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Exogenous, but not endogenous, nitric oxide increases proliferation rates in senescent human fibroblasts

Susanne Gansauge, Frank Gansauge, Andreas K. Nussler, Bettina Rau, Bertram Poch, Michael H. Schoenberg, Hans G. Beger*

Division of Molecular Oncology, Department of General Surgery, University of Ulm, Steinhövelstr. 9, 89075 Ulm, Germany

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Abstract We investigated the effects of endogenously produced and exogenously applied nitric oxide (NO) on cell proliferation rates and cell cycle regulation in senescent human fibroblasts (WI38). Induction of inducible nitric oxide synthase by tumor necrosis factor-α, interferon-γ and interleukin-1β inhibited cell proliferation and led to a G1 arrest. These effects were partially reversible by N^G-monomethyl-arginine (NMA). Addition of the NO donors sodium nitroprusside (SNP) or *S*-nitroso-*N*-acetyl-penicillamine (SNAP) increased cell proliferation rates as well as the S/G2 fraction. This points to a functional role of NO in cell cycle regulation and cell proliferation in human fibroblasts which depends on the mode of NO generation as well as the culture conditions used.

Key words: Fibroblast; Human; Nitric oxide; Proliferation

1. Introduction

Nitric oxide (NO) is a messenger molecule with complex biological activities contributing, among others, to immune regulation and inflammation. It also acts as a regulator of the vascular tone and as a neurotransmitter [1]. NO is synthesized enzymatically from L-arginine by at least three different NO synthases. The endothelial and neuronal isoforms are constitutively expressed; the third isoform has to be induced with stimuli including lipopolysaccharide and cytokines. Once expressed, this enzyme synthesizes large amounts of NO which is involved in the inhibition of T-cell proliferation, tumoricidal activity, suppression of cellular protein synthesis and oxidative damage [1–3]. Recently, it has been shown that inducible nitric oxide synthase (iNOS) mRNA and protein can be induced in mouse lung fibroblasts and rat fibroblasts by tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-γ (IFN-γ) leading to the release of large amounts of nitrate and nitrite implying the production of NO [4].

The effects of NO are different in diverse types of cells. Following exposure to exogenously applied NO, aortic smooth muscle cells as well as endothelial cells were inhibited in cell proliferation, whereas proliferation of K562 cells was not inhibited by NO [5–7]. By contrast, endogenously produced NO had cytoprotective functions in hepatocytes [8], whereas hematopoietic cells underwent apoptosis after induction of iNOS [9].

In the present study we show that exogenously applied NO increases proliferation in the human lung fibroblast cell line WI38 whereas endogenous production of NO by induction of iNOS inhibits cell proliferation.

2. Materials and methods

2.1. Culture conditions for stimulation assays

For all experiments WI38 human embryonal fibroblasts from passages higher than 38 were used. WI38 cells were seeded in 10 cm Petri dishes at a density of 1×10^6 cells/7 ml and cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM, Serva, Heidelberg, Germany) supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin and glutamine (Biochrom, Berlin, Germany). The cells were incubated at 37°C in 5% CO_2 atmosphere. Semiconfluent cultures were incubated with the extracellularly applied NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP, 1 mM) or sodium-nitroprusside (SNP, 1 mM) (both Sigma, Taufkirchen, Germany). For scavenging extracellularly applied NO 1×10^6 cells were incubated with either SNAP (1 mM) or SNP (1 mM) in culture medium containing human red blood cells (RBC) (culture medium:RBC = 6:1).

The endogenous NO production was induced with a cytokine mixture containing IFN-γ (200 U/ml, R&D Systems, Minneapolis, MN, USA), TNF-α (1000 U/ml, R&D Systems) and IL-1β (10 U/ml, Amersham, Braunschweig, Germany). Inhibition of iNOS activity was performed by addition of 1 mM N^G-monomethyl-arginine (NMA, Sigma).

For subsequent molecular analyses the cells were removed from the dishes by trypsinization or by mechanical scraping. All results were validated in at least three independent experiments.

2.2. Determination of nitrate and nitrite concentrations

To determine the amount of NO produced by human pancreatic carcinoma cell lines upon cytokine stimulation, 24 h after stimulation culture supernatants were assayed for the stable end products of NO oxidation, nitrite and nitrate, using an automated HPLC procedure based on the Griess reaction, as previously described [10].

2.3. Proliferation assay

The proliferation was determined by BrdU incorporation and subsequent BrdU detection using a commercially available BrdU-labeling and detection kit (Boehringer-Mannheim, Mannheim, Germany). The cells were processed according to the manufacturer's protocol.

