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ACTIVATION OF NADPH OXIDASE BY TRANSFORMING GROWTH FACTOR-BETA (TGF-β) IN HEPATOCYTES MEDIATES UP-REGULATION OF EPIDERMAL GROWTH FACTOR (EGF) RECEPTOR LIGANDS THROUGH A NF-κB-DEPENDENT MECHANISM

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KEY WORDS

Hepatocytes, TGF-beta, NF-kappaB, EGF, HB-EGF, TGF-alpha, ROS, NADPH oxidase, Nox

ABREVIATIONS

- DHE: hydroethidine
- DPI: Diphenyleneiodonium.
- EGF: Epidermal Growth Factor.
- EGFR: Epidermal Growth Factor Receptor
- EMSA: Electromobility Shift Assay.
- ERK: Extracellular Regulated Kinase.
- GSH: (glutathione, ethyl-ester form).
- HB-EGF: Heparin Binding EGF-like Growth Factor.
- H_2DCFDA: 2',7'-dichlorodihydrofluorescein diacetate
- JNK: Jun N-terminal Kinase.
- MAPK: Mitogen Activated Phospho Kinase.
- NF-κB: Nuclear Factor-Kappa B.
- NOX: NADPH Oxidase
- PI-3K: Phosphatidylinositol-3 Kinase.
- ROS: Reactive Oxygen Species.
- RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction.
- SBE: Smad Binding Element.
- TACE: TNF-alpha Converting Enzyme.
- TGF-α: Transforming Growth Factor-alpha.
- TGF-β: Transforming Growth Factor-beta.
- TβR-I: TGF-β Receptor Type I.

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ABSTRACT

The transforming growth factor-beta (TGF-β) induces survival signals in foetal rat hepatocytes through transactivation of the epidermal growth factor receptor (EGFR). The molecular mechanism is not completely understood, but both activation of the TACE/ADAM17 (one of the metalloproteases involved in shedding of the EGFR ligands) and up-regulation of transforming growth factor alpha (TGF-α) and heparin binding epidermal growth factor-like growth factor (HB-EGF) appear to be involved. Here we have analyzed the molecular mechanisms that mediate up-regulation of the EGFR ligands by TGF-β in foetal rat hepatocytes. The potential involvement of reactive oxygen species (ROS), an early signal induced by TGF-β, and the existence of an amplification loop triggered by initial activation of the EGFR, have been studied. Results indicate that diphenyleneiodonium (DPI) and apocynin, two NADPH oxidase inhibitors, and SB431542, inhibitor of the TGF-β receptor I (TβR-I), block up-regulation of EGFR ligands and Akt activation. Different members of the NADPH oxidase family of genes are expressed in hepatocytes, included *nox1*, *nox2* and *nox4*. TGF-β up-regulates *nox4* and increases the levels of Rac1 protein, a known regulator of both Nox1 and Nox2, in a TβR-I-dependent manner. TGF-β mediates activation of the NF-κB pathway, which is inhibited by DPI and is required for up-regulation of TGF-α and HB-EGF. In contrast, EGFR activation is not required for TGF-β-induced up-regulation of those ligands. Considering previous works that established the role of ROS in the apoptosis induced by TGF-β in hepatocytes, results presented here indicate that ROS might mediate both pro- and anti-apoptotic signals in TGF-β-treated cells.

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INTRODUCTION

The transforming growth factor beta (TGF-β) belongs to a family of structurally related polypeptide growth factors that play important roles in growth control, development and differentiation [1]. TGF-β inhibits growth and induces apoptosis in epithelial cells, contributing to suppress tumourigenesis. However, recent works have indicated that cell response to TGF-β is very complex, in particular in pre-neoplastic or tumour cells, where TGF-β might mediate both pro- and anti-apoptotic signals and induce epithelial-mesenchymal transition processes, contributing to tumour progression and dissemination [2].

One example of this complex response is that one observed in liver cells. TGF-β induces apoptosis in foetal rat hepatocytes, by a mechanism dependent on oxidative stress, mitochondrial release of cytochrome c and caspases activation [3]. However, TGF-β also induces survival signals in those cells [4], through transactivation of the epidermal growth factor receptor (EGFR), which is required for Akt phosphorylation and cell death rescue [5]. The balance between death and survival signals decides cell fate. Thus, a great proportion (around 60-70%) survive to the apoptotic effects of TGF-β and respond to this cytokine undergoing an epithelial-mesenchymal transition process, which increases their migratory capacity. Thus, understanding the molecular pathways that mediate TGF-β-induced hepatocyte survival might help in the design of new drugs that potentiate its suppressor effects. The molecular mechanisms that mediate EGFR transactivation by TGF-β are not completely understood. Activation of the TACE/ADAM17 (one of the metalloproteases involved in shedding of the EGFR ligands) produces the proteolysis and activation of the EGFR ligands that are anchoraged in the cell membrane [5]. Furthermore, TGF-β induces up-regulation of transforming growth factor alpha (TGF-α) and heparin binding epidermal growth factor-like growth factor (HB-EGF) [6]. Regulation of TGF-α expression by TGF-β had been previously proposed to occur in human colon carcinoma [7, 8] and endothelial [9] cells, however, up to now, nothing is known about the intracellular signal/s initiated by TGF-β that triggers this effect.

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One of the early events taking place in response to TGF-β in foetal rat hepatocytes is generation of reactive oxygen species (ROS), through activation of a NADPH-oxidase-like system and down-regulation of antioxidant genes [10]. With the increased understanding of the proliferation, survival and death signalling pathways, the exact role of ROS in regulating cellular functions is controversial. Growing evidence suggests that ROS within cells act as second messengers in intracellular signalling cascades which induce and maintain the oncogenic phenotype of cancer cells. However, it is also known that ROS can induce cellular senescence and cell death and can, therefore, act as anti-tumourigenic agents [11]. In this line of evidence, some important discoveries have underscored the critical role of ROS in the survival signals triggered by tumour necrosis factor-alpha (TNF-α) (notably in the TNF-α-mediated activation of nuclear factor-kappaB, NF-κB, and c-Jun N-terminal kinase, JNK), in addition to their known role in cell death (apoptotic and necrotic) pathways [12]. Interestingly, transactivation of EGFR by tumour necrosis factor [13] or endothelin-1 [14] would require ROS generation and different reports propose that ROS and oxidant stress might regulate the transcription of HB-EGF [15-17].

In the rat gastric epithelial cell line (RGM1 cells), oxidative and osmotic stress induce tyrosine phosphorylation of EGFR in the first minutes, followed by a marked increase in HB-EGF and amphiregulin transcripts [18], which suggests that EGFR might play a crucial role in the stress-induced expression of EGF-like growth factors in gastrointestinal cells. In addition, it is known that TGF-α is able to induce its own mRNA via a signal due to activation of the EGFR, acting predominantly at the post-transcriptional level [19].

