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The Influence of Surgical Preparation on Cyclic Nucleotide Synthesis in an Organ Culture of Human Saphenous Vein

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Objectives: *To investigate adenosine cyclic 3'5' monophosphate (cAMP) and guanosine cyclic 3'5' monophosphate (cGMP) synthesis in freshly isolated and surgically prepared human saphenous vein before and after culture.*
Setting: *Bristol Heart Institute, Bristol, U.K.*
Methods: *Freshly isolated and surgically prepared human saphenous vein was obtained from patients undergoing coronary artery bypass graft surgery. cAMP and cGMP synthesis, was assessed by radioimmunoassay in response to specific simulators in segments of saphenous veins after collection and following 14 days culture.*
Results: *Immediately after collection there was a significant reduction in the synthesis of cAMP (forskolin and prostaglandin E_1-stimulated) and cGMP (sodium nitroprusside-stimulated) in surgically prepared compared to freshly isolated saphenous veins. In contrast, following 14 days in culture, cAMP and cGMP synthesis was significantly elevated in surgically prepared compared to freshly isolated saphenous veins.*
Conclusions: *These data indicate that surgical preparation results in a marked reduction in cyclic nucleotide synthesis in saphenous vein which may be relevant to the pathophysiology of early vein graft failure. The normalisation of both cAMP and cGMP synthesis in surgically prepared veins following 14 days culture indicates that cyclic nucleotide synthesising capacity may not be a major determinant of neointima formation in this experimental model.*

Key Words: Cyclic nucleotides; Human saphenous vein; Organ culture

Introduction

Surgical preparation of saphenous veins for use as a conduit in vascular reconstructive procedures is known to result in structural and biochemical changes which are associated with the development of intimal hyperplasia.[1–6] Intimal hyperplasia essentially involves the migration of vascular smooth muscle cells (VSMCs) of medial origin to the luminal aspect of the graft where they continue to proliferate and secrete extracellular matrix proteins, thus forming a neointima which leads to stenosis, atherogenesis and eventually thrombotic occlusion of the graft.[7–9] Although the mechanisms underlying intimal hyperplasia remain to be fully clarified, it is well established that growth factors and metalloproteinases are important mediators of the initiation and progression of intimal hyperplasia.[7–9] Indeed, in an organ culture model of human saphenous veins it was found that surgical preparation elicited smooth muscle cell proliferation, neointima formation, mRNA expression for platelet derived growth factor (PDGF) and up-regulation of metalloproteinase activity.[10–12]

Endogenous systems that inhibit VSMC proliferation include prostacyclin (PGI_2) and nitric oxide (NO), the effects of which are mediated by adenylyl and guanylyl cyclases and thus the synthesis of adenosine cyclic 3'5' monophosphate (cAMP) and guanosine cyclic 3'5' monophosphate (cGMP), respectively.[9] Stable analogues of cAMP and cGMP (and the simulators of their synthesis) have been shown to inhibit proliferation and migration of cultured VSMCs.[13–16] More recently, in human saphenous veins in culture, neointima formation was inhibited by stable analogues of cAMP and cGMP.[5] It has also been shown that surgical preparation of human saphenous vein resulted in a marked reduction of PGI_2 synthesis, which indicated that this procedure elicits damage to the endothelium.[1] NO, also derived from the endothelium, stimulates cGMP synthesis in the smooth muscle component of blood vessels,[17] although little is known of NO-cGMP axis in vein grafts. Thus, in order to investigate further the impact of surgical preparation of saphenous veins, the synthesis of cAMP and cGMP in response to specific stimulators, was assessed in

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freshly isolated and surgically prepared saphenous veins immediately after collection and after 14 days organ culture.