2.4. RT-PCR of iNOS

For the molecular detection of iNOS total RNA was isolated from the cells as described previously. Reverse transcription was performed using the SUPERSCRIPT TM 2 Rnase H Reverse Transcriptase (Gibco, Berlin, Germany) according to the protocol of the manufacturer. PCR was carried out using the primers 5′-GCCTCGCTCTGGAAAGA-3′ and 5′-TCCATGCAGACAACCTT-3′, amplifying a 499 bp product [11] under the following conditions: 35 s at 96°C followed by 2 min at 56°C and 2 min at 72°C in a total of 30 cycles. β-Actin was used as an internal standard in each experiment for confirmation of an equal loading of the gel, according to a recently published protocol [12].

2.5. Determination of cell viability

For determination of cell viability, cells were removed by trypsinization and stained with Trypan blue (Sigma) and the numbers of viable and dead cells were determined by light microscopy.

*Corresponding author. Fax: (49) 731-502-7209

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2.6. Apoptosis assay

Apoptosis was determined by TUNEL reaction (TdT-mediated dUTP nick end labeling) using an in situ cell death detection kit (fluorescein) from Boehringer-Mannheim according to the manufacturer's protocol. In brief, following trypsinization, the cells were washed 2 times in PBS/1% BSA. After fixation with 4% paraformaldehyde in PBS for 30 min at room temperature, cells were washed in PBS/1% BSA. For permeabilization cells were incubated in 0,1% Triton X-100 for 2 min on ice. After two washing steps TUNEL reaction mixture was added and the cells were subsequently analysed by flow cytometry using an FACScan (Becton Dickinson, Germany).

2.7. DNA extraction for nucleosomal ladder

Cells were digested in a solution of 5% Sarcosyl, 50 mM Tris-HCl, and 10 mM EDTA containing 20 U of proteinase K at 50°C with shaking for 12 h. Digestion was continued for 1 h after addition of 10 μg of Dnase-free Rnase. After digestion, DNA was extracted sequentially with phenol and phenol/chloroform/isoamyl alcohol and chloroform and then ethanol precipitated and resuspended in 10 mM Tris-HCl (pH 8.0)/1 mM EDTA. Electrophoresis was carried out on 10 μg from each DNA sample on a 1.0% agarose gel containing 0.1 μg/ml ethidium bromide and photographed under UV light.

2.8. Cell cycle analysis

For cell cycle analysis, nuclei of the cells were isolated and stained with propidium iodide using the Cycle Test Plus (Becton Dickinson, Germany). Analysis was performed by flow cytometry using the Cell Quest programm (Becton Dickinson).

3. Results

3.1. iNOS is inducible by cytokine stimulation

Stimulation of WI38 cells with a cytokine mix containing INF-γ, IL-1β and TNF-α resulted in detectable iNOS levels as determined by RT-PCR (Fig. 1A). First signals were obtained 2 h after stimulation lasting for at least 24 h (data not shown). Measurement of nitrite and nitrate in the culture supernatant 24 h after cytokine stimulation revealed a significant increase in nitrite and nitrate which was largely blockable by addition of NMA ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 1B).

3.2. Exogenously applied NO but not endogenously produced NO increases proliferation in WI38 cells

Proliferation of WI38 cells was monitored by BrdU incorporation assays. After addition of cytokines proliferation decreased significantly as compared to negative controls, whereas this inhibition was reversed by addition of NMA, implicating that this proliferation inhibition was due to NO formation (Fig. 2). Exogenous addition of SNAP or SNP revealed a concentration dependent increase in proliferation during 24 h following stimulation. Low (0.1 mM) and high concentrations (5 mM) of either SNAP or SNP did not increase proliferation, whereas at a concentration of 1 mM SNAP or SNP proliferation of WI38 fibroblasts was significantly increased as compared to unstimulated cells. These effects of SNAP (1 mM) or SNP (1 mM) were reversed by the addition of human RBCs which scavenge NO radicals (Fig. 2).

