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Inhibitory effects of mesoionic 3-aryl substituted oxatriazole-5-imine derivatives on vascular smooth muscle cell mitogenesis and proliferation *in vitro*

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1 The effects of oxatriazole-type (GEA 3162 and GEA 5624) nitric oxide (NO) donors on mitogenesis and proliferation were studied in vascular smooth muscle cell (VSMC) culture. The effects of the GEA-compounds were compared with well-known NO-donors 3-morpholinosydnonimine (SIN-1) and S-nitroso-N-acetylpenicillamine (SNAP).

2 All NO-donors released NO and increased the production of cyclic GMP concentration-dependently. The production of cyclic GMP was inhibited by the guanylate cyclase inhibitor, ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one).

3 The NO-donors inhibited basal and serum-induced DNA synthesis concentration-dependently. The GEA-compounds were needed in concentrations 10 times lower than SIN-1 and SNAP. GEA 3162, SIN-1 and SNAP were also able to inhibit serum-induced cell proliferation. GEA 5624 was ineffective. The antimitogenic effect of NO-donors was not reduced by inhibiting the guanylate cyclase.

4 These results suggest that NO inhibits serum-induced DNA synthesis and proliferation of VSMC by a cyclic GMP-independent mechanism. The oxatriazole-type NO-donor GEA 3162 was found to be a more potent inhibitor of mitogenesis and cell proliferation than SIN-1 and SNAP.

Keywords: Nitric-oxide; proliferation; DNA synthesis; vascular smooth muscle cell; cyclic GMP; mesoionic 3-aryl substituted oxatriazoles

Introduction

Vascular smooth muscle cells (VSMC) have an important role in the pathogenesis of hypertension and atherosclerosis. In both these conditions there is an increase in medial smooth muscle mass in the vasculature. Nitric oxide (NO), acting as a local chemical messenger, regulates vascular tone and VSMC proliferation. NO relaxes VSMC by activation of soluble guanylate cyclase and further by elevation of intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP), which leads to vasodilatation. NO-induced increase in cyclic GMP has also been associated with an inhibition of VSMC proliferation (Garg & Hassid, 1989; Kariya *et al*., 1989).

In the cardiovascular system, NO is synthesized predominantly in the endothelium by constitutive nitric oxide synthase. NO from the endothelium maintains VSMC quiescence in intact vessel. Also VSMC are able to produce NO by themselves after stimulation by e.g. cytokines or lipopolysaccharides (Busse & Mülsch, 1990; Koide *et al*., 1993). Endothelial NO production is impaired in atherosclerosis and hypertension. It has also been shown that endothelial cells and VSMC from hypertensive rats release less NO than cells from normotensive rats (Malinski *et al*., 1993). NO releasing compounds have therefore emerged as a promising clinical treatment for controlling excessive VSMC proliferation observed in atherosclerosis and hypertension.

The aim of the present study was to compare the capability of the novel NO releasing compounds (GEA-compounds), to those of chemically different traditional NO-donors in the inhibition of VSMC mitogenesis measured as thymidine incorporation into newly synthesized DNA and cell proliferation. Furthermore, the role of cyclic GMP as a cellular signal system in the inhibitory mechanism was evaluated.

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Methods

Culture of rat vascular smooth muscle cells

Rat vascular smooth muscle cells were isolated by an explant-method (Ross, 1971) from the thoracic aortas of male Wistar rats (250-300 g). The aortas were cleaned of fat and connective tissue. The endothelium was removed mechanically followed by incubation of the aortas for 15 min at 37°C in the presence of collagenase (2 mg ml^{-1}), soybean trypsin inhibitor (0.375 mg ml^{-1}), elastase (0.125 mg ml^{-1}) and bovine serum albumin (2 mg ml^{-1}) in Dulbecco's modified Eagle's medium (DMEM) containing 15 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES). The tunica adventitia was removed and the remaining medial layer was cut into small pieces, and placed in tissue culture dishes. Fresh culture medium (DMEM/Ham's F-12, 1:1) containing antibiotics (penicillin 100 U ml^{-1}, streptomycin 0.1 mg ml^{-1}), HEPES (15 mM) and foetal calf serum (FCS, 15%) was gradually added during the first day. Dishes were left undisturbed for at least 7 days. The dishes were kept in a humidified atmosphere consisting of 95% air and 5% CO_2 at 37°C and subcultured weekly. Cells between passage 3 and 12 were used for experiments. The cells were positively identified microscopically by immunoperoxidase staining for smooth muscle specific α-actin (McGuire *et al*., 1993).

For experiments the cells were seeded at a density of 4×10^4 cells per well and grown in culture medium for 2–3 days. Confluent cells were washed three times with PBS (composi-

tion, mM: NaCl 136, KCl 2.6, Na_2HPO_4 7.8, KH_2PO_4 1.4) and were cultured in a serum-free medium (DMEM/Ham's F12, 1 : 1 containing antibiotics and HEPES) for 24 h to establish quiescence.

