

Short communication

SODIUM NITROPRUSSIDE, A NITRIC OXIDE DONOR, FAILS TO BYPASS THE BLOCK OF NEURONAL DIFFERENTIATION IN PC12 CELLS IMPOSED BY A DOMINANT NEGATIVE RAS PROTEIN

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Abstract: Nitric oxide (NO) is a mediator of a diverse array of inter- and intracellular signal transduction processes. The aim of the present study was to analyze its possible role as a second messenger in the process of neuronal differentiation of PC12 pheochromocytoma cells. Upon NGF treatment wild-type PC12 cells stop dividing and develop neurites. In contrast, a PC12 subclone (designated M-M17-26) expressing a dominant-negative mutant Ras protein keeps proliferating and fails to grow neurites after NGF treatment. Sodium nitroprusside (SNP), an NO donor, was found to induce the p53 protein and to inhibit proliferation of both PC12 and M-M17-26 cells, but failed to induce neuronal differentiation in these cell lines. Key signaling pathways (the ERK and Akt pathways) were also not affected by SNP treatment, and the phosphorylation of CREB transcription factor was only slightly stimulated. It is thus concluded from the results presented in this paper that NO is unable to activate signaling proteins acting downstream or independent of Ras that are required for neuronal differentiation.

Key words: Nitric oxide, Sodium nitroprusside, PC12 cells, Nerve growth factor, Neuronal differentiation, Dominant inhibitory Ras protein, p53 protein, ERK proteins, Akt protein, CREB protein

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Abbreviations used: cGMP – cyclic guanosine monophosphate; CREB – cAMP-responsive element binding protein; ERK – extracellular signal-regulated kinase; MAPK – mitogen-activated protein kinase; NGF – nerve growth factor; NO – nitric oxide; NOS – nitric oxide synthase; PI3K – phosphatidylinositol 3-kinase; SNP – sodium nitroprusside

INTRODUCTION

Nitric oxide (NO) is a small, diffusible, membrane-permeable regulatory molecule involved in diverse processes of intercellular and intracellular signaling [for reviews see 1, 2]. Endogenous NO is produced from L-arginine by members of the family of nitric oxide synthases (NOS): endothelial NOS, neuronal NOS and inducible NOS. NO, as a second messenger, exerts its effects through various mechanisms [2]. Activation of guanylate cyclase generates cyclic guanosine monophosphate (cGMP) that stimulates protein phosphorylation via protein kinase G. This pathway is not critical in differentiation [3, 4]. cGMP-independent mechanisms include covalent modifications of target proteins. S-nitrosylation of specific cysteine residues and nitration of proteins on tyrosine or tryptophan lead to modification of target protein conformation and thus modulation of their activity [for reviews see 5-7]. Nitrosylation of a large number of proteins which participate in differentiation has been described (Ras, extracellular signal-regulated kinase [ERK], cyclin D1, tubulin, etc) [8], although the physiological significance of nitrosylation of specific proteins in differentiation signaling remains to be clarified. The effects of NOS activation can be mimicked by NO donors. Sodium nitroprusside (SNP) was used as an NO donor in this study to analyze the possible role of NO in the process of neuronal differentiation.

As a cell culture model for neuronal differentiation, the PC12 rat pheochromocytoma cell line [9] has long been used to study nerve growth factor (NGF) signaling [for reviews, see 10, 11]. Naive PC12 cells resemble chromaffin cells that enter quiescence and then develop neurites upon NGF treatment. In NGF-stimulated PC12 cells activation of the Ras-ERK pathway [12] leads to induction of neuronal NOS [13] generating NO that has been described to inhibit cell proliferation [14]. The cessation of proliferation of PC12 cells caused by NGF treatment is accompanied by NO-mediated induction of p53 and the cyclin dependent kinase (Cdk) inhibitor p21WAF1 [15]. Activation of NOS and production of NO appear to be essential for NGF signaling: NOS inhibitors (*N_o*-Nitro-L-arginine methyl ester [L-NAME] and *s*-methylisothiourea) were found to attenuate neurite outgrowth caused by NGF treatment [4].