3.3. Endogenous NO induces a G1 arrest and apoptosis in WI38 fibroblasts

Induction of iNOS followed by production of NO induced a G1 arrest and 48 h after addition of the cytokines only 3.4% of the cells were in S/G2-phase (control of unstimulated cells: 11.2%, CM+NMA: 7.2%) (Fig. 3). Starting at 24 h after stimulation, apoptosis occurred in 8.5% of the cells stimulated with cytokines (TUNEL reaction), whereas addition of

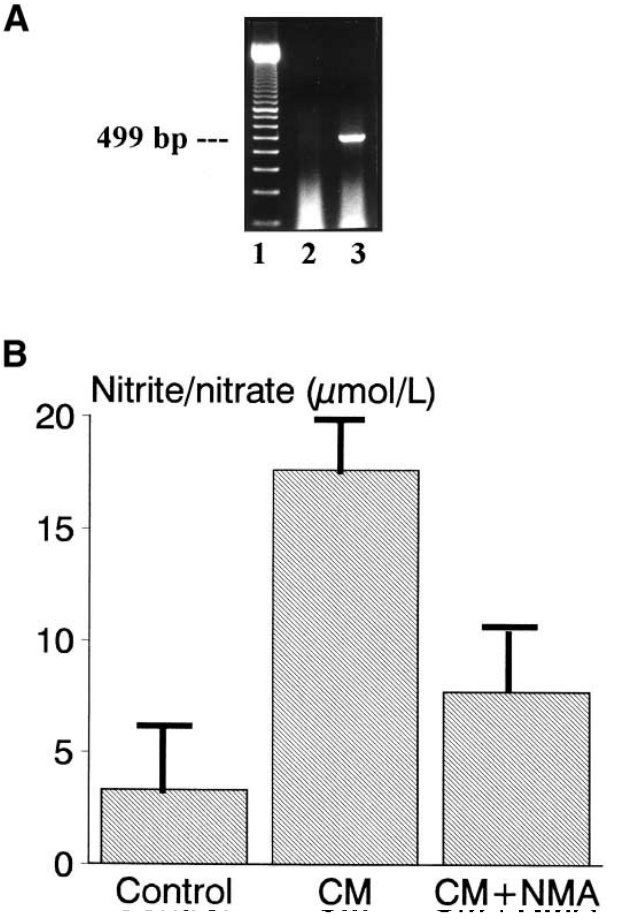

Fig. 1. A: Induction of iNOS mRNA in WI38 cells following stimulation with cytokines (TNF-α, IL-1β, IFN-γ). 4 h after stimulation with cytokines RT-PCR revealed a strong signal (lane 3). Lane 1: base marker, lane 2: control. B: Nitrite/nitrate concentrations of culture supernatants from WI38 cells incubated with cytokines (CM), and cytokines+NMA (CM+NMA). Nitrite/nitrate concentrations were significantly elevated after cytokine stimulation ($P < 0.001$). Experiments were performed in quadruplicate.

NMA reversed these effects (number of apoptotic cells: control, 24 h; 3.1% CM, 24 h; 8.5% CM+NMA, 24 h; 3.6% CM vs. control or CM+NMA, $P < 0.01$). After 48 h apoptosis occurred in 16.4% of the WI38 fibroblasts after CM stimulation and again addition of NMA to CM inhibited apoptosis (6.8% apoptotic cells). In controls 3.3% of the cells showed apoptotic features (CM vs. control or CM+NMA: $P < 0.01$) (Fig. 4). In all experiments the number of dead cells as determined by trypan blue staining did not differ significantly from the number of apoptotic cells.

3.4. Exogenous NO increases the S/G2-phase fraction in WI38 fibroblasts

Corresponding to the results from the BrdU incorporation assay, cell cycle analysis revealed an increase of the S/G2 fraction after addition of the exogenous NO donors SNAP or SNP. At a concentration of 1 mM SNAP or 1 mM SNP, 24 h after stimulation the S/G2 fraction increased after stimulation with SNAP or SNP and after 48 h 16.9% (SNAP) and 24.4% (SNP) (control 11.2%) of WI38 fibroblasts were in S/G2 phase (SNP vs. control: $P < 0.01$; SNAP vs. control: $P < 0.05$). These effects were reversed by the addition of RBCs (Fig. 5). Apoptosis or necrosis did not occur at a con-