Materials and Methods

Tissue preparation

Paired segments of freshly isolated and surplus surgically prepared saphenous vein were obtained from eight patients (median age, 58 years, range 43–72 years) undergoing coronary artery bypass graft surgery (CABG) and were processed for analysis and culture as previously described.[4,5,18] Freshly isolated segments were obtained immediately after dissection by a "no-touch" technique.[19] Surgically prepared segments had been subjected to side branch ligation, adventitial stripping and uncontrolled manual distension with patients heparinised blood and stored and transported to the laboratory in sterile RPMI 1640 culture medium with HEPES buffer (20 mmol/1, Flow Labs, Irvine, CA, U.S.A.) containing 4 IU/ml sodium heparin (CP Pharmaceuticals, Wrexham, U.K.) and 5 μg/ml amphotericin (Flow Labs). Papaverine was not included in the culture medium, as is usual, since this drug has been shown to possess phosphodiesterase inhibitory activity, an enzyme class that hydrolyses cyclic nucleotides.[20]

Procedures for tissue preparation and culture was essentially similar to that reported earlier. Veins were cut into segments for immediate biochemical analyses, histology or tissue culture.[4,5,18] For tissue culture, approximately 1 cm lengths were opened longitudinally and secured, endothelium upward onto polyester mesh, with minute stainless steel pins in glass Petri dishes containing set sylgard resin. Tissues were then cultured for 14 days in medium RPMI 1640 containing 2 g/1 sodium bicarbonate, 30% fetal bovine serum (Imperial Labs, Hants, U.K.), 100 mg/1 penicillin, 100 mg/1 streptomycin and 2 mmol/1 glutamine (Flow Labs). The tissues were maintained for 14 days at 37°C in 5% carbon dioxide in a humidified atmosphere incubator, culture medium being changed every 48 h.

Measurement of cyclic nucleotides

Freshly isolated and surgically prepared veins (day zero and after 14 days culture) were cut into approximately 2 mm square segments and placed in Dulbecco's Minimum Essential Medium (MEM; pregassed with 95/5; O_2/CO_2). Tissue segments were then incubated in MEM for 2 h at 37°C (with changes of medium every 30 min) to allow tissues to equilibriate and reach a steady state. Tissues were then transferred to polypropylene test tubes containing 250 μM isobutylmethylxanthine (an inhibitor of phosphodiesterase activity) and varying concentrations of either forskolin, PGE_1 (cAMP synthesis stimulators), sodium nitroprusside (NaNP), acetylcholine (Ach) or calcium ionophore A23187 (cGMP stimulators) dissolved in MEM. Tissues were then incubated at 37°C for 30 min. Reactions were stopped by the addition of 1M perchloric acid and tissues sonicated (3 × 30 s; Soniprep, MSE). Following centrifugation at 1000 *g* for 15 min, supernatants were taken and neutralised with 1M K_3PO_4. Aliquots were then taken and acetylated with triethylamine/acetic anhydride (1/2, v/v). Concentrations of cAMP and cGMP were measured using specific [^{125}I] — radioimmunoassay kits (Amersham International, Aylesbury, Bucks, U.K.).[20,21]

ATP, DNA and 6-oxo-$PGF_{1\alpha}$ measurements

Frozen vein segments of tissues were crushed under liquid nitrogen and the resultant powder extracted with perchloric acid as described previously.[4,5,18] The supernatant was neutralised and ATP measured using a bioluminescent assay.[22] The pellet was re-homogenised in perchloric acid, neutralised and centrifuged. After washing with ethanol to remove lipids the pellet was dried and DNA measured using a fluorimetric assay.[23] PGI_2 synthesis was assessed in zero time samples to establish the relative integrity of the endothelium. Segments of vessels were incubated in MEM or 1 h and supernatants taken for estimation of 6-oxo-$PGF_{1\alpha}$ by radioimmunoassay.[24]

Histology

Segments of all tissue samples were stored in phosphate buffered formalin for histological assessment (viz. presence of neointima) of cultured explants. The thickness of neointima in each sample (following 14 day culture) was measured with light microscopy (× 40 magnification) using a computerised image analyser system. Measurements were taken along the length of the opened internal surface of the vein (cross-sectional). Two to four fields were viewed, depending on the length of the sample, and six

measurements taken per field, from which the average of these readings was calculated.

Data expression and statistics

Data are expressed as fmoles of cAMP or cGMP per μg of DNA per min. Statistical analysis was carried out using Mann Whitney Rank sum test and linear regression analysis.