Sustained activation of the Ras protein is critical in NGF-induced neuronal differentiation: inhibition of Ras function results in blockade of neurite outgrowth [16]. In M-M17-26 cells, a cell line stably expressing a dominant negative mutant of H-Ras, NGF-mediated activation of the ERK pathway is strongly inhibited [17, 18]. Neither induction of p21WAF1 nor cessation of proliferation takes place after NGF treatment in this cell line [19, 20]. The blockade of NGF-induced differentiation can be overcome by combined treatment with NGF and the second messenger analog dibutyryl cyclic AMP (dbcAMP) or ionomycin (a Ca²⁺ ionophore) in this cell line [21]. Thus the activation of the cAMP or Ca²⁺/calmodulin pathways may activate signaling molecules that are able to bypass the block of ERK activation [22]. The

transcription factor CREB is a candidate to mediate this neuritogenic effect: it is phosphorylated on Ser133 by a series of protein kinases: protein kinase A, Akt, Ca²⁺/calmodulin-dependent kinase, p90Rsk, an ERK-activated protein kinase, protein kinase G and MSK1, a kinase activated during NGF treatment through the ERK and p38MAPK pathways [23, 24]. NO is known to activate both of these MAPKs in higher doses [25], to activate the cGMP-protein kinase G pathway [2] and to stimulate phosphorylation of CREB [26].

The aim of the present study was to determine whether long-term exposure of NGF-treated PC12 cells to an NO donor is able to overcome the differentiation blockade imposed by the dominant inhibitory Ras protein. We also analyzed the effect of SNP on some of the key proteins involved in signaling from the NGF receptor, acting downstream or independently of Ras. We found that NO alone or in combination with NGF is not able to overcome the block in Ras-ERK pathway activation and neurite formation.

MATERIALS AND METHODS

Cell culture

Wild-type PC12 and M-M17-26 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose supplemented with 5% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum (referred to as high serum medium). DMEM and sodium nitroprusside were purchased from Sigma-Aldrich (Budapest, Hungary), animal sera and NGF from Invitrogen (Paisley, Scotland, UK).

Neuronal differentiation assay

10³ cells per well were seeded in twenty-four-well plates. Next day the cultures were treated in high serum medium as indicated in the figures. The culture media were changed every three days. Treatments were carried out in duplicate. After the indicated times, 200 cells per well were counted. Cells having neurites longer than their diameter were scored as differentiated. Statistical significance was analyzed using Student's t-test ($p < 0.05$).

Proliferation assay

5x10³ cells per well were seeded in twenty-four-well plates. Next day the cultures were treated in high serum medium as indicated in the figures. The culture media were changed every three days. After six days, cells were trypsinized, collected and cell numbers were determined by hemocytometric quantitation. Statistical significance was analyzed using Student's t-test ($p < 0.05$).

Western blot analysis

5x10⁶ cells per plate were seeded in 100-mm plates, and treated on the next day as indicated in the figures. After treatment cells were harvested and protein extracts were prepared using the protocols provided with the antibodies. 30-50 µg protein extracts were electrophoresed in 10-15% polyacrylamide gels

and transferred to polyvinylidene difluoride (PVDF) membrane (Pierce Biotechnology, Rockford, IL, USA). Blots were treated with the appropriate antibodies and visualized using ECL reagent (Millipore Corporation, Billerica, MA, USA). The blots were visualized using the Kodak Image Station 440 gel documentation system (New England Nuclear, Boston, MA, USA). Anti-ERK1 and anti-p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-P-CREB (Ser133), anti-CREB, anti-P-Akt (Ser473) and anti-P-ERK antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Horse radish peroxidase conjugated secondary antibody (anti-rabbit) was purchased from Pierce Biotechnology (Rockford, IL, USA). Statistical significance was analyzed using Student's t-test ($p < 0.05$).