Measurement of nitrite

NO release from the NO-donors was estimated spectrophotometrically by using Griess reaction (Schmidt *et al*., 1992). Nitrite concentration of the cell supernatants was measured using sodium nitrite as a standard.

Measurement of cyclic GMP

Quiescent VSMC were incubated with serum-free medium containing cyclic GMP phosphodiesterase inhibitor (zaprinast, 100 μM) for 30 min and exposed to test agents for an additional 30 min. The effect of NO-donors on the cyclic GMP level during the 24 h incubation was determined in the absence or presence of zaprinast. Cyclic GMP was extracted with 0.1 M HCl and measured by radioimmunoassay after acetylation (Axelsson *et al*., 1988). Data were calculated on the basis of the cell protein content (Lowry *et al*., 1951).

DNA synthesis

The effects of NO-donors on mitogenesis were investigated by measuring [^{3}H]-thymidine incorporation into the newly synthesized DNA. Mitogenically quiescent VSMC were incubated for 20 h in the presence or absence of 5% FCS and test compounds. Treatments were repeated with freshly prepared solutions supplemented with [^{3}H]-thymidine (1 μCi ml^{-1}) for an additional 4 h. In the experiments where superoxide dismutase, oxyhaemoglobin and ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) were used, cells were treated by a single dose of the drugs and the experiment was terminated after 24 h by washing cells with ice-cold PBS, precipitation of the acid-insoluble material with 5% trichloroacetic acid and extraction of the DNA with 1 M NaOH. The radioactivity of an aliquot was measured by liquid scintillation counting.

Cell proliferation

The quiescent cells were cultured for 4 days in a medium supplemented with 5% FCS with or without the experimental compounds. Culture media containing the drugs were changed daily and the cell count was determined with haemocytometer at the beginning of the experiment, second and fourth experiment day.

Measurement of cell viability

Because NO, at high concentrations, has cytotoxic activity, the effects of NO-donors on cell viability was evaluated. Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan (Gross & Levi, 1992). At the end of the experiment, cells were incubated for 4 h with MTT (0.2 mg ml^{-1}). The medium was removed and cells were solubilized in DMSO. The reduction of MTT to formazan as a sign of cell respiration was quantitated by spectrophotometry at 550 nm. Cell viability was not diminished by the highest concentrations of the drugs tested. The cultures were also examined after each experiment by phase-contrast microscopy for evidence of cell detachment or changes in cell morphology.

Statistical analysis

Results are shown as mean $\pm$ s.e.mean. Statistical comparisons were performed by the use of unpaired, two-tailed Student's *t*-test or analysis of variance followed by Tukey-Kramer test when appropriate. Value of $P<0.05$ was considered to be statistically significant.

Materials

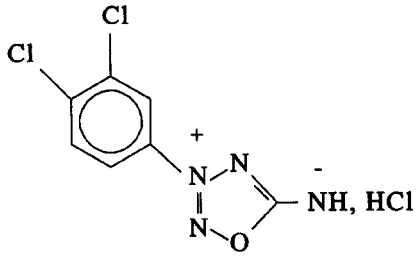
DMEM (Dulbecco's modified Eagle's medium), Ham's F-12 (Nutrient mixture Ham's F-12), collagenase (type 1A), elastase (type IIA from porcine pancreas), soybean trypsin inhibitor, bovine serum albumin (fraction V), zaprinast, haemoglobin (from bovine blood), superoxide dismutase (from bovine erythrocytes), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), monoclonal anti-α-smooth muscle actin and anti-mouse IgG peroxidase conjugate were from Sigma Chemical Co. (St. Louis, MO, U.S.A). ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) was purchased from Tocris Cookson (Bristol, U.K.). Cell culture dishes were obtained from Nunc (Copenhagen, Denmark). Foetal calf serum, HEPES and antibiotics were purchased from Gibco Europe (Paisley, U.K.). [^{3}H]-Thymidine and [^{125}I]-cyclic GMP were from Amersham International (Buckinghamshire, U.K.). The cyclic GMP antiserum was raised in rabbits in our laboratory and was found to cross-react less than 0.001% with related compounds (ADP, AMP, ATP, GDP, GMP, GTP) and less than 0.03% with cyclic AMP. The NO-donors, GEA-compounds (mesoionic oxatriazole derivatives), SIN-1 (3-morpholinosydnonimine) and SNAP (S-nitroso-N-acetylpenicillamine) were donated by Dr Gunnar Karup, GEA Ltd. (Copenhagen, Denmark). All NO-donor solutions were prepared daily immediately before use, kept cold and protected from light. Oxyhaemoglobin was prepared by the reduction of bovine haemoglobin with sodium hydrosulfite followed by purification using Sephadex G-25 column (Pharmacia, Uppsala, Sweden).

