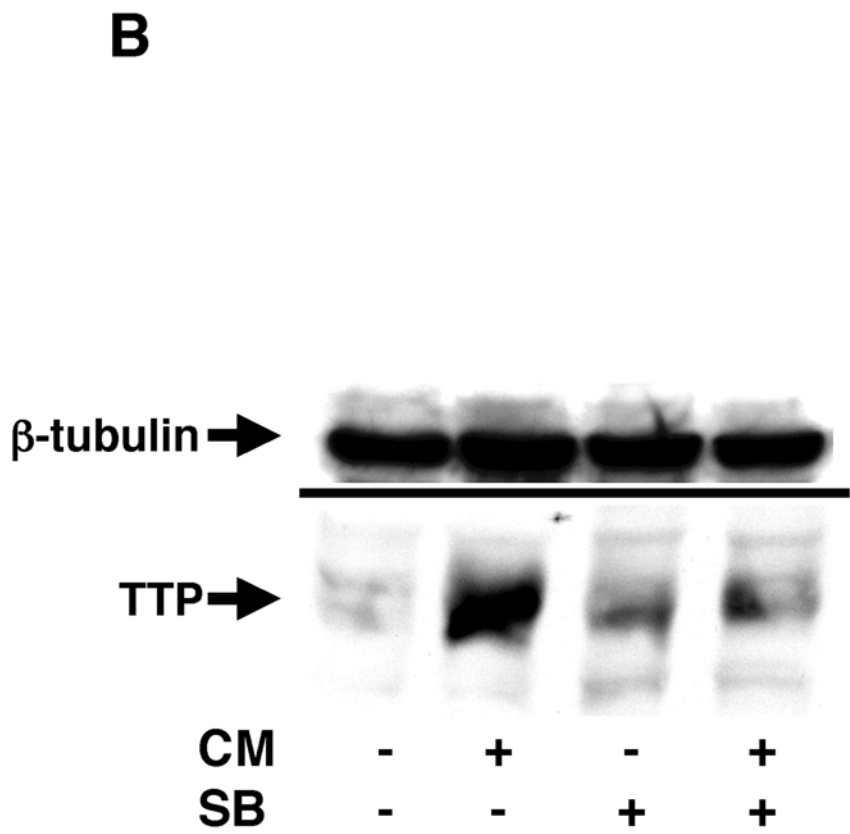
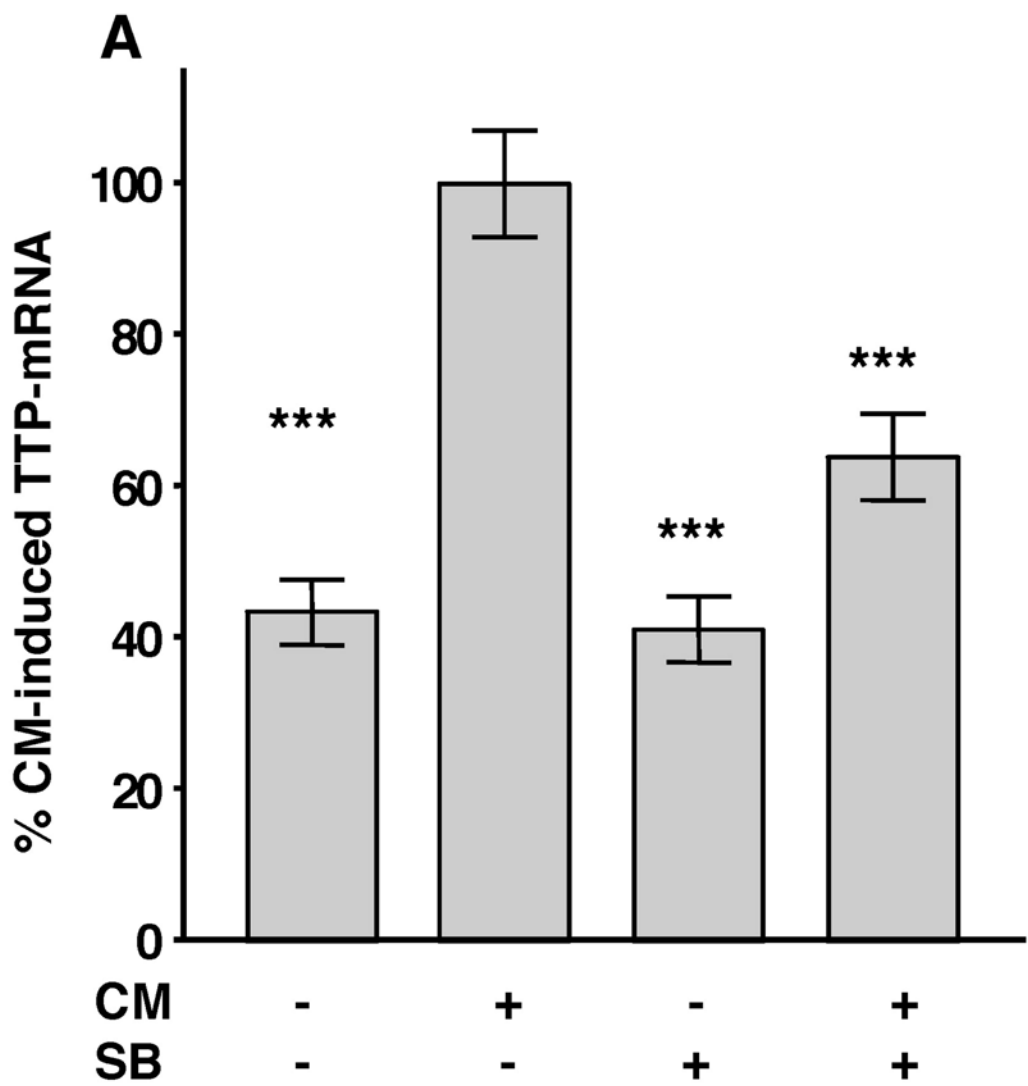
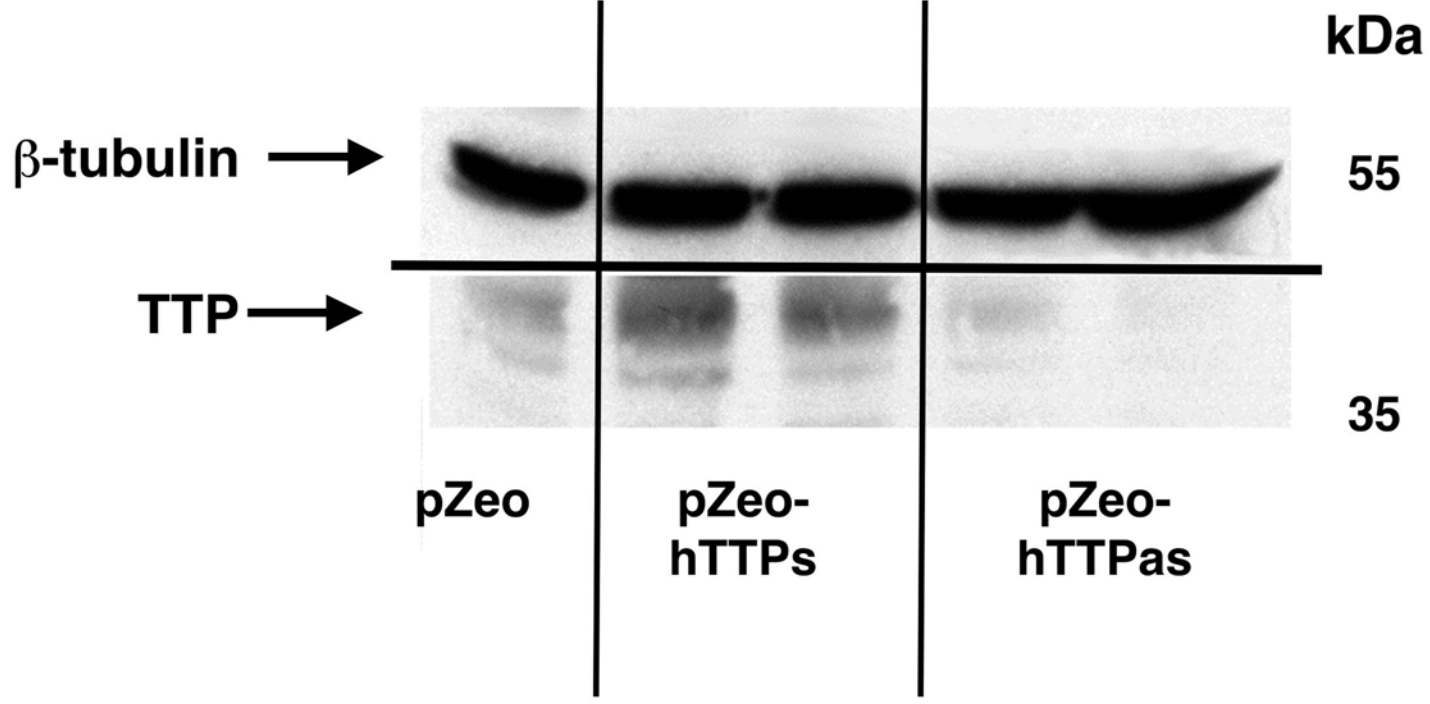




