Sucrose inhibits vascular smooth muscle cell proliferation/ migration – protein synthesis is maintained

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Abstract. We have previously shown that the onset of smooth muscle cell proliferation in tissue cultures is triggered independently of serum. The aim of the present study was to investigate if this process was affected by osmotic stress. Vascular explants from 8-month-old male rats were cultured under serum-free conditions using collagen I as migration substrate. Sucrose was added to the culture medium in concentrations varying from 1 to 3% (30–90 mOsM). Cell migration from aortic explants onto the culture dishes was totally inhibited at a sucrose concentration of 90 mOsM. A significant dose-dependent decline in proliferation was shown for cells in explants pulse labelled with ^3H -thymidine. In contrast, pulse labelling with ^{35}S -methionine revealed that protein synthesis was maintained in the presence of sucrose. The results indicate that osmotic pressure affects smooth muscle cell protein synthesis, proliferation and migration.

INTRODUCTION

Vascular smooth muscle cells (SMCs) are described by two phenotypes: the contractile quiescent cell and the dedifferentiated secretory cell (Chamley-Campbell *et al.* 1981; Thyberg *et al.* 1985). The dedifferentiated cells occur in atherosclerotic lesions as well as in lesions of injured blood vessels, where they proliferate, migrate and synthesize extracellular matrix components to stabilize the damage. In processes such as atherosclerosis or post-surgery repair, uncontrolled proliferation may lead to intima hyperplasia and narrowing of the lumen. Therefore, it is important to elucidate both how SMC activities are initiated and how to control SMC proliferation.

The SMCs reside in the vessel wall and are surrounded by a complex extracellular matrix (ECM). The ECM consists of both proteins and sugars forming a structure, which may be regarded as a gel. The components of the ECM are closely connected to the cells through receptor ligand interactions (Moiseeva 2001). One feature of the matrix is to regulate the hydrostatic pressure (Weinberg *et al.* 1997). The present study shows the impact of osmotic stress on SMC proliferation and protein synthesis. It is suggested that unbalanced hydrostatic conditions in the vessel wall may trigger an onset of SMC proliferation, and that osmotic pressure may attenuate this effect.

MATERIALS AND METHODS

Twelve 8-month-old male rats (White Wistar) were killed with an overdose of pentobarbital (100 mg/kg) injected into the peritoneum. The thoracic aorta was exposed, perfused, dissected out and transferred to Hanks' balanced saline solution (HBSS). Perfusion was performed by clamping the vessel below the aortic arch, and 4 ml of HBSS was injected into the aorta below the clamp. All further steps were performed in excess HBSS. Perivascular connective tissue was removed from the unopened aorta under a stereomicroscope. Ring segments of 0.5 mm in length were cut perpendicular to the longitudinal axis with a McIlwain tissue chopper. One ring segment was placed per culture well (each 4.6 cm², Life Technologies, Meda, Denmark) with one cut side facing the bottom of the well. The time from killing a series of three animals to incubation of the tissue was approximately 2 h. All buffers were supplemented with streptomycin (0.25 mg/ml)/penicillin (0.25 IU/ml).

Coating of culture dishes

Culture dishes were coated with collagen I (10 µg/cm²) according to the method described by NUNC, Roskilde Denmark. Collagen I from rat tail tendon was from Boeringer Mannheim, Mannheim, Germany.

Culture conditions

The ring segments were kept in the same medium throughout the experiment. RPMI-1640 medium (0.4 mL per well) was supplemented with FeCl₃ (0.1 µg/ml), HEPES buffer (1%), NaHCO₃ (0.9 g/l) and streptomycin (0.25 mg/ml)/penicillin (0.25 IU/ml). Cultures were added in various concentrations of sucrose (dissolved in medium) ranging from 30 to 90 mM. Sterile water was added in between the wells, the culture dishes were sealed with tape and incubated at 37 °C, 5% CO₂ at a humidity of 95%.

Pulse labelling with ³H-thymidine of cells inside the explants for autoradiography

³H-thymidine was used as a marker to detect early cellular activities inside the cultured tissue. Parallel cultures were incubated with either serum-free medium or serum-containing medium and pulse labelled with ³H-thymidine (37 kBq or 1 µCi/ml) for 0–24 and 24–48 h, respectively. Ring segments were removed after these periods of incubation, fixed in 4% Soerensen buffer (pH 7.3) for 6 h, dehydrated in ethanol, xylene, and embedded in paraffin. They were cut with a microtome in sections of 10 µm, placed on object glasses, de-paraffinized, dipped into emulsion (Amersham, Hoersholm, DK and developer from Kodak), and finally subjected to autoradiography for 2 weeks. To identify cells, the paraffin sections were then stained with an eosin/haematoxylin solution for 15–30 s. The sections were analysed under a stereo microscope.

