Nitric oxide inhibits irreversibly P815 cell proliferation: involvement of potassium channels

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Abstract. Nitric oxide (NO) has been shown to inhibit both normal and cancer cell proliferation. Potassium channels are involved in cell proliferation and, as NO activates these channels, we investigated the effect of NO on the proliferation of murine mastocytoma cell lines and the putative involvement of potassium channels. NO (in the form of NO donors) caused dose-dependent inhibition of cell proliferation in the P815 cell line inducing growth arrest in the mitosis phase. Incubation with NO donor for 4 or 24 h had a similar inhibitory effect on cell proliferation, indicating that this effect is irreversible. The inhibitory effect of NO was completely prevented by the blockade of voltage- and calcium-dependent potassium channels, but not by blockade of ATP-dependent channels. NO inhibition of cell proliferation was unaffected by guanylate cyclase and by cytoskeleton disruptors. Therefore, NO inhibits cell proliferation irreversibly via a potassium channel-dependent but guanylate cyclase-independent pathway in murine mastocytoma cells.

INTRODUCTION

Cell division and proliferation are basic events in the physiology of multicellular organisms. Like many other aspects of cell biology, proliferation is a tightly regulated series of events triggered by intra and extracellular signals (Nurse 2000). In some instances, these control mechanisms fail and a deranged cell proliferation becomes the basis of cancer growth (Johnson & Walker 1999).

Nitric oxide (NO) has been implicated in several physiological processes in the cardiovascular, immune and nervous systems. NO is synthesized in small amounts and for short time periods by the action of two constitutive and Ca^{2+} -dependent NO synthases (NOS), the endothelial and the neuronal isoforms. A third isoform (Ca^{2+} -independent and inducible, iNOS) produces much higher concentrations of NO over extended time periods (Moncada *et al.* 1991). Several effects of NO are mediated by guanylate cyclase activation (Ignarro 1991), whereas others are mediated by NO reaction with sulphhydryls from cysteine residues present in proteins and in low molecular weight thiols (Broillet 1999). One of the protein targets of NO reaction with thiols is potassium channels (Bolotina *et al.* 1994), which are also involved in cell proliferation (Wonderlin & Strobl 1996).

As there is an extensive literature indicating that NO has important inhibitory effects on cell proliferation in both normal (Sarkar & Webb 1998) and transformed cells (Hibbs *et al.* 1990),

we sought to investigate the relationship between cell proliferation, NO and potassium channels in mastocytoma cell lines.

MATERIALS AND METHODS

Cell culture

The mouse mastocytoma cell line P815 was obtained from the American Type Culture Collection (Rockville, MD). P815 and MCP-5 cells (Naveen *et al.* 1993) were maintained in plastic culture flasks in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM Hepes, pH 7.4 (referred to as DMEM), in a humidified incubator at 37 °C with a 5% CO₂ atmosphere. Every 3–4 days, cells were passaged by removing 90% of the supernatant and replacing it with fresh medium. For the experiments, cells were collected, centrifuged (250 × g, 10 min), washed once and resuspended in fresh medium. Cell viability was determined by Trypan Blue exclusion. A total of 5×10^4 viable cells/well were seeded in 200 µl medium in a 96-well plate. Test compounds were added in a maximum volume of 20 µl. For compounds soluble in DMSO, the same volume of this solvent was added to control wells. Cells were then returned to the incubator and left until the end of the experimental period.

Determination of cell number by crystal violet staining

A published method (Kueng *et al.* 1989) was modified as follows: cells were transferred to a V-shaped bottom 96-well plate, 20 µl of the crystal violet solution was added to each well to fix and stain cells and the plate was left for 30 min at 37 °C. The composition of the crystal violet solution was: 100 mg crystal violet, 30 ml ethanol, 50 ml formaldehyde (40% stock solution) and water to 100 ml. The following salts were dissolved in the solution to give final concentrations, in mM, of: NaCl 137, KCl 2.7, KH₂PO₄ 1.5 and NaHPO₄ 8.1, pH 7.4. This solution can be stored indefinitely in the refrigerator. The plate was centrifuged, the supernatant was removed and the excess dye was removed by two or three washes with deionized water until the supernatant was clear. Then, 200 µl of 1% (w/v) sodium dodecyl sulphate in deionized water were added to each well to solubilize the cell pellet, followed by homogenization and transferred to a flat-bottomed 96-well plate. The optical density was read at 550 nm in a plate reader. For evaluation of the correspondence between optical density and cell number, we conducted experiments in which known cell numbers were counted in a Neubauer chamber and processed for crystal violet staining.

