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SPECIAL REPORT

Nitric oxide is involved in the regulation of angiogenesis

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The *in vivo* model of the chick embryo chorioallantoic membrane (CAM) was used to study the involvement of nitric oxide (NO) in angiogenesis. The nitrovasodilator sodium nitroprusside (NaNP) and the amino acid, L-arginine, inhibited angiogenesis, assessed as both collagenous protein biosynthesis and vascular density. N^G-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor, increased both collagenous protein biosynthesis and vascular density, indicating that this agent promotes angiogenesis. These results suggest that NO may participate in the regulation of angiogenesis. Manipulation of NO synthesis therefore, may prove to be another approach for controlling angioproliferative diseases.

Keywords: Chick chorioallantoic membrane (CAM); nitric oxide (NO); N^G-mono-methyl-L-arginine (L-NMMA); angiogenesis; collagenous protein biosynthesis; sodium nitroprusside (NaNP)

Introduction Angiogenesis is, with few exceptions, a very slow process in the adult (Denekamp, 1982). It is accelerated however in a variety of proliferative states, including tumour growth and wound healing (Folkman, 1985; Leibovich, 1984). Although many angiogenic mediators have been identified, the exact sequence of events involved and the regulation of this process remain unclear. Major steps in angiogenesis include invasion of the vascular wall of the 'parent vessel' by activated endothelial cells which then proliferate, migrate and form the lumen of the new vessel. Nitric oxide (NO), is an endogenous vasoactive molecule with multiple properties (Moncada *et al.*, 1991). Recently, it was shown that nitrovasodilators including sodium nitroprusside (NaNP), inhibit endothelial cell proliferation via stimulation of guanylate cyclase (Garg & Hassid, 1989) suggesting that this effect was probably due to spontaneous NO formation by NaNP. Angiogenesis is intimately linked to endothelial cell proliferation. It was of interest therefore to investigate whether NO formation is involved in this process. In the present study the effects of N^G-monomethyl-L-arginine (L-NMMA) (Rees *et al.*, 1989), NaNP and L-arginine, were examined on the chick chorioallantoic membrane (CAM), an *in vivo* model of angiogenesis (Folkman, 1985). It is reported here that NO may be involved in the maintenance of the non-angiogenic status of the vascular endothelium and as such may be a target for the control of angioproliferative diseases.

Methods The *in vivo* CAM angiogenesis model, initially described by Folkman (1985) and modified as previously reported (Maragoudakis *et al.*, 1988) was used. Briefly, fresh fertilized eggs were incubated for 4 days at 37°C when a window was opened on the egg shell, exposing the CAM. The window was covered with tape and the eggs were returned to the incubator until day 9 when the test materials were applied. The test materials or vehicle and 0.5 μCi [U-^{14}C]-labelled proline, were placed on sterile plastic discs and were allowed to dry. The control discs (containing vehicle and radiolabelled proline) were placed on the CAM 1 cm away from the disc containing the test material. A solution of cortisone acetate (100 μg/ disc) was routinely incorporated in all discs in order to prevent an inflammatory response. The discs were inverted and placed on the CAM, the windows were covered and the eggs incubated until day-11 when assessment of angiogenesis took place.

Biochemical evaluation of newly formed vessels was performed by determining the extent of collagenous protein biosynthesis in the CAM lying directly under the discs (Maragoudakis *et al.*, 1988). Briefly, the area under the disc was cut off, placed in an appropriate buffer and protein biosynthesis was stopped. Non protein bound radioactivity was removed by washing with trichloroacetic acid. Pellets containing protein-bound radioactivity were resuspended and subjected to collagenase digestion. The resulting tripeptides corresponding to basement membrane collagen and other collagenous material synthesized by the CAM from [U-^{14}C]-proline, were counted and expressed as c.p.m. mg^{-1} protein.