Results

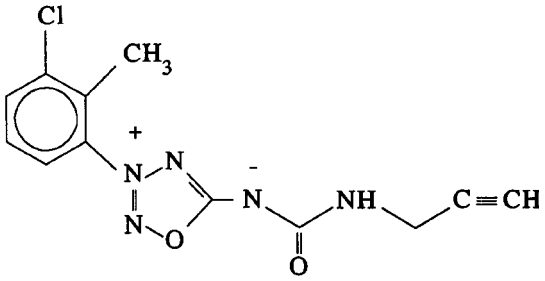
Nitrite production

The GEA-compounds (mesoionic 3-aryl substituted oxatriazole-5-imine derivatives; Figure 1), as well as SIN-1 (3-morpholinosydnonimine) and SNAP (S-nitroso-N-acetylpenicillamine), release NO spontaneously without enzymatic modification. The time-dependent generation of NO by different NO-donors (33 μM) in the absence of cells is shown in Figure 2. The compounds were incubated in the serum-free medium at 37°C for the indicated times and NO production was measured as the accumulation of its stable oxidation metabolite, nitrite, in the incubation medium. GEA 3162 and SIN-1 were the most effective compounds to release NO. After 60 min incubation the order of the compounds in their capability to release NO was GEA 3162 = SIN-1 > SNAP > GEA 5624.

The production of nitrite during the cell culture experiment was determined from incubation medium of the same cell preparations as the cyclic GMP production. All the drugs increased nitrite production concentration-dependently during the 30 min incubation (Table 1). The nitrite production was also measured during 24 h incubation of GEA 3162 and SNAP with VSMC. The nitrite production was complete within 30 min. The levels of nitrite remained constant during the 24 h experiment (data not shown).



GEA 3162



GEA 5624

Figure 1 The chemical structures of GEA 3162 and GEA 5624.

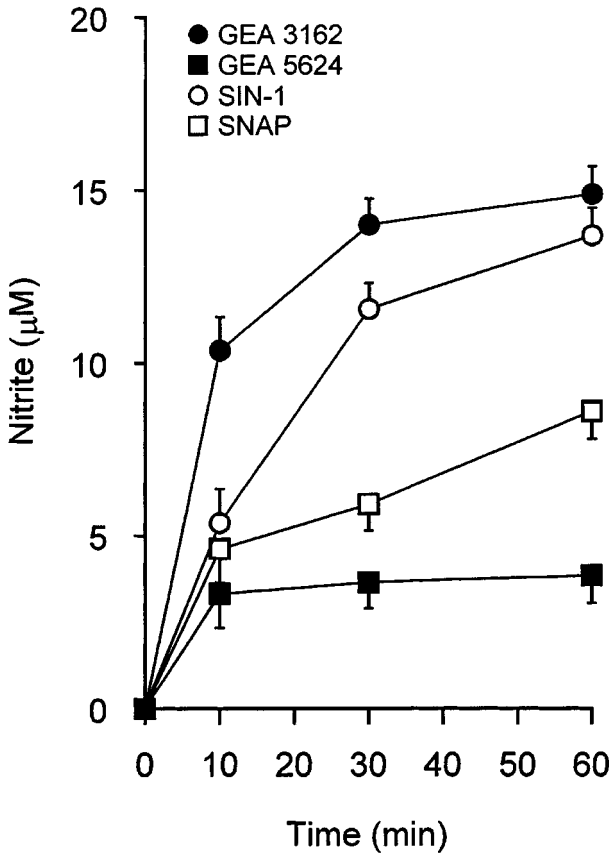


Figure 2 The time-dependent generation of nitrite from GEA 3162, GEA 5624, SIN-1 and SNAP. NO-donors (33 μM) were incubated at 37°C in serum-free medium without cells. Results are presented as the mean$\pm$s.e.mean of four different experiments containing two replicates in each experiment.

Cells were incubated 30 min with NO-donors in the presence of oxyhaemoglobin (50 μM), superoxide dismutase (100 U ml^{-1}) or the novel guanylate cyclase inhibitor, ODQ (10 μM). Oxyhaemoglobin reduced nitrite release from GEA 3162 (10 μM), GEA 5624 (33μM) and SNAP (333 μM) by 88% ($P<0.01$), 51% ($P<0.05$) and 42% ($P<0.05$), respectively. Oxyhaemoglobin was unable to decrease nitrite production of SIN-1 (333 μM). SIN-1 produced 50% more nitrite than SNAP (333 μM), which may exceed the capacity of oxyhaemoglobin to inactivate released NO. Superoxide dismutase and ODQ did not affect the nitrite accumulation caused by NO-donors (data not shown).