Mitotic analysis

Cells were incubated with 100 µM SNAP for 24 h and then cytocentrifuge slides were prepared and stained with May-Grünwald-Giemsa. The percentage of mitotic figures in 100 cells was counted under the oil immersion objective of a light microscope.

MTT assay

Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-yl)-2-5-diphenyltetrazolium bromide] assay (Van De Loosdrecht *et al.* 1991). Briefly, at the end of the experiment the plate was centrifuged, the supernatant was removed, fresh medium was added, the plate was centrifuged again and fresh medium containing 10% (v/v) of an MTT solution (5 mg/ml in PBS) was added. The plate was incubated for 3 h at 37 °C, centrifuged again and the supernatant was removed. This washing procedure was necessary to avoid MTT oxidation by NO. Then, 100 µl of a 0.04-N solution of isopropyl alcohol/HCl was added to each well and the plate was left to stand for 10 min.

Cell debris was removed by centrifugation and the clear supernatant was read at 550 nm in a plate reader. The optical density of control groups was taken as 100% viable cells. In preliminary experiments, cell viability evaluated by MTT and Trypan Blue assays yielded similar results.

Materials

Tissue culture media, serum and antibiotics were from Gibco (São Paulo, Brazil). S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and S-nitrosoglutathione (GSNO) were synthesized in our laboratory according to published methods (Field *et al.* 1978; Rockett *et al.* 1991). SIN-1 [3-(4-morpholinyl)-sydnimine-hydrochloride] was kindly donated by Dr K. Schonafinger (Hoechst Marion Roussel, Frankfurt am Main, Germany). Tetraethylammonium (TEA; a nonselective blocker of potassium channels), 4-aminopyridine (4-AP; a selective blocker of voltage-sensitive potassium channels) and glibenclamide (GB; a selective blocker of ATP-dependent potassium channels) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Charybdotoxin (ChTx; a blocker of high conductance, calcium-activated and voltage-dependent potassium channels), iberiotoxin (IbTx; a selective blocker of high conductance calcium-activated potassium channels) and apamin (a selective blocker of the small conductance, calcium-activated potassium channels) were from Alomone Laboratories (Jerusalem, Israel). All solutions were prepared in culture medium except glibenclamide, which was dissolved in DMSO, and the toxins, which were dissolved in PBS containing 5 mg/ml bovine serum albumin.

Statistical analysis

Data are expressed as mean $\pm$ SEM of triplicate wells. Each experiment was repeated at least twice with similar results. Statistical significance was analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's posthoc *t*-test. A *P*-value of less than 0.05 was considered significant.

RESULTS

Validation of the crystal violet method

As can be seen in Fig. 1, there was a linear correlation between the logarithm of the cell number and the optical density as assessed by the crystal violet method. This method is easy to perform, cheap and more appropriate for large numbers of samples, than direct counting.

Anti-proliferative effect of NO on murine mastocytoma cell lines

NO can be cytostatic (Peunova & Enikolopov 1995) or cytotoxic (Albina & Reichner 1998) depending on its concentration. Therefore, to be sure that the effect of NO was due to inhibition of proliferation and not to a cytotoxic effect, P815 cells were incubated with a large range of SNAP concentrations from 10 to 1000 μ M for up to 48 h. Cell death was evident only at concentrations of 180 μ M or higher of SNAP (Costa & Assreuy, unpublished results). Therefore, in all further experiments, the maximal SNAP concentration employed was of 100 μ M. The NO donor induced an arrest in the cell cycle ($0.7 \pm 0.3\%$ versus $19 \pm 0.3\%$ of cells in mitosis in control and 100 μ M SNAP, respectively, after 24 h incubation) with most of the cells in metaphase (Fig. 2).

