Identification of p54^{nrb} and the 14-3-3 Protein HS1 as TNF-α-Inducible Genes Related to Cell Cycle Control and Apoptosis in Human Arterial Endothelial Cells

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TNF-α plays a pivotal role in inflammation processes which are mainly regulated by endothelial cells. While TNF-α induces apoptosis of several cell types like tumor cells, endothelial cells are resistant to TNFa mediated cell death. The cytotoxic effects of TNF-α on most cells are only evident if RNA or protein synthesis is inhibited, suggesting that de novo RNA or protein synthesis protect cells from TNF-α cytotoxicity, presumably by NF-κB mediated induction of protective genes. However, the cytoprotective genes involved in NF-κB dependent endothelial cell survival have not been sufficiently identified. In the present study, the suppression subtractive hybridization (SSH) method was employed to identify rarely transcribed TNF-α inducible genes in human arterial endothelial cells related to cell survival and cell cycle. The TNF-α-induced expression of the RNA binding protein p54^{nrb} and the 14-3-3 protein HS1 as shown here for the first time may contribute to the TNF-α mediated cell protection of endothelial cells. These genes have been shown to play pivotal roles in cell survival and cell cycle control in different experimental settings. The concerted expression of these genes together with other genes related to cell protection and cell cycle like DnaJ, p21^{cip1} and the ubiquitin activating enzyme E1 demonstrates the identification of new genes in the context of TNF-α induced gene expression patterns mediating the prosurvival effect of TNF-α in endothelial cells.

Keywords: Endothelial cells, HS1, p54, Suppression subtractive hybridization, TNF-α

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Introduction

Tumor necrosis factor is a pleiotropic cytokine produced by many cell types, including macrophages, monocytes, smooth muscle cells, lymphoid cells and fibroblasts, that has been shown to mediate inflammatory, proliferative, cytostatic and cytotoxic effects in a variety of cell types. A primary cellular target of TNF is the vascular endothelium where it both initiates inflammatory responses by enhancing adhesion molecule expression and cytokine secretion, and inhibits endothelial cell growth responsible for the pathogenesis of atherosclerosis, cardiovascular dysfunction and tumor growth inhibition (Pober and Cotran, 1990). An important component in this process is the transcription factor NF-κB, which is rapidly activated following binding of TNF to the TNF-R1 receptor (Heller and Kronke, 1994). NF-κB activation depends on its release from a cytoplasmic inhibitor of NF-κB (IκBα), followed by its translocation to the nucleus, where it binds to cognate sequences in the promoter region of multiple genes. Upon stimulation, endogenous IκBα is typically phosphorylated, followed by its proteolytic degradation through the ubiquitin-proteasome pathway (Baeuerle and Baichwal, 1997; Barnes and Karin, 1997). Among important target genes of NF-κB in endothelial cells are those encoding inflammatory and chemotactic cytokines such as interleukin 1 (IL-1), IL-6, IL-8; cell adhesion molecules such as E-selectin, ICAM-1, MadCAM-1 and VCAM-1; and proinflammatory enzymes such as inducible nitric oxide synthase and cyclooxygenase-2 (Baeuerle and Henkel, 1994). The concerted expression of these genes leads to the inflammatory response of activated endothelial cells.

Many studies have demonstrated a growth inhibitory effect of TNF-α on vascular endothelial cells leading to delayed re-endothelialization after injury and atherosclerosis (Frater-Schroder *et al.*, 1987; Yilmaz *et al.*, 1998; Kishore *et al.*, 2003). TNF-α induced growth inhibition is mediated by inhibiting the cell cycle which is caused by enhanced

expression of the CDK inhibitor p21[cip1], a key regulator of the G1 check point of the cell cycle, beside other CDK inhibitors like p16[INK4a] and p27[kip1], by enhanced expression of the tumor suppressor gene p53, retention of the retinoblastoma gene in its active state and suppression of the E2F1 transcription factor (Lopez-Marure *et al.,* 2000; Dormond *et al.,* 2002).

Considerable attention has been paid to the apoptotic pathway elicited by TNF. Activation of the TNF receptor 1 results in cell death by recruitment of a complex of proteins to the cell membrane including TRADD, FADD/Mort1 and MACH/FLICE which culminates in the activation of cysteine proteases (rev. in (Aggarwal, 2000)). Although tumor cells and virally infected cells are susceptible to TNF-induced cell death, many normal cells are not. Human endothelial cells which play a pivotal role in modulating the inflammatory response are not directly killed by TNF-α (Pohlman and Harlan, 1989; Secchiero *et al.,* 2003). The protection of TNF induced cell death is mediated by NF-κB since its inhibition by overexpression of its specific inhibitor IκBα sensitizes cells, including endothelial cells, to TNF-mediated apoptosis (Beg and Baltimore, 1996; Liu *et al.,* 1996; Van Antwerp *et al.,* 1996). Furthermore, the cytotoxic effects of TNF-α on most cells are only evident if RNA or protein synthesis is inhibited. The data suggest that de novo RNA or protein synthesis protect cells from TNF-α cytotoxicity, presumably by NF-κB mediated induction of protective genes (Wong *et al.,* 1989). The cytoprotective genes involved in NF-κB dependent endothelial cell survival have not been sufficiently identified. Beg and Baltimore (Beg and Baltimore, 1996) demonstrated that one such anti-apoptotic gene, A20, is induced in RelA^{+/+} 3T3 cells after TNF-α treatment but it is not induced in RelA^{-/-} 3T3 cells, indicating an NF-κB dependent expression. However, transfection of A20 into RelA^{-/-} 3T3 cells is unable to prevent cell death, suggesting that other genes may be required to protect cells from TNF-α cytotoxicity. Chu *et al.* (1997) showed that the human inhibitor of apoptosis protein 2 (IAP2) protected TNF-treated Hela cells. Recently, Karsan *et al.* (1996) demonstrated that the Bcl-2 homologue A1 was induced by TNF-α and that overexpressed A1 protected endothelial cells from TNF-α and actinomycin D-mediated cytotoxicity.

