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NITRIC OXIDE-GENERATING VASODILATORS INHIBIT MITOGENESIS AND PROLIFERATION OF BALB/C 3T3 FIBROBLASTS BY A CYCLIC GMP-INDEPENDENT MECHANISM

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Summary: The purpose of this study was to investigate the effects of nitric oxide-generating vasodilators and 8-bromo-cGMP on serum-induced mitogenesis in BALB/c 3T3 fibroblasts that lack soluble guanylate cyclase activity. Two such vasodilators, S-nitroso-N-acetylpenicillamine and isosorbide dinitrate, decreased the incorporation of (^{3}H)thymidine in these cells dose-dependently whereas 8-bromo-cGMP was ineffective at concentrations of up to 10 mM. Moreover, S-nitroso-N-acetylpenicillamine also inhibited cell proliferation, consistent with the data on (^{3}H)thymidine incorporation. S-nitroso-N-acetylpenicillamine had no effect on cGMP accumulation, confirming previous studies that these cells lack soluble guanylate cyclase activity. Hemoglobin and $FeSO_4$/ascorbate, agents that inhibit the actions of nitric oxide, both decreased S-nitroso-N-acetylpenicillamine-induced antimitogenesis, supporting the view that this effect was related to the generation of nitric oxide. The antimitogenic activity of S-nitroso-N-acetylpenicillamine was unlikely to be the expression of nitric oxide-induced degradation of serum mitogens, as indicated by the decrease of the antimitogenic activity on prolonged preincubation of SNAP in serum-containing medium. We conclude that nitric oxide-generating vasodilators inhibit serum-induced mitogenesis and cell proliferation in BALB/c 3T3 fibroblasts by a cGMP-independent mechanism.

Several recent studies have demonstrated that vasoactive agents that increase cGMP, such as atrial natriuretic hormone or NO, inhibit mitogenesis and cell proliferation of mesangial and vascular smooth muscle cells in culture (1-6). The antimitogenic effect of NO is mimicked by 8-bromo-cGMP, suggesting that it is mediated by cGMP as the second messenger (1,2,4,6). NO also inhibits the proliferation of tumor cells, but the relationship of this effect to cGMP metabolism has not yet been addressed (7,8).

That the actions of NO are not necessarily linked with cGMP was established recently by Brüne and Lapetina who observed that NO activates an endogenous ADP-ribosyl transferase in platelets and several other tissues (9). This action appeared to be independent of cGMP as it was not mimicked by cell-permeant cGMP analogues.

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ABBREVIATIONS: DMEM, Dulbecco's modified essential medium; IBMX, 3-isobutyl-1-methylxanthine; ISDN, isosorbide dinitrate; SDS, sodium dodecylsulfate; SNAP, S-nitroso-N-acetylpenicillamine.

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Previous studies have shown that BALB/c 3T3 fibroblasts lack soluble guanylate cyclase activity (10). These cells thus appear to be an attractive model to investigate potential cGMP-independent effects of NO. In this communication we document that NO-generating vasodilators inhibit serum-induced thymidine incorporation and cell proliferation in BALB/c 3T3 fibroblasts and that these effects are independent of cGMP.

MATERIALS AND METHODS

BALB/c 3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and were used between subcultures 66 and 74. SNAP was synthesized according to the method of Field et al (11) by the reaction of $NaNO_2$ and N-acetylpenicillamine as previously described (1,2). ISDN and fatty acid-free bovine serum albumin were obtained from Sigma (St. Louis, MO). Insulin, transferrin and selenium were purchased from Collaborative Research (Bedford, MA). DMEM and fetal bovine serum were purchased from GIBCO (Grand Island, NY).

For thymidine incorporation studies, BALB/c 3T3 fibroblasts were seeded at a density of ~$2x10^4/cm^2$ and cultured for 2-5 days in DMEM supplemented with 10% fetal bovine serum, insulin (5 μg/ml), transferrin (5 μg/ml) and selenous acid (5 ng/ml) plus penicillin (100 U/ml) and streptomycin (100 μg/ml). To achieve quiescence, the cells were washed, 3 times, with serum-free medium and cultured for 2 days in DMEM supplemented with 0.5% bovine serum albumin. To investigate the effect of various agents on mitogenesis, quiescent cells were cultured for 24 h in DMEM, with or without 5% serum supplementation and containing or lacking experimental agents. Finally, cells were incubated for 2 h in freshly prepared corresponding media, additionally supplemented with ~1μCi (methyl-^3H) thymidine (7-20 Ci/mmol, New England Nuclear, Boston, MA), to measure DNA synthesis by thymidine incorporation. The experiments were terminated by washing the cells with 1 mM Ca-supplemented phosphate buffered saline, precipitation of acid-insoluble material with 10% trichloroacetic acid and extraction of DNA with 0.1% SDS/0.5 N NaOH. The radioactivity of an aliquot of the extract was measured by scintillation counting.