Anti-proliferative effects of NO donors

Next, we performed experiments designed to assess whether the antiproliferative effect of SNAP might be shared by other NO donors. The results are depicted in Fig. 3. As a positive control for

Figure 1. Validation of the crystal violet method in comparison with counting in Neubauer chambers. Known numbers of P815 viable cells (determined in Neubauer chambers) were plated in triplicate wells of a 96-well plate in 200 μ l medium. Each group was then fixed and stained with crystal violet solution, washed with distilled water, homogenized in SDS 1% (w/v) and centrifuged. The optical density was read at 550 nm in a plate reader. Each point represents the mean $\pm$ SEM of triplicates. When no error bar is visible, it was covered by the symbol. The x axis is in log scale and the correlation coefficient was 0.886.

Figure 2. SNAP arrests P815 cells in mitosis. Cells were incubated with SNAP (100 μ M) for 24 h and the mitotic figures were counted in slides stained with May-Grünwald-Giemsa as described in Materials and Methods section. Photographs were taken under oil immersion. Bar represents 20 μ m, total magnification $\times 1000$.

the absence of cell proliferation, we used the protein synthesis inhibitor cycloheximide which, in spite of completely blocking cell proliferation, did not induce cell death (as assessed by MTT or Trypan Blue assays), at least up to 48 h incubation. SNAP caused a concentration- and time-dependent inhibition of P815 cell proliferation, with maximal effect being achieved at 100 μ M/48 h incubation. A similar profile was seen for GSNO, although with a lower potency. Sodium nitroprusside also inhibited P815 cell proliferation although, unlike SNAP and GSNO, it was cytotoxic to P815 cells (43 and 48% cell death at 10 and 100 μ M, respectively, after 48 h incubation, $n = 3$). The antiproliferative effect of SNAP was not cell-specific, as proliferation

Figure 3. Concentration- and time-dependent effect of NO donors on P815 cell proliferation. Cells (5×10^4 cells/well; 200 μ l medium) were seeded and incubated with NO donors or medium for the indicated time periods. Then, cultures were fixed and stained with crystal violet solution, washed, homogenized and the optical density was read at 550 nm, as detailed in Materials and Methods section. In all panels, open circles represent cells incubated with medium alone and the closed diamonds represent cells incubated with cycloheximide (18 μ M). (a) SNAP at 10 (closed circles), 30 (squares) or 100 μ M (triangles). (b) GSNO at 100 (closed circles), 300 (squares) or 1000 μ M (triangles). (c) SNP at 1 (closed circles), 10 (squares) or 100 μ M (triangles). Points represent mean $\pm$ SEM of triplicates. When no error bar is visible, it was covered by the symbol. Similar results were obtained in at least two identical experiments. All curves in closed symbols were statistically different from the control curve, as determined by one-way analysis of variance (ANOVA) followed by Bonferroni's posthoc *t*-test.

Figure 4. Effect of SNAP on the proliferation of P815 and MCP-5 mastocytoma cell lines. P815 cells (a) and MCP-5 (b) (5×10^4 cells/well; 200 μ l medium) were seeded and incubated with SNAP at the indicated concentrations, cycloheximide (Cy; 18 μ M) or medium alone for 24 h. Cells were then fixed and stained with crystal violet solution, washed, homogenized and the optical density was read at 550 nm. Similar results were obtained in at least two identical experiments. Bars are mean $\pm$ SEM of triplicate wells. The symbol * means significant differences ($P < 0.05$) from each respective control group, using one-way analysis of variance (ANOVA) followed by Bonferroni's posthoc *t*-test.

of another mastocytoma cell line was also inhibited in a similar fashion to P815 cells (Fig. 4). In addition, the effects of SNAP and GSNO could be entirely attributed to NO as both NAP (the non-nitrosylated parent compound of SNAP) and GSH (the non-nitrosylated parent compound of GSNO) had no effect on cell proliferation (Table 1).