Cyclic GMP production

The production of cyclic GMP in VSMC culture by NO-donors during 30 min incubation in serum-free medium in the presence of cyclic GMP-selective phosphodiesterase inhibitor (zaprinast, 100 μM) is shown in Table 1. The stimulation of cyclic GMP production was less in the case of GEA-

Table 1 The release of NO from NO-donors measured as nitrite and the effect of NO-donors on cyclic GMP production in VSMC

Treatment	*Concentration* (μM)	*Nitrite production* (μM)	*Cyclic GMP production* (fmol/μg protein)
Control		0.3$\pm$0.1	1.1$\pm$0.2
GEA 3162	3.3	1.5$\pm$0.1	3.4$\pm$0.5**
	10	3.8$\pm$0.2**	4.2$\pm$0.5***
	33	12$\pm$1.3***	2.9$\pm$0.4**
GEA 5624	10	1.8$\pm$0.4*	2.6$\pm$0.6
	33	3.3$\pm$0.7***	5.1$\pm$2.4*
	100	7.0$\pm$0.3***	10$\pm$3.0***
SIN-1	100	29$\pm$1.1***	15$\pm$4.3***
	333	86$\pm$3.9***	31$\pm$10***
	1000	267$\pm$9.6***	46$\pm$6.7***
SNAP	100	15$\pm$1.6***	37$\pm$12***
	333	31$\pm$3.0***	48$\pm$14***
	1000	67$\pm$3.6***	55$\pm$11***

VSMC were incubated with the NO-donors for 30 min in the presence of zaprinast (100 μM). Results are expressed as the means$\pm$s.e.mean of four to seven different experiments containing two replicates in each experiment. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared to the basal (control) nitrite or cyclic GMP production.

Table 2 The effect of oxyhaemoglobin, superoxide dismutase and ODQ on cyclic GMP production in VSMC

Treatment	*+oxyHb*	*+SOD*	*+ODQ*
GEA 3162 (10 μM)	3$\pm$1***	255$\pm$26***	10$\pm$1***
GEA 5624 (33 μM)	9$\pm$5***	388$\pm$21***	25$\pm$5***
SIN-1 (333 μM)	77$\pm$20	167$\pm$23*	6$\pm$2***
SNAP (333 μM)	22$\pm$7***	165$\pm$20*	8$\pm$4***

The DNA synthesis of VSMC was induced by 5% FCS and the cells were incubated for 24 h in the presence of NO-donors, zaprinast (100 μM) and 50 μM oxyhaemoglobin (oxyHb), 100 U ml^{-1} superoxide dismutase (SOD) or 10 μM ODQ. Results are expressed as percentage of cyclic GMP production in the presence of NO-donor alone (means$\pm$s.e.mean) of five to eight different experiments containing two replicates in each experiment. *$P<0.05$ and ***$P<0.001$ compared to cells treated with NO-donor alone.

compounds in comparison to SIN-1 and SNAP. The effects of oxyhaemoglobin, superoxide dismutase and ODQ on the cyclic GMP production are shown in Table 2. Oxyhaemoglobin and ODQ decreased and superoxide dismutase increased NO-donors induced cyclic GMP production. The intracellular cyclic GMP-level was also examined over the 24 h incubation period with NO-donors. In the presence of GEA 3162 (10 μM) cyclic GMP was elevated up to the 6 h time point when compared to the corresponding control. In the presence of SNAP (333 μM) the concentration of cyclic GMP persisted elevated during entire 24 h incubation time (Figure 3). ODQ effectively prevented the activation of guanylate cyclase induced by NO-donors.

Effect of NO-donors on DNA synthesis

Incubation of VSMC with 5% FCS for 24 h stimulated thymidine incorporation by 9 fold compared to serum-free medium. The effects of the mesoionic oxatriazole derivatives (GEA 3162, GEA 5624), and the old NO-donors SIN-1 and SNAP on basal and 5% FCS induced DNA synthesis were compared (Figure 4). Incubation of VSMC with NO-donors produced concentration-dependent decrease in DNA synthesis. The effect was more marked in basal DNA synthesis. The GEA-compounds were more potent than SIN-1 and SNAP. SIN-1 and SNAP were needed in much higher (about 10 fold) concentrations than the GEA-compounds to inhibit thymidine incorporation into the newly synthesized DNA.

Oxyhaemoglobin reduced GEA 3162, SIN-1 and SNAP induced inhibition of thymidine incorporation (Table 3). The effect of GEA 5624 was not diminished by oxyhaemoglobin. Superoxide dismutase and ODQ had no effect on thymidine incorporation. The effect of ODQ on DNA synthesis was also examined after 1, 6 and 12 h incubation. In Figure 3 is presented the effect of GEA 3162 and SNAP on thymidine incorporation in the presence or absence of ODQ. ODQ had no effect on DNA synthesis during the 24 h incubation. The result was similar in the presence of GEA 5624 and SIN-1 (data not shown).