To determine the effect of SNAP on cell proliferation, cells were seeded at densities ranging from $3.2x10^3/cm^2$ to $7.6x10^3/cm^2$, and cultured for 2 days in DMEM supplemented with 10% fetal bovine serum. Cells were then cultured for 2 days in serum-free DMEM supplemented with 0.5% bovine serum albumin, to achieve quiescence. This was followed by culture, for 4 days, in DMEM supplemented with 5% fetal bovine serum, containing or lacking various concentrations of SNAP. The culture medium was changed daily and, on appropriate days, the cells were dissociated with trypsin-EDTA and counted by hemocytometer. It should be noted that we tested only a limited range of SNAP concentrations in cell proliferation experiments because, in these longer-term experiments, SNAP induced cell detachment, at concentrations greater than 600 μM.

To determine the effect of SNAP on cyclic nucleotide levels, cells were incubated with or without varying concentrations of SNAP in physiologic salt solution of the following composition (in mM): 135 NaCl, 5 KCl, 1 Na_2HPO_4, 0.5 $MgSO_4$, 1.8 $CaCl_2$, 10 glucose, 10 HEPES, 0.025% fatty acid-free bovine serum albumin, pH 7.4. Incubations were terminated by aspirating the medium and cellular cyclic nucleotides were extracted with 0.1 N HCl, as described previously (12). The time of incubation for these experiments was 10 min and this was based on the finding that shorter incubation times gave similar results and therefore a 10 min incubation was routinely used. Cyclic AMP and cGMP levels were measured by radioimmunoassay, according to the procedure of Brooker et al. (13).

RESULTS

Results shown in figure 1 indicate that two structurally dissimilar vasodilators, SNAP and ISDN, sharing the capacity to generate NO (14), dose-dependently inhibited serum-induced thymidine incorporation in BALB/c 3T3 fibroblasts. In contrast, 8-bromo-cGMP which inhibited mitogenesis in aortic smooth muscle cells had no significant effect on thymidine incorporation in fibroblasts, even at concentrations as high as 10 mM (figure 2).

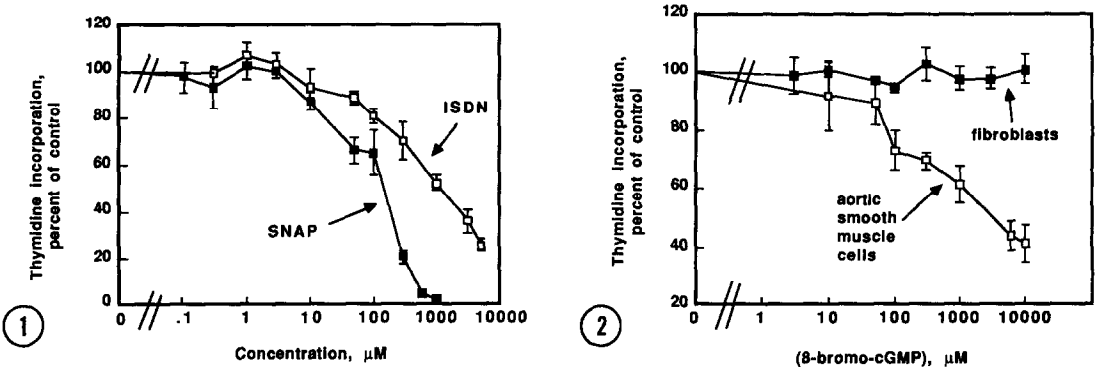

Figure 1: Concentration-response relationships for the inhibition of 5% fetal bovine serum-stimulated (^{3}H)thymidine incorporation into BALB/c 3T3 fibroblasts by SNAP (closed squares) or ISDN (open squares). Results are the mean±SE from 3 experiments, each in quadruplicate, and are expressed as percent of control, defined as (^{3}H)thymidine incorporation in the presence of 5% serum. Incorporation of (^{3}H)thymidine in control experiments ranged from 1.3×10^4 cpm/well to 5.5×10^4 cpm/well. The effect of both vasodilators was statistically significant, as determined by the nonparametric Kruskal-Wallis H test ($p<0.01$).

Figure 2: Concentration-response relationship for the effect of 8-bromo-cGMP (closed squares) on 5% serum-stimulated (^{3}H)thymidine incorporation into BALB/c 3T3 cells. Also shown, for the purpose of comparison, is the inhibitory effect of 8-bromo-cGMP in rat aortic smooth muscle cells (open squares). Results are the mean±SE from 3 experiments, each in quadruplicate, and are expressed as percent of control, defined as (^{3}H)thymidine incorporation in the presence of 5% serum.