Table 1. Effects of N-acetyl-penicillamine (NAP), reduced glutathione (GSH) and SIN-1 on P815 cell proliferation

Compound (μM)	Optical density (550 nm)
None	0.61 ± 0.03
NAP 10	0.64 ± 0.05
100	0.59 ± 0.02
GSH 10	0.57 ± 0.03
100	0.72 ± 0.02
SIN-1 10	$0.31 \pm 0.02^a \#$
100	$0.30 \pm 0.07^a \#$

P815 cells were seeded, incubated with compounds for 24 h (NAP and GSH) or 48 h (SIN-1), fixed and stained with crystal violet, as detailed in Materials and Methods section. Data are mean $\pm$ SEM of triplicates. ^a $P < 0.05$ versus none (ANOVA followed by Bonferroni's posthoc *t*-test). #Indicated that the cell viability was reduced by 35–40% after 48 h incubation, as assessed by Trypan Blue exclusion (see Results section).

Table 2. Lack of effect of guanylate cyclase inhibition by ODQ on SNAP-induced decrease in P815 cell proliferation

Compound (μM)	Optical density (550 nm)
None	1.21 ± 0.04
SNAP 100	0.76 ± 0.02^a
ODQ 1	1.21 ± 0.04
10	1.11 ± 0.01
SNAP 100 + ODQ 1	0.71 ± 0.03^a
SNAP 100 + ODQ 10	0.81 ± 0.05^a

P815 cells were seeded, incubated with the compounds for 24 h, fixed and stained with crystal violet, as detailed in Materials and Methods section. Data are mean $\pm$ SEM of triplicates. ^a $P < 0.05$ versus none (ANOVA followed by Bonferroni's posthoc *t*-test). Cell viability was $> 95\%$ in all groups at the end of the experiment.

Effects of SIN-1 on P815 cell proliferation

The mixed NO/superoxide anion donor SIN-1 was tested against P815 proliferation. This compound substantially decreased cell proliferation (Table 1). However, simultaneous viability assay showed that SIN-1 also induced a substantial cell death (31 and 39% cell death at 10 and 100 μM , respectively, after 48 h of incubation, $n = 3$).

Effects of soluble guanylate cyclase inhibition on the antiproliferative effect of NO donors

Several NO effects are mediated by activation of guanylate cyclase and cGMP production (Ignarro 1991). The inhibitory effect of SNAP (~40%) on P815 cell proliferation was unaltered by the selective soluble guanylate cyclase inhibitor ODQ (Table 2). In addition, it seems that this cell line does not require guanylate cyclase activity for its normal proliferation, as ODQ did not affect cell proliferation when tested in the absence of the NO donor (Table 2).

Irreversibility of NO-induced inhibition of P815 cell proliferation

As depicted in Fig. 5, the same degree of cell proliferation inhibition as that induced by incubating SNAP with P815 cells for 24 h was obtained when the NO donor was left in contact with the cells for only 4 h.

Effects of cytoskeleton disrupting agents on the antiproliferative effect of NO donors

As NO interferes with the cytoskeleton (Eiserich *et al.* 1999), and because the co-ordinated reorganization of this structure is essential for cell proliferation (for example, see Sorger *et al.*

Figure 5. Irreversibility of SNAP-induced inhibition of proliferation in P815 cells. Cells (5×10^4 cells/well; 200 µl medium) were seeded and incubated with SNAP, cycloheximide (Cy; 18 µM) or medium for 4 h (closed bars) or for 24 h (open bars). Cultures were then fixed, stained with crystal violet solution, washed, homogenized and the optical density was read at 550 nm, as detailed in Materials and Methods section. Each bar represents mean $\pm$ SEM of triplicates. Similar results were obtained in at least two identical experiments. The symbols * and # mean significant differences ($P < 0.05$) from each respective control group and the symbol § means significant difference compared to cycloheximide incubated for 4 h, using one-way analysis of variance (ANOVA) followed by Bonferroni's posthoc *t*-test.