Results

When assessed at day zero there was a marked reduction in both cAMP (forskolin and PGE_1-stimulated) and cGMP (NaNP-stimulated) in surgically prepared compared to freshly isolated saphenous vein segments (Figs. 1–3). Acetylcholine and A23187 exerted no effect on cGMP synthesis, either in freshly isolated or surgically prepared tissues or in cultured tissue. Endothelial denudation as an explanation for this was discounted since PGI_2 synthesis was unaffected in the freshly isolated tissues (90 ± 15 [mean ± S.D.] pg 6-oxo-$PGF_{1\alpha}$/μg DNA/min) but was markedly diminished in surgically prepared vein segments (28 ± 5 [mean ± S.D.] pg 6-oxo-$PGF_{1\alpha}$μg DNA/min; $p < 0.001$).

Following 14 days culture, histology confirmed the presence of a measurable neointima: neointimal thickness (median microns [range]) in freshly isolated was 49 [0–70] and in surgically prepared; 71 g [0–96]; $p < 0.05$.

Following 14 days culture, cAMP synthesis and cGMP synthesis by freshly isolated vein segments, in response to PGE_1 and forskolin was similar to that at day zero (Figs. 4–6). However, cAMP and cGMP synthesis was significantly greater in surgically prepared veins compared to similarly cultured freshly isolated veins.

There was a highly significant correlation between the concentrations of ATP and maximal concentrations

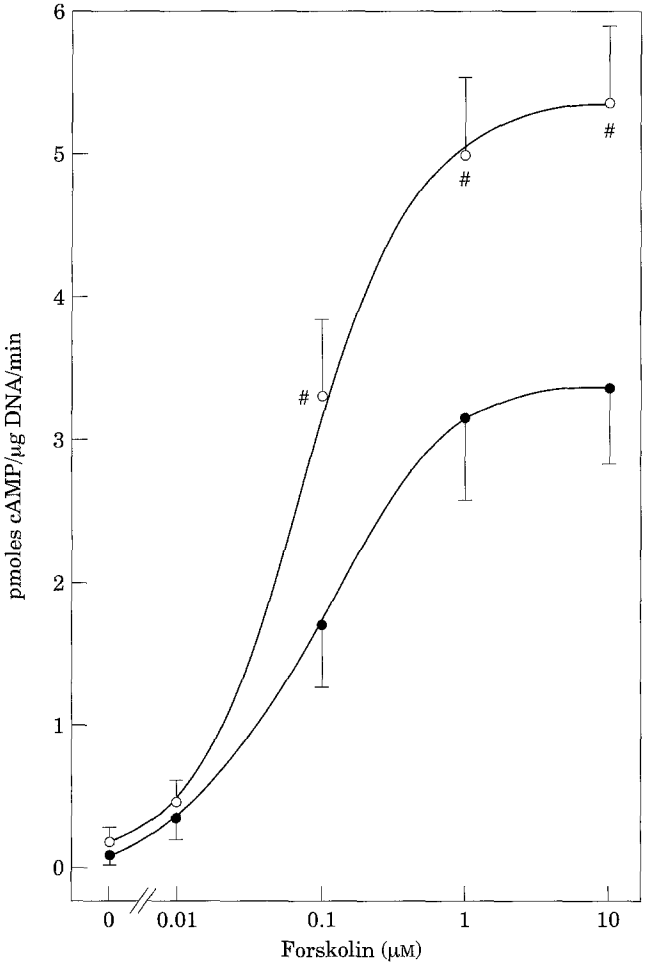

Fig. 1. Effect of forskolin on cAMP synthesis by human saphenous vein immediately after collection: (○) freshly isolated and (●) surgically prepared. Each point = mean ± S.D.; $n = 8$; # $p < 0.001$.