Pulse labelling

Parallel cultures were incubated in medium supplemented with either ³H-thymidine or ³⁵S-methionine (37 kBq/ml). The cultures were labelled from time 0 with steps of 24 h. After the respective labelling periods explants were removed from the cultures, rinsed in 20 ml of PBS buffer for 5 min, dried on filter paper for another 5 min, transferred to separate Eppendorf tubes, and frozen at –20 °C until use. Isotopes were obtained from Amersham, UK. The experiments were repeated three times.

Preparation for scintillation counting

Eppendorf tubes each containing one explant were added to 0.4 ml of NaOH (0.5 M) and incubated at 60 °C for 2 h. Each vial containing the dissolved tissue was added 0.4 ml of HCl (0.5 M) and sonicated (3 × 15 s). The protein content was estimated from 0.2 ml of the suspension

from each vial (Bio-Rad protein assay, Bio-Rad Laboratories, Herlev, Denmark). Bovine serum albumin taken through this process showed a loss of 5–10%. The remaining 0.6 ml was added to 0.2 ml of TCA (tri-chloric acid) supplemented with 1 mM of non-radioactive methionine or thymidine corresponding to the labelling isotope. The final concentration of TCA was 8% (w/v). From this solution 0.4 ml was applied to filters and rinsed with excess ice-cold TCA (vacuum pressure). The filters were dried, transferred to scintillation tubes, added to 3 ml of scintillation liquid, and CPM was measured in a scintillate counter (average count 10 min per vial). CPM per vial was corrected for protein content and plotted against time.

Early protein synthesis

Ring segments were pulse labelled with 74 kBq ^{35}S -methionine for 0–4, 4–12 h, and up to 36 h with steps of 8 h and prepared as described above.

Immunostaining

In the present study, cells migrated onto the bottom of the culture wells were identified as SMCs by using the streptavidin peroxidase immunostaining method. The cells were fixed in 4% Soerensen buffer (pH 7.3) for 1 h and immunostained by anti SMC-actin (1 : 200). Antibodies were from DAKO, Roskilde, Denmark and 3,3-diaminobenzidine tetrahydrochloride dihydrate were from Fluka, Buchs SG, Switzerland.

Cell counting

The number of cells that had migrated from the ring segments onto the bottom of the culture wells was obtained from parallel cultures. Cells in these cultures (12 days old) were immunostained as described above and counted in an inverted phase contrast microscope (220 $\times$) using a counting frame in the ocular.

Osmolarity

At 90 mM (3% sucrose), the osmolarity in the medium was measured to be the same as with 10% FCS (v/v).

Statistics

Data were analysed by two sided Student's *t*-test. $P_{2\alpha} < 0.05$ was considered significant (Graph Pad Instat, Graphpad Software).

RESULTS

In the present study the impact of sucrose on vascular SMC proliferation and protein synthesis *in vitro* was investigated. The experiments were performed with serum-free medium.

Early protein synthesis and proliferation

Ring segments were transferred to the culture dishes and subjected to pulse labelling. ^{35}S -methionine incorporation revealed that the protein synthesis was active within 0–4 h from incubation. The rate of incorporation in the following 8-h intervals was the same up to 28 h and increasing up to 36 h (Fig. 1). In contrast, autoradiography studies of ^3H -thymidine incorporation under the same conditions revealed that there was a lag of 24 h before cell nuclei were positively labelled (data not shown).

Figure 1. Early protein synthesis. Cells in ring segments were from time of incubation pulse labelled with 74 kBq ³⁵S-methionine in serum-free medium (0–4 h) and after labelling were monitored at 8-h intervals. The data are presented with the mean and SE ($n = 6$ per group).

Figure 2. Anti α -actin immunostaining of smooth muscle cells migrated onto the bottom of the culture dishes (bar represents 40 μ m).

Migration of cells in sucrose containing medium

In cultures incubated without sucrose, SMCs migrated from the explants onto the bottom of the collagen coated culture dishes after 2–3 days (Fig. 2). In a period of 12 days this number reached $7 \times 10^3 \pm 1500$ cells/well. At 30 and 60 mM, sucrose cell migration was estimated to be $5 \times 10^3 \pm 750$ and $1-2 \times 10^3$ cells/well, respectively. At a concentration of 90 mM, sucrose migration of cells from explants onto the bottom of the culture dishes was totally inhibited.