In an effort to identify TNF-inducible genes related to apoptosis and cell cycle control in vascular endothelial cells, the present study used a recently developed method, termed suppression subtractive hybridization (SSH) (Diatchenko *et al.,* 1996; Stier *et al.,* 2000). This method combines a high subtraction efficiency with an equalize representation of differentially expressed sequences. It is based on a specific form of PCR permitting exponential amplification of cDNA´s which differ in abundance, whereas amplification of sequences of identical abundance in two populations are suppressed. After PCR-amplification the subtracted cDNA fragments are cloned into a TOPO-TA cloning vector and subsequent sequenced. Here, we describe the identification of TNF-α-inducible genes related to cell cycle control and cell protection by SSH in human umbilical arterial endothelial cells, mediating the anti-apoptotic and anti-proliferative effects of TNF on endothelial cells.

Materials and Methods

Isolation and characterization of human umbilical arterial endothelial cells (HUAEC) Endothelial cells were isolated from human umbilical cord arteries as previously described (Ko *et al.,* 1995) and cultivated on human fibronectin coated culture dishes in Medium 199 supplemented with 20% FCS, 10 mg/ml heparin and 30 µg/ml crude ECGF. For characterization of these cells the presence of von Willebrand factor (vWF) was demonstrated by indirect immunoperoxidase staining for vWF.

Isolation of poly(A)+RNA and cDNA synthesis The suppression subtractive hybridization was performed between unstimulated HUAEC (driver) and HUAEC stimulated for 4 h with tumor necrosis factor-α (TNF-α [50 ng/ml]) (tester). Total RNA was extracted by TRI-Reagent (Sigma, St. Louis, USA) according to the manufacturer's recommendations. Polyadenylated RNA was isolated with the Poly(A)Quick-Kit (Stratagene, Heidelberg, Germany). mRNA quantification was perfromed by spectrophotometry and the integrity of the mRNA was examined by electrophoresis of 1 µg mRNA on a 1% agarose gel stained with ethidium bromide. The further steps were performed according to the PCR-Select™cDNA Substraction Kit (Clontech, Palo Alto, USA). For the first-strand cDNA synthesis 1 µg of the isolated tester- and driver-poly(A)+RNA was incubated with the cDNA synthesis primer for 2 min at 70°C and rapidly chilled on ice. The reverse transcription reaction was performed in 1 × RT-buffer (50 mM Tris-HCl, 6 mM MgCl₂ and 75 mM KCl) with 1 mM dNTP and 200 U MMLV Reverse Transciptase for 1.5 h at 42°C. Following this reaction the second strand cDNA synthesis was immediately performed. In 1 × second strand buffer (100 mM KCl, 10 mM Ammoniumsulfate, 5 mM MgCl₂, 0.15 mM β-NAD, 20 mM Tris-HCl, 0.05 mg/ml bovine serum albumin) with 1 mM dNTP´s, DNA polymerase I 0.3 U/µl, RNAse H 0.01 U/µl and *E. coli* DNA ligase 0.06 U/µl the first strand cDNA was incubated at 16°C for 2 h. The double stranded cDNA was blunted by the addition of T4 DNA polymerase for 30 min at 16°C. The reaction was stopped by addition of EDTA/glycogen followed by the precipitation of the ds cDNA. To obtain shorter, blunt-ended molecules, the second strand cDNA was digested by Rsa I at 37°C for 1.5 h.

Adaptor ligation and subtractive hybridization The adaptor ligation was perfomed only with the reverse transcripted and digested mRNA of the tester cDNA. The cDNA of the unstimulated HUAEC (driver cDNA) remained unligated. One half of the tester ds cDNA was ligated with adaptor 1 (2 µM): the other half with adaptor 2 (2 µM) in a ligation buffer (50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 1 mM DTT, 1 mM ATP and 5% polyethylene glycol) with 0.05 U T4 DNA ligase at 16°C overnight. The reaction was stopped by EDTA/glycogen and the ligase was inactivated by heating the samples at 72°C for 5 min. For the first hybridization an excess of driver cDNA was added to each tester

cDNA (ligated with adaptor 1 or 2) in separate samples. After denaturation at 98°C for 1.5 min, the first hybridisation was performed in a hybridisation buffer at 68°C for 8 h. For the second hybridization, the two samples of the first hybridisation were mixed together without denaturation at 68°C overnight. The reaction was stopped by adding 200 µl of a dilution buffer and heating at 75°C for 7 min.