According to this, the aim of this work has been to analyze the molecular mechanisms that mediate up-regulation of the EGFR ligands HB-EGF and TGF-α by TGF-β in foetal rat hepatocytes. The possible involvement of ROS, induced by a NADPH-oxidase system, and/or the existence of an amplification loop triggered by the initial activation of the EGFR have been studied. Results indicate that TGF-β receptor I (TβR-I/Alk5)-dependent activation of NADPH-oxidase mediates up-regulation of EGFR ligands by a mechanism that does not require EGFR activity, but depends on a functional NF-κB pathway.

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EXPERIMENTAL

Materials

Human Recombinant TGF-β1, AG1478, apocynin, PD169316, PD98059, SN-50 were from Calbiochem (La Jolla, CA, USA). Culture media was from Invitrogen-Gibco (Carlsband, CA, USA). Foetal bovine serum was from Sera Laboratories International (Cinder Hill, UK). EGF was a gift of Serono Lab (Madrid, Spain). Monoclonal anti-β-Actin antibody, diphenyleneiodonium chloride (DPI), glutathione (ethyl ester form), superoxide dismutase, catalase, rapamycin and SB431542 were from Sigma (Madrid, Spain). LY294002 and SP600125 were from Alexis Biochemicals (Lausen, Switzerland). The fluorescent probes 2´,7´-dichlorodihydrofluorescein diacetate (H_2DCFH-DA) and hydroethidine (DHE) were from Molecular Probes (Eugene, OR, USA). Anti-Akt (CS-9272), anti-phospho Akt (Ser473) (CS-9271), and anti-phospho Smad-2 (Ser465/467) (CS-3101) antibodies were from Cell Signalling Technology (Beverly, MA, USA). NF-κB-p65 antibodies, used in Western blot (SC-109) or EMSA experiments (SC-109X) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Rac1 (Upstate 05-389) was from Upstate (Lake Placid, NY, USA). The secondary antibody Alexa Fluor 488-conjugated anti-rabbit immunoglobulin (A11001) was from Molecular Probes (Eugene, OR, USA).

Cell isolation and culture

Foetal hepatocytes were obtained by collagenase disruption of 20-day-old foetal Wistar rats and cultured in non-coated plastic dishes with arginine-free, ornithine-supplemented, M-199 medium as previously described [4]. After attachment and serum starvation for 12-24 hours, factors were added. Inhibitors were added 30 min before TGF-β.

Western blot analysis

Total protein extracts were obtained as described previously [5], separated by SDS electrophoresis in 12% polyacrylamide gels and transferred to PVDF membranes. After blocking with a 5% non-fat milk TTBS solution the membranes were incubated overnight with the corresponding antibody in a 0.5% non-fat milk TTBS solution (1:5000 for β-actin, and 1:1000 for others). After washing and incubating the membrane

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with an appropriate peroxidase-conjugated antibody (1:5000) for 1 hour at room temperature, antibody binding was revealed using ECL (GE-Healthcare). β-actin is used as loading control.

Reverse transcriptase-polymerase chain reaction analysis (RT-PCR)

Total RNA was obtained using the RNeasy Kit (Quiagen). Complementary DNA was generated by the SuperScript First-Strand Synthesis System for RT-PCR using oligo(dT) as primer.

Rat specific primers sequences for PCR reactions were used:

snail: 5′-GCAGCTGGCCAGGCTCTCGGTGGC-3′ (forward),
5′-GTAGCTGGGTCAGCGAGGGCCTCC-3′ (reverse).

tgf-α: 5′-TGGTGCAGGAAGAGAAGC-3′ (forward),
5′-TGACAGCAGTGGATCAGC-3′ (reverse).

hb-egf: 5′-CGGTGGTGCTGAAGCTCTTTC -3′ (forward),
5′-TGGTAACCAGGGAGGCAGTG-3′ (reverse).

nox 1: 5´-GGATCACAACCTCACCTTCC-3´ (forward), 5´-GCTGCATACATCACTGTCACG-3´ (reverse);

nox 2: 5´-TCAAGTGTCCCCAGGTATCC-3´ (forward), 5´-CTTCACTGGCTGTACCAAAGG-3´ (reverse);

nox 4: 5´-TTACTACTGCCTCCATCAAGC-3´ (forward),
5´-GGAATGATTGGATGTCTCTGC-3´ (reverse)

rac1: - 5´-TGC GTT CCC TGG AGA GTA CAT CC-3´ (forward),
5´-TTG AGT CCT CGC TGT GTG AGT GC-3´ (reverse)

albumin: 5´-CTGCCGATCTGCCCTCAATAGC (forward),
5´-GTGCCCACTCTTCCCAGGTTTCT-3´ (reverse)

Products were obtained after 30-35 cycles of amplification and 60-65ºC of annealing temperature and were electrophoresed in 0.8-1.2 % agarose gels. Expression of *albumin* was analyzed as loading control. The –RT channel, lacking any kind of RNA, is shown as a specificity control.

NADPH-oxidase Activity assay

Cells were detached by trypsinization, pelleted by centrifugation at 2500*g* for 5 minutes at 4ºC and resuspended in PBS. NADPH-oxidase was analyzed as described previously [10]. Briefly, cells were incubated with 250μM NADPH. NADPH consumption was

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monitored by the decrease in absorbance at λ=340 nm for 5 min. Specific oxidase activity was measured by monitoring the rate of consumption of NADPH inhibited by adding 10μM diphenyleneiodonium (DPI) 30 min before the assays. An aliquot of cells was lysed with SDS and used to determine the protein concentration. Results are expressed as picomol of oxidized NADPH per minute per milligram of protein.

Measurement of intracellular reactive oxygen species

The oxidation-sensitive fluorescent probes 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and hydroethidine (DHE) were used to analyze the intracellular content of peroxides and superoxide anions, respectively. Cells were detached by trypsinization, resuspended in PBS and incubated during 30 min with 5 μM H_2DCFDA or 20 μM DHE. Fluorescence intensity was measured by using a FACScan flow cytometer (Becton.Dickinson, San Jose, CA, USA). For each analysis, 10,000 events were recorded.

Electromobility Shift Assay (EMSA)

Specific probes for DNA-protein interaction analysis were used:

NF-κB Binding Element, corresponding to the NF-κB motif in the iNOS promoter: 5´-tcgaCCAACTGGGACTCTCCCTTTGGGAACA-3´ (forward), 5´-tcgaTGTTCCCAAAGGGAGAGTCCCAGTTGG-3´ (reverse)

Smad Binding Element, corresponding to the consensus sequence: 5´-agctGGAGTATGTCTAGACTGACAAGTTAC-3´ (forward), 5´-agctGTACATTGTCAGTCTAGACATACTCC-3´ (reverse)

AP-1 Binding Element, corresponding to the collagenase TPA responsive element: 5´-agctTGATGAGTCAGCCG-3´ (forward) 5´-gatcCGGCTGACTCATCA-3´ (reverse).