Cells in the ring segments pulse labelled with ³H-thymidine

Sucrose added to the growth medium in various concentrations (30–90 mM) inhibited incorporation of ³H-thymidine in a dose-dependent manner (Fig. 3). Within the first 24 h of incubation, no incorporation took place, therefore these results were not included. To test if proliferation in cultures with sucrose increased later during the incubation period, explants were pulse labelled

Figure 3. Pulse labelling with ^3H -thymidine. Aortic ring segments were cultured in serum-free medium for a period of 0–120 h in the presence of 0–90 mM sucrose (0–3%). Cultures with 90 mM sucrose (3%) were further incubated up to 144 h. Cells were pulse labelled with 37 kBq ^3H -thymidine at 24-h intervals. At 60 and 90 mM (2 and 3%) sucrose there was a significant dose-dependent inhibition of proliferation in the incubation period. The data are presented with the mean and SE ($n = 7$ –8 per group).

with ^3H -thymidine for a further 24 h (120–144 h). Proliferation did not increase in this period (Fig. 3).

Protein synthesis of pulse labelled cells in the ring segments

Under the influence of sucrose, ^{35}S -methionine incorporation was significantly higher than in cultures without sucrose within the first 24 h. From 24 to 72 h the protein synthesis tended to be stimulated in cell cultures supplemented with 60 mM (2%) sucrose. From 72 to 96 h the stimulation of protein synthesis was significant at 60 mM (Fig. 4).

DISCUSSION

The osmotic active compound sucrose revealed two effects: (1) the proliferation rate was lowered, and (2) protein synthesis was either stimulated or unaffected. Furthermore, migration of SMCs was inhibited at a sucrose concentration at 90 mOsM. These results may suggest that an injury initiate the onset of SMC activities in three phases: (1) protein synthesis, (2) proliferation, and (3) migration.

The cell membrane is thought to be almost impermeable to sucrose, which creates an osmotic pressure. Sucrose was used to investigate if osmotic stress would influence the initial activation of SMCs. High concentrations of sucrose (270 mOsM) have been shown to damage cells and cell-to-cell connections in vascular tissue (Daniel *et al.* 1987). However, the highest concentration used in the present study was three times lower. Furthermore, sucrose in a concentration of 3% contributes to the medium with a 90 mOsM pressure, which corresponds to a serum-concentration of 10%. At 90 mOsM sucrose, no cells migrated onto the culture dishes, and proliferation in the tissue was almost abolished. However, the rate of protein synthesis in

Figure 4. Protein synthesis. Aortic ring segments were cultured in serum-free medium in a period of 0–96 h on collagen-I coated culture dishes. Cells were pulse labelled with 37 kBq ^{35}S -methionine at periods of 24 h in the presence of 0–90 mM sucrose (0–3%). Within 0–24 h a significant increase in protein synthesis was found at 30 mM (1%) and 60 mM (2%) sucrose. The data are presented with the mean and SE ($n = 7$ –8 per group).

treated cultures was the same as in non-treated cultures. Therefore, proliferation seems to play a role prior to migration.

Changed hydrostatic forces in the ECM may trigger an injury stimulus. It was suggested that oedema induced a weakening of proteoglycans (PG) resulting in interactions with other components of the ECM (Negrini *et al.* 1996). Furthermore, it was shown that enzymatic digestion of heparin PG initiated a local dedifferentiation of SMCs (Chamley-Campbell *et al.* 1992). It is thus not unlikely that changes of fluid/ECM ratio may initiate the onset of SMC activities. It was shown *in vivo* that, 6 h after arterial injury, there was loss of the regular pericellular distribution of both heparin sulphate and chondroitin sulphate, which was associated with a significant expansion in the extracellular space (Bingley *et al.* 2001). At the cellular level, compounds with osmotic features such as urea and sucrose in equimolar concentrations lead to a sustained cell shrinkage and inhibited Ca^{2+} current through voltage-sensitive Ca^{2+} channels in a dose-dependent manner (Sasaki *et al.* 1986; Wagner *et al.* 2000). These findings suggest that, despite an increased extracellular space, the osmotic effect reduces the cell volume. Combined with the present results, this suggests that a reduction in cell volume may be controlling the initial onset of proliferation.

Inhibited influx of extracellular Ca^{2+} induced by sucrose reduces the activity of Ca^{2+} -dependent kinases, reducing both proliferation (Means 1994) and migration (Pauly *et al.* 1995). Results in the present report suggest that cell proliferation in the explants takes place prior to migration, indicating that inhibition of proliferation and, at the same time, maintenance of protein synthesis, may be due to a sucrose induced G_1/S arrest. Such an arrest has been shown in cells, where an influx of extracellular Ca^{2+} was inhibited (Berridge 1995). A possible explanation for the augmented protein synthesis may be the result of a synchronizing effect in the cell cycle, induced by sucrose, although this is not clear.

In summary, the present report demonstrates that sucrose with its osmotic features suppresses proliferation and at the same time maintains protein synthesis. A possible effect of

hydrostatic interaction with the ECM leading to initiation of the cells may be attenuated by exposing the cells to osmotic stress by sucrose. These findings may reflect a key event in initiation of cells in the vascular wall leading to pathological conditions.

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