Nerve growth factor exposure promotes tubular epithelial–mesenchymal transition via TGF-β1 signaling activation

Donatella Vizza*, Anna Perri*, Giuseppina Toteda, Simona Lupinacci, Francesca Leone, Paolo Gigliotti, Danilo Lofaro, Antonella La Russa, and Renzo Bonofiglio

Department of Nephrology Dialysis and Transplantation, Research Center Kidney and Transplantation, Annunziata Hospital, Cosenza, Italy

Abstract

Clinical studies showed that renal expression and serum levels of nerve growth factor (NGF) are increased in renal diseases characterized by progressive fibrosis, a pathologic process in which TGF-β1 mediates most of the key events leading to tubular epithelial–mesenchymal transition (EMT). However, the pathogenic role of high NGF levels has not yet been elucidated. In this study, we found that in tubular renal cells, HK-2, NGF transcriptionally up-regulated TGF-β1 expression and secretion and enhanced cell motility by activating EMT markers via its receptors, TrkA and p75NTR. Interestingly, we observed that TGF-β1–SMAD pathway activation and the up-regulation of EMT markers NGF-induced were both prevented when knockdown of TGF-β1 gene occurred and that the pretreatment with an antibody anti-NGF reversed the nuclear translocation of p53MAD3/SMAD4 complex. Collectively, our results demonstrated that NGF promotes renal fibrosis via TGF-β1-signaling activation, suggesting that in kidney diseases high NGF serum levels could contribute to worsen renal fibrosis.

Introduction

Renal fibrosis, characterized by glomerulosclerosis and tubule-interstitial fibrosis, is the final common manifestation of a wide variety of chronic kidney diseases leading to the complete destruction of kidney parenchyma and end-stage renal failure, requiring dialysis or kidney transplant (Eddy, 2005). Several cellular pathways, including mesangial and fibroblast activation, as well as tubular epithelial–mesenchymal transition (EMT), have been identified as the major approaches for the generation of the matrix-producing cells in diseased conditions. However, the molecular pathway leading to fibrogenic cell activation are not yet completely elucidated (Cho, 2010; Harris & Neilson, 2006). Recently, the pathologic significance of tubular EMT in renal fibrosis has become increasingly recognized (Kalluri & Neilson, 2003; Liu, 2004). Broad agreement exists that tubular epithelial cells in vitro can undergo EMT, characterized by loss of epithelial features and acquisition of mesenchymal markers, under the activation of various profibrotic cytokines, particularly transforming growth factor-β1, TGF-β1, that, together with its downstream signaling, mediates all the key events that occur in tubular EMT (Bottinger & Bitzer, 2002; Humphreys et al., 2010; Lamouille et al., 2014; Miettinen et al., 1994; Xie et al., 2003; Yang & Liu, 2001). In vivo studies demonstrated that in kidney diseases, mainly characterized by excessive ECM accumulation, a significant increased expression of TGF-β1 is detectable in the glomeruli and the tubule-interstitium (Jones et al., 1991, 1992; Wahl et al., 1987). TGF-β1 is a multifunctional cytokine belonging to growth factor superfamily that exerts its biological functions through the activation of a canonical TGF-β/SMAD pathway (Lu et al., 2010) and/or a non-canonical signaling, including MAPK pathways, JNK, p38 and PI3K cascade (Derynick & Zhang, 2003).

Studies conducted by Anzano et al. reported that growth factors are able to induce a transforming phenotype in certain untransformed fibroblast target cells (Anzano et al., 1983). Moreover, Micera et al. showed that the chronic exposure to nerve growth factor (NGF) mediates the pro-fibrotic process occurring in conjunctiva during pathological condition, via TGF-β1-induction (Micera et al., 2005). NGF is a secretory protein, belonging to the neurotrophin family, that plays a critical role in the development, survival and function of cells localized within and outside the peripheral and central nervous system (Connor & Dragunow, 1998; Levi-MONTALCINI, 1987). NGF promotes its biological effects through the activation of two distinct receptor types located on the surface of the neurotrophin-responsive cells: the tropomyosin-related kinase A (TrkA) receptor and the receptor p75 for neurotrophins (p75NTR), belonging to the death receptor family (SOFRONIEW et al., 2001). In vivo studies reported that in several
in EMT of tubular renal cells

Furthermore, our previous clinical studies showed that NGF serum levels, as well as its renal expression, were increased in some progressive renal diseases and in kidney transplant, both histologically characterized by glomeruli and tubulo-interstitial fibrosis (Antonucci et al., 2009; Bonfiglio et al., 2007; Gigliotti et al., 2013).

Therefore, beginning our clinical findings, the aim of this study is to carry out in vitro studies, using human proximal tubular renal cell line, to investigate whether NGF is involved in EMT of tubular renal cells via TGF-β1-activation, assuming that NGF could participate to the mechanism of renal fibrosis.

Methods

Reagents and antibodies

Keratinocyte-SFM, epidermal growth factor (EGF), bovine pituitary extract (BPE), penicillin/streptomycin (P/S), NGF and TRIZOL reagent were from Invitrogen (Carlsbad, CA). Enzyme-linked immuno-sorbent (ELISA) kit was purchased from R&D Systems (Minneapolis, MN).

Phosphate buffered saline, aprotinin, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), sodium orthovanadate, NP-40, K252A, cyclohexamide (cx), Coomassie Brilliant Blue were from Sigma Aldrich (Milan, Italy). Sybr Green universal pcr master mix was from Bio-Rad (Hercules, CA).