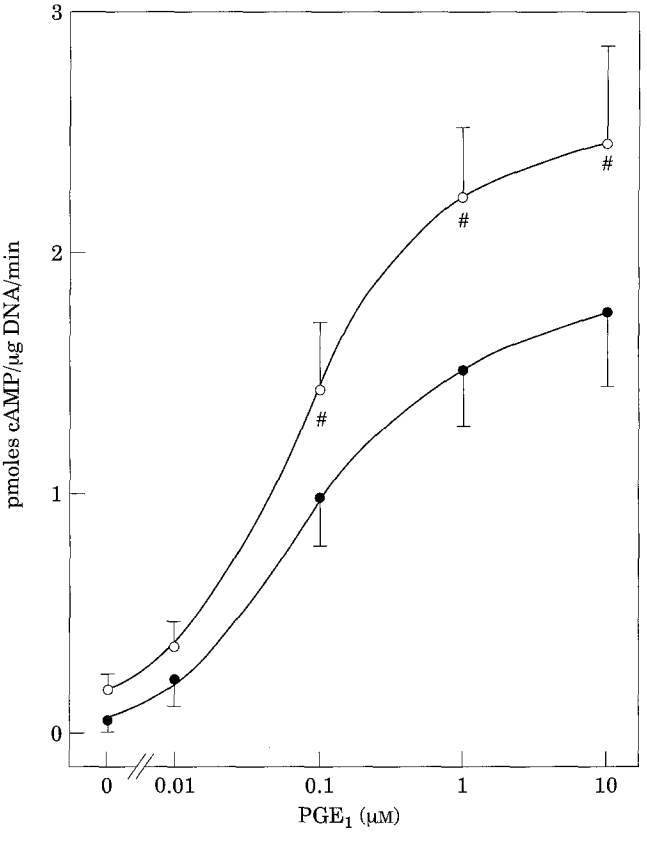

Fig. 2. Effect of prostaglandin E1 on cAMP synthesis by human saphenous vein immediately after collection: (○) freshly isolated and (●) surgically prepared. Each point = mean ± S.E.M.; $n = 8$; # $p < 0.001$.

of cAMP in freshly isolated and surgically prepared vein at day zero as determined by linear regression analysis (y = 0.011x + 12.4, R = 0.6, $p < 0.01$) but not after 14 days culture.

DNA concentrations (μg/mg wet weight [mean ± S.D., $n = 8$]) were as follows: Day 0; freshly isolated,0.53 ± 0.18; surgically prepared, 0.445 ± 0.098 ($p < 0.01$). Day 14: freshly isolated, 0.51 ± 0.24; surgically prepared, 0.355 ± 0.18 ($p < 0.01$). ATP concentrations (pmoles/μg DNA [mean ± S.D., $n = 8$]) were as follows. Day 0: freshly isolated, 448 ± 55; surgically prepared 148 ± 35 ($p < 0.01$). Day 14, freshly isolated: 260 ± 36; surgically prepared: 269 ± 38 (not significant).

Discussion

The present study firstly demonstrates that routine surgical preparation of saphenous veins for coronary arterial bypass grafting into the coronary arterial bed markedly reduces both cAMP and cGMP synthetic capacity. This initial and marked reduction of cAMP and cGMP synthesis may be a further factor implicated in early vein graft failure. The significant correlation between cAMP and ATP concentrations indicates that the diminished synthesis of cAMP in surgically prepared veins is due to a reduction of substrate levels (viz. ATP). Previous work has demonstrated that ATP is markedly reduced in surgically prepared saphenous veins compared to freshly isolated veins, which was confirmed in the present study.[3] This reduction has been ascribed to both diminished cell viability and to alterations of nucleotide metabolism.[1–5] Thus, the initial reduction of cAMP and cGMP synthesis may be a reflection simply of cell death, with no reduction of cyclase activity in viable cells and as such have no relevance to intima formation.

