NERVE GROWTH FACTOR ENHANCES THE NEPHROTOXIC EFFECTS EXERTED BY CYCLOSPORINE IN TUBULAR RENAL CELLS

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BACKGROUND AND AIM

Nerve growth factor (NGF) is a neurotrophin promoting cell growth, differentiation, survival and death through two different receptors: TrkA and p75. Respect to the beneficial effects exerted by NGF in many chronic neurodegenerative diseses, NGF serum concentrations increase during many inflammatory and autoimmune diseases, in some kidney disease and, particularly, in renal transplant. Our study was raised from basic evidence indicating that Cyclosporine A-inhibition of calcineurin-Nuclear Factor of Activated T cells (NFAT) pathway increases NGF expression levels. Therefore, the aim of our study is to investigate the involvement of NGF and its receptors in nephrotoxic effects exerted by Cyclosporine A (CsA) in tubular renal cells.

MATERIALS AND METHODS

We evaluated in human proximal tubular epithelial cells, HK-2, treated with CsA or NGF, alone or in combination, vitality cell, gene and protein expression of NGF receptors, apoptosis and cell cycle regulators by MTT assay, real time PCR-assay and Western blot analysis, respectively.

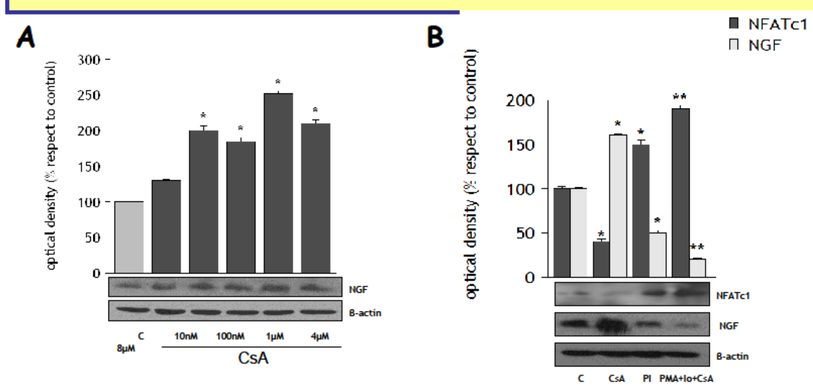

Figure 1. CSA up-regulates NGF protein levels via NFATc1. **(A)** HK-2 cells were untreated (c) or treated for 48h with CsA 10nM-100nM-1μM-4μM-8μM before lysis. Equal amounts of total cellular extract were analyzed for NGF levels by western blotting. β-Actin was used as loading control. Bars represent the means ± SD of 3 separate experiments between NGF/β-Actin levels in which band intensities were evaluated as density arbitrary units and expressed as percentages of the control (100%). *p<0.05 vs c. **(B)** WB of NFATc1 and NGF in total extracts from HK-2 cells treated with CsA 10nM, PMA 100nM+Io 2.5μM and CsA+PMA+Io. β-Actin was used as loading control. The upper panels represent the means ± SD of 3 separate experiments between NFATc1/β-Actin and NGF/ β-Actin levels in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control (100%). *p<0.05 vs c; **p<0.05 vs CsA-treated cells. All immunoblots show a single representative of 3 separate experiments.