Antibodies against TGFβ-1 (sc-146), Vimentin (Sc-6260), N-cadherin (sc-7939), E-cadherin (sc-8426), GAPDH (sc-25778), Lamin B (sc-6217), total and phosphorylated JNK (Thr183/Tyr185) (sc-571, sc-6254), total and phosphorylated MAPK P42/44 (THR202/TYR204) (sc-29283, sc-7383), ECL system (sc-2048), goat anti mouse IgG-HRP (sc-2005), goat anti rabbit IgG-HRP (sc-2004) were from Santa Cruz Biotechnology (Santa Cruz, CA). Total and phosphorylated p38 (pY180/pY182) (1544-1, 1229-1) were from Epitomics (Burlingame, CA). Total SMAD4 (ab40750), total and phosphorylated SMAD3 (S423/S425) (ab40854, ab52903) and FSP-1 (S100A4) were from Abcam (Cambridge, UK). Alexa Fluor 488 goat anti-rabbit IgG (H+L) (A11008) and anti-mouse (A11001) were from Life-Technologies (Carlsbad, CA). DAPI Fluorescent Stain (D9542) was from Sigma Aldrich (Milan, Italy). Antibody anti-NGF was a gift from Dr. Aloe Luigi (Institute of Cellular Biology and Neurobiology, National Research Council, Rome, Italy).

Cell culture

Human renal proximal tubular cells, HK-2 (ATCC CRL-2190), were grown in Keratinocyte-SFM containing 5 ng/ml EGF, 0.05 mg/ml BPE and 1 mg/ml P/S. Before each experiment, cells were growth-arrested in serum-free medium supplemented with 5% of complete medium for 24 h and then treated as reported in Results section.

ELISA assay

Conditioned medium obtained from HK-2 cells treated with 5–1000 ng/ml NGF was collected at 24 h after the treatments, centrifuged (1200 rpm) for 10 min and frozen at −80°C until analyzed. The levels of secreted TGF-β1 were determined by an enzyme-linked immuno-sorbent assay (ELISA) (R&D Systems, Minneapolis, MN), that quantify the activated TGF-β1 human concentrations. The sandwich ELISA employed a monoclonal antibody utilized as coating antibody and a polyclonal antibody conjugated with horseradish peroxidase. Standards and samples were bound to both antibodies. Finally, a substrate solution was added for color development and the absorbance was measured with a microplate reader (Dynatech 5000, Germany) at 490 nm. TGF-β1 levels were quantified by comparison with a standard curve using increasing concentrations of human TGF-β1. The sensitivity of the assay was up to 0.7 pg/ml.

Wound-healing scratch assays

HK2 cells monolayer will be scraped and treated as indicated. Wound closure will be monitored; cells were fixed, stained for 15 min with Coomassie Brilliant Blue and then washed in cold PBS. Then wound-healing will be photographed at 96 h of NGF exposure. Images are representative of three different experiments.

Motility assay

Cells synchronized in serum-free media for 24 h were dispersed with versene, washed twice and processed as previously reported (De Amicis et al., 2014) using Boyden chambers. After 96 h of incubation the upper chamber was removed. The migrated cells were counted using an inverted microscope.

Immunoblot analysis and immunoprecipitations

Cells were treated as indicated and then subjected to total protein extraction (Vizza et al., 2013). Nitrocellulose membrane was probed with TGF-β1 antibody (1:300). As internal control, all membranes were subsequently stripped (0.2 M glycine, pH 2.6, for 30 min at room temperature) of the first antibody and reprobed with anti-GAPDH antibody (1:10000) (Santa Cruz Biotechnology). The antigen–antibody complex was detected by incubation of the membranes for 1 h at room temperature with peroxidase-coupled goat anti-rabbit (1:2000) or anti-rabbit (1:7000) or donkey anti-goat (1:3000) IgG and revealed using the enhanced chemiluminescence system (Santa Cruz Biotechnology). Blots were then exposed to film (Kodak film, Sigma-Aldrich, St. Louis, MO). For nuclear and cytoplasmic extracts cells were washed in cold PBS and then 300 µl of hypotonic buffer (10 mM Hepes pH 7.9, 1.5 mM MgCl2, 10 mM KCl, protease inhibitors) was added. Cell lysis was followed by microscopy until 90% of the cells were lysed. Following centrifugation (1000 g,
NGF involvement in tubular EMT

4°C, 10 min), the supernatant was referred to as cytoplasmic fraction. The pellet containing nuclei was resuspended in high salt buffer [20 mM Hepes pH 7.9, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, protease inhibitors]. Extraction of nuclear proteins was achieved by vortexing this solution thoroughly, incubating for 30 min on ice and subsequent centrifugation (25,000g, 4°C, 20 min). The collected supernatant represents the nuclear fraction. The purity of the cytoplasmic and nuclear fractions was confirmed by immunoblotting with an anti-lamin serum. Equal amounts of cytosolic and nuclear proteins were resolved by 8% SDS-PAGE and probed with antibodies directed against total and phosphorylated MAPK P42/44 (1:1000), total and phosphorylated JNK (1:500), total and phosphorylated SMAD3 (1:5000), total SMAD4 (1:5000), GAPDH (1:10000), Lamin B (1:3000).

For co-immunoprecipitation experiments, we used 1 mg of nuclear and cytoplasmic protein extracts and 2 μg of SMAD3 or SMAD4 antibodies overnight, followed by protein A/G precipitation. Equal amounts of cell extracts and coimmunoprecipitated protein were subjected to SDS polyacrylamide gel electrophoresis, as described earlier (Vizza et al., 2013). Membranes were probed with anti-SMAD4 or SMAD3 antibodies, respectively. The bands of interest were quantified by Image J densitometry scanning program.