Table 3. Effects of cytochalasin B and colchicine on SNAP-induced inhibition of P815 cell proliferation

Compound (µM)	Optical density (550 nm)
None	0.87 ± 0.03
SNAP 100	0.53 ± 0.01^a
Cytochalasin B 1	1.00 ± 0.07
Cytochalasin B 10	0.86 ± 0.00
SNAP 100 + Cytochalasin B 1	0.54 ± 0.06^a
SNAP 100 + Cytochalasin B 10	0.46 ± 0.04^a
Colchicine 1	0.85 ± 0.01
Colchicine 10	0.72 ± 0.04
SNAP 100 + Colchicine 1	0.56 ± 0.02^a
SNAP 100 + Colchicine 10	0.55 ± 0.01^a

P815 cells were seeded and incubated with the compounds for 4 h with or without SNAP. Cells were then washed, resuspended in medium, incubated for a further 20 h, fixed and stained with crystal violet, as detailed in Materials and Methods section. Data are mean $\pm$ SEM of triplicates. ^a $P < 0.05$ compared to row None (ANOVA followed by Bonferroni's posthoc *t*-test). Cell viability was $> 95\%$ in all groups at the end of the experiment.

1997), we tested whether cytochalasin B (which depolymerizes microfilaments) and colchicine (which depolymerizes microtubules) affected SNAP-induced inhibition of P815 cell proliferation. The results of these experiments are shown in Table 3. SNAP-induced inhibition of cell proliferation ($\sim 40\%$ inhibition in relation to control) was not affected by the two compounds, at least within the 4-h period in which SNAP and the compounds were coincubated with the cells. As expected, when cells were incubated with cytochalasin B or colchicine (in the absence of the NO donor), for 24 h, proliferation was completely prevented (data not shown).

Figure 6. Effect of potassium channel blockers on SNAP-induced inhibition of P815 cell proliferation. Cells (5×10^4 cells/well; 200 μ l medium) were seeded and incubated with the compounds or medium for 24 h. Cultures were then fixed and stained with crystal violet solution, washed, homogenized and the optical density was read at 550 nm, as detailed in Materials and Methods section. In all panels, open circles represent the effect of SNAP alone at the indicated concentrations. (a) SNAP plus tetraethylammonium (TEA) at 10 μ M (triangles), 100 μ M (inverted triangles) and 10 mM (squares); (b) SNAP plus 4-aminopyridine (4-AP) at 1 μ M (triangles), 10 μ M (inverted triangles) and 100 μ M (squares); (c) SNAP plus glibenclamide (GB) at 10 μ M (triangles), 100 μ M (inverted triangles) and 1000 μ M (squares); (d) SNAP plus charybdotoxin (ChTx) at 100 nM (triangles); SNAP plus iberiotoxin (IbTx) at 100 nM (inverted triangles) and SNAP plus apamin at 100 nM (squares). Symbols represent mean $\pm$ SEM of triplicates. When no error bar is visible, it was covered by the symbol. Similar results were obtained in at least two identical experiments. All curves in closed symbols are statistically different ($P < 0.05$) from SNAP control curve (open symbols), except those depicted in (c) (SNAP plus glibenclamide), as determined by one-way analysis of variance (ANOVA) followed by Bonferroni's posthoc *t*-test.

Effect of potassium channel blockers on SNAP-induced inhibition of P815 cell proliferation

Potassium channels have been shown to be important for cell cycle progression (Wonderlin & Strobl 1996). Therefore, we examined the role of potassium channels in the antiproliferative effect of SNAP. TEA (a nonselective blocker of potassium channels) opposed SNAP-induced inhibition of cell proliferation (Fig. 6a). 4-aminopyridine (a blocker of voltage-sensitive potassium channels) displayed a similar effect in reversing the SNAP-induced inhibition (Fig. 6b), but glibenclamide (a blocker of ATP-dependent potassium channels) failed to do so (Fig. 6c). Charybdotoxin, iberiotoxin, and apamin reversed SNAP-induced inhibition of cell proliferation, indicating that potassium channel subtypes affected by these substances are also involved (Fig. 6d). None of the potassium channel blockers caused cytotoxicity or affected cell proliferation by themselves, at least within the range of concentrations employed (data not shown).