PCR amplification The primary PCR was used to selectively amplify the differentially expressed sequences and performed with 1 µl of the diluted suppression hybridized cDNA in a total volume of 25 µl (400 nM of each primer, 0.2 mM of each dNTP, 0.5 µl of Advantage KlenTaq polymerase mix (Clontech), 1 × PCR buffer). PCR was performed on a thermal cycler (Perkin Elmer 480) with the following parameters: 75°C for 7 min, 30 cycles at 94°C for 30 sec, 68°C for 30 sec and 72°C for 1.5 min. 1 µl of the amplified product was used as a template in secondary PCR for 30 cycles with nested PCR primer1, nested PCR primer2 in the same concentrations of PCR-buffer, dNTP and Taq-polymerase as used in the first PCR under following conditions: 94°C for 30 sec, 68°C for 30 sec and 72°C for 1.5 min.

Detection of subtracted cDNA fragments and reamplification Detection of subtracted cDNA fragments was performed on a horizontal polyacrylamid electrophoresis-chamber (Pharmacia-Biotech, Freiburg, Germany). 7 µl of the amplified cDNA were separated on a long-run gel (ETC, Kirchentellinsfurt, Germany) in three slots. The gel was subsequently cut in the second lane and the first part was stained with silver. Visible bands were cut out in the unstained part of the gel and the efficiency was detected by silver staining of this remaining part of the gel. The excised bands were used as a template in another PCR under the same conditions and with the same primers used for the secondary PCR. The results of this amplification were shown on polyacrylamid-gel.

Cloning and sequencing of the subtracted cDNA fragments The products of the reamplificated cDNA fragments were inserted into pCR II cloning vector (TOPO-TA Cloning-Kit, Invitrogen, Carlsbad, USA) and transformed into Tp10' cells (Invitrogen, San Diego, USA). The bacteria were plated on ampicillin-containing agar plates, which were overlaid with 1.4 µg β-X-Gal and 100 µM IPTG, at 37°C overnight. White colonies were picked and transferred into a well of a 96-well multititer plate containing 200 µl LB-medium with 50 mg/ml ampicillin and subsequently incubated for 4 h at room temperature. The bacteria were diluted in H$_2$0 and lysed by heating to 100°C for 10 min. Five µl of each sample were used to PCR amplify the cloned inserts in 50 µl reactions (standard PCR buffer (Perkin Elmer, Norwalk, USA), 200 µM dNTP, 2 U Taq polymerase, 10% DMSO) using 10 µM of M13-APfor and M13-APrev primers (5'-primer: 5'-GCT ATT ACG CCA GCT GGC GAA AGG GGG ATG TG-3', 3'-primer: 5'-CCC CAG GCT TTA CAC TTT ATG CTT CCG GCA CG-3') which flank the multiple cloning site of pCRII.1 under the following conditions: 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. Seven µl of the PCR-product were loaded on a horizontal polyacrylamid-gel to confirm the correct size of the cloned PCR fragment. DNA sequencing was performed by automated means at the MWG-Biotech (Ebersberg, Germany). Nucleic acid homology searches were performed using the BLAST program through e-mail servers at the US-american National Center for Biotechnology Information (Altschul *et al.*, 1997).

Reverse transcription/polymerase chain reaction (RT/PCR) of detected sequences To confirm the induction and differential expression of the detected sequences, RT-PCR analyses were performed (Ko *et al.*, 1995). 5'- amd 3'-primers were designed for

**Table 1.** Location and sequences of the optimal primers

primers	PCR-fragment size (bp)	Sequences	location on cDNA
5'-GAPDH	287	5'-GCCAAAAGGGTCATCATCTC-3'	406-425
3'-GAPDH	287	5'-GTAGAGGCAGGGATGATGTTC-3'	672-692
5'-Ubi E1	398	5'-CCTGCATCTGGACTATGTG-3'	2406-2424
3'-Ubi E1	398	5'-GGATGATCTTCCCTGCAATC-3'	2784-2803
5'-Cdc2	236	5'-GATCTCCAGAAGTATTGCTGG-3'	635-655
3'-Cdc2	236	5'-GCTTCCTGGTTTCCATTTGG-3'	851-870
5'-p21^{cip1}	322	5'-GATTAGCAGCGGAACAAGG-3'	1728-1746
3'-p21^{cip1}	322	5'-GAGCTGTGAAAGACACAGAAC-3'	2029-2049
5'-p54^{nrb}	260	5'-GGTGAGAATTCAGGCAAACG-3'	2150-2169
3'-p54^{nrb}	260	5'-CCCAAGATTGTACTAGGCC-3'	2391-2409
5'-HS1	404	5'-GTGTGCAGTACTGCTGTAG-3'	1027-1045
3'-HS1	404	5'-CCTGTTGCCACAGATACAC-3'	1412-1430
5'-DnaJ	363	5'-CAGATAGGACCTGGAATGG-3'	572-590
3'-DnaJ	363	5'-GGTTCGGTTGTCAAGAGTAG-3'	915-934
5'-EF-1a	215	5'-CTTACACACTGGGTGTGAAAC-3'	472-492
3'-EF-1a	215	5'-GAACCAAGGCATGTTAGCAC-3'	667-686