Effect of NO-donors on cell growth

Treatment of quiescent VSMC with 5% FCS induced cells to proliferate. Cells were incubated for 4 days in the presence of NO-donors. GEA 3162 (10 μM), SNAP (333 μM) and SIN-1 (1000 μM) inhibited FCS-induced VSMC proliferation (Figure 5) by 33, 47 and 52%, respectively. In contrast, GEA 5624 (33 μM) was ineffective. Higher concentration could not be used because of the cytotoxicity.

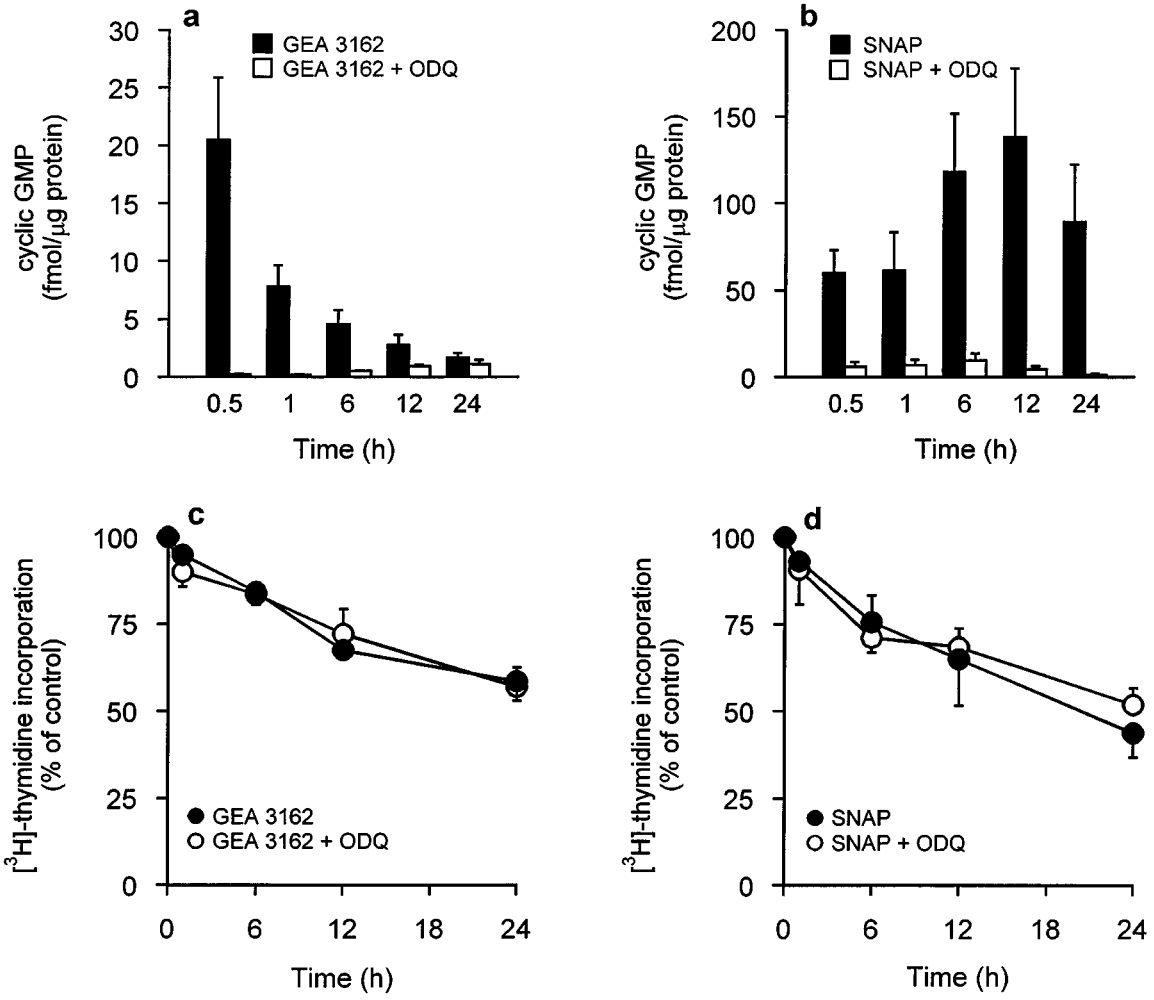


Figure 3 The effect of ODQ (10 μM) on intracellular concentration of cyclic GMP (in the absence of zaprinast) and [^{3}H]-thymidine incorporation in the presence of GEA 3162 (10 μM) (a; c) and SNAP (333 μM) (b; d). VSMC were incubated for 24 h in cell culture medium containing 5% FCS. [^{3}H]-Thymidine (0.5 μCi ml^{-1}) was added to the medium for determination of DNA synthesis. Results are presented as the mean$\pm$s.e.mean of five different experiments containing two replicates in each experiment.