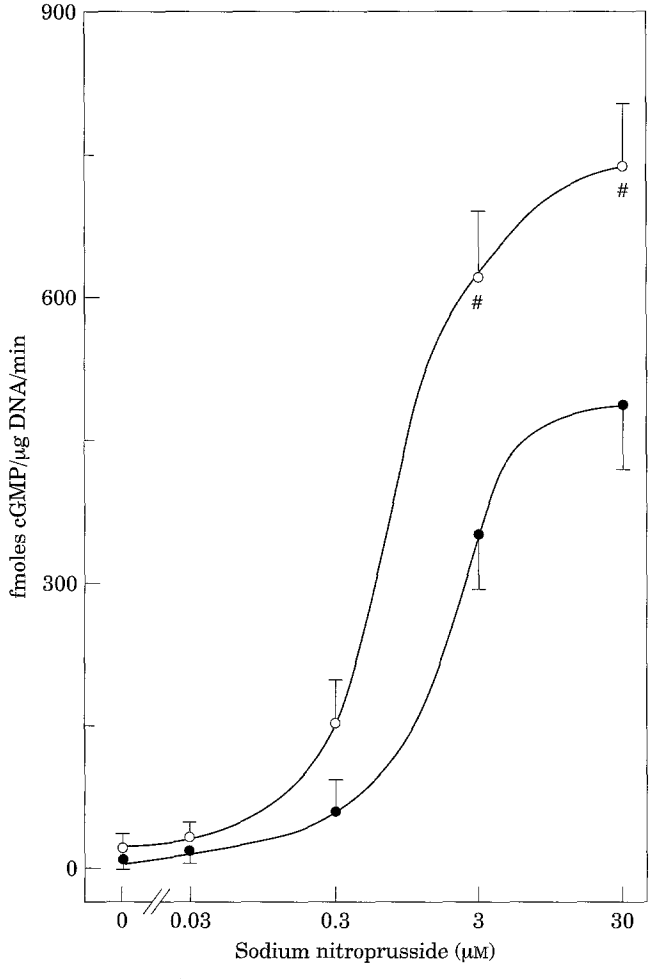

Fig. 3. Effect of sodium nitroprusside on cGMP synthesis by human saphenous vein immediately after collection: (○) freshly isolated and (●) surgically prepared. Each point = mean ± S.E.M.; $n = 8$; # $p < 0.001$.

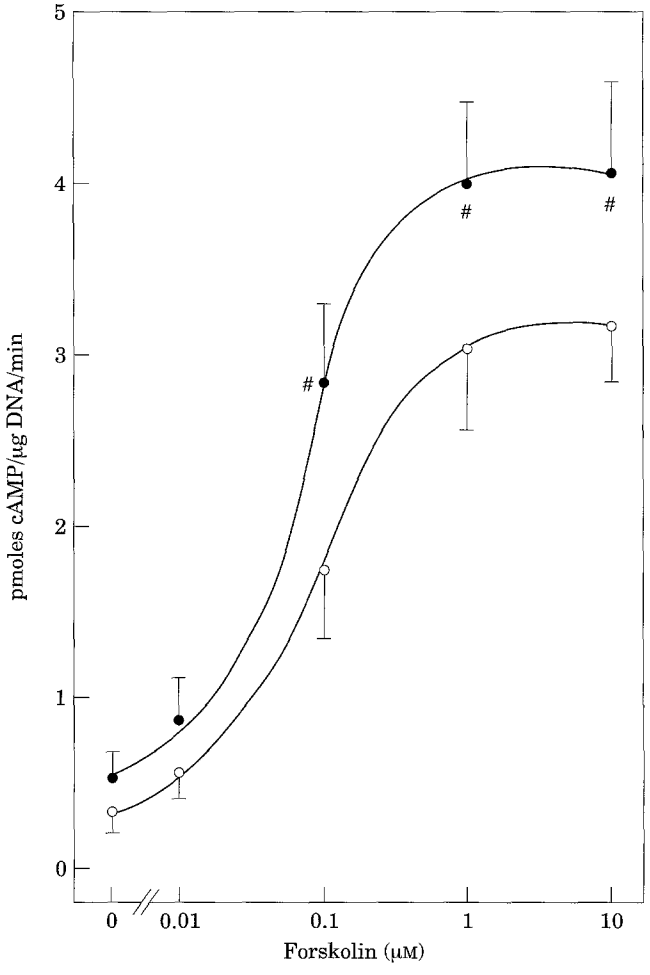

Fig. 4. Effect of forskolin on cAMP synthesis by human saphenous vein following 14 day organ culture: (○) freshly isolated and (●) surgically prepared. Each point = mean ± S.D.; $n = 8$; # $p < 0.001$.