each of the detected sequences (Table 1), and synthesized by MWG-Biotech (Ebersberg, Germany). PCR conditions were optimized by varying the annealing temperature and time as well as the Mg^{2+} and K^+ concentration of the PCR buffer. Total RNA (4 µg) of unstimulated HUAEC and HUAEC stimulated with TNF-α [50 ng/ml] was incubated with moloney murine leukemia virus (MMLV) reverse transcriptase (200 U/µl) in a total reaction volume of 40 µl containing 1 × RT buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$); 10 mM dithiothreitol; 0.5 mM dNTP; 50 U RNA guard and 100 pmol random hexamer primer for 5 min at 25ºC, 5 min at 30ºC, 90 min at 37ºC and 5 min at 95ºC. 1 µl of the RT sample was used for PCR amplification in a final volume of 50 µl containing 1 × PCR buffer (Perkin Elmer); 0.2 mM dNTP; 20 pmol of each primer and 2,5 U Taq polymerase. The samples were covered with mineral oil and the reaction was conducted in a thermal cycler (Perkin Elmer 480) using the following conditions: 5 min at 94ºC; 25 to 30 cycles with 94ºC for 1 min; 60ºC for 1 min and 72ºC for 1.5 min; extension at 72ºC for 20 min. 20 µl of each

sample were loaded onto a 1.5% agarose gel and stained with ethidium bromide.

Determination of the exponential phase of the RT-PCR   To avoid artificial results of the PCR, the amplification of the reversed transcribed RNA was performed for each sequence with the constructed primers in the presence of 0.1 µCi a-[³²P]-CTP. After 15, 20, 25, 30, 35 and 40 cycles, samples were removed and loaded on an ethidium bromide stained 1.5 % agarose gel. Under UV-light, the bands were cut out and melted in 500 µl H_2O. The relative amounts of amplified DNA were determined by Cerencov counting in a scintillation counter (Beckmann, Düsseldorf, Germany). The amounts of radioactivity were plotted as a function of the number of cycles.

Densitometric analysis   Densitometric analysis was performed on a 2-dimensional scanning densitometer (Biometra, Göttingen, Germany) using the ScanPack software version 14.1 A 27. The
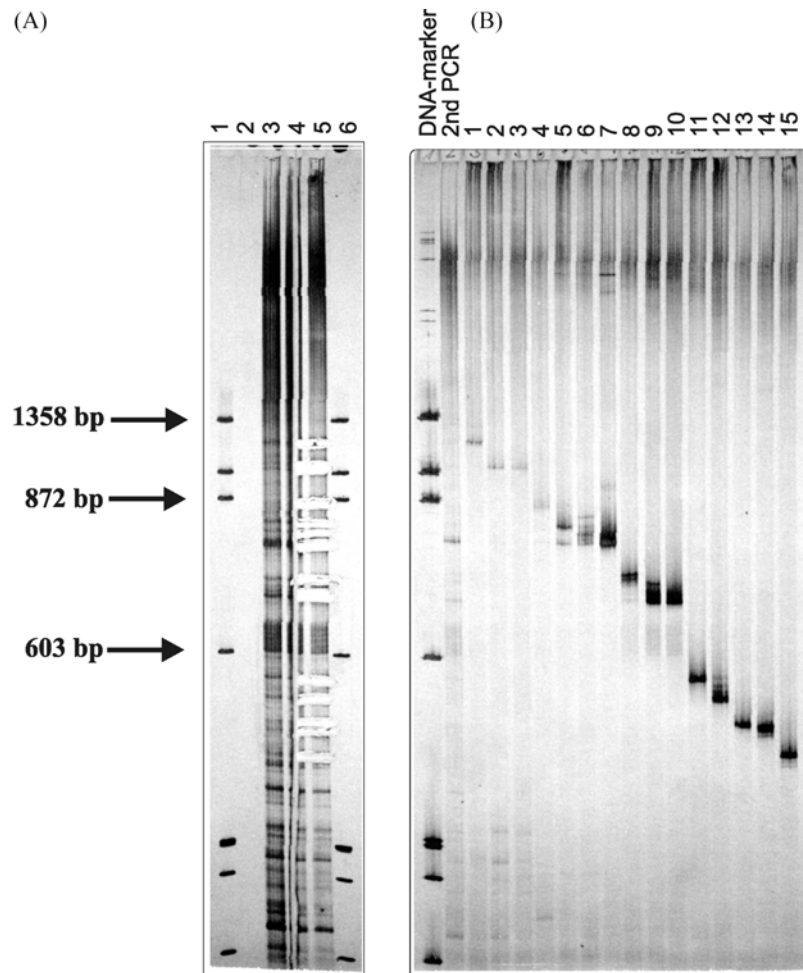
Fig. 1. Results of SSH. A: Reverse transcribed mRNA of HUAEC stimulated with TNF-α for 4 h subtracted against reverse transcribed mRNA of non-stimulated HUAEC according to the SSH protocol. Lane 2 shows the PCR products of the first PCR and lane 3-5 show the product of the second PCR. Distinct cDNA fragments were detected by polyacrylamide electrophoresis and excised from the gel (lane 5). Lanes 1 and 6: HaeIII digest of FX174 DNA. B: results of the reamplifications of the cut subtracted cDNA fragments. The 15 singel PCR products of the second PCR were reamplified and an aliquot of each sample were loaded on the gel (lane 3-17). Lane 1: HaeIII digest of ΦX-174 DNA, lane 2: PCR products of the second PCR.