Fig. 3



**Fig. 4**  
**A**



**Fig. 4**  
**B**

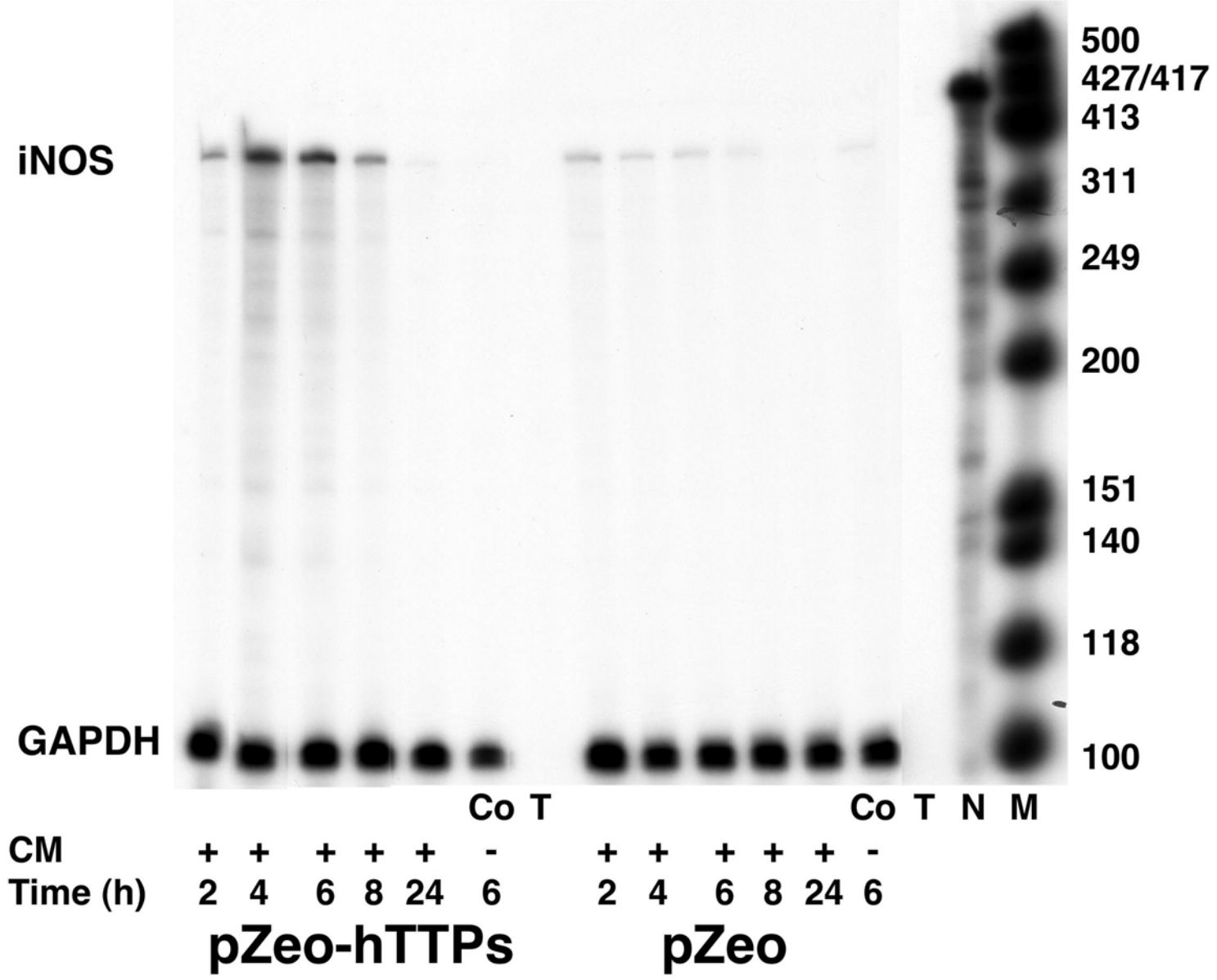
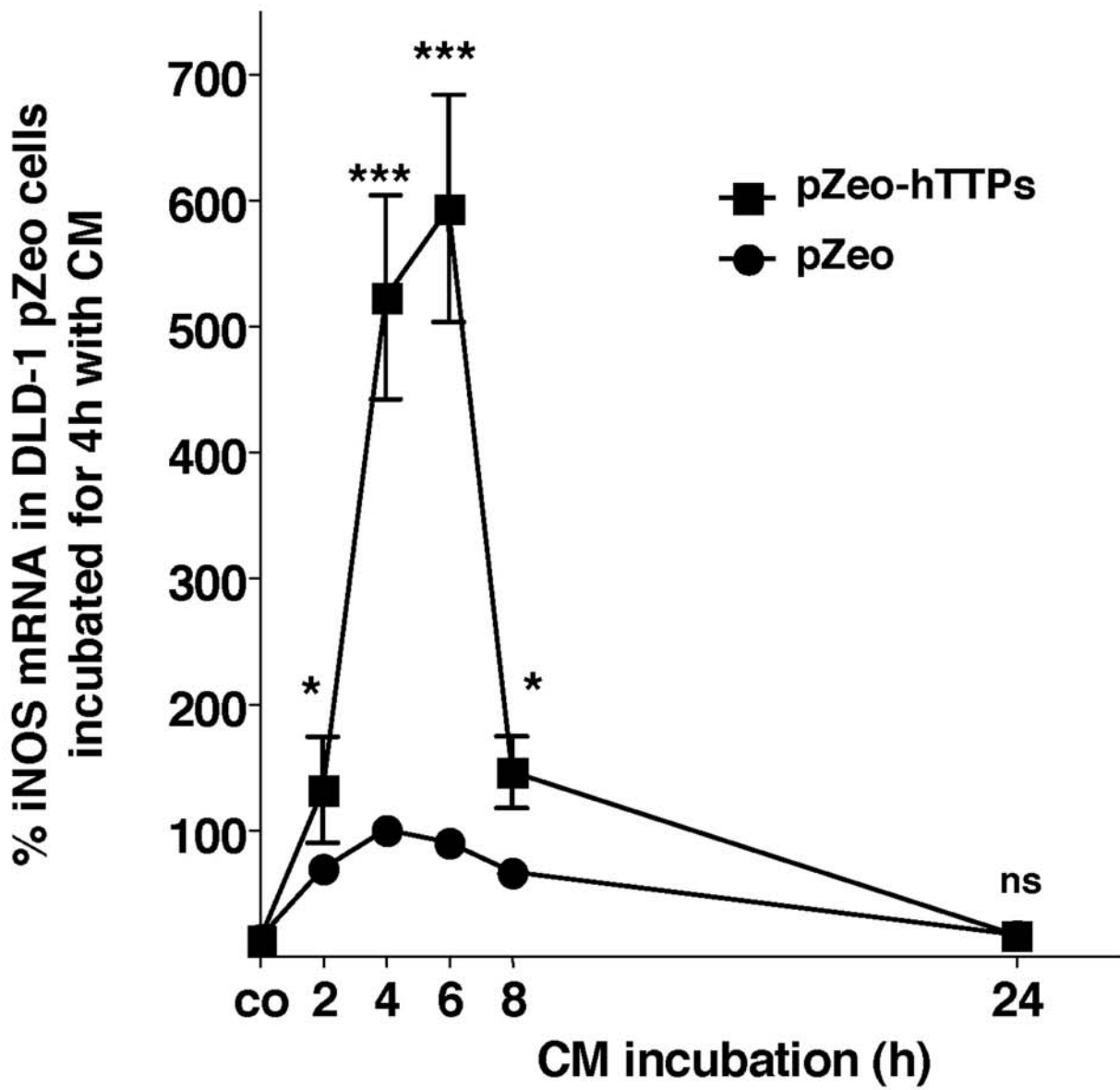