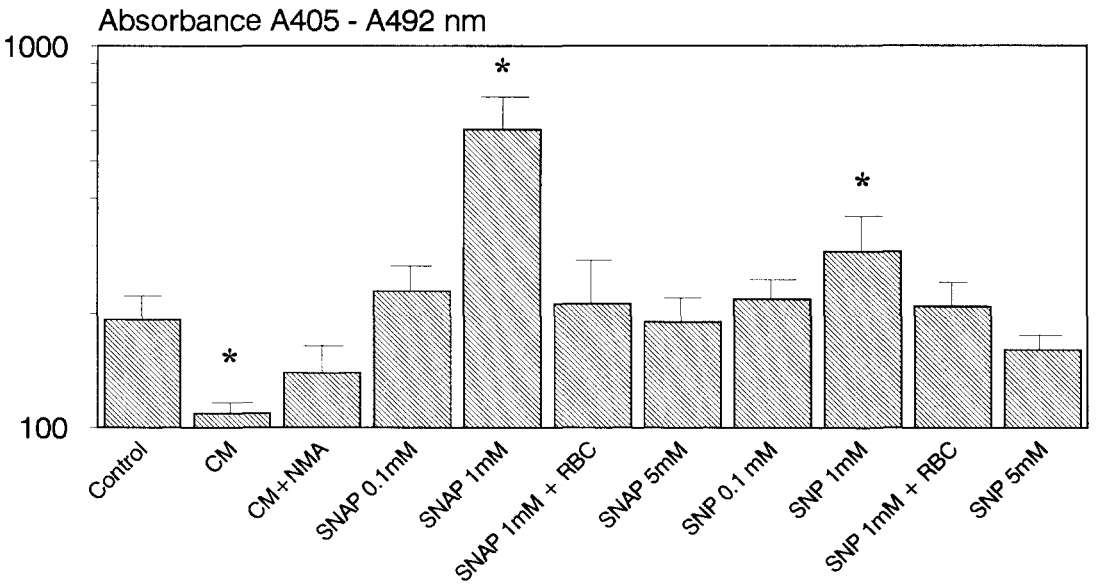

Fig. 2. BrdU incorporation assay of WI38 cells cultured for 24 h under various conditions. After cytokine stimulation (CM) proliferation was significantly reduced, whereas addition of 1 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP) or 1 mM nitroprusside (SNP) led to an increased proliferation (*$P < 0.001$ as compared to control). RBC: red blood cells.

centration of 1 mM SNAP or SNP. At higher concentrations of either SNAP (5 mM) or SNP (5 mM) apoptosis occurred in 14.4% (SD 3.1) and 16.2% (SD 2.7), respectively, whereas the number of dead cells was higher (5 mM SNAP, 21.3% (SD 4.5); 5 mM SNP, 20.1% (SD 5.3)), implying that direct toxic effects as well as apoptotic changes were caused by these high concentrations of exogenously applied NO.

4. Discussion

Thus far, the effects of NO on cell proliferation are different in several reports. In regard to endogenous NO production via induction of iNOS, the majority of reports demonstrated that the induction of iNOS leads to a growth arrest followed by apoptosis as shown in hematopoietic cells [9], peritoneal macrophages [13], cortical cells [14], mastocytoma cells [15], colon cancer cell lines [11] and pancreatic cancer cells [16]. However, transfection of iNOS in the human colon carcinoma cell line DLD-1 resulted in accelerated growth and better neovascularization of DLD-1 tumors grown in nude mice [17]. Recent reports also provide evidence for cytoprotective functions of NO in hepatocytes [8] and in endothelial cells [18]. In a rat ischemia–reperfusion study it was shown, that NO was not able to prevent ischemic damage but accelerated morphological repair and enhanced cell proliferation [19]. Similar results were obtained by Benrath and coworkers who could demonstrate a reduction in cell proliferation and wound healing in UV-damaged skin when iNOS activity was blocked by NMA [20].

In our study we were able to demonstrate that iNOS is inducible in the human embryonal fibroblast cell line WI38 resulting in the production of NO. This endogenous NO production led to a decreased cell proliferation and apoptosis in these cells. Since these effects were only partially inhibited by the addition of NMA one has to be aware that, besides the iNOS induction, other processes that influence cell proliferation and apoptosis probably have been initiated by the cytokines used. Interestingly, addition of the extracellular NO donors SNP and SNAP increased cell proliferation rates and did not induce apoptosis. In a previous report Firnhaber and Murphy exposed WI38 cells to exogenous NO donors [21]. In this study extracellularly applied NO had only limited effects on DNA synthesis and proliferation. Although we used