Table 2. Differentially expressed genes of known function

Locus	Accession	Identified gene	Location
HSQAE1	X56976	ubiquitin activating emzyme E1	2562-2762
HSCDC2	X05360	Cdc2 kinase	629-1049
HUMSDI1A	L26165	cyclin-dependent kinase inhibitor p21	1877-2027
HSY11298	Y11298	p54^{nrb}	922-1131
HSEF1AR	X03558	Elongation factor 1α	415-835
HSHS1RNA	X57347	HS1 protein	1027-1507
HUMDNAJA	D13388	DnaJ	545-992
HUMVCAM1V	M30257	VCAM-1	1763-2310
HUMIL8A	M12830	IL-8	3556-4021
HSMCP1	X14768	MCP-1	122-528
HUMMAD3A	M69043	IκBα	1140-1481
AF000652	AF000652	Syntenin	1515-1734

ethidium bromide stained gels were photographed and the densitomeric results of gene expression were standardized to that of GAPDH expression of the same reverse transscribed RNA sample.

Statistics Data are presented as means $\pm$ SD. Statistical analysis of data was performed using the Mann-Whitney U test.

Results

In an effort to identify TNF-inducible genes in primary vascular endothelial cells related to apoptosis and cell cycle control, the mRNA of HUAEC stimulated with TNF-α (tester) and non-stimulated HUAEC (driver) was isolated, reverse transcribed into cDNA and digested with RsaI. For the SSH procedure, two different adaptors were separately ligated to the digested tester cDNA. After the first hybridization of the two different tester cDNA with an excess of denaturated driver-cDNA in separate samples and a second hybridization step, the subsequent PCR amplification was performed without denaturation. Following polyacrylamide electrophoresis, subtracted cDNA fragments of different size were detected (Fig. 1). The visible subtracted cDNA fragments were excised from the gel and reamplified. As shown in Fig. 1B, discrete cDNA fragments could be resolved by this procedure. The reamplified cDNA fragments were inserted into a cloning vector sequenced using M13 AP-primer. Nucleic acid homolgy searches identified 15 different cDNAs from which 12 sequences represented differentially expressed genes of known functions (Table 2).

The genes identified by SSH could be classified into two groups. Five of the genes encoded proteins involved in the regulation of proinflammatory events such as vascular cell adhesion molecule-1 (VCAM-1), IL-8, monocyte chemoattractant protein-1 (MCP-1), IκBα, and syntenin a novel PDZ motif containing adaptor protein. The second group of genes were involved in cell cycle regulation, growth regulation, protein synthesis and apoptosis. These cDNAs encode the cyclin-dependent kinase inhibitor p21^{cip1}, Cdc2 kinase, ubiquitin activating enzyme E1, elongation factor 1a, HS1 protein a 14-3-3 protein family member and the heat shock protein DnaJ/Hsp40. Interestingly, we also found that p54^{nrb}, a novel RNA binding protein, which is involved in transcription and pre-mRNA splicing, was stimulated by TNF-α.

To confirm whether the genes for p54^{nrb} and the other cell cycle and apoptosis related genes were indeed inducible by TNF-α as suggested by the previous SSH data, we performed detailed semi-quantitative PCR-analyses. In the first experiments, the exponential amplification phases for these genes were determined by radioactively labeled RT-PCR. Further amplification was then performed using 26 PCR cycles for p21^{cip1}, p54^{nrb}, DnaJ and HS1, 27 cycles for ubiquitin activating enzyme E1, 30 cycles for Cdc2, and 23 cycles for EF-1a. As detected by RT-PCR, stimulation of HUAEC with TNF-α caused a time dependent elevation of the transcripts specific for p21^{cip1}, Cdc2, EF-1a, p54^{nrb}, ubiquitin activating enzyme E1, DnaJ and HS1 (Fig. 2). In contrast, the expression of GAPDH was not altered upon TNF-α stimulation, confirming that equal amounts of RNA had been used. Densitometric analyses of the ethidium-bromide-stained gels of three further independent experiments for each gene product revealed a 4-7 fold increase of mRNA expression in response to stimulation with TNF-α (Fig. 3). Furthermore, we demonstrate that expression of p54^{nrb} and HS1, whose transcriptional regulation has not been studied so far, follows a kinetic similar to other TNF-α inducible gene products, suggesting that it might presumably represent a NF-κB target.