After annealing each forward and reverse oligonucleotides, 0.5 μg were radiolabelled using the Klenow Enzyme (Roche) and [$^{\alpha 32}$P] dCTP (GE-Healthcare). Radiolabelled oligonucleotides were purified using MicroSpin SC-200 HR columns (GE-Healthcare) and used as probes. Radiolabelling was measured by liquid scintillation counting. Nuclear protein extracts were obtained as described previously [4]. For specificity controls, 10 μg of protein extract were incubated for 15 min at 4ºC with anti-p65 antibody (2 μg) or with unlabeled oligo (100 ng). After this, 10 μg of all nuclear protein extracts (specificity controls included) were incubated with 150,000 counts per million

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of the appropriate probe and 0.5 μg/μl of Polydeoxyinosinic Deoxycytidylic Acid in a 40 mM Hepes pH=8, 0.2 mM EDTA, 0.1 M ClNa, 80 mM KCl, 10 mM Cl_2Mg, 10% glycerol, 2 mM DTT binding solution for 30 minutes at 4ºC. DNA-protein complexes were separated in 6% non-denaturing polyacrylamide gels in 0.5× TBE (Tris–borate–EDTA) at room temperature. Gels were dried and complexes were visualized by autoradiography.

Immunocytochemistry

Cells were cultured in non-coated, glass coverslips. After washing with PBS, cells were fixed with 4% paraformaldehyde in PBS for 12 min at room temperature, permeabilized with a 0.1% triton X-100, 0.1% BSA, PBS solution for 3 min, blocked with 1% BSA and 10% goat serum in PBS for 1 hour and incubated with the anti-p65 antibody (1:400) in 1% BSA, PBS solution, overnight at 4ºC. After washing, cells were incubated with Alexa Fluor 488-conjugated anti-rabbit (1:200) in a 1% BSA, PBS solution, for 1 hour at room temperature. Coverslips were embedded in Vectashield (Vector Laboratories) with DAPI. Cells were visualized in an Olympus BX-60 microscope with the appropriate filters. Representative images were taken with a Spot 4.3 digital camera and software and edited in Adobe Photoshop.

RESULTS

Activation of the TGF-β receptor I and a NADPH oxidase system is required for the increase in TGF-α and HB-EGF transcript levels induced by TGF-β in foetal rat hepatocytes.

EGFR activation is crucial for the survival of foetal hepatocytes against the pro-apoptotic signals induced by TGF-β because it mediates the activation of the PI-3K/Akt signalling pathway in response to TGF-β treatment [4]. As we have previously shown, inhibition of the EGFR activity with the specific inhibitor AG1478 completely blocked the activation of the PI-3K/Akt survival pathway (Fig. 1A), leading to a massive apoptosis of foetal hepatocytes [5]. Analysis of the mRNA levels of the EGFR ligands, TGF-α and HB-EGF, by RT-PCR after TGF-β treatment revealed that this cytokine was

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promoting an increase in the expression of these genes (Fig. 1B), which might account for activation of the EGFR pathway. We first wanted to know if this event was being triggered by the canonical TGF-β signalling pathway through the TGF-β Receptor I (TβR-I, ALK 5), so we used a specific TβR-I inhibitor (SB431542). This compound completely blocked the up-regulation of both TGF-α and HB-EGF. Next, we analyzed the possible implication of ROS production in the up-regulation of EGFR ligands. For this, we cultured the cells in the presence of a flavoprotein inhibitor: diphenyleneiodonium (DPI), which completely blocked TGF-β-induced ROS production in previous studies of our group [10]. As shown in Fig. 1C, DPI blocked the up-regulation of both TGF-α and HB-EGF. In contrast, the expression of a well-known target of TGF-β, Snail, was blocked by the TβRI inhibitor, but not by DPI, indicating that its effect was not unspecific. At the protein level, DPI also blocked the activation of Akt without affecting Smad-2 phosphorylation, demonstrating again that DPI is not impairing classical TGF-β signalling. TβR-I inhibitor completely abolished both Akt activation and Smad-2 phosphorylation (Fig. 1D). These data suggest that both up-regulation of EGFR ligands and activation of the PI-3K/Akt pathway need the activation of the TβR-I and ROS production.

A detailed timing analysis showed that TGF-β induces an early activation of a NADPH-oxidase system in foetal rat hepatocytes (Fig. 2A), coincident with an increase in the intracellular content of superoxide anion (analyzed with DHE, by flow cytometry) and peroxide (analyzed with H_2DCFDA, by flow cytometry) (Fig. 2B). Adding superoxide dismutase (SOD) to the culture medium completely blocked the increase in peroxides (Fig. 2C), indicating that superoxide anion is the first ROS specie produced, as expected if a NADPH oxidase system is involved. Different members of the NADPH oxidase family of genes are expressed in these cells, included *nox1*, *nox2* and *nox4* (Fig. 3A), in agreement with previous results obtained in adult hepatocytes [20]. TGF-β1 up-regulated *nox4* and increased the protein levels of Rac1 (Fig. 3B), a known regulator of Nox2 [21] and some other members of the family. Increase in Rac1 protein levels does not correlate with an increase in its transcript levels (Fig. 3C), which would indicate a posttranscriptional regulation. Both, upregulation of *nox4* and increase in Rac1 protein levels, were blocked in the presence of the TβR-I inhibitor (Fig 3D). Since DPI is not a specific NADPH-oxidase inhibitor, because it inhibits flavoproteins, we next tested a more selective NADPH-oxidase inhibitor, apocynin. Apocynin completely blocked the

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early NADPH-oxidase activation induced by TGF-β (Fig. 4A) and was able to abolish the up-regulation in the expression of TGF-α and HB-EGF genes (Fig. 4B), thus reaffirming the role of NADPH-oxidases in the control of EGFR ligands expression. A complete inhibition in TGF-β-mediated Akt phosphorylation was observed when fetal hepatocytes were cultured in the presence of apocynin (Fig. 4C). Furthermore, adding catalase or superoxide dismutase (SOD) to the culture medium also impaired TGF-β-mediated Akt activation. The addition of a permeable form of glutathione blocked this activation, too, reinforcing the essential role of ROS-mediated signalling in the TGF-β-regulated survival signals in fetal rat hepatocytes.

The NF-κB pathway, downstream ROS, is required for TGF-α and HB-EGF up-regulation.