Fig. 4  
C



**Fig. 4**  
**D**

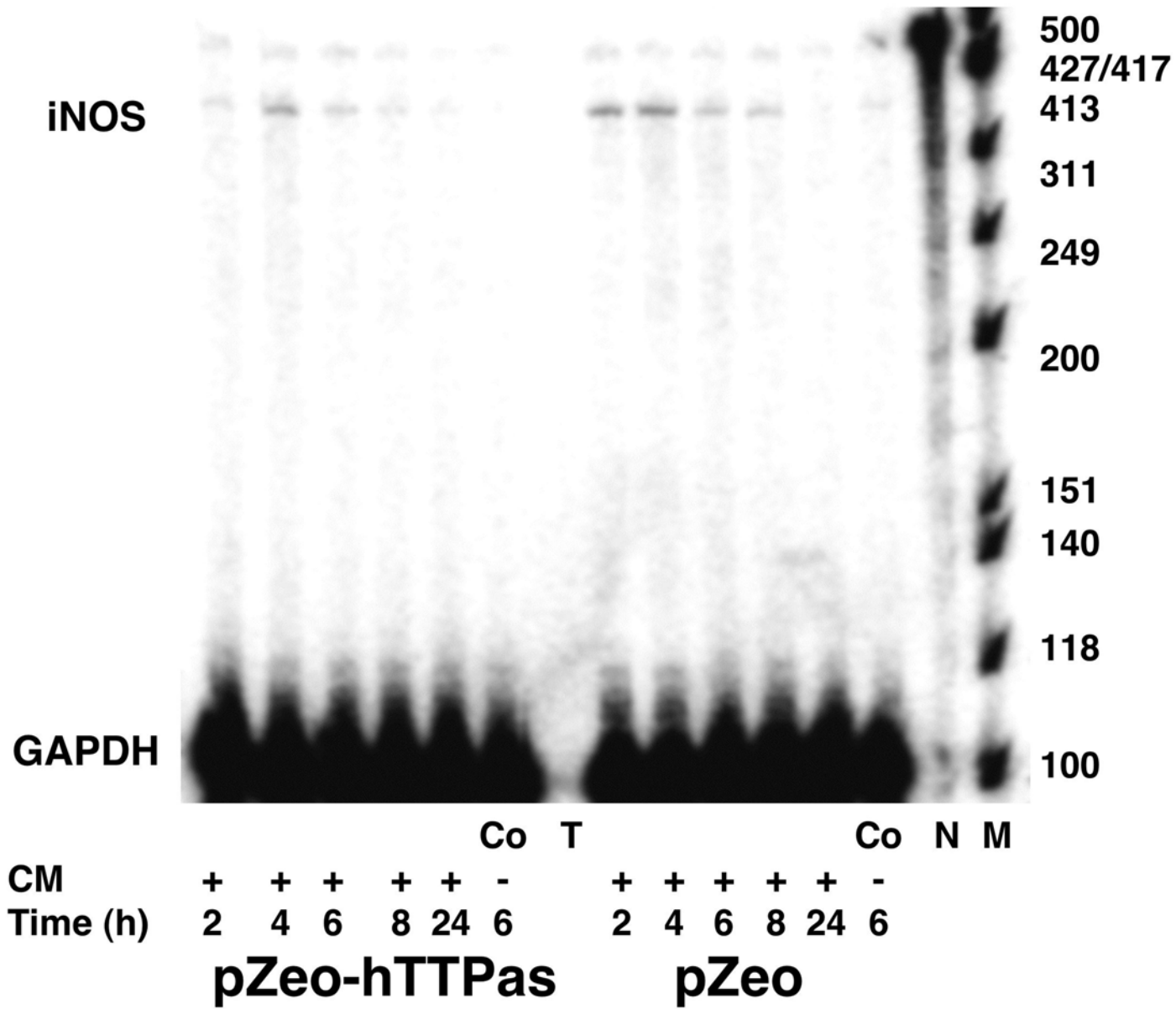


Fig. 4