RESULTS AND DISCUSSION

Non-toxic concentrations of SNP inhibit cell proliferation and induce the p53 protein in a Ras-independent manner

Cessation of proliferation upon NGF treatment is an important factor in NGF-induced neurite formation in PC12 cells [27]. Inhibition of Ras function eliminates this anti-mitogenic effect of Ras [19]. We wanted to find out if non-toxic concentrations of SNP can provide the cell-cycle arrest required for subsequent neurite outgrowth. 50 μ M SNP induced decreased cell proliferation of both wild-type PC12 and M-M17-26 cells after 6 days of treatment without signs of cytotoxicity (Fig. 1A). As NO generated from SNP is known to induce the p53 protein [28], we tested whether this induction is Ras-dependent.

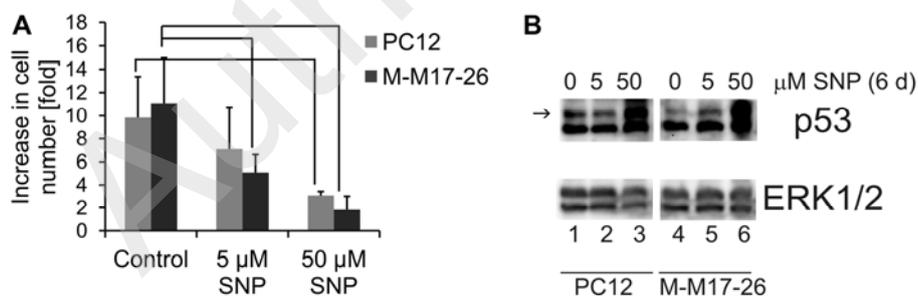


Fig. 1. The effect of long-term SNP treatment on cell proliferation (A) and on the expression of p53 protein (B) of wild-type PC12 and M-M17-26 cells. Cells were cultured without treatment or were treated with 5 μ M or 50 μ M SNP for 6 days as indicated in the figures. A – The total number of cells was determined after 6 days of SNP treatment. Significant differences ($p < 0.05$) are indicated. B – Plates were harvested at the end of treatment and cell lysates were processed for Western blot analysis with anti-p53 antibody. The full-length, transcriptionally active isoform of p53 is indicated by the arrow. Anti-ERK 1/2 antibody was used as a loading control.

Cessation of proliferation by SNP was accompanied by strong and prolonged induction of the p53 protein both in wild-type PC12 cells and in the M-M17-26

subclone (Fig. 1B). The transcriptionally active isoform of p53 is indicated by an arrow in Fig. 1B. The lower band may correspond to an alternatively spliced variant that has altered transcription factor activity [29]. SNP thus triggers cell cycle arrest Ras-independently, presumably via a p53-mediated mechanism.

SNP is not able to overcome the blockade of NGF-induced differentiation in M-M17-26 cells

PC12 cells stop proliferating as they undergo neuronal differentiation following NGF treatment [27]. Cell cycle arrest is mediated by the production of NO [14]. In contrast, M-M17-26 cells continue to proliferate and fail to differentiate following NGF treatment [19, 20]. In order to test whether NO is able to overcome the inhibition of neuritogenesis by the dominant negative Ras protein, the effect of combined SNP and NGF treatment on the differentiation of M-M17-26 cells was analyzed. In the parental PC12 cell line SNP alone did not induce neuronal differentiation (Fig. 2A). Although in an earlier study two days of combined treatment with an NO donor (sodium nitrite or SNP) and NGF increased the number of neurites two-fold in wild-type PC12 cells compared to cells treated with NGF alone [30], in our study SNP did not affect neuritogenesis: after six days of treatment there was no significant difference in the rate of differentiated cells treated only with NGF, or NGF and low (0.5 or 5 μ M) concentrations of the NO donor (Fig. 2A). 50 μ M SNP significantly ($p < 0.05$) inhibited NGF-induced differentiation. A possible explanation for this observation is that SNP, like other nitric oxide donors, inhibits ATP synthesis [31] even in non-toxic concentrations (J.V., unpublished result) and this can lead to inhibition of differentiation. In M-M17-26 cells neither NGF [16] nor SNP alone were able to induce neurite formation. Furthermore, combined treatment with SNP and NGF failed to overcome the blockade of NGF-induced differentiation imposed by the dominant negative mutant Ras protein (Fig. 2B).