Next, we wondered whether the increased EGFR ligands expression was being triggered by redox-sensitive signalling pathways. Semiquantitative RT-PCR assays with RNA from cells treated with specific inhibitors of JNK (SP600125) and p38MAPK (PD169316) showed that these proteins were not involved (Figs. 5A and 5B). However, a NF-κB specific inhibitor (SN-50) that selectively impairs the translocation of NF-κB active complexes into the nucleus, was able to almost completely block the increased expression of TGF-α and HB-EGF (Fig. 5C), thus indicating the possible involvement of this pathway. TGF-β activated NF-κB in a quick fashion, as can be seen in the EMSA time course analysis shown in figure 6A. This effect was blocked by the TβR-I inhibitor, implicating the classical TGF-β signalling pathway in the processes studied. Nuclear translocation of the p65 subunit after TGF-β treatment is shown in an immunocytochemistry assay (Fig. 6B). While in the control cells p65 is located in the cytoplasm, in the TGF-β-treated cells p65 staining completely co-localize with the nuclei staining. As ROS are well-known activators of NF-κB, we analyzed the possibility that ROS were doing so in our system. For that purpose we used the flavoprotein inhibitor DPI observing that it was able to completely impair the TGF-β-mediated NF-κB activation (Fig 6C). To further assess the lack of interaction between DPI and TGF-β signalling pathway, transcriptional binding of Smads to their binding DNA element (Smad Binding Element, SBE) was also analyzed. DPI did not affect the TGF-β-stimulated DNA binding activity of Smads (Fig. 6C) nor the translocation of

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Smad2 and Smad3 to the nucleus, analyzed by Western blot (results not shown). AP-1 is shown as a transcription factor neither modulated by TGF-β nor by ROS at short times. These results demonstrate that NF-κB activation mediates the increased expression of TGF-α and HB-EGF induced by TGF-β, and that ROS are responsible for this activation.

Activation of the EGFR pathway, phosphatidylinositol-3 kinase or extracellular regulated kinases (ERKs) are not required for up-regulation of TGF-α or HB-EGF by TGF-β in foetal rat hepatocytes.

Results from our and other groups had established that EGF can impair TGF-β-induced apoptosis in some different ways, including activation of the PI-3K pathway. Moreover, EGF is also capable of increasing the expression of EGFR ligands (Fig. 7A). For these reasons, we wondered whether EGFR could be collaborating with ROS in activating NF-κB to modulate the expression of its own ligands. Contrary to our expectations, the EGFR seemed not to be involved. Using a selective EGFR inhibitor, AG1478, we could not observe neither a blocking effect on NF-κB activity, analyzed by EMSA (Fig. 7B), nor on the expression levels of TGF-α and HB-EGF (Fig. 7C), indicating that EGFR does not trigger the early up-regulation of its own ligands. As expected, EGFR does not affect TGF-β signalling through Smads proteins, as the specific EGFR inhibitor does not affect the Smads transcriptional binding to their Smad Binding Element (SBE) (Fig. 7B). To better sustain this result, we analyzed the implication of possible effectors downstream EGFR activation, such as phosphatidylinositol-3 kinase (PI-3K), Extracellular Regulated Kinases (ERKs) or mTOR. Using specific inhibitors for these proteins (LY294002 for PI-3K, PD 98059 for ERKs and rapamycin for mTOR) we analyzed the expression levels of EGFR ligands, finding that neither of these proteins seemed to be involved (Fig. 7D). All these data support the idea that EGFR is not necessary for TGF-β-mediated up-regulation of TGF-α and HB-EGF, and that other signalling pathways linked to EGFR activation, such as PI-3K, ERKs or mTOR are not required either.

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DISCUSSION

TGF-β is a potent regulator of epithelial cell proliferation, through inhibiting growth and inducing apoptosis [1]. Indeed, loss of TGF-β-mediated growth restraint has been shown to be associated with an increased risk of transformation [1]. However, there is also evidence that high production and/or activation of TGF-β in tumours can enhance cancer progression by autocrine and/or paracrine mechanisms [2]. It is worthy to note that TGF-β was originally isolated as an agent able to induce anchorage-independent growth of mouse fibroblasts [22]. Results presented in this paper indicate that, in addition to its role in inducing apoptosis in hepatocytes [3], TGF-β controls the expression of EGFR ligands, such as TGF-α or HB-EGF, which contributes to transactivate the EGFR pathway that regulates anti-apoptotic signals, such as activation of the Akt pathway (Fig. 1 A and B). Thus, TGF-β controls both pro- and anti-apoptotic signals, as previously suggested [4], in foetal rat hepatocytes. The balance among these signals control cell fate and a great percentage of cells (around 70%) survive (ref. 4 and results not shown).

Although previous reports have proposed that TGF-β might regulate the expression of EGFR ligands [6-9] nothing was known about the intracellular signal/s initiated by TGF-β that triggers this effect. Here we propose that activation of NADPH-oxidase, TβR-I-dependent, is necessary for this effect. Different results support this hypothesis: 1) up-regulation of TGF-α and HB-EGF (TβR-I-dependent) is inhibited by DPI (Fig. 1) and apocynin (Fig. 4), two known inhibitors of the NADPH-oxidase systems; 2) TGF-β early activates a NADPH-oxidase pathway, which is coincident with up-regulation of the gene *nox4*, member of the NADPH-oxidase family, and increase in Rac1 protein levels, a well-known activator of Nox1 and Nox2 (21) (Figs. 2, 3); 3) activation of the TβR-I is required for both the increase in *nox4* mRNA transcripts and the enhancement of Rac1 protein levels (Fig. 3).

Different intracellular signals are controlled by oxidative stress, with later effects in gene transcription. Among them, p38MAPK has been proposed to be activated by ROS in TGF-β-treated foetal rat hepatocytes [23]. p38MAPK is particularly interesting, because a previous report has indicated that activation of p38MAPK might regulate the expression of an autocrine survival factor by TGF-β in mesenchymal cells [24]. JNK is

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another signal under redox regulation which might contribute to regulate gene transcription through the AP-1 transcription factor [25]. However, results presented in Fig. 5 exclude the involvement of p38MAPK or JNK in the upregulation of EGFR ligands by TGF-β in hepatocytes. Furthermore, AP-1 DNA binding activity is not modulated by TGF-β at short times (Figs. 6 and 7).

NF-κB has been known to be redox regulated for some time and is a direct target for oxidation that can affect its ability to bind to DNA [26]. Many kinases involved in direct or indirect activation of NF-κB are affected by oxidants and therefore, have the potential to alter NF-κB [27, 28]. Furthermore, recent results have evidenced that NF-κB might be activated by NADPH oxidases in different cell types [29-31]. NF-κB plays important roles during liver neoplastic development through transcriptional regulation of pro-survival genes, such as Bcl-x_L or XIAP [32]. Interestingly, the NF-κB / Bcl-X_L / XIAP axis potently counteracts the TGF-β-induced apoptosis [33] and exert a general cytoprotective role on preneoplastic hepatocytes [34]. Here we propose that a NADPH oxidase-dependent activation of NF-κB might mediate regulation of the expression of EGFR ligands by TGF-β, which might act increasing proliferation and preventing apoptosis of liver cells. Results that support this hypothesis are: 1) SN-50, a known inhibitor of the nuclear translocation of NF-κB, blocks up-regulation of TGF-α and HB-EGF (Fig. 5C); 2) TGF-β induces an early activation of the NF-κB DNA binding, which is dependent on TβR-I activation, and correlates with nuclear translocation of the RelA /p65 subunit (Figs. 6A, B); 3) DPI, the NADPH-oxidase inhibitor, impairs NF-κB activation (Fig. 6C). Previous reports have proposed a crucial role for NF-κB in the control of EGFR ligands transcription. Thus, NF-κB activity is required for platelet-activating factor (PAF) mediated up-regulation of HB-EGF in monocytes [35]. NF-κB is also involved in HB-EGF expression in non-parenchymal liver cells after partial hepatectomy [36]. Interestingly, the hepatitis C virus core protein promotes proliferation of human hepatoma cells through up-regulation of TGF-α transcription via activation of NF-κB [37]. Thus, NF-κB might control EGFR ligands transcription, either directly, or indirectly, by acting on NF-κB target genes whose products are required for TGF-α/HB-EGF transcriptional induction. It has been recently suggested that Smad4 plays a role in the transcriptional activation of NF-κB by TGF-β in colon cancer cells [38]. As shown in Figs. 1, 4, 6 and 7, NADPH oxidase inhibitors do not affect either Smad2