One notable finding was that A23187 and acetylcholine, both stimulators of NO synthesis in the endothelium of other vascular tissues,[17] were without effect on cGMP synthesis in freshly isolated human saphenous veins, in which the endothelium remains intact. These data indicate that cholinergic receptors linked to NO synthase (NOS) and/or calcium–stimulated NOS (constitutive) may not be present, or is extremely low, in the endothelium of human saphenous veins. Furthermore, in saphenous vein segments, cGMP synthesis was stimulated by nitroprusside (obviates NO synthesis since it breaks down to generate NO in aqueous solution).[25] This demonstrates that guanylyl cyclase is present in the vein segments and as such the lack of effect of acetylcholine and A23187 cannot be ascribed to an absence of this enzyme. This apparent lack of endothelial NOS activity may render the saphenous vein susceptible to complications associated with graft failure, since NO inhibits platelet and lecocyte adhesion and VSMCs proliferation.[17,26]

Culture of freshly isolated saphenous vein segments for 14 days did not result in impairment of cAMP and cGMP synthesis and patterns of responses to all stimulators were similar to those obtained at day zero. Notwithstanding the physiological relevance, these data consolidate the validity of using this particular model to investigate the mechanisms controlling cyclic nucleotide synthesis. From a methodological point of view, therefore, measurement of cyclic nucleotides constitutes an alternative biochemical parameter for the assessment of viability of cultured vein explants.

Cyclic nucleotides regulate a number of key functions associated with intimal hyerplasia: platelet and leucocyte adhesion and activity, smooth muscle hyperplasia, proto-oncogene expression, extracellular matrix protein turnover, cholesterol metabolism etc.[9] Thus, the increased synthetic capacity of both cAMP and cGMP in surgically prepared compared to freshly isolated saphenous veins, following 14 day culture, was somewhat unexpected. Previous studies have demonstrated that surgical preparation of these vessels results in formation of a neointima,[4,5] which in turn has recently been shown to be inhibited by cyclic

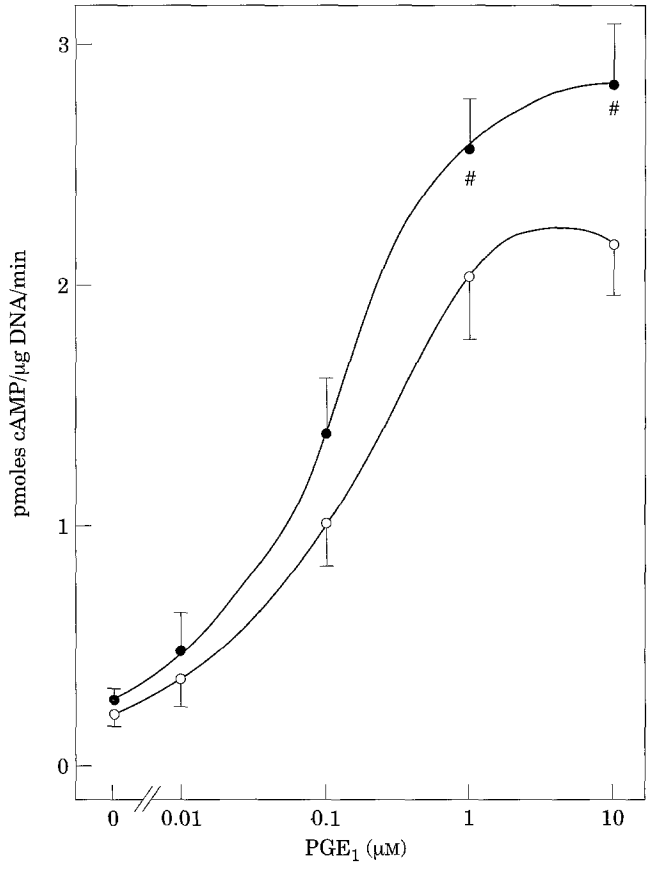

Fig. 5. Effect of prostaglandin E1 (PGE1) on cAMP synthesis by human saphenous vein following 14 day culture: (○) freshly isolated and (●) surgically prepared. Each point = mean ± S.D.; $n = 8$; # $p < 0.001$.