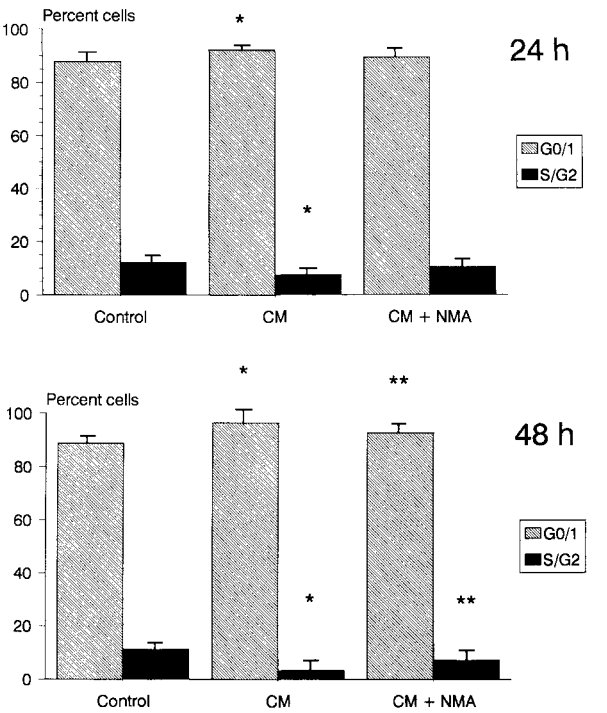

Fig. 3. Cell cycle analysis of WI38 cells 24 and 48 h after induction of endogenous NO production. At 24 h following CM stimulation the S/G2 fraction was reduced to 7.6% (control: 12.2%) and 48 h following CM stimulation the S/G2 fraction was reduced to 3.4% (control: 11.2%) indicating a G1 arrest. Inhibition of iNOS by addition of NMA partially reduced the G1 arrest (CM+NMA, 24 h; 10.4% S/G2, 48 h; 7.2% S/G2) (*$P < 0.01$ as compared to control; **$P < 0.05$ as compared to CM).