Real-time RT-PCR assays

HK-2 cells were grown in 6-well plates to 70–80% confluence and exposed to treatments as indicated. Total RNA was isolated from cells with TRIZOL reagent (Invitrogen) according to the manufacturer’s protocol. The purity and integrity of the RNA was confirmed both spectroscopically and electrophoretically. Analysis of gene expression was performed using real-time reverse transcription PCR.

cDNA was synthesized from 2 μg of total RNA with random hexamer primers using the High capacity cDNA Archive Kit (Applied Biosystems, Applera Italia, Monza, Milano, Italy). Real-time PCR was performed using SYBR Green Universal PCR Master Mix with 0.1 mmol/l of each primer in a total volume of 30 μl reaction mixture following the manufacturer’s recommendations. Negative control containing water instead of first strand cDNA was used. Each sample was normalized on its GAPDH mRNA content.

Primers were used for the amplification were:
(TGF-β1): forward 5’-ATCTTCTGAAAACACTAAAGGCTCG-3’ and reverse 5’-ACCTTCTAGATGTAGTCCCGC-3’ (Vimentin): forward 5’-GAGAACTTGGCCTTTGAAGC-3’ and reverse 5’-GGTCTGCTGTAGGGCGCAATC-3’ (N-Cadherin): forward 5’-ACAGTGGCCACCTACAAAGG-3’ and reverse 5’-CCGAGATTGGGTGGTGATAATG-3’ (FSP-1): forward 5’-TGCGGGAAGAGCAGATAGG-3’ and reverse 5’-TGAGAGCGGCCAGGTGAAAAA-3’ (E-Cadherin): forward 5’-TGCCCCGAAAATGGAAGG-3’ and reverse 5’-GTGGATGTGGCATAATGCC-3’ (GAPDH): forward 5’-CCACCTCCTCCACCTTTGC-3’ and reverse 5’-CATACCCAGAAAATGAGCCTTGAC-3’

The relative gene expression levels were normalized as previously described (Vizza et al., 2013).

Transfection assay

HK-2 cells were treated with 100 nM of validated stealth p75NTR targeted for the human p75NTR mRNA sequence RNAi (Invitrogen ID: 1299001), with siRNA targeted for the human TGF-β1 mRNA sequence (Ambion ID: s14055) or with a negative control RNAi (Invitrogen ID: 452001) that does not match with any human mRNA. Cells were transfected using Lipofectamine 2000 according to protocol outlined in the product manual. Efficiency of knockdown for each gene and the negative control was determined by Western blot analyses (data not shown). After 48 and 24 h of incubation with the respective siRNA complexes the cells were serum starved overnight, treated with NGF for an additional period of 48 h and then total RNA was extracted as above described.

MTT assay

Cell viability was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium (MTT) assay. HK2 cells (4 × 10⁶ cells/ml) were grown in 24-well plates and exposed to treatments as indicated for 96 h in serum-free medium added with 5% of complete medium. A 100 μl of MTT (2 mg/ml, Sigma) was added to each well, and the plates were incubated for 2 h at 37°C. Then, pure DMSO was added to solubilize the absorbance as measured at a test wavelength of 570 nm in Beckman Coulter.

Observation of morphologic change

The morphologic changes of the HK-2 cells were observed using the reserved microscope. The photography was taken using a Leica microscope image system (Leica, Mannheim, Germany).

Immunofluorescence assay

For immunofluorescence assay (IF), HK-2 cells were grown on glass cover slips and then fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 0.5% BSA and then incubated overnight with: antibody anti-FSP-1, anti-Vimentin, anti-N-cadherin, anti-E-cadherin, anti-total and phosphorylated SMAD3, anti-total SMAD4 (Santa Cruz Biotechnology). Next day, the slides were incubated with secondary antibody anti-rabbit IgG FITC-conjugated (1:100) (Invitrogen) at RT. The cellular localization of protein was studied with fluorescence microscope with 20× magnification.

Results

NGF exposure up-regulates TGFβ-1 secretion and expression levels in a transcriptional dependent-manner in HK-2 cells

Since it has been reported that NGF contributes to fibrotic disorders regulating TGFβ-1 expression by both transcriptional and post-transcriptional mechanisms (Chen et al., 2014; Cosgaya & Aranda, 1995), we investigated the potential ability of NGF to modulate TGF-β1 expression and secretion in our in vitro model. To this aim, firstly we performed ELISA assay using conditioned medium of cultured HK-2 cells treated for 24 h with increasing concentrations of NGF (from 5 to 500 ng/ml). As reported in Figure 1(A), our results...
showed that NGF induced TGF-β1 secretion in cultured medium in a dose-dependent manner, with a maximum effect observed at 100 ng/ml (Figure 1A).

Next, by real-time RT-PCR and WB analysis, we evaluated the mRNA (Figure 1B, left panel) and protein (Figure 1B, right panel) expression levels of TGF-β1. As shown in Figure 1(B) a significant up-regulation of TGF-β1 was detected after 24 h of NGF exposure (100 ng/ml) that was maintained up to 48 h (data not shown). In order to confirm that NGF transcriptionally up-regulates TGF-β1, HK-2 cells were exposed for 24 h to NGF with or without the protein synthesis inhibitor cycloheximide (CX) (50 μM). In this experimental conditions, we observed that CX prevented the enhancement of TGF-β1 mRNA (Figure 1C, left panel) and protein (Figure 1C, right panel) expression, suggesting that TGF-β1 protein induced upon NGF stimulus is ex novo synthesized (Figure 1C).