**E**

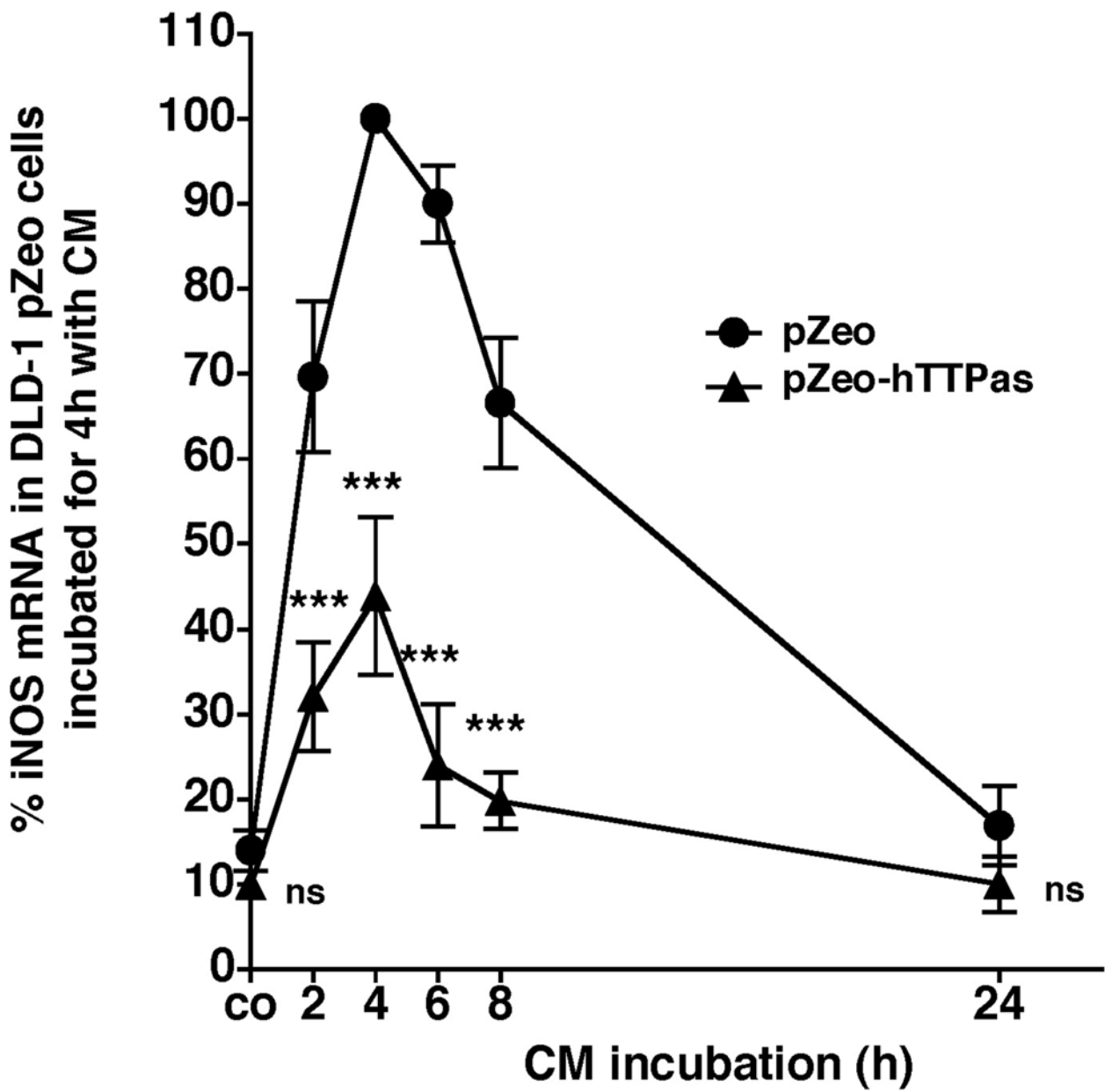
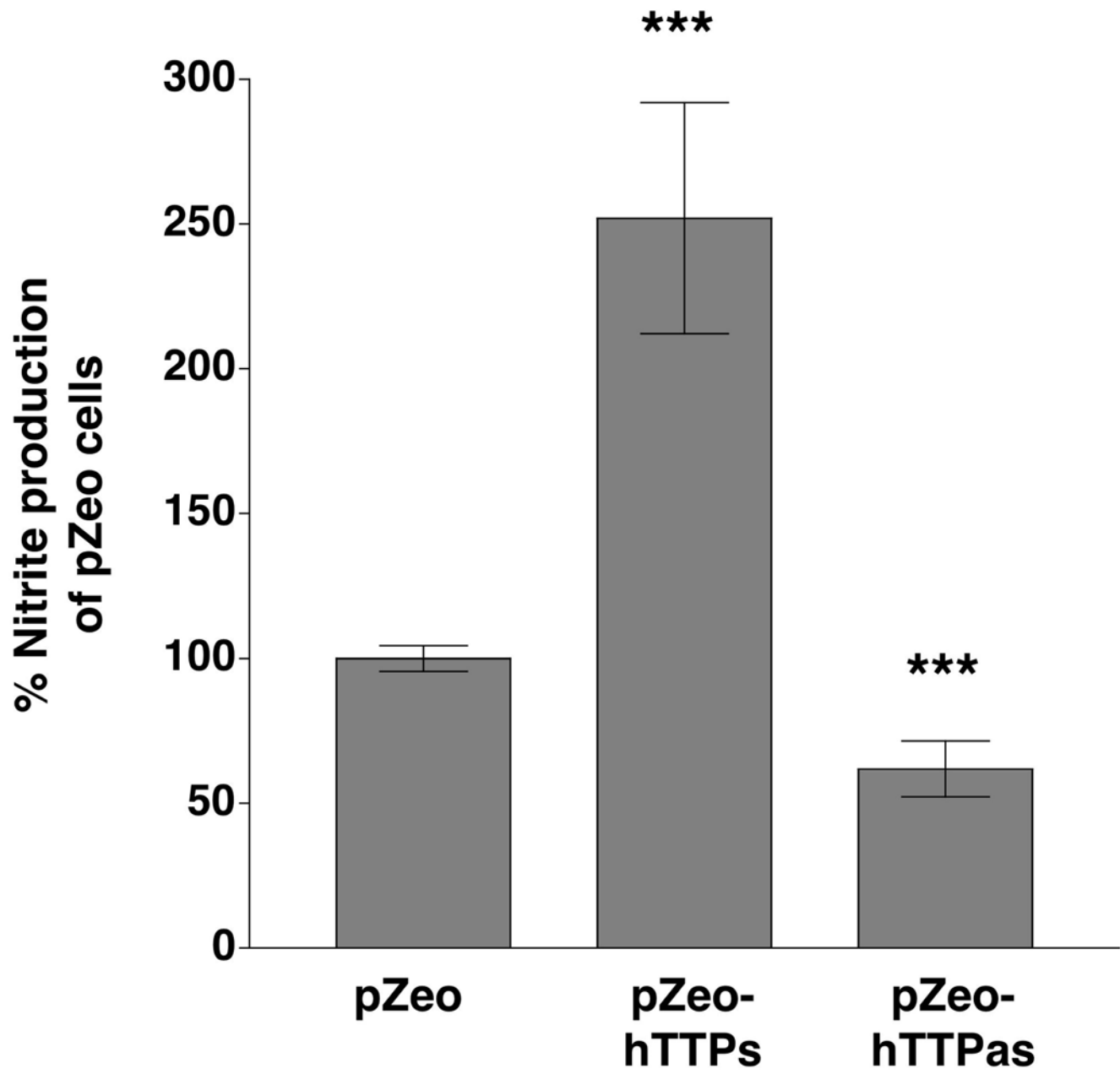