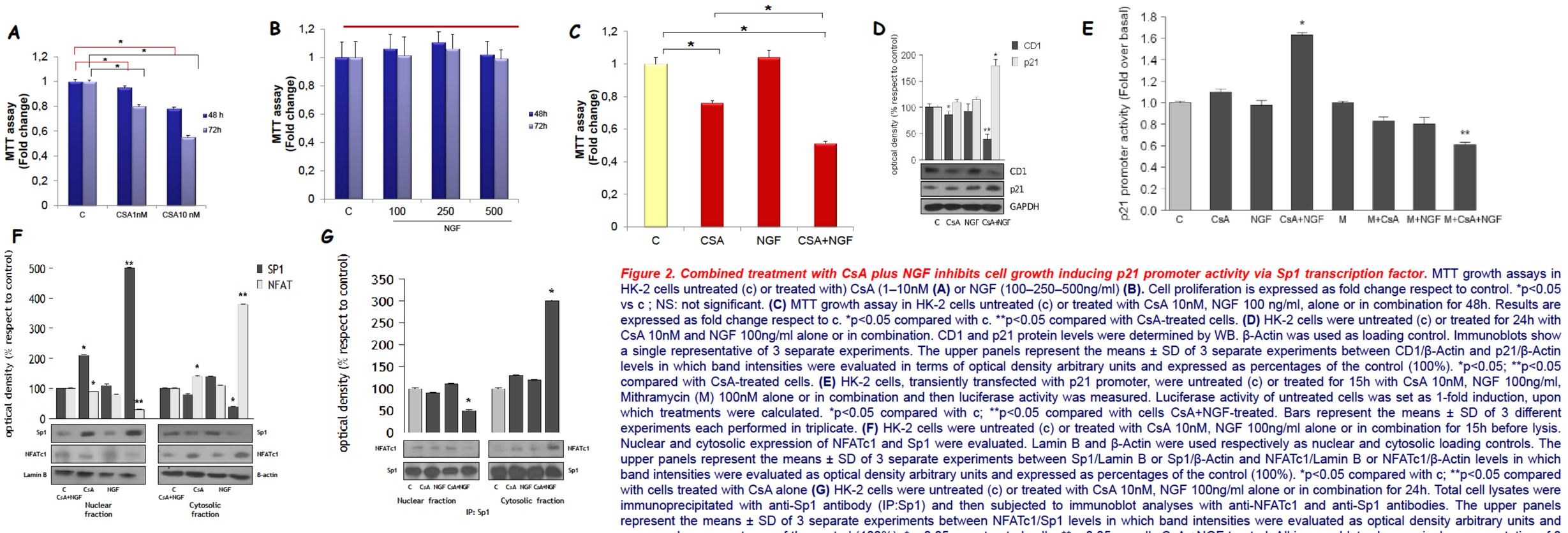

Figure 2. Combined treatment with CsA plus NGF inhibits cell growth inducing p21 promoter activity via Sp1 transcription factor. MTT growth assays in HK-2 cells untreated (c) or treated with) CsA (1–10nM **(A)** or NGF (100–250–500ng/ml) **(B).** Cell proliferation is expressed as fold change respect to control. *p<0.05 vs c ; NS: not significant. **(C)** MTT growth assay in HK-2 cells untreated (c) or treated with CsA 10nM, NGF 100 ng/ml, alone or in combination for 48h. Results are expressed as fold change respect to c. *p<0.05 compared with c. **p<0.05 compared with CsA-treated cells. **(D)** HK-2 cells were untreated (c) or treated for 24h with CsA 10nM and NGF 100ng/ml alone or in combination. CD1 and p21 protein levels were determined by WB. β-Actin was used as loading control. Immunoblots show a single representative of 3 separate experiments. The upper panels represent the means ± SD of 3 separate experiments between CD1/β-Actin and p21/β-Actin levels in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control (100%). *p<0.05; **p<0.05 compared with CsA-treated cells. **(E)** HK-2 cells, transiently transfected with p21 promoter, were untreated (c) or treated for 15h with CsA 10nM, NGF 100ng/ml, Mithramycin (M) 100nM alone or in combination and then luciferase activity was measured. Luciferase activity of untreated cells was set as 1-fold induction, upon which treatments were calculated. *p<0.05 compared with c; **p<0.05 compared with cells CsA+NGF-treated. Bars represent the means ± SD of 3 different experiments each performed in triplicate. **(F)** HK-2 cells were untreated (c) or treated with CsA 10nM, NGF 100ng/ml alone or in combination for 15h before lysis. Nuclear and cytosolic expression of NFATc1 and Sp1 were evaluated. Lamin B and β-Actin were used respectively as nuclear and cytosolic loading controls. The upper panels represent the means ± SD of 3 separate experiments between Sp1/Lamin B or Sp1/β-Actin and NFATc1/Lamin B or NFATc1/β-Actin levels in which band intensities were evaluated as optical density arbitrary units and expressed as percentages of the control (100%). *p<0.05 compared with c; **p<0.05 compared with cells treated with CsA alone **(G)** HK-2 cells were untreated (c) or treated with CsA 10nM, NGF 100ng/ml alone or in combination for 24h. Total cell lysates were immunoprecipitated with anti-Sp1 antibody (IP:Sp1) and then subjected to immunoblot analyses with anti-NFATc1 and anti-Sp1 antibodies. The upper panels represent the means ± SD of 3 separate experiments between NFATc1/Sp1 levels in which band intensities were evaluated as optical density arbitrary units and expressed as percentages of the control (100%). *p<0.05 vs untreated cells; **p<0.05 vs cells CsA +NGF-treated. All immunoblots show a single representative of 3 separate experiments.