NGF exposure promotes EMT in HK-2 cells

Although, several in vitro studies showed that NGF could promote EMT process (Micerca et al., 2001, 2005, 2006, 2007), to date nothing is reported about the effect of NGF exposure in tubular EMT. Firstly, in order to evaluate whether NGF promotes EMT process in HK-2 cells, we performed wound-healing scratch and trans-well migration assays. As reported in Figure 2(A), we found that after 96 h NGF greatly induced the wound closure compared to the untreated cells. Concomitantly we observed that in the same experimental conditions, NGF induced a significant migration of HK-2 cells (Figure 2B, left panel). To investigate whether NGF-induced change in cell migration was caused by an enhancement of cell proliferation, we used MTT assay that revealed that NGF exposure did not affect cell vitality (Figure 2B, right panel). Next, to explore whether upon NGF treatment HK-2 cells increased their motility because they acquired a mesenchymal phenotype, we analyzed, by real-time PCR and Western blot analysis, the modulation of key markers involved in EMT process. Therefore, we treated HK-2 cells with NGF for 48 h. Our results showed a significant genic (Figure 2C) and protein (Figure 2D) up-regulation of mesenchymal markers as Vimentin, N-cadherin, FSP1, and a down-regulation of the major component of adherens junctions, E-cadherin. These results were confirmed by immunofluorescence staining (Figure 2E).

NGF exposure mediates the tubular EMT via both TrkA and p75NTR receptors

Apart from the well-established role of NGF in modulating cell growth, differentiation, survival and death, recent studies provided evidence for a direct involvement of the neurotrophin in the fibrotic process, through its receptors, TrkA and p75NTR (Chen et al., 2014; Micerca et al., 2007). It is well known that TrkA mainly drives proliferation, differentiation and survival, while p75NTR triggers differentiation and apoptosis (Frade et al., 1996; Yoon et al., 1998). Therefore, we investigated whether the up-regulation of TGF-β1 expression, promoted by NGF in our experimental model, is mediated by TrkA and/or p75NTR. To this aim, HK-2 cells were transiently transfected with p75NTR RNAi and then exposed to NGF for 48 h (Figure 3A). As shown in Figure 3(B), the up-regulation of TGF-β1 NGF-induced was abrogated in terms of mRNA and protein levels when knockdown of p75NTR gene occurred (Figure 3B, top panel). Interestingly, in HK-2 cells exposed to NGF for 48 h, the pretreatment with K252A, an alkaloid acting as a potent and selective inhibitor of TrkA tyrosine kinase (Angeles et al., 1998), prevented the genic and protein up-regulation of TGF-β1 expression, suggesting that both receptors cooperate in modulating the cytokine expression upon NGF stimulus (Figure 3B, bottom panel). At this point, we evaluated, in the same experimental conditions, the role of TrkA and p75NTR in modulating EMT process triggered by neurotrophin exposure. Therefore, in HK-2 cells pretreated as above reported and then exposed to NGF treatment for 48 h, we evaluated the gene expression of EMT markers by real-time PCR. Our results showed that when HK-2 cells were treated with NGF knockdown of p75NTR gene occurred, the up-regulation of Vimentin, N-cadherin and FSP1 was reversed, while the down-regulation of epithelial marker E-cadherin was restored (Figure 3C). Interestingly, our data demonstrated that in HK-2 cells exposed to NGF, the pretreatment with K252A significantly down-regulated the mesenchymal markers and induced a marked up-regulation of E-cadherin (Figure 3D), highlighting that, although both receptors are involved in tubular EMT promoted by NGF exposure, the contribution of NGF-TrkA signaling is crucial to activate the biological effect.

NGF promotes EMT of HK-2 cells via TGFβ-1

In agreement with that reported in literature, showing that NGF could promote fibrosis activating TGF-β1 (Bitzer et al., 1998; Bottinger & Kopp, 1998; Cosgaya & Aranda, 1995; Haas et al., 2009), we tested, in our cellular model, the role of TGF-β1 NGF-induced in the observed tubular EMT process. Firstly, we observed that the exposure to NGF for 96 h caused morphologic changes in HK-2 cells that acquired an elongated fibroblastoid morphology as reported in Figure 4(B). Concomitantly, we investigated whether upon NGF treatment, HK-2 cells phenotype was changed when TGF-β1 knocked-down gene occurred (Figure 4A). Interestingly, as reported in Figure 4(B), in the above-reported experimental conditions, we did not observe the fibroblastoid morphology induced by NGF exposure. Next, HK-2 cells transiently transfected with TGF-β1 RNAi for 24 h (Figure 4C) and then treated with NGF, were used to investigate the gene expression profiles of Vimentin, N-cadherin, FSP1 and E-cadherin by real-time PCR. As shown in Figure 4(C), TGF-β1 gene silencing abrogated the up-regulatory effects induced by NGF on Vimentin, N-cadherin and FSP1 mRNA expression, while the expression of E-cadherin was restored. No changes of EMT genes expression were observed after transfection of cells with a control siRNA upon identical experimental conditions.

Our results underline a direct involvement of TGF-β1 in the modulation of EMT markers observed in HK-2 cells upon NGF exposure.