Fig. 4

F



**Fig 5**

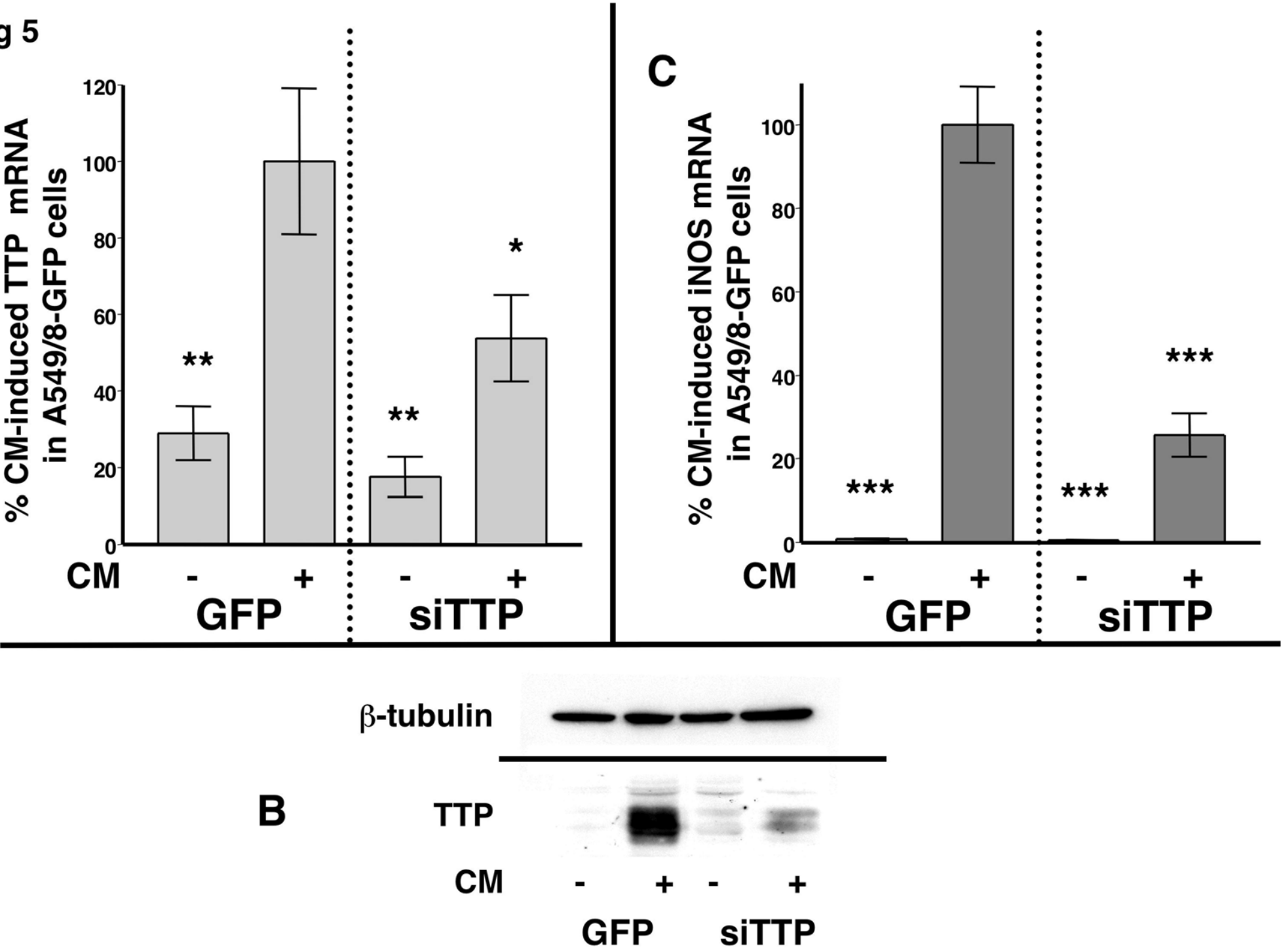




Fig. 6

A