Discussion

Although TNF-mediated death has gained much attention, many recent studies suggest that TNF also activates a cell survival pathway that protects against its apoptotic effects in different cell types. While the death pathway in endothelial cells does not require new gene expression, the inducible survival pathway is abrogated by RNA or protein synthesis
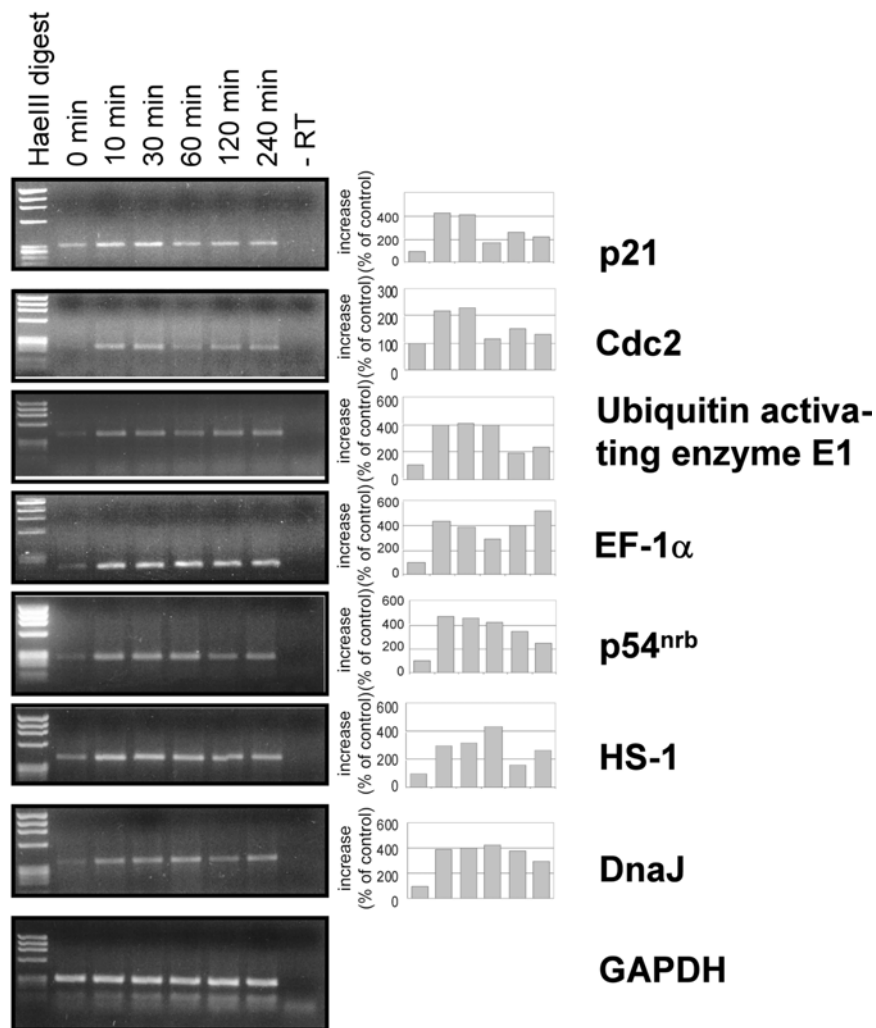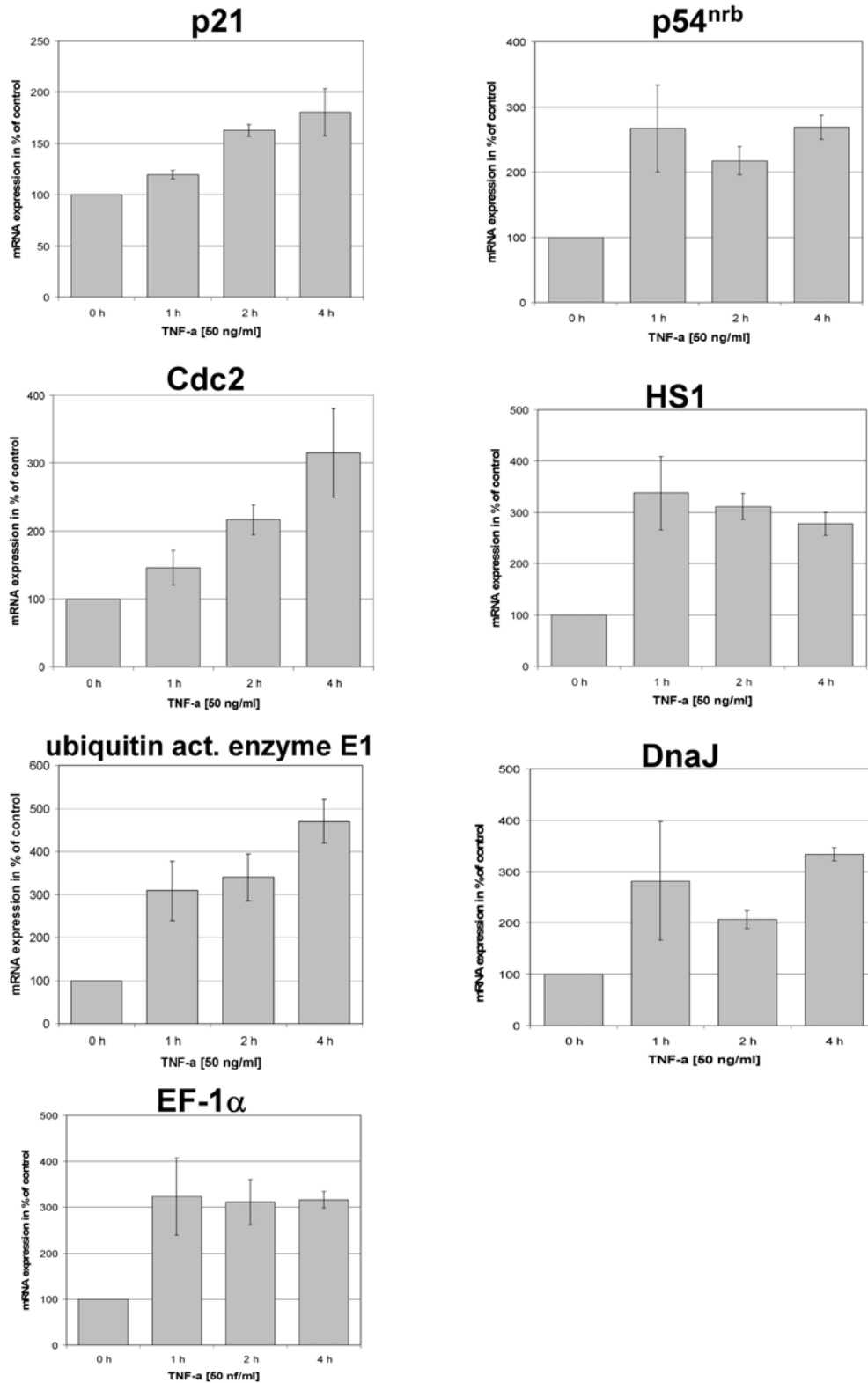
Fig. 2. Expression of p21cip1, Cdc2 kinase, ubiquitin activating enzyme E1, elongation factor 1α, HS1, DnaJ and p54nrb specific mRNA in response to TNF-α stimulation. HUAEC were incubated in medium alone or treated with 50 ng/ml TNF-α for the indicated time. Total RNA was isolated and RT-PCR was performed as described. The molecular size of the resulting PCR products are shown on the left. As a control for the same amount of RNA a RT-PCR with GAPDH primer was performed (lower panel). The relative quantities of the specific PCR products were determined by densitometry and normalized with the density of the GAPDH profiles (right panels).