TGFβ-1/SMAD pathway activation mediates the pro-fibrogenic effect of NGF in HK-2 cells

It is well known that TGF-β1 regulates its downstream genes through a canonical SMAD-mediated signaling and a
non-canonical signal transduction cascades including MAPK, JNK and p38 pathways (Derynck & Zhang, 2003). Therefore, we aimed to identify the main signaling by which the activated TGF-β1 upon NGF treatment promotes EMT of tubular cells. Firstly, we explored the contribution of non-canonical pathway using cytosolic extracts of HK-2 cells exposed to short time NGF treatment (15–30 min–1 h–2 h). Our results showed that after 15 min, NGF modestly up-regulated pMAPK and pJNK but it did not induce a significant phosphorylation p38 pathway (Figure 5A). Next, we focused on the TGF-β1-canonical pathway to establish whether activated TGF-β1 upon NGF treatment, could promote the nuclear translocation of SMAD complexes involved in the regulation of transcription of target genes. To this aim, we performed nuclear and cytosolic extracts obtained from HK-2 cells subjected to short exposure to NGF as reported above, to evaluate the phosphorylation status as well as the total levels of SMAD3 and SMAD4. Our results,
Figure 2. NGF exposure increase HK-2 cells motility up-regulating EMT markers. (A) Serum-starved HK-2 cells were scrape wounded and then treated with NGF (100 ng/ml). Images are representative of the process of wound-healing after 96 h of treatment. (B) Left panel: HK-2 cells were serum starved for 24 h. Synchronized cells were then seeded on polylysine-coated Boyden chambers and treated with NGF. Columns mean number of migrated cells after 96 h of NGF treatment of three independent experiments. *p < 0.05 compared with untreated cells (−). Right panel: MTT growth assay in HK-2 cells serum starved for 24 h and then treated for 96 h with NGF as indicated. Cell proliferation is expressed as fold change respect to untreated cells (−/C0). NS: not significant. (C) Vimentin, N-cadherin, FSP1 and E-cadherin levels in terms of mRNA and protein (D) content of HK-2 exposed for 48 h to NGF. Each sample was normalized to its GAPDH content. The values represent the means ± s.d. of three different experiments each performed in triplicate. *p < 0.05 versus untreated cells (−). Numbers over the blots represent the average fold change versus untreated cells (−/C0). The immunoblot shows a single representative of three separate experiments. (E) HK-2 cells were cultured and untreated (−) or treated with NGF for 48 h and then immunostained for Vimentin or N-cadherin or FSP1 or E-cadherin (green fluorescence); nuclei were counterstained with DAPI. (Negative) cells were incubated replacing the anti-Vimentin or N-cadherin or FSP1 or E-cadherin antibodies by normal mouse IgG utilized as negative control (data not shown). Original magnification: ×20.
Figure 3. NGF exposure promotes tubular EMT via TrkA and p75NTR receptors. (A) mRNA expression of p75NTR in HK-2 cells transfected for 48 h with siRNA targeted human p75NTR mRNA sequence or with a control siRNA. GAPDH was used as loading control. *p<0.05 versus C. (B) TGF-β1 levels in terms of mRNA (left panel) and protein (right panel) content of HK2 cells (upper panel) transfected for 48 h with siRNA targeted human p75NTR mRNA sequence or with a control siRNA or (lower panel) pre-treated for 48 h with K252A (200 nM) and then exposed to NGF for 48 h. Each sample was normalized to its GAPDH content. The values represent the means ± s.d. of three different experiments each performed in triplicate. Numbers on top of the blots represent the average fold change versus untreated cells normalized for GAPDH content. (C, D) Vimentin, N-cadherin, FSP1 and E-cadherin mRNA content evaluated by real-time RT-PCR, after NGF exposure in HK2 treated as above described. Each sample was normalized to its GAPDH mRNA content. *p<0.05 versus C, **p<0.05 versus cells treated with NGF.
obtained by Western blot analysis (Figure 5B) and immunofluorescence staining (Supplementary Figure 1) showed that phosphorylated SMAD3 was detected in the cytosolic and in the nuclear fraction after 15 min of NGF stimulus, reaching a maximum after 1 h of treatment (Figure 5B). Referring to SMAD 4, we detected significant levels of protein in the nucleus already in the absence of NGF. Moreover, according to that reported in the literature, by co-immunoprecipitation assay, we observed that NGF administration induced the association between SMAD3 and SMAD4 in the cytosolic compartment (Figure 5C, left panel). Interestingly, we observed that the short NGF exposure promoted the nuclear translocation of the complex SMAD3/SMAD4 (Figure 5C, right panel). To confirm that the SMAD signaling was mainly activated by TGFβ-1, we investigated the cytosolic and nuclear content of pSMAD3 and total SMAD4 using HK-2 cells transfected with TGF-β1 RNAi. As reported in Figure 5(D), WB analysis showed that in the presence of TGF-β1 RNAi, the phosphorylation of SMAD3 and the content of SMAD4 were significantly mitigated with respect to that detected in un-transfected cells (Figure 5B), suggesting that TGF-β1 up-regulated by NGF signaling could mediate the tubular EMT via activation of its canonical pathway.