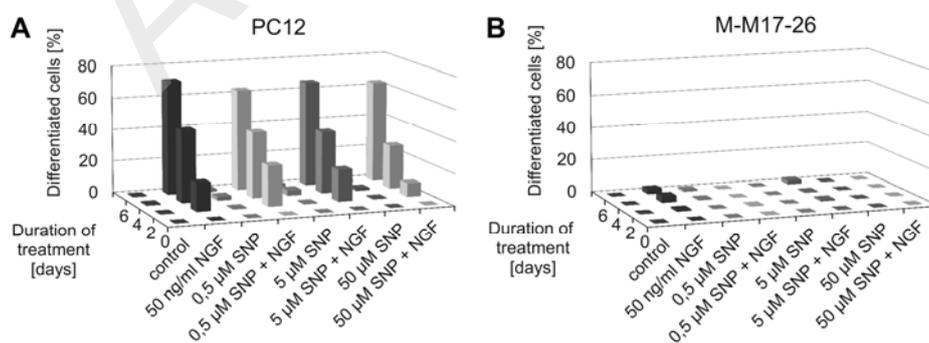


Fig. 2. The effect of SNP on NGF-induced neuronal differentiation of wtPC12 (A) and M-M17-26 cells (B). Cells were grown without treatment or treated with either 0.5, 5 or 50 μ M SNP or with NGF (50 ng/ml) alone or in combination as indicated, and scored for neurite outgrowth every second day.

SNP is not able to overcome the blockade of NGF-induced ERK activation in M-M17-26 cells

The cause of the failure of differentiation in M-M17-26 cells may be the consequence of the lack of activation of signaling protein(s) that mediate neuronal differentiation. Early events during signal transduction from the NGF receptor are essential to mediate the more delayed phenotypic effects (differentiation, survival, etc.) of NGF in PC12 cells. Neuritogenesis in PC12 cells requires the early, sustained activation and nuclear translocation of ERK, an important downstream effector of Ras proteins [32, 18]. Therefore, the ability of SNP to cause ERK phosphorylation in M-M17-26 cells was tested. 30 min of NGF treatment, as expected, strongly stimulated ERK phosphorylation in wild-type PC12 cells (compare lanes 1 and 4 in Fig. 3A and B), and it was almost completely blocked in the M-M17-26 clone (lane 7 vs lane 10). In contrast, non-toxic concentrations (5 and 50 μ M) of SNP alone hardly affected the level of ERK phosphorylation in PC12 (lanes 2 and 3 in Fig. 3A and B) and in M-M17-26 cells (lanes 8 and 9). In the parental PC12 cell line the NGF-caused strong ERK phosphorylation was not influenced by low concentrations of SNP (lanes 5 and 6) and SNP was not able to bypass the block of NGF-induced ERK phosphorylation in M-M17-26 cells (lanes 11 and 12). NO was reported to nitrosylate Ras proteins on Cys118 that helped the activation of Ras by promoting the dissociation of GDP from it [33, 34]. However, this modification is not sufficient to activate the dominant negative Ras protein as it does not bind Raf following treatment with an NO donor [35]. The results presented in Fig. 3 are in agreement with these earlier findings.

SNP does not influence NGF-mediated Akt phosphorylation

In contrast to the Ras-ERK pathway, the phosphatidylinositol 3-kinase (PI3K) signaling route is stimulated by NGF in PC12 cells in a Ras-independent manner [36]. Although activation of this pathway is not sufficient for NGF-induction of neurite growth, it still may contribute to the process of neuronal differentiation. Therefore, the early effect of NGF and/or SNP was tested on the phosphorylation of Akt protein kinase, a key component of the PI3K pathway (Fig. 3A and B). As expected, NGF caused Ras-independent phosphorylation of Akt after 30 min of NGF treatment (compare lanes 1, 4, 7 and 10). SNP did not stimulate significantly Akt phosphorylation if used alone (lanes 2, 3, 8 and 9) or in combination with NGF (lanes 5, 6, 11 and 12). We may thus conclude that the NGF-stimulated activation of two essential protein kinases of NGF signaling, ERK, a downstream effector of Ras, and Akt, acting Ras-independently, is not influenced by NO in PC12 cells.