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phosphorylation or Smad DNA-binding activity. Smads might contribute to induce expression of *nox4*, and/or activate NF-κB. Further work is necessary to completely understand whether both ROS elevation and Smad involvement are critical for the transcriptional induction of the EGFR ligands by TGF-β in hepatocytes.

It is worthy to note that, although different data in the literature point out the existence of an autocrine loop of EGFR ligands that is up-regulated as soon as the EGFR is activated [18,19], this pathway does not appear to be involved in the early up-regulation of TGF-α or HB-EGF addressed by TGF-β in hepatocytes. Thus, in spite of the strong effect of extracellular EGF on the expression of TGF-α and HB-EGF (Fig. 7A), the EGFR inhibitor AG1478 does not impair neither the activation of NF-κB by TGF-β, nor the upregulation of the EGFR ligands (Fig. 7). Of course, we cannot exclude the possibility that the EGFR pathway might contribute to the amplification or maintenance of EGFR ligands expression at later times.

In summary, results presented in this manuscript indicate that activation of NADPH-oxidase by TGF-β, which correlates with up-regulation of *nox4* and increase in Rac1 protein levels, mediates anti-apoptotic signals in foetal rat hepatocytes through up-regulation of EGFR ligands and transactivation of the EGFR pathway. Since previous works have clearly established the role of ROS in the apoptosis induced by TGF-β in different cell types, included hepatocytes [39], results presented here indicate that ROS might mediate both pro- and anti-apoptotic signals in TGF-β-treated hepatocytes. Our results also support the important role played by NF-κB, downstream ROS, to control EGFR ligands up-regulation. It is worthy to note that IKK-β-deficient mice die at mid-gestation from uncontrolled liver apoptosis, a phenotype that is remarkably similar to that of mice deficient in both the RelA (p65) and NF-κB1 (p50/p105) subunits of NF-κB [40]. Mice lacking IKK-β specifically in both hepatocytes and hematopoietic-derived Kupffer cells exhibit reduced hepatocyte regeneration and diminished induction of hepatomitogens [41].Taking these results together, it is possible to hypothesize that NF-κB might play an essential role in regulating EGFR ligands under different physiological and pathological situations of the liver.

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Figure 1

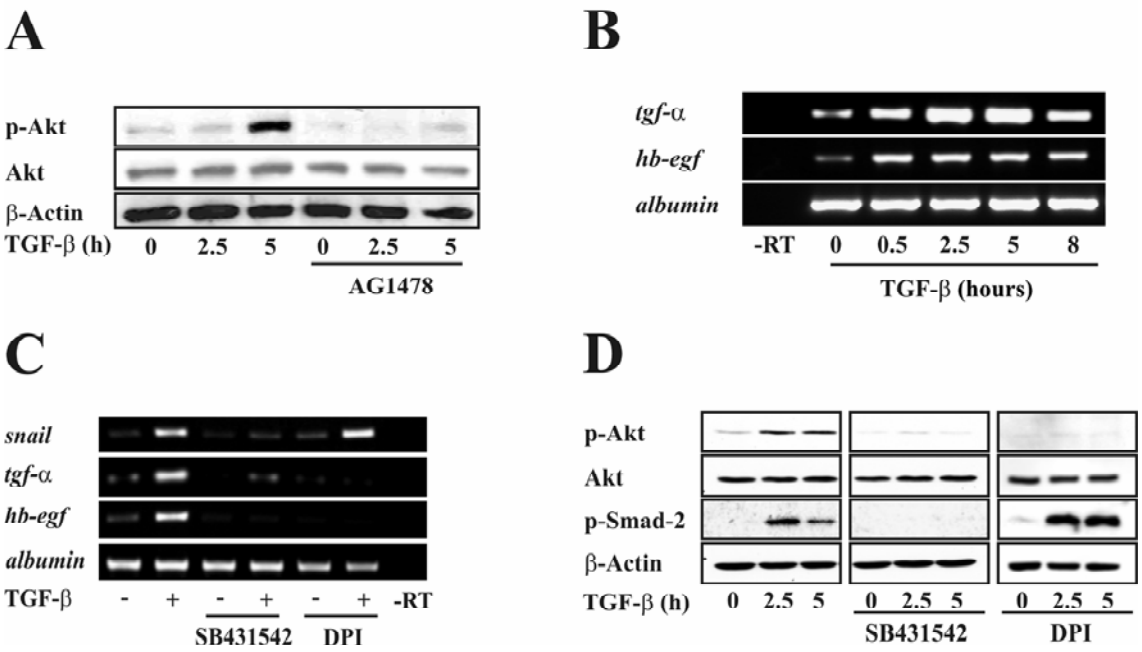

Figure 1. ROS production and TβR-I are required for up-regulation of EGFR ligands by TGF-β.

Hepatocytes were incubated in the presence or absence of TGF-β (1 ng/ml), the EGFR inhibitor AG1478 (10 μM), the TβR-I inhibitor SB431542 (10 μM) and/or the ROS scavenger, flavoprotein inhibitor, diphenyleneiodonium, DPI (20 μM), at different times, as indicated (3 h in C). Protein extracts were obtained to study the activation state of Akt by Western blot (A and D). Smad-2 activation was also studied in D. Total RNA was obtained to study the expression of *tgf-α* and *hb-egf* (B and C) by RT-PCR. Expression levels of *snail* were also analyzed in C. In all cases, a representative experiment of at least four is shown.