Finally, to further confirm the crucial role exerted by NGF in promoting the activation of TGF-β1–SMAD signaling, HK-2 cells were pre-treated for 72 h with an antibody against NGF and then subjected to short time NGF exposure. Time course assay showed that the pSMAD3 levels, as well as

Figure 4. EMT process is mediated by TGF-β1 NGF-induced: (A) mRNA expression of TGF-β1 in HK-2 cells transfected for 48 h with siRNA targeted human TGF-β1 mRNA sequence or with a control siRNA. GAPDH was used as loading control. *p<0.05 versus untreated cells (−). (B) Representative photomicrograph shows the morphology of HK-2 cells transfected for 48 h with siRNA targeted human TGF-β1 mRNA sequence or with a control siRNA, starved for 24 h and then untreated (−) or treated with NGF (100 ng/ml) for 96 h. (C) mRNA contents of Vimentin, N-cadherin, FSP1 and E-cadherin evaluated by real-time RT-PCR in HK2 cells trasfected for 24 h with TGF-β1 RNAi or with a control siRNA and then untreated (−) or treated for 48 h with NGF. Results obtained were normalized to its GAPDH mRNA content. The values represent the means ± s.d. of three different experiments each performed in triplicate. *p<0.05 versus untreated cells (−), **p<0.05 versus cells treated with NGF.
Figure 5. NGF exposure activates canonical TGF-β1 signaling: (A) Immunoblot analysis showing phosphorylated and total levels of MAPK, JNK and p38 in cytosolic extracts of HK2 treated from 5 min to 2 h with NGF. GAPDH was used as loading control. (B) Phosphorylated and total content of SMAD3 and total levels of SMAD4 of nuclear and cytosolic protein fraction of HK-2 cells treated as above reported. GAPDH and Lamin B were used as loading control. (C) (Upper panel) phosphorylated and total content of SMAD3 and total levels of SMAD4 of cytosolic and nuclear proteins exposed to NGF for 30 min and 1 h. GAPDH and Lamin B were used as loading control. (Lower panel) cytosolic and nuclear protein extracts of HK2 cells treated as above were immunoprecipitated using anti-SMAD3 (IP: SMAD3) and anti-SMAD4 (IP: SMAD4) antibodies, respectively and resolved in SDS polyacrylamide gel electrophoresis. Immunoblotting was performed using anti-SMAD4 and anti-SMAD3 antibodies, respectively. Levels of phosphorylated SMAD3 and total non-phosphorylated SMAD3 and SMAD4 proteins were measured in cytosolic and nuclear extracts of HK2 transfected for 24 h with TGF-β1 RNAi or with a control siRNA (D) or in HK2 pre-treated for 72 h with an antibody anti-NGF neutralizing NGF effects (E) and then subjected to short time NGF exposure. GAPDH and Lamin B were used as loading control. All immunoblots show a single representative of three separate experiments. Numbers on top of the blots represent the average fold change versus untreated cells normalized for GAPDH or Lamin B content.

DOI: 10.3109/08977194.2015.1054989

NGF involvement in tubular EMT
SMAD4 content, was almost entirely reversed in nuclear and cytosolic fractions obtained from HK-2 treated with antibody anti-NGF (Figure 5E) respect to that detected in un-pretreated cells (Figure 5B).

Discussion
In the present study, we provided the first evidence that NGF, mainly through canonical TGF-β1 signaling activation, promotes EMT of human tubular epithelial cells, HK-2.

In vitro and in vivo studies showed that NGF contributes to the repair process or fibrotic disorders by inducing the expression of TGF-β1 at both transcriptional and post-transcriptional levels (Blitstein-Willinger, 1991; Cosgaya & Aranda, 1995; Micera et al., 2005), suggesting that these factors exert cooperative or overlapping effects on wound repair or fibrotic processes (Micera et al., 2006).