SNAP failed to increase cGMP levels in BALB/c 3T3 fibroblasts in either the presence or absence of the cyclic nucleotide phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (fig.3). In contrast, SNAP potently increased cGMP levels in renal mesangial cells (fig.3). Thus, in addition to being unresponsive to 8-bromo-cGMP, BALB/c 3T3 fibroblasts appeared to lack soluble guanylate cyclase activity, in agreement with previous studies (10). SNAP also did not significantly alter cAMP levels in these cells (fig. 3).

To further investigate the role of NO in SNAP-induced antimitogenesis, we used agents that inhibit the effects of NO. As indicated in table 1, the antimitogenic effect of SNAP was inhibited by hemoglobin as well as $FeSO_4$/ascorbate, substances that have been shown previously to inhibit the effects of NO (8,15).

The generation of the free radical NO may induce the degradation of mitogens in serum, an effect that could account for the antimitogenic effect of SNAP or ISDN. To investigate this possibility, we took advantage of the fact that SNAP is labile in aqueous media ($t_{1/2} \sim 5$ h) (16) and demonstrated that its antimitogenic effect was unlikely to be the expression of the degradation of serum mitogens by NO by verifying that preincubation of SNAP (100 μM) in 5% serum for 24 h induced a decrease of its antimitogenic effect, relative to the same concentration of freshly prepared SNAP-containing medium (24 h incubated medium: 25.9±1.7% inhibition; freshly prepared medium: 76.3±1.7% inhibition). Thus, most of the antimitogenic effect of SNAP is unlikely to be due to the degradation of serum mitogens by SNAP or to the presence of SNAP degradation products. The residual antimitogenic effect could be due to the activity of undegraded SNAP, SNAP-degradation products

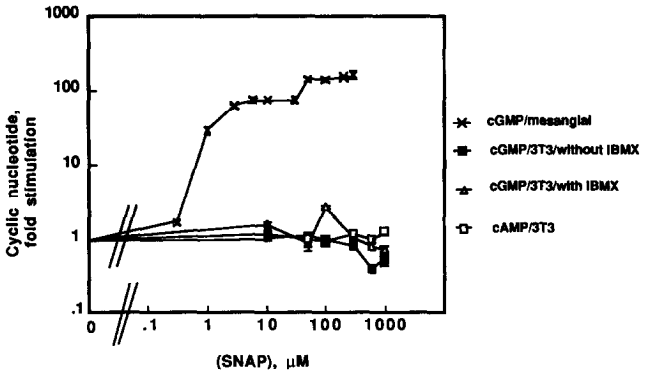

Figure 3: Effect of SNAP on cGMP accumulation, in BALB/c 3T3 fibroblasts, in the absence (closed squares) or presence of 1 mM IBMX (triangles). Also shown is the effect of SNAP on cAMP levels in fibroblasts (open squares) and on cGMP levels in mesangial cells (crossed symbols). Cells were incubated for 10 min in physiological salt solution, followed by extraction of cyclic nucleotides and quantitation by radioimmunoassay, as described in text. Results are expressed as fold stimulation over control incubations lacking SNAP and represent mean±SE of quadruplicate determinations from single experiments. Similar results were obtained in 1 to 2 other experiments.

or relatively minor degradation of serum mitogens by NO. These results are similar to those of our previous experiments in which SNAP also did not induce major degradation of agents that enhance aortic smooth muscle or renal mesangial cell mitogenesis (1,2).

Since decreased thymidine incorporation is not necessarily associated with inhibition of cell proliferation, we investigated the effect of SNAP on the proliferation of BALB/c 3T3 fibroblasts. As depicted in figure 4, SNAP dose-dependently inhibited serum-induced cell proliferation. That the antiproliferative effect of SNAP was not the expression of cytotoxicity was demonstrated by four independent criteria including the lack of staining of the cells by trypan blue, the lack of release of lactate dehydrogenase into the supernatant medium and the lack of cell detachment from the culture surface (results not shown). In a single experiment, we also observed that removal of SNAP

Table 1: Effect of hemoglobin or ascorbate/$FeSO_4$ on SNAP-induced inhibition of mitogenesis in BALB/c 3T3 cells

Effector	Thymidine incorporation (% of control)
SNAP	3.1±0.8
SNAP + Hemoglobin	46.3±2.4*
SNAP	11.7±0.6
SNAP + ascorbate/$FeSO_4$	49.5±2.2*

Cells were incubated in the presence of 5% serum, with or without various effectors, as described in text. Control is defined as thymidine incorporation induced by 5% serum. Concentrations of effectors were as follows: SNAP (300 μM), hemoglobin (10 μM), ascorbate (2 mM), $FeSO_4$ (100 μM). Treatment with hemoglobin alone or the combination of $FeSO_4$ and ascorbate alone did not significantly affect thymidine incorporation. Results are the mean ± SE from one series of experiments, in quadruplicate. Similar results were obtained in a second series of experiments. Asterisks indicate $p<0.005$, relative to results obtained in the presence of SNAP alone, via unpaired Student's t-test.