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Figure 2

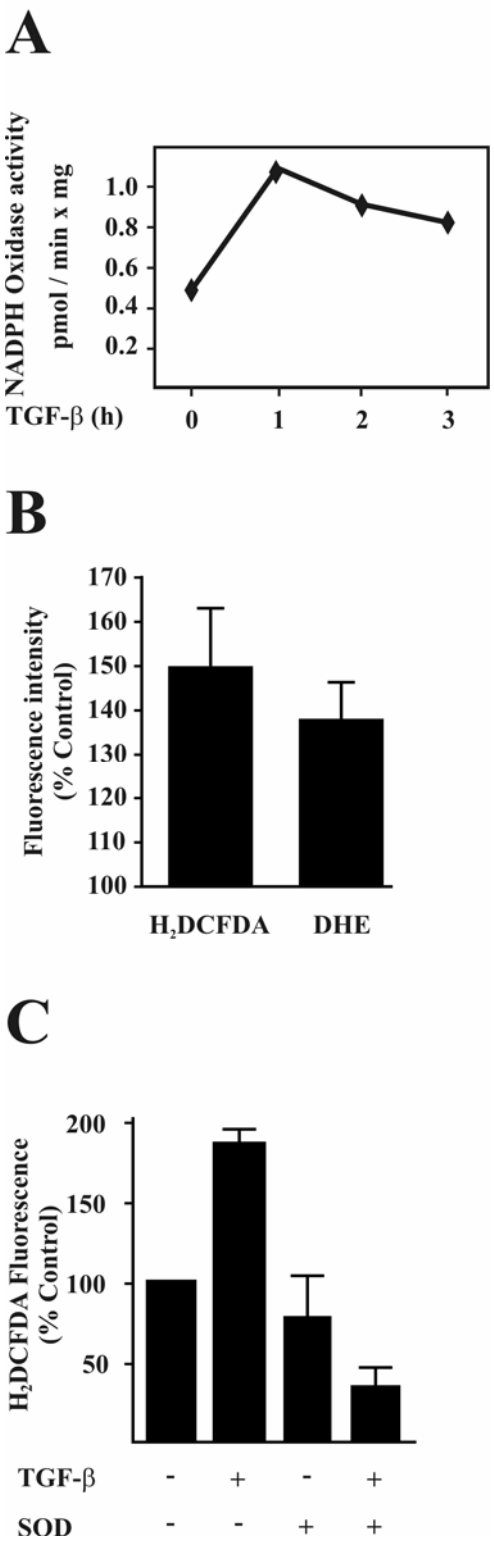

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Figure 2. TGF-β activates a NADPH-oxidase system in hepatocytes, which produces an increase in the intracellular content of superoxide anion and peroxide. A. Cells were incubated with TGF-β (1 ng/ml) at different times. NADPH-oxidase activity assay was performed as described in the Materials and Methods section. A representative experiment of three is shown. B. Intracellular content of reactive oxygen species was analyzed, as described in the Material and Methods section, in hepatocytes treated with TGF-β (1 ng/ml) during 3 h. Results are expressed as percentage of control, untreated, cells and are the mean±S.E.M. of 5 different experiments. C. Intracellular content of peroxides were analyzed in cells incubated for 3 h in the absence or presence of TGF-β (1 ng/ml) and/or 300 units/ml of superoxide dismutase. Results are expressed as percentage of control, untreated, cells and are the mean±S.E.M. of 3 different experiments.

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Figure 3

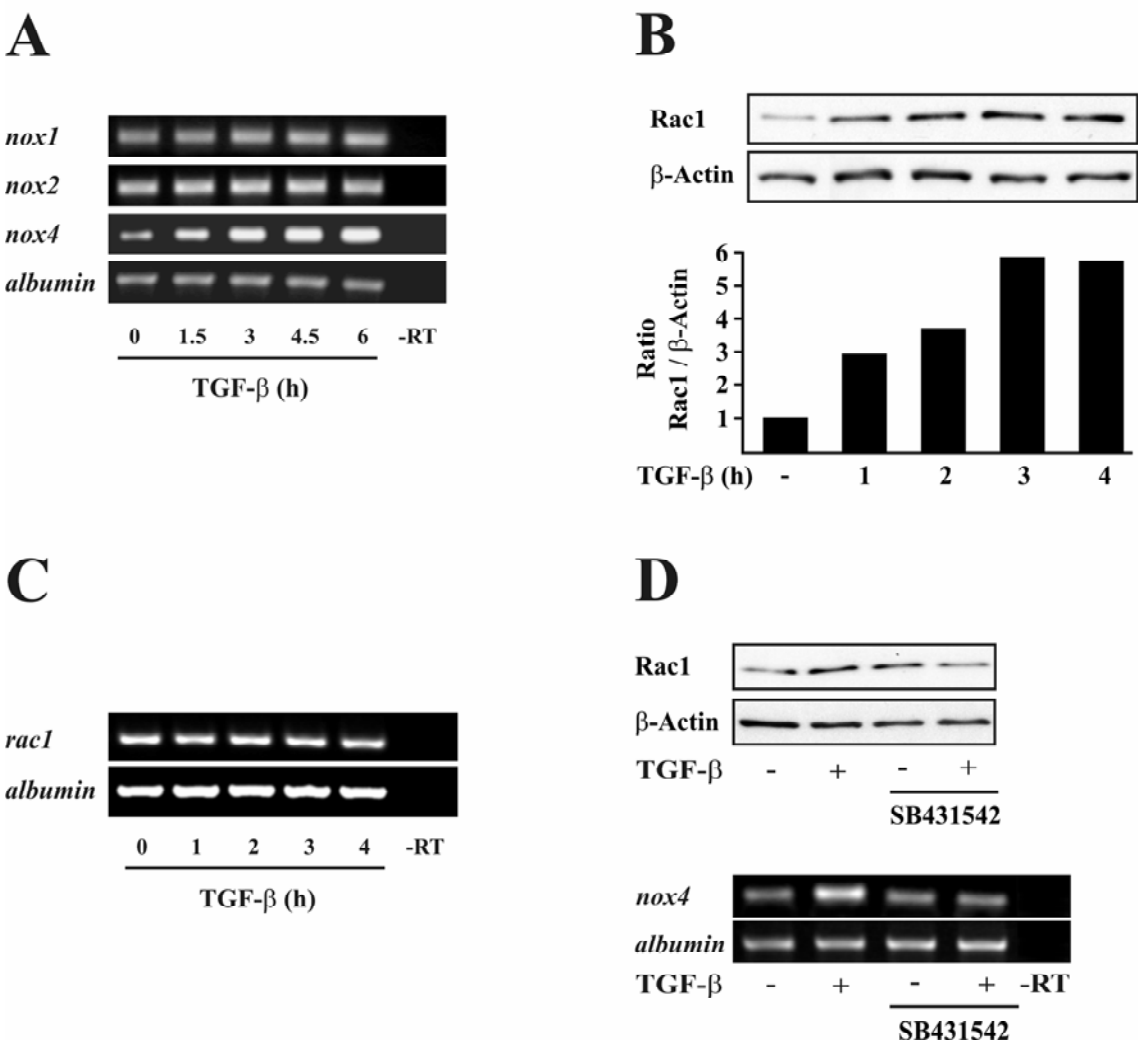

Figure 3. TGF-β upregulates *nox4* mRNA transcripts and Rac1 protein levels. After treatment of hepatocytes with TGF-β (1 ng/ml) at different times (as indicated): A. RT-PCR analysis was carried out to study expression levels of *nox1*, *nox2* and *nox4*; B. Rac1 protein levels were analyzed by Western blot; and C. RT-PCR was performed to analyze *rac1* transcript levels. D. Effect of the TβR-I inhibitor SB431542 (10 μM) on TGF-β-mediated increase in *nox4* transcripts and Rac1 protein levels (3 h of TGF-β, 1 ng/ml treatment). In all the cases, a representative experiment of at least three is shown.