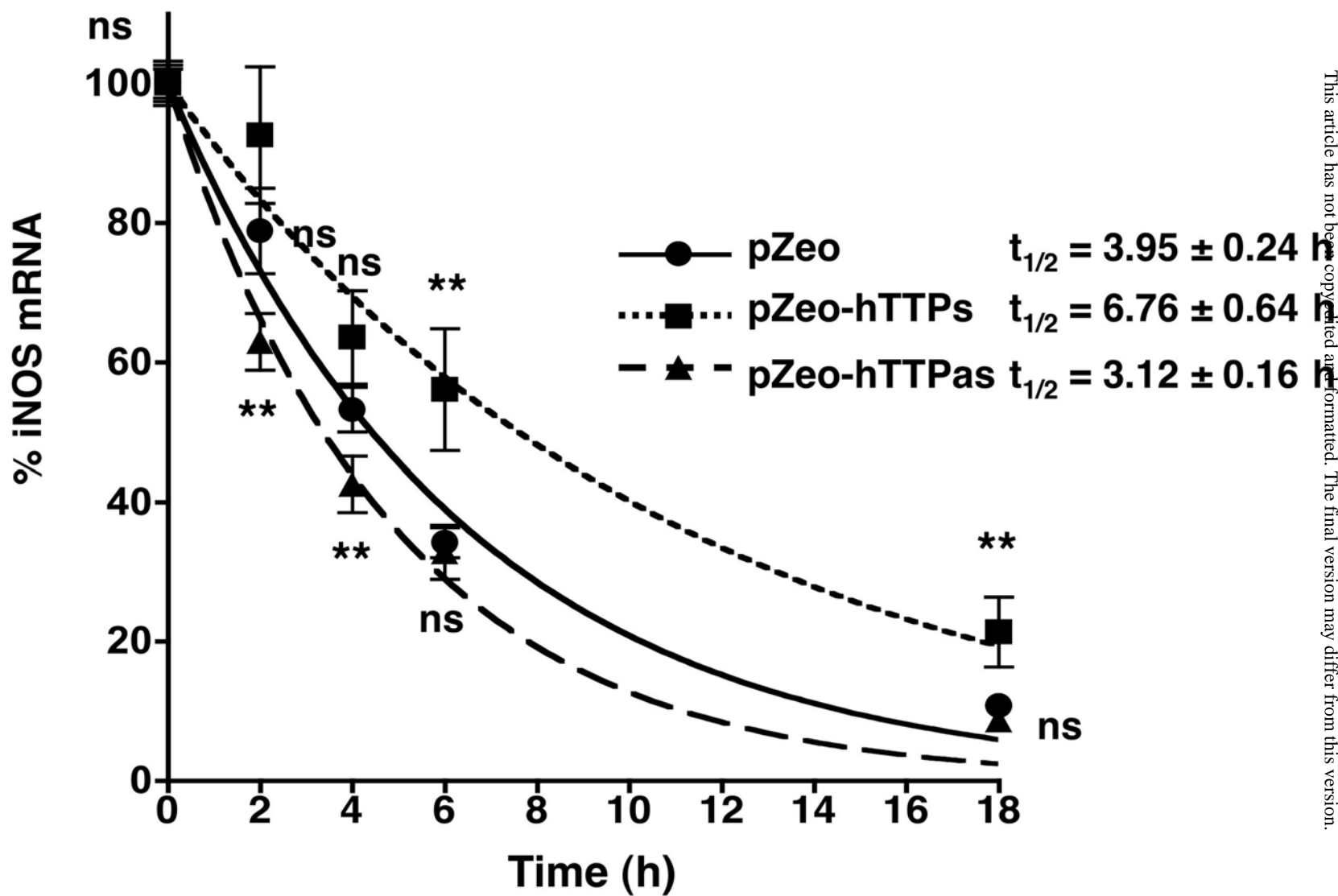


Fig. 6

**B**

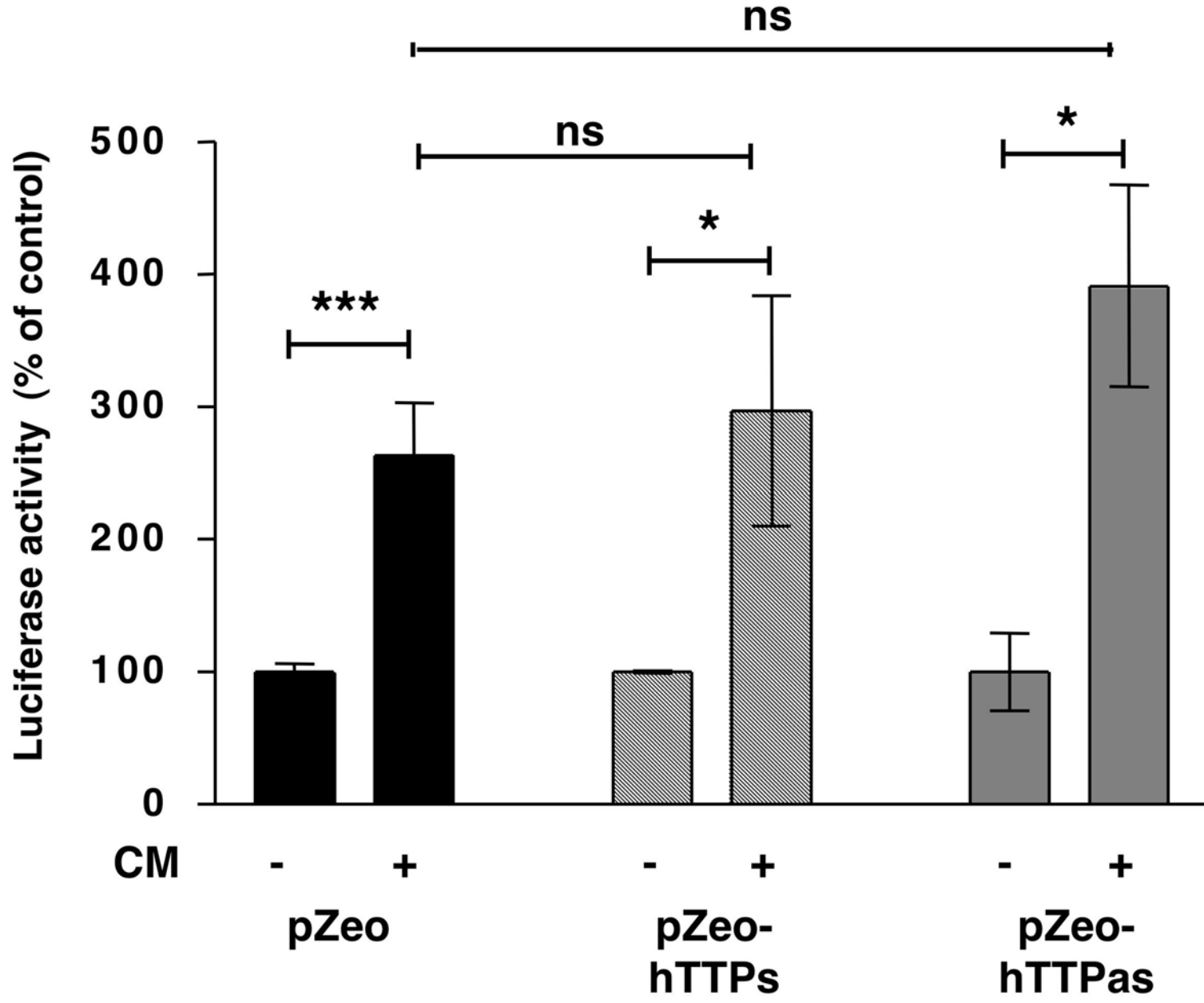