inhibitors, indicating a transcriptional regulation of the anti-apoptotic pathway (Pohlman and Harlan, 1989). The aim of this study was to identify TNF-inducible genes in arterial endothelial cells in the context of gene expression patterns related to endothelial cell growth, cell cycle regulation and apoptosis. The recent description of a novel equalizing cDNA subtraction method, termed suppression subtractive hybridization (SSH) (Diatchenko *et al.,* 1996) provides the technical basis for this kind of comparison. In this study, we identified 15 differentially expressed genes in activated endothelial cells, from which 80 % were differentially expressed upon TNF-α stimulation. Five of the genes were involved in the control of inflammatory response in activated endothelial cells such as VCAM-1, IL-8, MCP-1, IκBα and syntenin, whereas seven of the detected genes, which we have analyzed here in more detail, were related to the regulation of cell cycle, protein

synthesis and survival. By several time course studies using RT-PCR analyses we show that expression of the latter genes was indeed rapidly activated by TNF-α, which further demonstrate the reliability of the SSH approach for the investigation of differential gene expression.

The concerted expression of p21cip1, Cdc2, ubiquitin activating enzyme E1 and EF-1a as shown in this study is crucially involved in cell cycle control of endothelial cells by TNF-α. It is generally assumed that TNF-α inhibits the proliferation of vascular endothelial cells, which is associated with an accumulation of cells in the G1 phase of the cell cycle by retention of the tumor suppressor retinoblastoma gene product (RB) in its active state and enhanced expression of key regulators of cell cycle progression like p53 and the inhibitor of the cyclin dependent kinase p21cip1 (Frater-Schroder *et al.,* 1987; Yilmaz *et al.,* 1998; Lopez-Marure *et*

Fig. 3. Statistical analysis of p21^{cip1}, Cdc2 kinase, ubiquitin activating enzyme E1, elongation factor 1α, HS1, DnaJ and p54^{nrb} gene expression after TNF-stimulation. The individual RT-PCR profiles from three independent experiments were subjected to densitometry and statistically analyzed with regard to GAPDH expression. Data of gene expression are presented as means ± S.D.

al., 2000; Dormond et al., 2002; Secchiero et al., 2003).