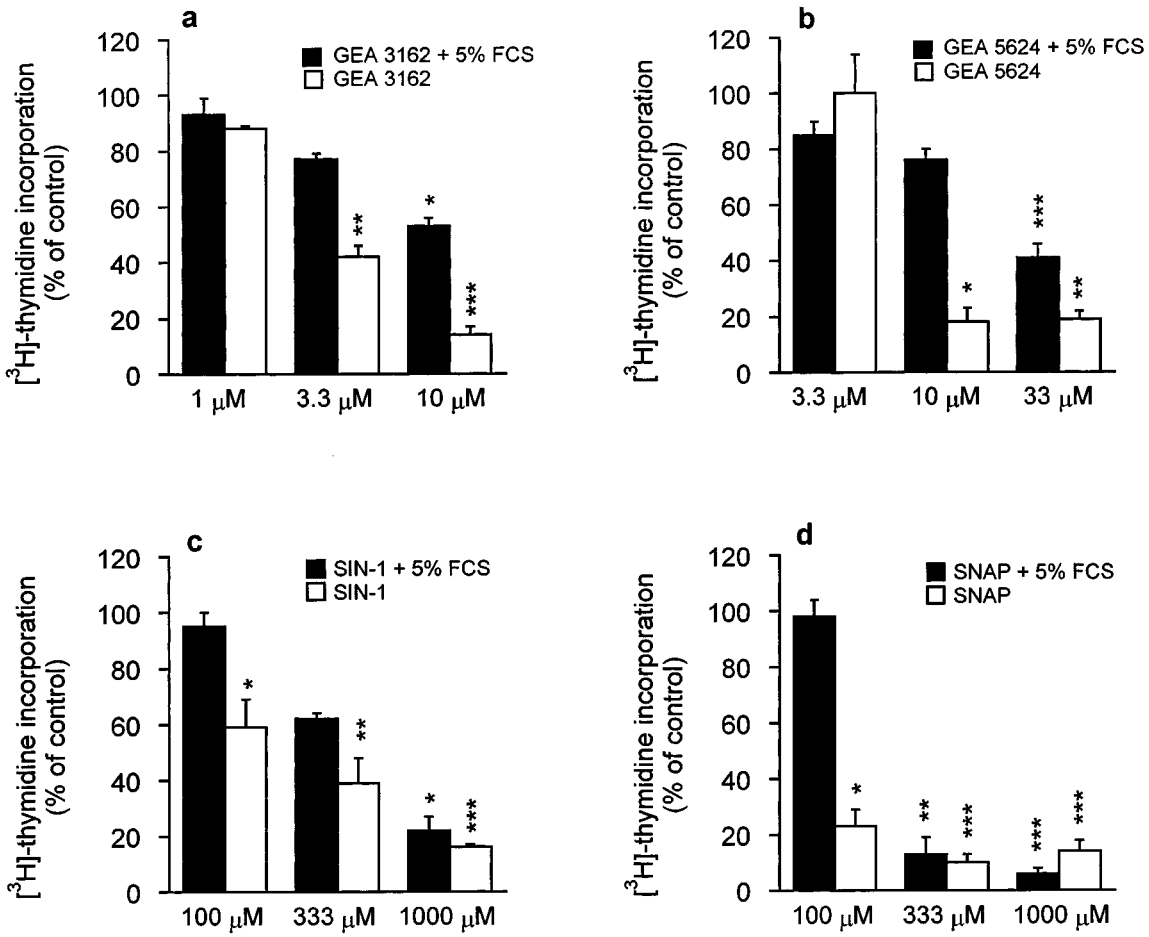


Figure 4 The effect of the NO-donors on DNA synthesis ([^{3}H]-thymidine incorporation). VSMC were incubated for 24 h in the presence of different NO-donors. Solid columns represent the effect of NO-donors on 5% FCS-induced DNA synthesis and open columns the effect of NO-donors on unstimulated DNA synthesis. Results are expressed as the percentage of the respective control (vehicle), mean ± s.e.mean of four to six different experiments containing two replicates in each experiment. $*P<0.05$, $**P<0.01$ and $***P<0.001$ compared to the respective control.

Discussion

The increased growth of vascular smooth muscle cells is involved in the etiology of hypertension and atherosclerosis. NO is proposed to be one of the main candidates of endothelium-derived inhibitors of VSMC growth (Lee *et al.*, 1995). In recent studies, it has been shown that NO and NO-donors inhibit proliferation of several cell types including VSMC (Mooradian *et al.*, 1995, Rizvi & Myers, 1997). Therefore, administration of NO-donors could be a potent treatment for compensation of the endothelial incapability to produce endogenous NO.