Fig. 7

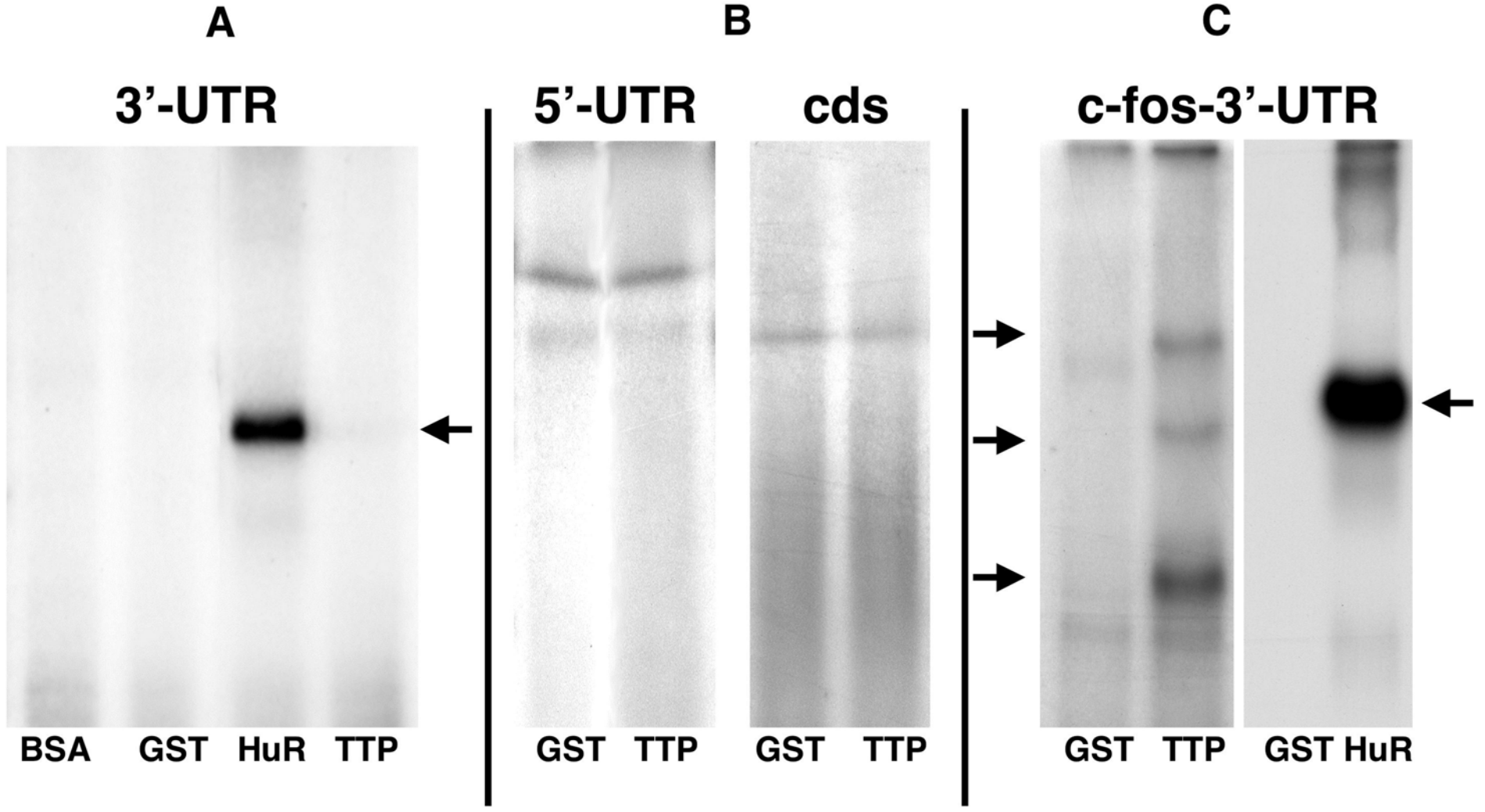


Fig. 8

A

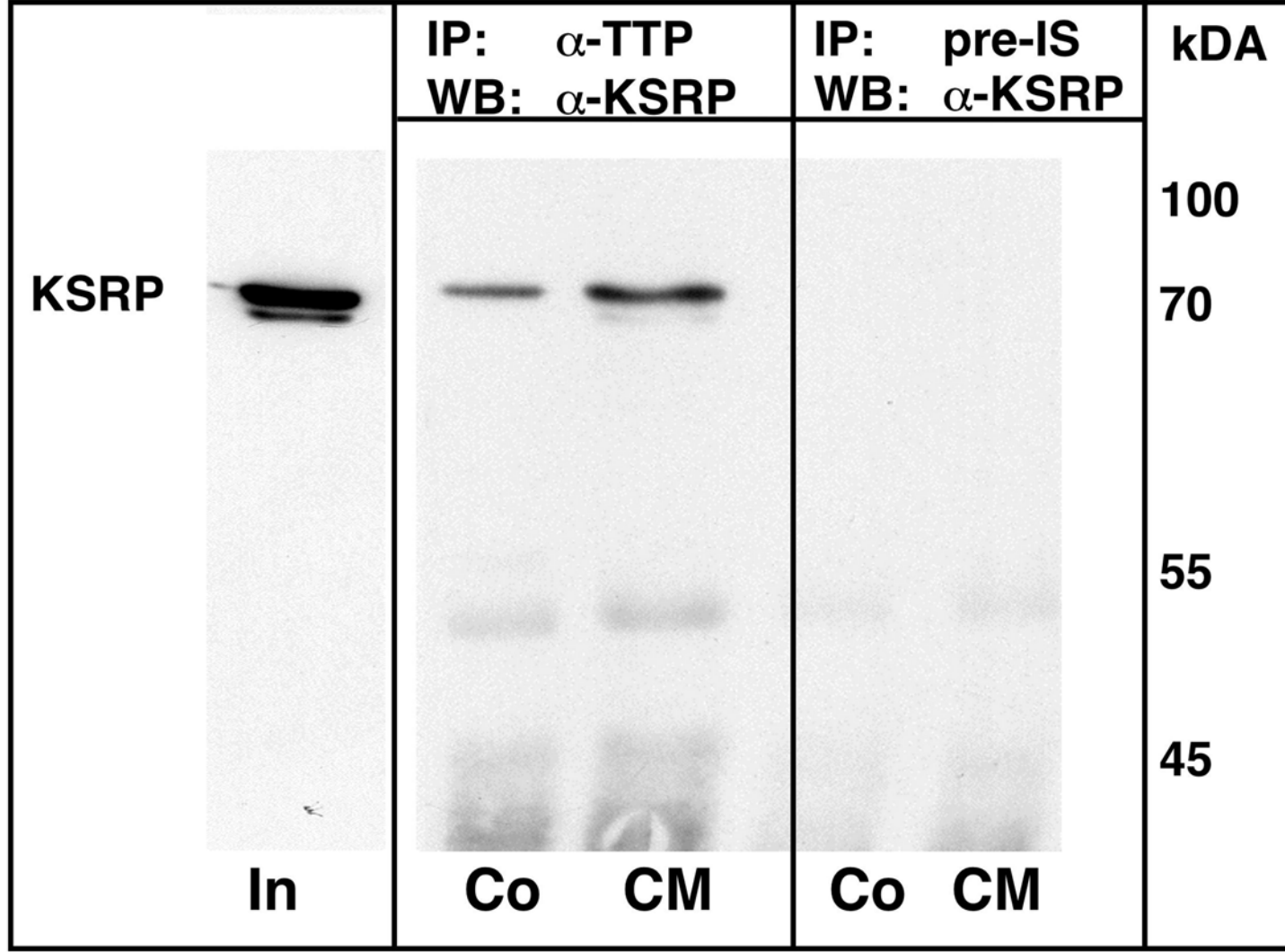