SNP is not able to overcome the blockade of NGF-induced CREB phosphorylation in M-M17-26 cells

Since several signaling pathways converge on CREB (e.g. the cGMP-protein kinase G, cAMP, Ca⁺⁺-calmodulin, PI3K-Akt pathways) [24], some of them being potential targets of NO, we tested the combined effect of SNP and NGF on early CREB phosphorylation. Basal phosphorylation of CREB is two-fold higher in M-M17-26 cells than in PC12 cells. Short-term SNP treatment caused a slight Ras-dependent increase in CREB phosphorylation (compare lanes 1 vs 2 or 3 in Fig. 3A and B). As expected, NGF treatment increased CREB phosphorylation in PC12 cells (compare lanes 1 and 4). Combined treatment with SNP and NGF had no synergistic effect on CREB phosphorylation in these cell lines (lanes 4 vs 5, 6, and 10 vs 11, 12). CREB phosphorylation thus did not increase in M-M17-26 cells even upon combined NGF and SNP treatment.

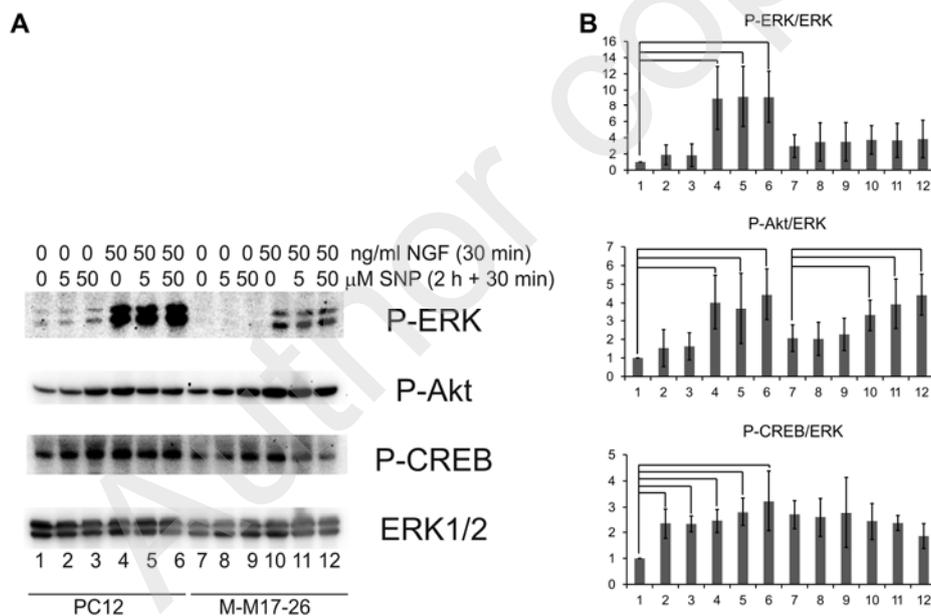


Fig. 3. The effect of short-term NGF and SNP treatment on the phosphorylation of ERK, Akt, and CREB in wild-type PC12 and M-M17-26 cells. Cells were left untreated or were pretreated with 5 μM or 50 μM SNP for 2 hours. NGF treatment was performed for the next 30 minutes in the presence or absence of SNP as indicated in the figure. Plates were harvested at the end of treatment and cell lysates were processed for Western blot analysis with antibodies specific for P-ERK, P-Akt and P-CREB. Anti-ERK 1/2 antibody was used as a loading control (A). Experiments to analyze the short-term effect of SNP and NGF on ERK, Akt and CREB phosphorylation were repeated at least 3 times, and subjected to densitometric analysis. Samples are numbered as in chart A (B). Significant changes ($p < 0.05$) are indicated. There are no significant changes in the amounts of Akt or CREB vs ERK upon NGF and/or SNP treatment (data not shown).

The results presented in this paper thus indicate that NO, although triggering mitotic arrest presumably by the induction of the p53 protein, fails to synergize with NGF to bypass the block of neuronal differentiation caused by inhibition of Ras function. This failure can be explained by the inability of NO to stimulate proteins acting in NGF signaling downstream (ERK) or independently of Ras (Akt).

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