DISCUSSION

Nitric oxide has been reported as being both cytostatic and cytotoxic towards different mammalian cell types and tumour cell lines. For instance, NO inhibits proliferation of aortic vascular smooth muscle cells (Sarkar & Webb 1998) and leukaemia cell lines (Hibbs *et al.* 1990). Here we showed that the proliferation of two mouse mastocytoma cell lines was inhibited by exposure to NO donors. This effect was concentration-dependent and seems to be attributable to NO alone as NAP, the non-nitrosylated parent compound of SNAP, and GSH, the parent compound of GSNO, were devoid of any such effect. GSNO displayed essentially the same antiproliferative effect as SNAP, although it was less potent. This can be explained by the slower kinetics of NO release from GSNO ($t_{1/2}$ of 3 h) when compared to SNAP ($t_{1/2}$ of 1.3 h) (Arnelle & Stamler 1995). Sodium nitroprusside, considered to be a faster NO releaser ($t_{1/2}$ of minutes), also inhibited cell proliferation. However, SNP caused a substantial reduction in cell viability and thus the observed decrease in cell proliferation may be a consequence of a cytotoxic effect. In contrast, SNAP and GSNO did not cause cytotoxic effects, at least within the range of concentrations and observation times employed here.

The inhibitory effect of SNAP on P815 cell proliferation seems to be related to an interference with mechanisms involved in progression through the M phase as arrested cells were mostly seen in mitosis, specifically in metaphase.

Throughout the experimental period studied (up to 48 h), the proliferation of P815 cells exposed to SNAP was consistently below control levels. Taking into account that most NO has already been released from SNAP after 24 h in culture conditions, these results indicate that the continuous presence of NO does not seem to be critical for inhibition of cell proliferation. In fact, an incubation period as short as 4 h is enough for SNAP to inhibit cell proliferation (see below).

Several cytotoxic effects of NO have been attributed to its very fast reaction with superoxide anion yielding peroxynitrite, a short-lived and powerful oxidant species (Beckman & Koppenol 1996). Although originally described as an NO donor, SIN-1 was later shown to concurrently generate NO and superoxide and, thus, has also been employed as a peroxynitrite donor (for example Ma *et al.* 1997). Although this compound inhibited P815 cell proliferation, it also caused a substantial reduction in cell viability. Therefore, SIN-1-induced inhibition of cell proliferation may have resulted from a cytotoxic effect. Moreover, as the cytotoxic effect of SIN-1 was not shared by either SNAP or GSNO, this suggests that peroxynitrite does not mediate the SNAP or GSNO antiproliferative effect on P815 cells.

Guanylate cyclase is one of main targets of physiological concentrations of NO. Increased cGMP synthesis mediates NO-induced vasodilatation and antiaggregatory actions, as well as

its effects on neurotransmission (Ignarro 1991). In smooth muscle cells and in fibroblasts, NO inhibition of cell proliferation is a cGMP-mediated mechanism (Garg & Hassid 1989). In our system, however, ODQ, a potent and selective inhibitor of soluble guanylate cyclase, failed to affect the SNAP antiproliferative effect. Cyclic GMP can also be formed by the particulate guanylate cyclase (Garbers 1991) and cGMP originating from any of the two cyclases activates cGMP-dependent protein kinases (PKG; Ruth 1999). In this regard 8-Br-cGMP, a membrane permeant analogue of cGMP, did not cause any changes in cell proliferation (control 0.87 ± 0.08 versus 0.61 ± 0.03 units of absorbance at 550 nm). Therefore, the antiproliferative effect of NO on the P815 murine mastocytoma cell line seems to be independent of soluble guanylate cyclase activation, of cGMP formation and of PKG activation. Whether the difference in NO effects can be attributed to the fact that some studies were performed with primary cells whereas others, such as our own, were carried out with tumour cell lines remains to be established.