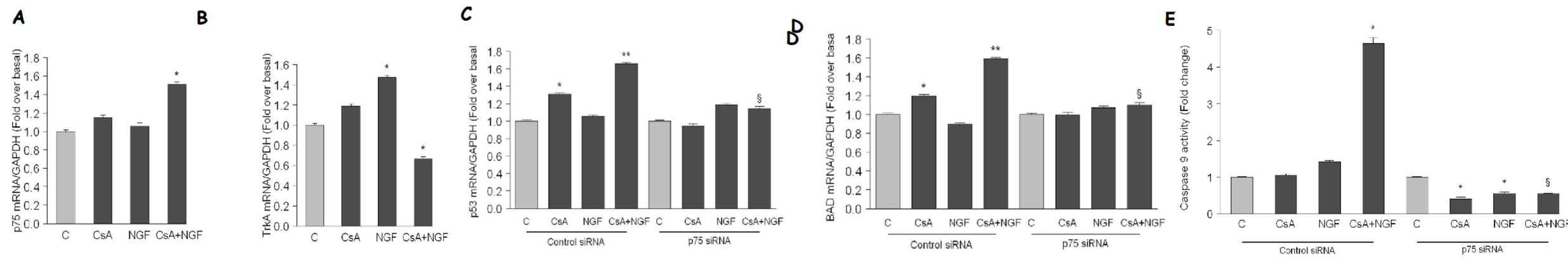

Figure 3. Combined treatment with CSA plus NGF activates intrinsic apoptosis via p75NTR. Real time RT-PCR analysis of $p75^{NTR}$ **(A)** and $TRkA^{NTR}$ **(B)** mRNA treated for 24h with CsA 10nM and NGF 100ng/ml alone or in combination. *p<0.05 compared with c; **p<0.05 compared with cells treated with CsA alone. p53 **(C)** and BAD **(D)** mRNA levels in HK-2 cells transfected with $p75^{NTR}$-siRNA or control-siRNA for 72 hours followed by CsA 10nM and NGF 100ng/ml treatment alone or in combination for 24 hours. *p<0.05 compared to untreated cells (c), **p<0.05 compared with CsA-treated cells; § p<0.05 compared with cells CsA+NGF-treated and transfected with control-siRNA. All mRNA sample were normalized to its GAPDH mRNA content. Bars represent the means ± SD of 3 independent experiments, each performed in triplicate. **(E)** caspase 9 activity in HK-2 cells transfected for 72 h with siRNA targeted human $p75^{NTR}$ mRNA sequence or with a control siRNA and then treated with CsA 10nM and NGF 100ng/ml alone or in combination for 72h. *p<0.05 combined-treated versus untreated cells; § p<0.05 compared with cells transfected with a control siRNA and treated with CsA+NGF; NS: not significant. The results represent the means ± SD of 3 independent experiments, each performed in triplicate.

RESULTS

Our results showed that in HK-2 cells combined treatment with CsA+NGF induced a significant reduction in cell viability concomitantly with a down-regulation of Cyclin D1 and up-regulation of p21 levels respect to cells treated with CsA alone. Moreover functional experiments showed that the co-treatment significantly up-regulated human p21promoter activity by involvement of the Sp1 transcription factor, whose nuclear content was negatively regulated by activated NFATc1. In addition we observed that the combined exposure to Cyclosporine A + nerve growth factor promoted an up-regulation of p75NTR and its target genes, p53 and BAD leading to the activation of intrinsic apoptosis. Finally, the chemical inhibition of p75NTR down-regulated the intrinsic apoptotic signal.

CONCLUSIONS

Our study demonstrate two new mechanisms by which NGF promotes growth arrest and apoptosis in tubular renal cells exposed to CsA, suggesting that NGF serum levels may be monitored during CsA treatment.

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