For morphological evaluation, eggs were treated as above in the absence of radiolabelled proline. At day 12, eggs were flooded with 10% buffered formalin and the plastic discs removed. An area around the disc was cut off, placed on a glass slide and the vascular density index was measured by the method of Harris-Hooker *et al.* (1983).

For each egg, total collagenous protein biosynthesis and vascular density under the disc containing the test material were expressed as a percentage of the control disc containing vehicle. Results were compared by paired *t* test.

Materials Fertilized eggs were obtained from Ioannina, Greece. Collagenase type VII from clostridium histolyticum, cortisone acetate, L-arginine and sodium nitroprusside were purchased from SIGMA Chem. Co. (Poole). L-[U-^{14}C]-proline, specific activity 273 mCi mmol^{-1} was purchased from New England Nuclear Inc. N^G-monomethyl-L-argine (L-NMMA) was a gift from Dr S. Moncada.

Results Sodium nitroprusside from 0.1–7.5 μg/ disc caused a dose-dependent decrease in collagenous protein biosynthesis (Figure 1a) which ranged from 11.8 ± 3.1% to 29.5 ± 4.4% of control (n = 5-22). Furthermore, morphological evaluation of the effect of NaNP (2.5 μg/ disc) on the CAM showed that this compound caused a 23.4 ± 1.9% (n = 10) decrease in vascular density (Figure 1b). The NO synthase inhibitor L-NMMA (Rees *et al.*, 1989) from 1.0–27.0 μg/ pellet caused a dose-related increase in collagenous protein biosynthesis (Figure 1c) ranging from 5.6 ± 5.5% to 32.0 ± 8.4% of control (n = 7–37). A similar result was observed when the effect of L-NMMA (27 μg/disc) on the CAM was assessed morphologically, i.e. a 30.8 ± 5.8%, (n = 10) increase in vascular density was observed (Figure 1d). The inactive analogue D-NMMA, when present at the same doses, had no effect (data not shown). L-Arginine, the natural precursor of NO, at a dose of 2.3 μg/ disc, caused a decrease in collagenous protein

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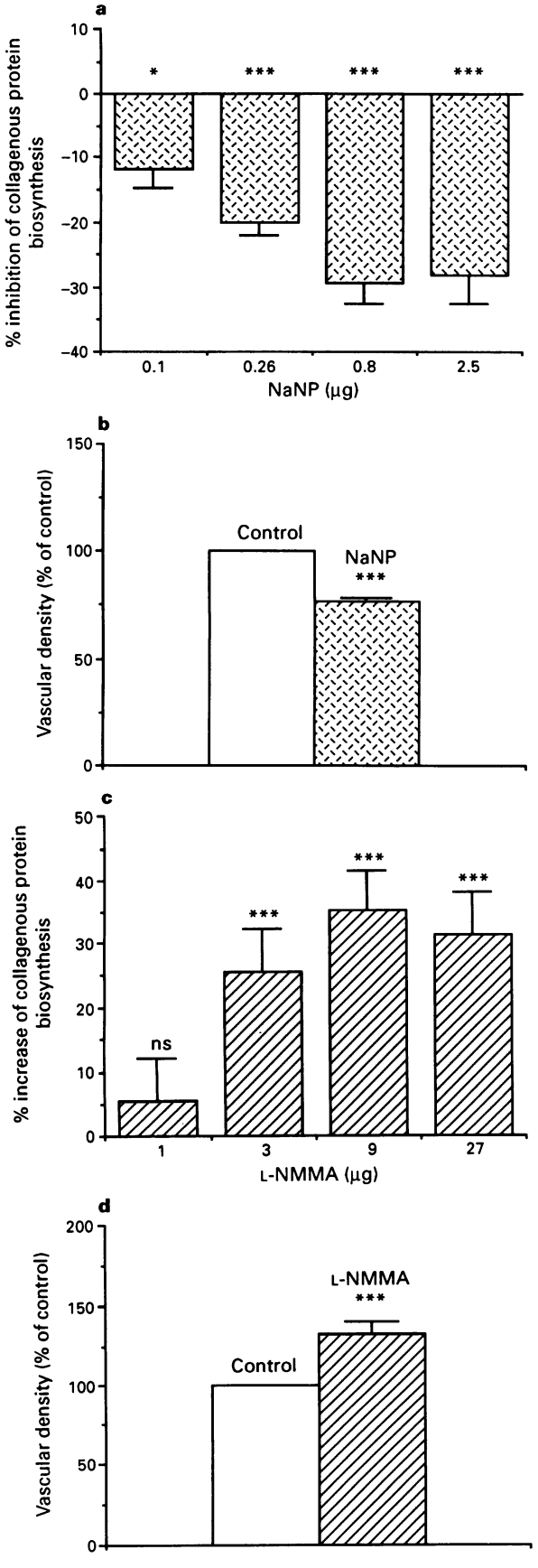