We investigated the effects of the novel oxatriazole-type NO-donors (GEA-compounds) on DNA synthesis and proliferation of VSMC. These mesoionic 3-aryl substituted oxatriazole-5-imine derivatives release NO and have vasodilator, antiplatelet and fibrinolytic activities (Corell *et al.*, 1994; Karup *et al.*, 1994; Kankaanranta *et al.*, 1996). They are also effective in reducing blood pressure (Nurminen & Vapaatalo, 1996) and inhibiting the growth of tumor and hematopoietic cells (Vilpo *et al.*, 1994, 1997).

In the present study, GEA 3162, an unsubstituted imine derivative, was effective in reducing both DNA synthesis and cell proliferation in cultured VSMC. GEA 5624, an urea derivative was able to reduce DNA synthesis but not proliferation. The well-established NO-donors, SIN-1 and SNAP, reduced FCS-induced DNA synthesis and proliferation in agreement with the earlier studies (Garg & Hassid, 1989; Dubey & Overbeck, 1994). However, in comparison to the GEA-compounds they were needed in at least ten times higher concentrations to reduce DNA synthesis. Cultured VSMC are also able to synthesize DNA through autocrine mechanisms in

Table 3 The effect of oxyhaemoglobin, superoxide dismutase and ODQ on DNA synthesis in VSMC

Treatment	*NO-donor alone*	*+oxyHb*	*+SOD*	*+ODQ*
GEA 3162 (10 μM)	64±8	96±18*	66±9	62±9
GEA 5624 (33 μM)	61±6	57±6	61±8	59±7
SIN-1 (333 μM)	87+5	105±5*	99±6	77±10
SNAP (333 μM)	22±8	114±10***	17±4	26±10

The DNA synthesis of VSMC was induced by 5% FCS and the cells were incubated for 24 h in the presence of NO-donors and 50 μM oxyhaemoglobin (oxyHb), 100 U ml^{-1} superoxide dismutase (SOD) or 10 μM ODQ. Results are expressed as percentage of respective control (means ± s.e.-mean) of seven different experiments containing two replicates in each experiment. $*P<0.05$ and $***P<0.001$ compared to cells treated with NO-donor alone.

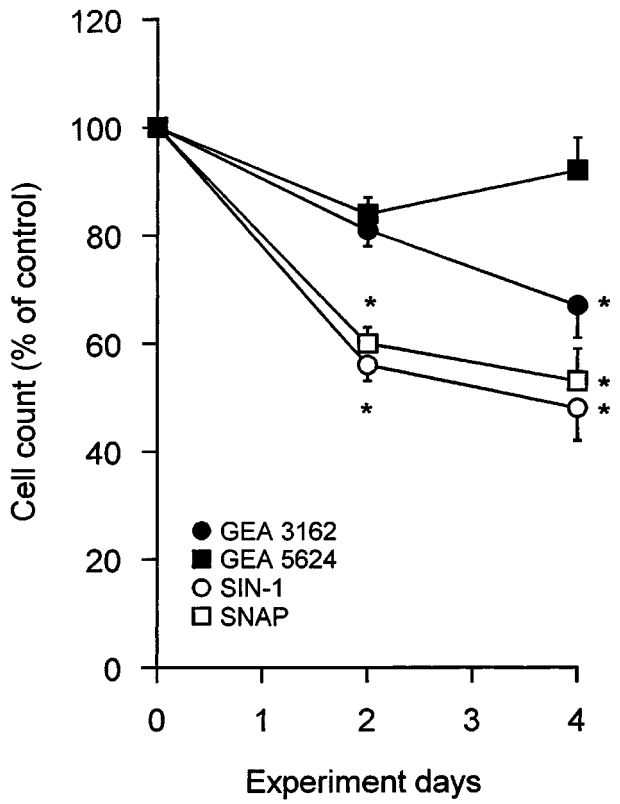


Figure 5 The effect of the NO-donors on VSMC proliferation. Growth arrested VSMC were treated daily with GEA 3162 (10 μM), GEA 5624 (33 μM), SIN-1 (1000 μM) or SNAP (333 μM) in the presence of 5% FCS. Results are expressed as the percentage of the respective control (vehicle), mean $\pm$ s.e.mean of four to seven different experiments containing two replicates in each experiment. $^*P<0.05$ compared to the respective control.

the absence of exogenous growth factors (Nilsson *et al*., 1985). All the NO-donors studied were more effective in decreasing basal DNA synthesis than the FCS-stimulated one. Reasons for this may include binding of NO-donors to medium supplements (e.g. serum proteins), the presence of growth factors in the serum leading to higher concentrations of NO required to inhibit proliferation (Garg & Hassid, 1989) or accelerated NO oxidation by free radicals in the serum (Dubey, 1993). The inhibitory effect of NO-donors on DNA synthesis and proliferation was not caused by cytotoxicity since NO-donors did not affect cell viability.