NO has been shown to affect the cytoskeleton (Clancy *et al.* 1993). As this structure is essential for cell proliferation, it is possible that the antiproliferative effect of SNAP might be related to effects on the cytoskeleton. The lack of effect of cytochalasin B and colchicine on SNAP-induced inhibition in cell proliferation indicates that microtubules and microfilaments do not seem to be involved in the antiproliferative effect of SNAP, at least in the onset of this effect during the first 4 h of incubation.

NO (either released by LPS-induced NOS or infused over a short period of time) has been shown to decrease rat vascular sensitivity towards vasoconstrictors (Terluk *et al.* 2000; Silva-Santos *et al.* 2002). An important point regarding this effect is that it persisted for several hours after NO had disappeared from the system, indicating that these NO effects are long-lasting. The results shown here indicate that the inhibition of proliferation caused by NO does seem to belong to the long-lasting category of effects. A brief incubation of 4 h with SNAP rendered cells incapable of proliferation for at least a further 20 h. The irreversibility of the SNAP effect cannot be attributable to a cytotoxic effect as (i) cell viability was not reduced, and (ii) wash out of cycloheximide after 4 h of incubation was promptly followed by proliferation, with cells reaching numbers identical to the control group (see Fig. 5).

There are several reports in the literature suggesting that ion channels, particularly potassium channels, are involved in cell proliferation (Wonderlin & Strobl 1996). NO has been shown to affect potassium channels (Bolotina *et al.* 1994). As potassium channels have been shown to be involved in the long-lasting actions of NO on vascular reactivity, we wondered whether this mechanism might play a role in the antiproliferative effect of NO. Our results indicate that, indeed, potassium channels are involved as TEA, a nonselective potassium channel blocker, prevented the onset of SNAP-mediated inhibition. Screening with selective blockers of the different potassium channel subtypes indicated that voltage-dependent and calcium-dependent, but not ATP-dependent, potassium channels are important for the SNAP antiproliferative effect. Three points regarding these findings are important. Firstly, the effective concentrations of potassium channel blockers needed to prevent NO effects are well below the effective concentration range for potassium channel blockade usually found in the literature. Secondly, in the absence of SNAP none of the potassium channel blockers used in this report affected P815 cell proliferation, even at the concentrations usually found in the literature. Thirdly, none of the potassium channel blockers affected cell viability. Although some of the potassium channel blockers used in the present report (such as charybdotoxin and iberiotoxin) are accepted in the literature as being selective for some channel subtypes, most of the blockers are not selective and thus electrophysiological experiments are needed to confirm which ion channels and their subtypes are affected by NO. Therefore, although some classes of potassium channels are important effectors of the NO antiproliferative effect, they do not seem to be important for normal cell proliferation. This is in contrast with other

reports, describing potassium channels as being essential for cell proliferation (Wonderlin & Strobl 1996; Rane 1999). As for the mechanism by which NO interferes with voltage- and calcium-dependent potassium channels, our results, as they stand, do not allow any further conclusions. However, one likely possibility is related to the observation that NO reacts avidly with thiols, yielding S-nitrosothiols (Stamler *et al.* 1992). Potassium channels have cysteine sulphhydryls in their amino acid sequence (Bolotina *et al.* 1994) and, thus, it is possible that S-nitrosylation of key channel protein residues induces conformational changes which would keep the channel in the open state, leading to the arrest of cell division.

Although our data suggest that NO is exerting at least part of its antiproliferative effect through potassium channels, some limitations of our results should be kept in mind. For instance, part of the NO antiproliferative effect may be related to its direct action on DNA causing nitro-guanine formation and thus limiting cell proliferation (Hsieh *et al.* 2001). Although it is reasonably well-accepted in the literature that NO donors are a convenient source of NO, only the use of NO gas directly in the culture could confirm that the antiproliferative effect of NO donors is definitely due to NO. Finally, some effects of S-nitrosothiols may be related not to NO release but, rather, to a direct effect on relevant targets (Lipton *et al.* 2001).

In any event, a better understanding of the relationship among NO, potassium channel activation and cell proliferation may in future lead to the development of improved strategies for the clinical management of cancer and other diseases in which cell proliferation is abnormal.

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