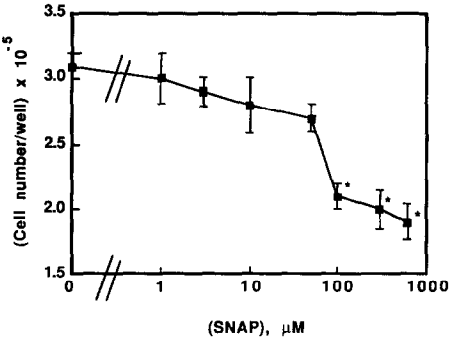

Figure 4: Concentration-response relationship for the inhibition of BALB/c 3T3 cell proliferation by SNAP. Quiescent cells were cultured for 4 days in the presence or absence of varying concentrations of SNAP plus 5% fetal bovine serum, followed by cell detachment and counting, as described in text. Results are the mean±SE of one experiment, in quadruplicate. The effect of SNAP was statistically significant as determined by analysis of variance ($F=9.80$, $df=7,24$, $p<0.001$). Asterisks indicate significantly different values, relative to those found in the absence of SNAP, via Scheffé's post-hoc F-test. A second such experiment yielded similar results.

reversed the inhibition of cell proliferation, consistent with the lack of cytotoxicity (results not shown). It should also be noted that even a concentration of SNAP as high as 1 mM did not induce overt cytotoxicity in the relatively short-term thymidine incorporation experiments although concentrations of SNAP greater than 600 μM may have been cytotoxic in the longer-term cell proliferation experiments, as manifested by cell detachment.

DISCUSSION

We and others have shown that cGMP and cGMP-elevating agents, including NO, inhibit the proliferation of vascular and renal cells in culture (1-6). The principal novel finding presented in the current communication is that NO inhibits serum-induced mitogenesis and cell proliferation in BALB/c 3T3 fibroblasts that lack soluble guanylate cyclase activity and that are unresponsive to a cell-permeant analogue of cGMP. The concentration-response relationships for inhibition of (^{3}H)thymidine incorporation and cell proliferation were quite similar to each other, although the full range of SNAP concentrations could not be tested in proliferation experiments due to cell detachment that occurred over the 4 day experiments, at concentrations of SNAP greater than 600 μM. The notion that the antimitogenic activity of NO-generating vasodilators was mediated by the generation of NO was supported by the findings that two structurally different nitric oxide-generating agents, SNAP and ISDN, both inhibited mitogenesis, and that established antagonists of the effects of NO, $FeSO_4$/ascorbate and hemoglobin, inhibited the antimitogenic activity of SNAP. Thus, our observations indicate that NO modulates mitogenesis in BALB/c 3T3 fibroblasts by a mechanism that is independent of cGMP. The inhibitory effect of SNAP is also not attributable to altered cAMP, based on the invariance of cAMP levels in response to the vasodilator, or to the degradation of serum mitogens, based on the disappearance of the antimitogenic effect upon preincubation of SNAP in serum-supplemented medium.

The recent findings of Brüne and Lapetina, on NO induced ADP-ribosylation of a 39 kDa protein in platelets and several other tissues may be relevant to the current findings, inasmuch as this effect also occurred by a cGMP-independent process (9). Thus, a potential mechanism that could mediate the effects of NO in 3T3 fibroblasts is enhancement of ADP-ribosylation of specific proteins. Also relevant to this aspect may be recent findings indicating that pertussis toxin inhibits epidermal growth factor, phorbol ester or insulin-stimulated DNA synthesis in BALB/c 3T3 fibroblasts by a mechanism dependent on ADP-ribosylation of the guanine nucleotide binding regulatory protein $G_{i\alpha}$ (17). However, the studies of Brüne and Lapetina failed to identify a guanine nucleotide binding regulatory protein as substrate for NO-enhanced ADP-ribosylation, in platelets (9). Additional studies will therefore be required to establish the relationship, if any, between NO-induced ADP ribosylation and the antimitogenic effect of NO.

In summary, NO inhibits the mitogenesis and proliferation of BALB/c 3T3 fibroblasts by a cGMP-independent mechanism which differs from that in vascular smooth muscle and mesangial cells in which this effect, at least in part, appears to be mediated by cGMP as the second messenger (1,2).

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