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Figure 4

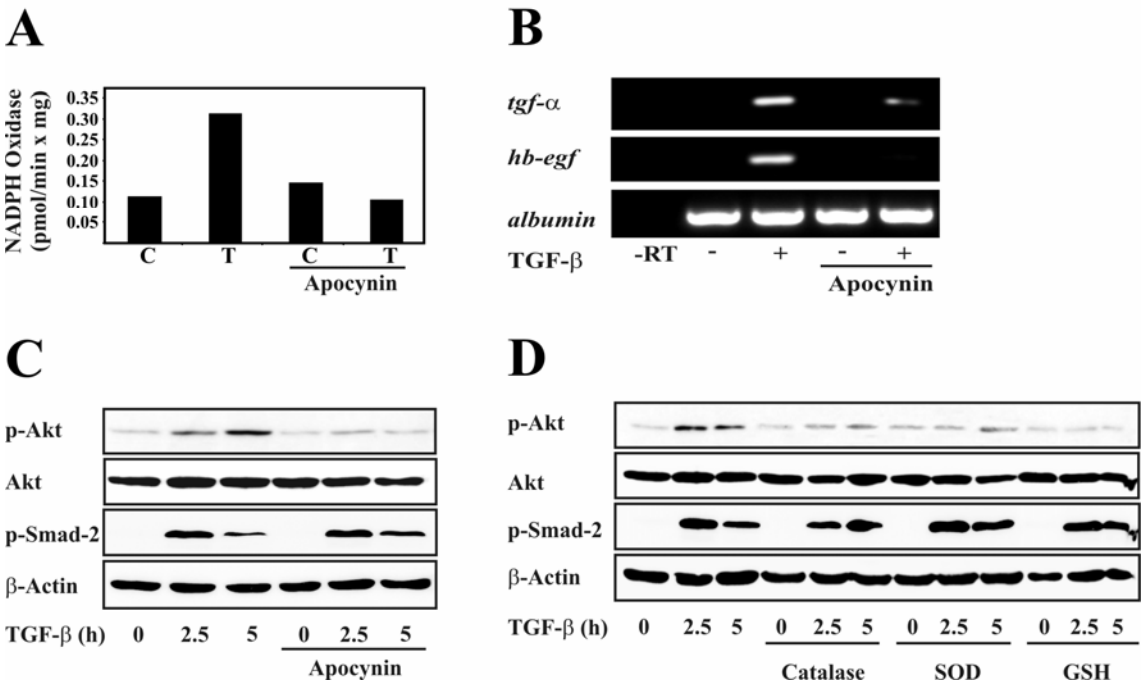

Figure 4. Apocynin, a selective NADPH-oxidase inhibitor, impairs TGF-β-induced *tgf-α* and *hb-egf* up-regulation. Apocynin and antioxidants block Akt activation. A. The effect of apocynin (300 μM) over NADPH-oxidase activity was analyzed in the absence (C) or presence (T) of TGF-β (1 ng/ml) after 3 hours of treatment. B. Expression levels of *tgf-α* and *hb-egf* were analyzed by RT-PCR after cell treatment with or without TGF-β (1 ng/ml; 3 h) in the presence or absence of apocynin (300 μM). C. Effect of apocynin on Akt activation (Western blot analysis) in cells treated with TGF-β (1 ng/ml) and/or apocynin (300 μM) during 3 h. D. Effect of the antioxidants Catalase (300 units/ml), SOD (300 units/ml) and GSH (0.5 mM, Glutathion-ethyl-ester, a cell-permeable form of the reduced glutathione) on TGF-β-induced Akt activation (1 ng/ml TGF-β, 3h; Western blot analysis). A representative experiment of at least three is shown in all cases.

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Figure 5

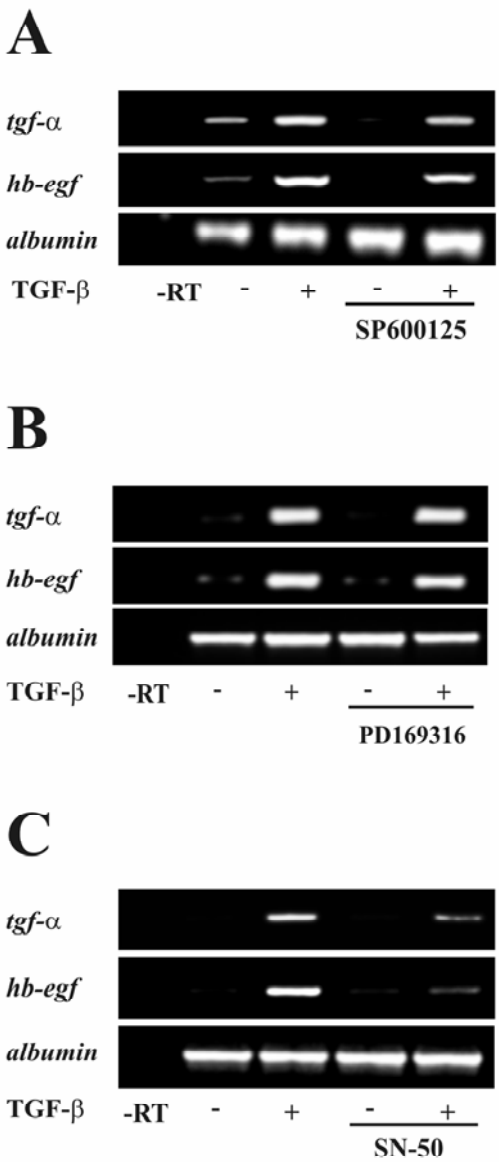

Figure 5. NF-κB activation is necessary for TGF-β-induced up-regulation of EGFR ligands.

Expression levels of *tgf-α* and *hb-egf* were analyzed by RT-PCR in cells cultured in the presence or absence of TGF-β (1 ng/ml; 3h), with or without the JNK inhibitor SP600125 (40 μM) (A), the p38MAPK inhibitor PD169316 (800 nM) (B) or the inhibitor of the nuclear translocation of NF-κB, SN-50 (50 μM) (C). In all cases, a representative experiment of at least three is shown.