The findings of the current study could contribute to elucidate the role exerted by the increased NGF serum levels that we previously observed in patients affected by chronic kidney diseases, in the fibrotic renal process (Antonucci et al., 2009; Bonofilio et al., 2007; Gigliotti et al., 2013). Indeed, our results showed that in tubular renal cells the exposure to NGF increased the synthesis of TGF-β1 in a transcriptional manner. Interestingly, by ELISA assay that quantify the activated human TGF-β1 concentrations, we observed that the treatment with NGF, as well as promoting the synthesis and the activation of the cytokine, also promotes the activation of latent pre-formed TGF-β1. It has been demonstrated that TGF-β1 represents the key mediator of glomerular and interstitial fibrosis detectable in patients affected by chronic kidney diseases (Bottinger & Bitzer, 2002; Yang et al., 1997). In vitro studies showed that the treatment with TGF-β1 of normal mouse breast epithelial cells changes the cuboidal shape to an elongated spindle, accompanied by a decrease in epithelial markers and an increased expression of mesenchymal markers (Meulmeester & Ten Dijke, 2011; Wendt et al., 2009). In agreement with these data, in our experimental model, we observed that the exposure to NGF, concomitantly to induce TGF-β1 expression, caused cellular phenotypic alteration, increased HK-2 cells motility and up-regulated genic and protein expressions of mesenchymal markers as Vimentin, N-cadherin and FSP1, down-regulating the epithelial marker E-cadherin. These data suggested that NGF acts as a pro-fibrotic factor in tubular renal cells and prompted us to investigate the involvement of its receptors in the observed biological effect. It is well known that the actions exerted by NGF, including fibrosis process, are mediated by its receptors, TrkANTR and p75NTR (Sofroniew et al., 2001). Interestingly, through the inhibition of TrkANTR or p75NTR obtained as reported in the results section, we demonstrated that the induction of TGF-β1 and EMT markers promoted by NGF treatment was mediated by the cooperation of both receptors, although we observed that TrkANTR signaling seems mainly involved in promoting EMT of tubular cells with respect to p75NTR signaling. Up until today, we have not yet explored the molecular mechanisms that could explain these findings and we believe that this is a limitation of our study. However, we could speculate that the over-expression of p75NTR could be reached after a long term of NGF exposure, as suggested by Micera et al. (2001). In addition, in renal biopsies obtained from patients affected by fibrotic kidney diseases showing high NGF serum levels, we observed a strong expression of p75NTR in areas of interstitial fibrosis (Bonofilio et al., 2007). Therefore, we did not exclude that the sharp up-regulation of p75NTR/axis could be triggered by NGF during the latest stages of fibrosis process and that, almost in the early stages, the biological effects were conducted by TrkANTR signaling in cooperation with the p75NTR expressed by HK-2 cells. Therefore, further in vitro studies will be needed to elucidate these aspects. As widely demonstrated in other experimental models, showing that TGF-β1 and NGF signaling are closely linked (Blitstein-Willinger, 1991; Cosgaya & Aranda, 1995; Micera et al., 2005, 2006), we confirmed that in our cellular system, NGF promotes tubular EMT by up-regulating TGF-β1, because the activation of EMT process was prevented when knocked down of TGF-β1 gene occurred. In the literature it is reported that TGF-β1 exerts its biological activities by a canonical and/or a non-canonical pathway that can cross-talk and mutually modulate each other (Chen et al., 2012; Gupta et al., 2011; Zhang 2009). Essential elements of the post-receptor signaling machinery for TGF-β1 is the pathway restricted SMADs, SMAD2 and SMAD3, and the common mediator SMAD4. The activated TGFβ-1, binding to TGFβ-receptor type I, promotes the phosphorylation of cytoplasmic mediators belonging to the SMAD family that, as complexes, acts as transcription factors, binding DNA either directly or in association with other DNA binding proteins to regulate target genes expression (Massague & Chen, 2000; Massague & Wotton, 2000; Piek et al., 1999). Our results provided evidence that NGF-mediated activation of TGFβ-1 signaling, predominantly involves the canonical pathway, since, NGF exposure promoted a strong phosphorylation of SMAD3 that shuttled into nucleus as complex with SMAD4, regulating the transcription of specific target genes. The fact that in basal conditions we detected SMAD4 in the nuclear compartment, but not SMAD3, regardless of ligand stimulation, is in agreement with the findings that SMAD4 continuously shuttles between cytoplasm and nucleus (Pierreux et al., 2000). Lutz et al. reported that in pheochromocytoma cells, which represent a model system unresponsive to TGF-β1, NGF stimulation leads to activation of SMAD pathway that did not occur via phosphorylation of SMAD3, but by promoting translocation of SMAD3 into nucleus where it forms an heteromeric complex with SMAD4, leading to the activation of SMAD-dependent reporter genes (Lutz et al., 2004). Our findings suggested that in HK-2 cells, NGF activates SMAD proteins through the TGF-β1 triggered mechanism, since the phosphorylation of SMAD3 and the nuclear translocation of the complex SMAD3/SMAD4 were both reversed in the presence of TGF-β1 siRNA or using an antibody against NGF that selectively inhibits the binding of the neurotrophin to its receptors. However, although we detected a modest up-regulation of pERK and pJNK, we cannot exclude that non-canonical pathway may be also involved in the tubular EMT process promoted by NGF via TGF-β1 activation. Interestingly, Yang et al. recently provided news insights into mechanisms underlying the transcriptional regulation of N-Cadherin expression in TGF-β-induced EMT, demonstrating that in non-small cell lung cancer cells, TGF-β1-activated SMAD3/4 complex, interacting with a
specific SMAD-binding element in N-Cadherin promoter, induced the up-regulation of N-Cadherin expression (Yang et al., 2015). Therefore, further studies will be needed to investigate the molecular mechanisms by which TGF-β1 activated by NGF, could modulate the transcriptional activity of the promoters of investigated genes involved in the EMT process.

Conclusions

In conclusions, related to translational aspects, our data could highlight the contribution of NGF in renal fibrosis, allowing us to establish whether the elevated NGF serum levels, as well as the enhanced renal expression of NGF and its receptors, detected in patients affected by fibrotic kidney diseases, could be considered as an early prognostic markers of renal fibrosis and, consequently, as a novel therapeutic strategy to prevent or mitigate renal fibrosis.

Acknowledgements

We thank Dr Ernestina De Francesco, Department of Pharmacy and Sciences of Nutrition and Health, University of Calabria, for help with immunofluorescence assay.