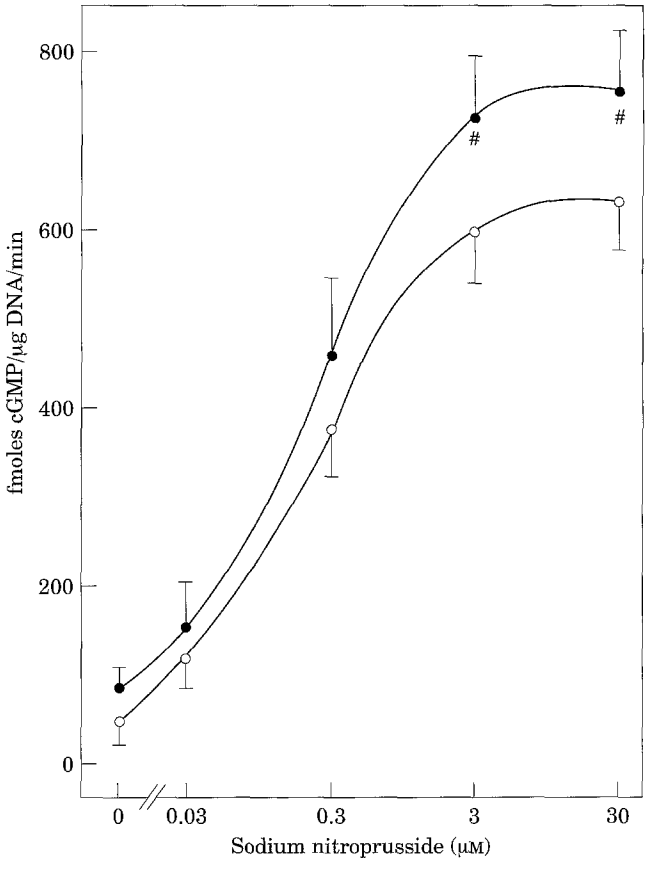

Fig. 6. Sodium nitroprusside–stimulated cGMP synthesis by human saphenous vein following 14 day culture; (○) freshly isolated and (●) surgically prepared. Each point = mean ± S.E.M.; $n = 8$; # $p < 0.001$.

nucleotide analogues.[5] The present data indicates that cyclic nucleotide synthesising capacity may not be a major determinant of neointima formation, at least in this experimental model. However, impairment could occur upstream of these second messengers, e.g. diminished release of PGI_2 and NO and signal transduction systems that link these primary messengers to the cyclases. Furthermore, it has also recently been demonstrated that both adenylyl and guanylyl cyclase activity is significantly reduced in experimental porcine saphenous vein grafts.[27] The difference between these *in vitro* and *in vivo* models is that *in vivo*, grafts are subjected to mechanical forces including arterial pressure, shear stress and pulsaltile flow as well as the impact of platelet and leucocyte release substances. These cells, which adhere to vein grafts, release a battery of potent promitogenic substances that have been widely implicated in neointima formation and include peptide growth factors, interleukins, leucotrienes, vascoconstrictors and proteases. The present *in vitro* system excludes the impact of such extrinsic factors, and as such constitutes a limitation to the model.

In conclusion, the present study demonstrates that surgical preparation of saphenous vein for use as a conduit in vascular reconstructive procedure results, acutely, in a marked reduction of cyclic nucleotide synthesis which probably reflects marked cell death. These data consolidate that manipulation of vein grafts prior to implantation should be minimalised, without compromising the integrity of the surgical procedure. After 14 days in culture, however, cyclic nucleotide synthesising capacity recovered indicating that prolonged supression of cAMP and/or cGMP synthesis may not be an important factor in intimal hyperplasia, at least in this *in vitro* model. On this basis the administration of cAMP and cGMP analogues may not be an effective strategy in inhibiting intimal hyperplasia.

Acknowledgements

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