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Figure 6

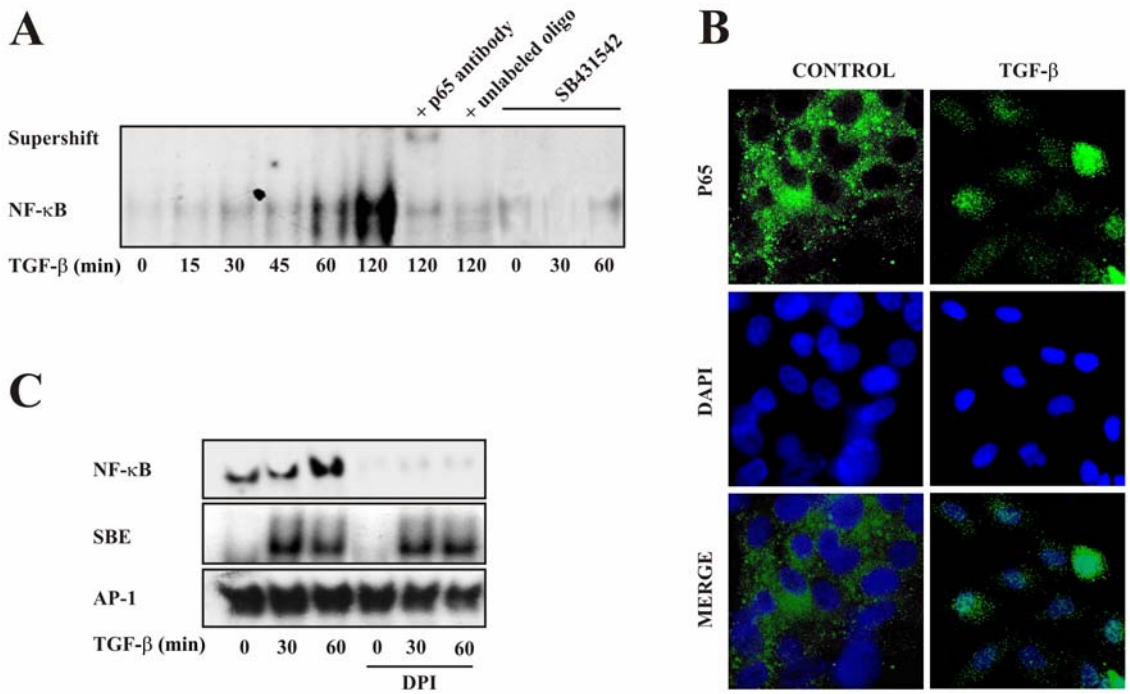

Figure 6. ROS mediate TGF-β-induced NF-kB activation.

A. Following cell incubation with TGF-β (1 ng/ml) at different times in the presence or the absence of the TβR-I inhibitor SB431542 (10 μM), nuclear protein extracts were obtained. Binding activity of NF-κB was analysed by an Electromobility Shift Assay as described in the Material and Methods section. Specificity controls were carried out by incubating nuclear extracts with an anti-p65 antibody and with unlabeled oligo. A representative experiment of three is shown. B. Cellular localization of the p65 subunit of NF-κB before (control) and after treatment of hepatocytes with TGF-β (1 ng/ml) was analysed by fluorescent microscopy immunocytochemistry (B), using DAPI for nuclear staining. A representative experiment of five is shown. C. The effect of DPI on the binding activity of NF-κB, Smad Binding element (SBE) and AP-1 was carried out by a Electromobility Shift Assay. For this, cells were incubated with TGF-β (1 ng/ml) in the presence or the absence of DPI (20 μM) and nuclear protein extracts were collected. A representative experiment of two is shown.

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Figure 7

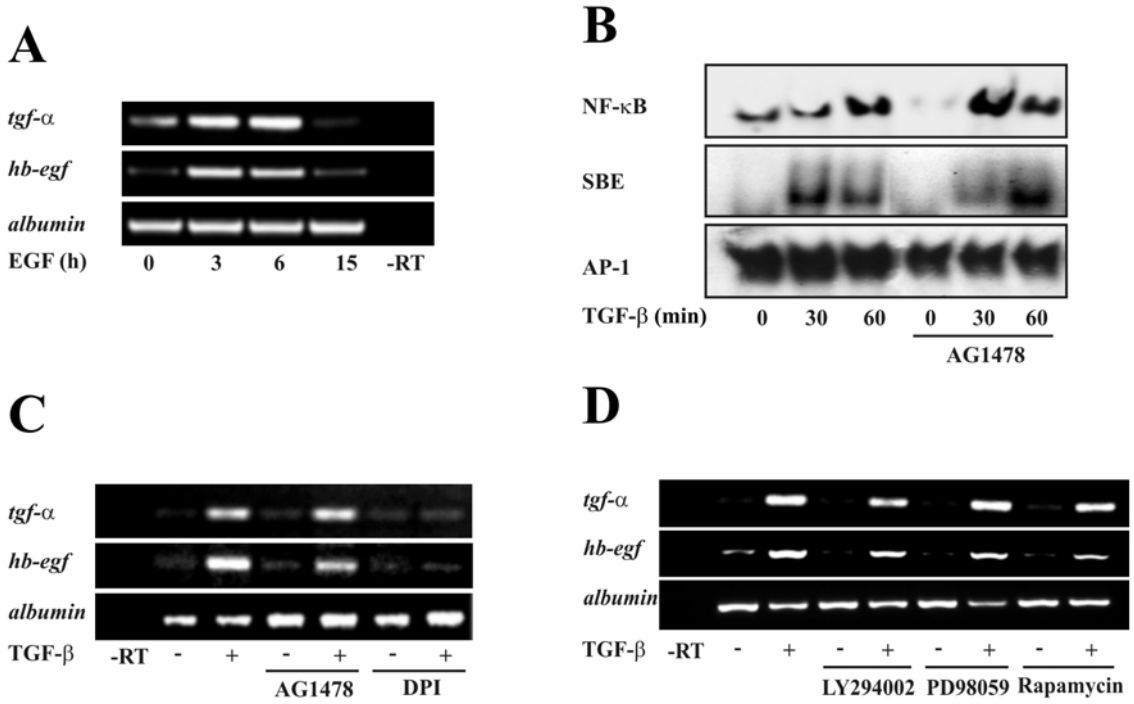

Figure 7. EGFR is not required for TGF-β-induced up-regulation of its own ligands.

A. Time course analysis of the effect of EGF (20 ng/ml) on EGFR ligands expression by RT-PCR. B. After treatment of cells with TGF-β (1 ng/ml) at different times in the presence or absence of AG1478 (10 μM), nuclear extracts were collected and the DNA binding activity of NF-κB, Smad Binding Element (SBE) and AP-1 was analyzed by EMSA. A representative experiment of two is shown. C and D. The effect of the EGFR inhibitor AG1478 (10 ng/ml), DPI (20 μM), the PI-3K inhibitor LY294002 (10 μM), the ERKs inhibitor PD98059 (50 μM) and the mTOR inhibitor rapamycin (10 μM) on the expression of EGFR ligands in cells cultured in the presence or absence of TGF-β (1 ng/ml, 3h) was analyzed by RT-PCR. A representative experiment of at least three is shown.

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