Declarati0n of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

Aloe L, Rocco ML, Bianchi P, Manni L. 2012. Nerve growth factor: From the early discoveries to the potential clinical use. J Transl Med 10:239.
Angeles TS, Yang SX, Steffler C, Dionne CA. 1998. Kinetics of trkA tyrosine kinase activity and inhibition by K-252a. Arch Biochem Biophys 349:267–274.
Antonucci MT, Bonofiglio R, Papalia T, Caruso F, Caroleo MC, Mancuso D, Aloe L. 2009. Nerve growth factor and its monocyte receptors are affected in kidney disease. Nephron Clin Pract 111: c21–c28.
Anzano MA, Roberts AB, Smith JM, Sporn MB, De Larco JE. 1983. Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type alpha and type beta transforming growth factors. Proc Natl Acad Sci USA 80:6264–6268.
Bitzer M, Sterzel RB, Bottinger EP. 1998. Transforming growth factor-beta in renal disease. Kidney Blood Press Res 21:1–12.
Blixtstein-Willinger E. 1991. The role of growth factors in wound healing. Skin Pharmacol 4:175–182.
Bonini S, Lambiase A, Bonini S, Angelucci F, Magrini L, Manni L, Aloe L. 1996. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. Proc Natl Acad Sci USA 93:10955–10960.
Bonofiglio R, Antonucci MT, Papalia T, Romeo F, Capocasale G, Caroleo MC, Di Fausto V, Aloe L. 2007. Nerve growth factor (NGF) and NGF-receptor expression in diseased human kidneys. J Nephrol 20:186–195.
Bottinger EP, Bitzer M. 2002. TGF-beta signaling in renal disease. J Am Soc Nephrol 13:2600–2610.
Bottinger EP, Kopp JB. 1998. Lessons from TGF-beta transgenic mice. Miner Electrolyte Metab 24:154–160.
Chen JC, Lin BB, Hu HW, Lin C, Jin WY, Zhang FB, Zhu YA, et al. 2014. NGF accelerates cutaneous wound healing by promoting the migration of dermal fibroblasts via the PI3K/Akt-Rac1-JNK and ERK pathways. Biomed Res Int 2014:547187.
Chen XF, Zhang HJ, Wang HB, Zhu J, Zhou WY, Zhang H, Zhao MC, et al. 2012. Transforming growth factor-beta1 induces epithelial-to-mesenchymal transition in human lung cancer cells via PI3K/Akt and MEK/Erk1/2 signaling pathways. Mol Biol Rep 39: 3549–3556.
Cho MH. 2010. Renal fibrosis. Korean J Pediatr 53:735–740.
Connor B, Dragunow M. 1998. The role of neuronal growth factors in neurodegenerative disorders of the human brain. Brain Res Brain Res Rev 27:1–39.
Cosgay JM, Aranda A. 1995. Nerve growth factor regulates transforming growth factor-beta 1 gene expression by both transcriptional and posttranscriptional mechanisms in PC12 cells. J Neurochem 65: 2484–2490.
De Amici F, Perri A, Vizza D, Russo A, Panno ML, Bonfiglio D, Giordano C, et al. 2013. Epigallocatechin gallate inhibits growth and epithelial-to-mesenchymal transition in human thyroid carcinoma cell lines. J Cell Physiol 228:2054–2062.
Derynk R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425:577–584.
Eddy AA. 2005. Progression in chronic kidney disease. Adv Chronic Kidney Dis 12:353–365.
Fradé JM, Rodríguez-Tebar A, Barde YA. 1996. Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 383:166–168.
Gigliotti P, Lofaro D, Leone F, Perri A, Vizza D, Papalia T, Bonofiglio R. 2013. High nerve growth factor blood concentration in renal transplantation: A new prognostic marker? Transplant Proc 45: 2654–2656.
Gupta J, Robbins J, Jilling T, Seth P. 2011. TGFbeta-dependent induction of interleukin-11 and interleukin-8 involves SMAD and p38 MAPK pathways in breast tumor models with varied bone metastases potential. Cancer Biol Ther 11:311–316.
Haas SL, Fitzner B, Jastreboff W, Wiercinska E, Gaitaniti H, Jesnowski R, Lohr JM, et al. 2009. Transforming growth factor-beta1 induces nerve growth factor expression in pancreatic stellate cells by activation of the ALK-5 pathway. Growth Factors 27:289–299.
Harris RC, Neilson EG. 2006. Toward a unified theory of renal progression. Annu Rev Med 57:365–380.
Heese J, Beck FK, Behrens MH, Pluss K, Fieribeck W, Huwiler A, Muhl H, et al. 2003. Effects of high glucose on cytokine-induced nerve growth factor (NGF) expression in rat renal mesangial cells. Biochem Pharmacol 65:293–301.
Humphreys BD, Lin SL, Kobayashi A, Hudson TE, Nowlin BT, Bonventre JV, Valierus MT, et al. 2010. Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. Am J Pathol 176:85–97.
Jones CL, Buch S, Post M, McCulloch L, Liu E, Eddy AA. 1991. Pathogenesis of interstitial fibrosis in chronic purine aminonucleoside nephropathy. Kidney Int 40:1020–1031.
Jones CL, Buch S, Post M, McCulloch L, Liu E, Eddy AA. 1992. Renal extracellular matrix accumulation in acute puromycin aminonucleoside nephrosis in rats. Am J Pathol 141:1381–1396.
Kalluri R, Neilson EG. 2003. Epithelial–mesenchymal transition and its implications for fibrosis. J Clin Invest 112:1776–1784.
Lamouille S, Xu J, Derynk R. 2014. Molecular mechanisms of epithelial–mesenchymal transition. Nat Rev Mol Cell Biol 15: 178–196.
Levi-Montalcini R. 1987. The nerve growth factor 35 years later. Science 237:1154–1162.
Liu Y. 2004. Epithelial to mesenchymal transition in renal fibrogenesis: Pathologic significance, molecular mechanism, and therapeutic intervention. J Am Soc Nephrol 15:1–12.
Lu L, Wang J, Zhang F, Chai Y, Brand D, Wang X, Horwitz DA, et al. 2010. Role of SMAD and non-SMAD signals in the development of Th17 and regulatory T cells. J Immunol 184:4295–4306.
Lutz M, Kriegstein K, Schmitt S, Momoki P, Sebald W, Wizenmann A, Knaus P. 2004. Nerve growth factor mediates activation of the Smad pathway in PC12 cells. Eur J Biochem 271:920–931.
Massague J, Chen YG. 2000. Controlling TGF-beta signaling. Genes Dev 14:627–644.
Massague J, Wotton D. 2000. Transcriptional control by the TGF-beta pathway in breast tumor models with varied bone metastases potential. J Cell Physiol 228:2054–2062.
Meulmeester E, Ten Dijke P. 2011. The dynamic roles of TGF-beta in renal disease. Kidney Blood Press Res 21:1–12.
Nguyen H, et al. 2003. Effects of high glucose on cytokine-induced nerve growth factor (NGF) expression in rat renal mesangial cells. Biochem Pharmacol 65:293–301.
Supplementary material available online
Supplementary Figure 1