Fig. 8

**B**

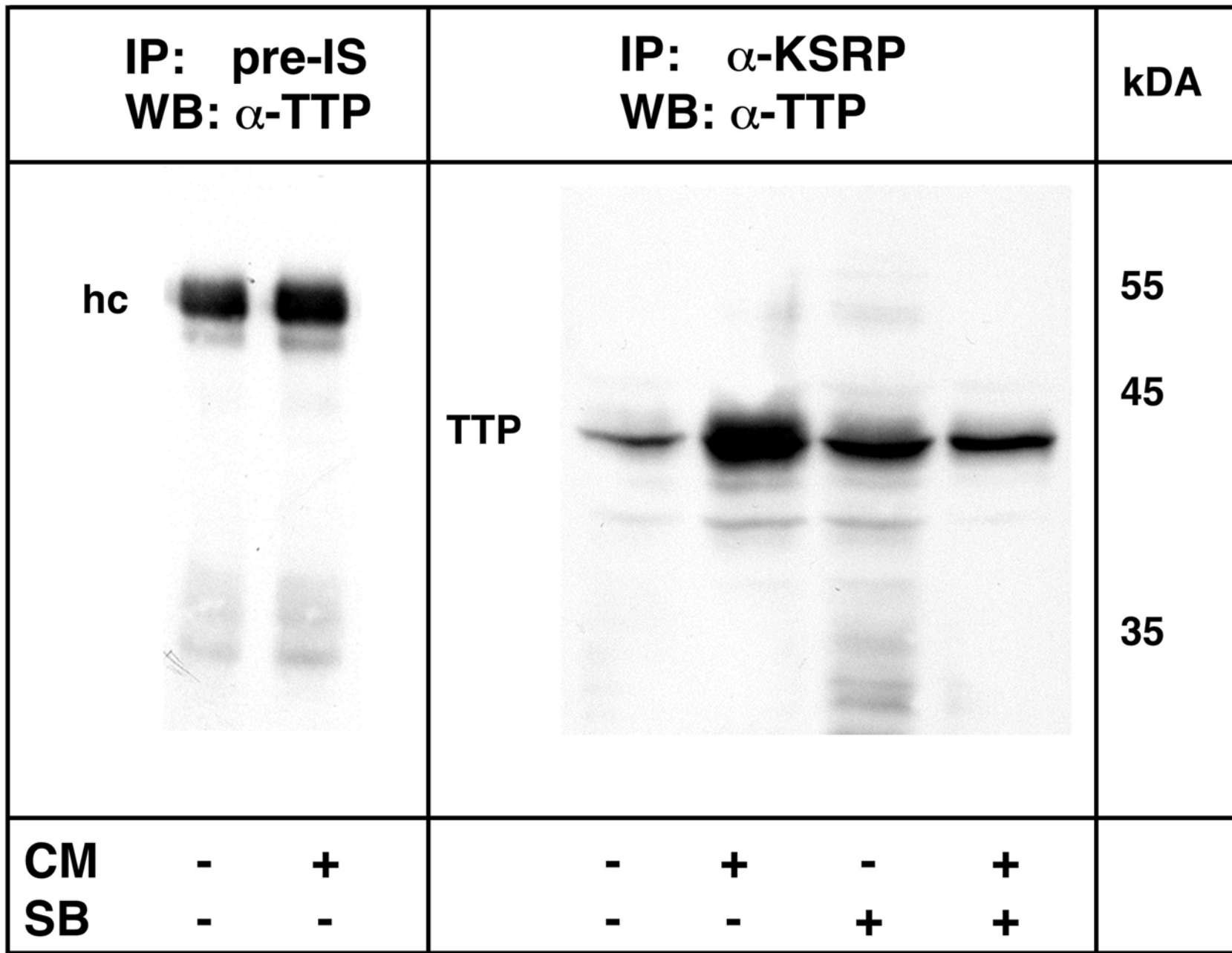


Fig. 8

C