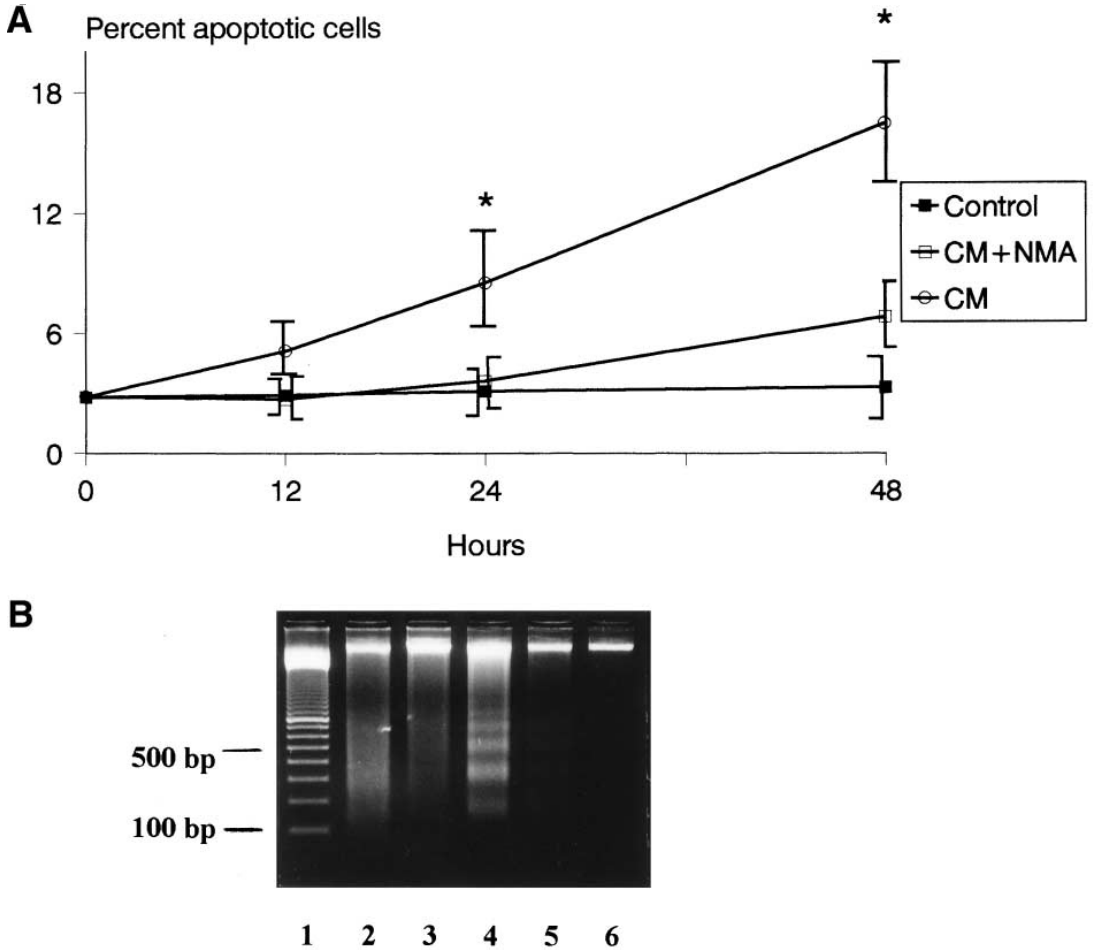

Fig. 4. A: Induction of apoptosis in WI38 fibroblasts after induction of endogenous NO production. After 48 h 16.4% of the cells underwent apoptosis after iNOS induction by cytokines whereas addition of NMA inhibited these effects (6.8%). $^{*}P < 0.01$. B: DNA integrity analysis of WI38 fibroblasts 48 h after treatment with 1 mM SNAP (lane 2), 1 mM SNP (lane 3), cytokines (lane 4), and cytokines plus NMA (lane 5). DNA laddering was only observed after induction of iNOS (lane 4). Addition of NMA inhibited the DNA laddering. Lane 1: base marker; lane 6: control (culture medium only).

the same cell line and the same NO donors we observed an increase in proliferation. Besides slight differences in model systems the major differences between our study and this previous report [21] were the age and the culture conditions we were using. All the WI38 cells used were from passages higher than the 38th passage, in all experiments cells were not growing logarithmically, and experiments were performed when semiconfluent layers were grown. Since WI38 cells are not transformed they have a finite lifetime of 50 ± 10 population doublings [22]. For that reason, the WI38 cells used represent a senescent fibroblast type with low spontaneous proliferation rates. Thus our study cannot be extrapolated to predict the effects of NO on other models of growth where their effects may be less [21]. In our experiments, DNA synthesis was

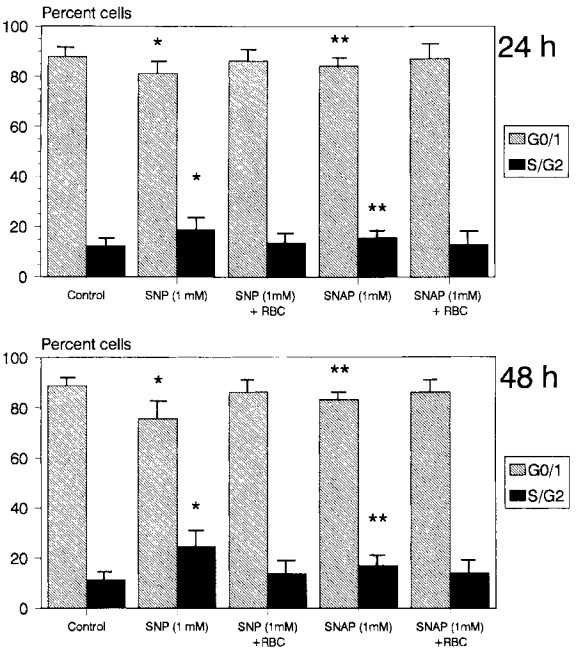

Fig. 5. Cell cycle analysis of WI38 cells 24 and 48 h after addition of SNP or SNAP. 24 h following addition of SNP or SNAP the S/G2 fraction increased to 18.8% and 15.7%, respectively (control: 12.2%) and 48 h following stimulation the S/G2 fraction increased to 24.4% and 16.9%, respectively (control: 11.2%) corresponding to the cell proliferation experiments (Fig. 2) ($^{*}P < 0.01$ as compared to control; $^{**}P < 0.05$ as compared to control). The increase in the S/G2 fraction after addition of SNP or SNAP was inhibited by red blood cells (RBC).

stimulated when senescent WI38 cells were exposed to exogenous NO, but it is unknown whether the same signalling pathways also stimulate DNA synthesis in transformed cells or during logarithmic growth. In any case, it may be worth noting that in parallel to our results regarding proliferation due to NO stimulation, the proliferative effects of oxygen radicals were also often demonstrated in quiescent or in transformed cells [23,24] whereas in logarithmically growing cells oxygen radicals reduced proliferation rates [21,25,26].

The rate of NO generation that increases proliferation rates in lung fibroblasts appears to be far above the amount they produce endogenously, making it unlikely that these cells regulate their own growth via NO production. However, lung fibroblasts may occasionally be exposed to higher levels of NO such as cigarette smoke and other types of air pollution, as well as NO generated by other cells within the lung, providing stimuli for fibroblast proliferation.

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