biosynthesis of 22.9 ± 5.0%, compared to control, ($n = 10$, $P < 0.01$). The same amount of L-arginine caused a 9.8 ± 3.0%, ($n = 5$, $P < 0.05$), decrease in vascular density. Larger doses (up to 21 μg) did not cause a further decrease (data not shown) in either parameter.

Discussion In the present study it was shown that NaNP, a vasolidator which generates NO spontaneously (Ignarro *et al.*, 1981), caused a significant depression of angiogenesis as shown by decreases in collagenous protein biosynthesis and vascular density in the CAM. The amino acid L-arginine, the endogenous precursor for NO formation (Moncada *et al.*, 1991), had a similar albeit smaller effect. L-NMMA a specific inhibitor of NO synthase (Rees *et al.*, 1989) (but not the inactive analogue D-NMMA) caused a significant increase in collagenous protein biosynthesis and vascular density. These results suggest that NO negatively regulates the angiogenic process. The modest antiangiogenic effect caused by L-arginine (particularly when vascular density was assessed) is not unexpected since exogenous L-arginine is unable to compete with the endogenous amino acid which may be presented to the enzyme more efficiently (Rees *et al.*, 1989). The extent of increase of angiogenesis caused by L-NMMA, was around 30% and may reflect the extent to which NO may participate in the angiogenic process in the CAM under the present experimental conditions. The experiments described here were performed in the presence of cortisone in order to prevent an inflammatory response in the CAM, leading to artifacts. However, corticosteroids have been shown to inhibit the inducible form of the NO synthase (O'Connor & Moncada, 1991). This may lead to an underestimation of the role of NO of angiogenesis. Efforts are being made to devise experiments where the contribution of this form of the enzyme will be studied, without compromising the reproducibility of the system.

Angiogenesis is a phenomenon of pathophysiological importance which proceeds in a sequence of distinct steps involving proliferative and migratory phenomena (Folkman, 1985). NO may act directly in the CAM to inhibit endothelial cell proliferation as it does in messangial cells (Garg & Hassid, 1989). On the other hand NO may act indirectly by preventing endogenous mediators of cell growth to be released and/or act as mitogens. In conclusion, modulation of NO formation appears to have an effect on angiogenesis and under normal conditions, may play a role in maintaining the vascular endothelium in a quiescent state. It is possible that in 'angiogenic' disease states where excessive angiogenesis is evident, the synthesis or availability of NO is compromised, thus, tipping the balance towards the initiation of proliferative events in the vessel wall. This new role for NO may offer an alternative way of controlling angiogenesis-associated disease.

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Figure 1 Effect of sodium nitroprusside (NaNP) and of N^G-monomethyl-L-arginine (L-NMMA) on angiogenesis in the chick chorioallantoic membrane (CAM) expressed as (a,c) collagenous protein biosynthesis (CPB) and (b,d) vascular density. Results are expressed as mean ± s.e. of the mean% of control and are compared by Student's paired t test. $^*P < 0.05$; $^{**}P < 0.02$ and $^{***}P < 0.01$. The number of observations n is indicated in the text.

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