An interesting finding was the identification of the HS1-

protein as a TNF-α inducible gene in vascular endothelial cells. So far, the transcriptional regulation of the HS1-protein

by TNF-α in vascular endothelial cells has not been studied. The HS1 protein is part of the 14-3-3 protein family of conserved phophoserine/phosphothreonine-binding molecules that control the function of a wide array of cellular proteins (van Hemert *et al.*, 2001). Through protein-protein interactions, subcellular localization of proteins and enzyme activation, 14-3-3 proteins participates in the regulation of diverse cellular processes, including cell-cycle control, signal transduction, apoptosis and stress response. The 14-3-3 proteins regulate several enzymes controlling the cell cycle. The Cdc2 kinase is kept directly or indirectly through activation of Cdc2 inhibitors in an inactive form by the 14-3-3 proteins thereby preventing the G2 to M transition (Honda *et al.*, 1997; Laronga *et al.*, 2000; Wang *et al.*, 2000). The transcription factor p53 essential for cell cycle arrest is activated by 14-3-3 proteins leading to an enhanced expression of p21[cip1] and 14-3-3 proteins (Hermeking *et al.*, 1997; Waterman *et al.*, 1998). Therefore, 14-3-3 proteins have an inhibitory effect on the progression through the cell cycle leading to an inhibition of the G1 to S (p53) and the G2 to M (Cdc2) transition. Like in cell cycle control, the 14-3-3 proteins have major function in the control of apoptosis inhibiting the proapoptotic effect of TNF-α beside other direct effects on the regulation of apoptotic proteins. The 14-3-3 proteins stabilize A20 a zink finger protein that inhibits TNF-induced apoptosis (Vincenz and Dixit, 1996; Lademann *et al.*, 2001), while the apoptosis signal-regulating kinase 1 (ASK1), activated by death-inducing stimuli such as TNF-α, is inhibited by association with 14-3-3 proteins (Zhang *et al.*, 1999; Xing *et al.*, 2000; Liu *et al.*, 2001). Furthermore, the survival factor Interleukin-3 leads to an association of 14-3-3 proteins with the Bad-protein resulting in the inhibition of Bad proapoptotic activity (Zha *et al.*, 1996; Subramanian *et al.*, 2001). The ability of the 14-3-3 protein to inhibit several proapoptotic proteins while enhancing the function of prosurvival proteins support the idea that 14-3-3 acts to promote cell survival. As a differentially expressed protein in HUAEC induced by TNF-α it might counteract the proapoptotic effect of TNF-α on endothelial cells.

In the context of TNF-α induced activation of endothelial cells, the identification of the differentially expressed p54[nrb] (p54) gene described here for the first time is of great interest. p54 with high homology to the PSF splicing factor has a high affinity for RNA via its N-terminus and can bind pre-mRNA and RNA implying a role in RNA processing (Dong *et al.*, 1993; Shav-Tal and Zipori, 2002). As a binding protein of single stranded RNA through its RRM motifs it mediates the nuclear retention of defective RNAs (Zhang and Carmichael, 2001) and in splicing of several RNAs. Furthermore, p54 is known as a transcription factor activating the expression of several genes (Basu *et al.*, 1997). Thus, transcription and RNA processing appear to be intimately connected nuclear processes that share common factors like p54 (Xing *et al.*, 1993; Yang *et al.*, 1993). Of interest, essential pre-mRNA splicing factors like PSF have been shown to regulate the

transcription of heat shock proteins upon activation of cells suggesting an stabilizing effect of these genes on gene transcription under conditions of stress. B52, the drosophila homologue to mammalian splicing factors like p54, is recruited to heat shock loci. As transcription of heat shock genes is increased during heat shock activation, the distribution of the protein across the locus increases in close proximity to the transcription machinery of RNA-polymerases (Champlin and Lis, 1994). Such a coupling of transcription and splicing implies a mechanism for the ordered joining of exons and translation efficiency.

In our experiments, we found that p54 expression was upregulated in TNFa stimulated HUAEC in conjunction with the expression of heat shock proteins like DnaJ, 14-3-3 proteins, ubiquitin related genes and endothelial cell activation. It may be speculated that p54 is needed for the proper expression of proteins including heat shock proteins leading to a stabilization of the transcription machinery, protein production and protein function, promoting the survival of endothelial cells under conditions of TNF-α induced cell stress. The fact that the survival promoting response to TNF-α requires an ongoing gene expression and protein synthesis in contrast to the induction of apoptosis, raises the possibility that TNF may induce synthesis of antiapoptotic proteins. The concerted expression of genes involved in different mechanisms of cell protection, like DnaJ, 14-3-3, ubiquitin activating enzyme and p54[nrp] may contribute to the survival promoting response counteracting the apoptosis inducing influences of TNF-α on vascular endothelial cells, probably mediated by NF-κB activation.

In sum, we show that the technique of SSH allows the rapid identification of differentially expressed genes in the context of gene expression patterns. Beside the induction of adhesion molecules and cytokines, we could detect a variety of genes related to cell cycle control and apoptosis. The detection of the heat shock protein DnaJ/Hsp40, the 14-3-3 protein HS1 and the RNA binding protein p54[nrp], first described in this study, might give new insides in the NF-κB mediated survival promoting effects of TNF-α in vascular endothelial cells, counteracting TNF-induced apoptosis. Further studies are required to define the intracellular mechanisms of the newly detected genes in the context of endothelial cell activation and cell protection by TNF-α.

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