Mooradian *et al*. (1995) have suggested that the NO release rate and the inhibited proliferation are related. They found that the antiproliferative effect is greatest when NO is delivered slowly from the compound. In our study, the unsubstitued 5-imine derivative, GEA 3162, which at physiological pH is rapidly hydrolyzed and releases NO, was the most effective substance to inhibit DNA synthesis and proliferation. The half life of imine types of GEA-compounds is 2–5 min and of urea types several hours in rat plasma (Karup *et al*., 1994). SIN-1 and SNAP are relatively stable NO-donors and they were found to reduce proliferation. The nitrite concentration in culture medium was stable from 30 min to 24 h time point in the presence of GEA 3162 and SNAP, which indicates that NO was already released at the beginning of the experiment.

GEA 5624 was able to reduce DNA synthesis in the 24 h experiment. In the proliferation experiment it had a slight inhibitory effect on cell proliferation after 2 days treatment, but this effect was abolished on fourth day. It is possible that the increased cell number during the experiment has decreased the capacity of GEA 5624 to inhibit cell proliferation. Additionally, contrary to the other NO-donors used, GEA 5624 is poorly soluble in water.

The mechanism by which NO inhibits cell growth is not fully known. NO activates soluble guanylate cyclase which leads to increased cyclic GMP production. The elevation of intracellular cyclic GMP, and also cyclic AMP, has been shown to inhibit VSMC proliferation (Assender *et al*., 1992). However, relatively high concentrations of cyclic GMP are needed to inhibit FCS-induced growth (Garg & Hassid, 1989; Dubey, 1993), which suggest that the antiproliferative effects of NO are only partly mediated by cyclic GMP (Southgate & Newby, 1990; Cornwell *et al*., 1994b). This is in agreement with our results, which show that SIN-1 and SNAP inhibited DNA synthesis at the concentrations, when cyclic GMP was elevated over 30 fold. GEA-compounds, on the other hand, reduced DNA synthesis at concentrations which increased cyclic GMP only 5 fold. Furthermore, the blocking of cyclic GMP formation by the guanylate cyclase inhibitor, ODQ, did not diminish the inhibitory effects of NO-donors on the DNA synthesis. ODQ is a novel compound, which has been shown to inhibit soluble guanylate cyclase in vascular tissue without affecting NO itself (Garthwaite *et al*., 1995; Moro *et al*., 1996). Oxyhaemoglobin, which inactivates NO, reduced cyclic GMP production induced by NO-donors. Oxyhaemoglobin also prevented the reduction of DNA synthesis, except in the case of GEA 5624.

Superoxide anions, which are spontaneously generated in aqueous media, can alter NO levels by inactivating NO (Rubanyi & Vanhoutte, 1986). SIN-1 is known to be a combined NO and superoxide anion donor on the contrary to GEA 3162 which is a pure NO-donor. This difference may have an influence on the potencies of SIN-1 and GEA 3162 to induce NO-mediated events (Moilanen *et al*., 1993). In contrast to SIN-1, SNAP does not release detectable amounts of superoxide anions (Ioannidis & de Groot, 1993). Although superoxide dismutase (SOD), which catalyzes the degradation of superoxide anions, increased the concentration of cyclic GMP, it did not affect DNA synthesis in our experiments.

Cyclic GMP activates specific cyclic GMP-dependent protein kinase (cyclic GMP kinase), which is the major receptor protein for cyclic GMP in VSMC. The expression of cyclic GMP kinase is dependent of cell density and decreases in passaged VSMC (Cornwell *et al*., 1994b). These findings have to be considered when investigating the relationships between NO and cyclic GMP in culture conditions. However, the role of cyclic GMP kinase is still unclear. It has been suggested that activation of cyclic AMP kinase, rather than cyclic GMP kinase, by cyclic GMP might take part of NO-dependent inhibition of VSMC proliferation (Cornwell *et al*., 1994a). NO releasing drugs may also have effects unrelated to cyclic GMP on VSMC proliferation. NO activates cyclo-oxygenase (Alanko *et al*., 1995) and inhibits several enzymes including ribonucleotide reductase (Kwon *et al*., 1991) and thymidine kinase (Garg & Hassid, 1993) which are involved in growth regulation. Differences in the inhibitory potencies between the tested compounds may reflect differences in the NO releasing mechanisms. It is also possible that GEA-compounds have NO-independent effects on VSMC growth.

In conclusion, in the present study it has been shown that the novel mesoionic oxatriazole derivative GEA 3162 is able to inhibit DNA synthesis and proliferation of aortic vascular smooth muscle cells at low concentration in comparison to the old NO-donors, SIN-1 and SNAP. GEA 5624 was able to inhibit mitogenesis but not proliferation. ODQ, the specific guanylate cyclase inhibitor, reduced NO-donor induced cyclic

GMP elevation, but did not affect DNA synthesis. Therefore, cyclic GMP dependent events may have a minor role in the reduction of DNA synthesis and proliferation induced by NO releasing compounds.

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