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# Heparin inhibits phorbol ester-induced ornithine decarboxylase gene expression in endothelial cells

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**Abstract Glycosaminoglycans regulate angiogenesis by affecting the availability of different growth factors for the endothelial cell (EC). However, little is known about the molecular and functional consequences resulting from direct interaction of these polyelectrolytes with the EC. Here we show that heparin markedly inhibited serum-stimulated DNA synthesis and ornithine decarboxylase (ODC) mRNA expression in human endothelial cells (HEC). About 50% of the serum effect on DNA synthesis and ODC gene expression was prevented by the selective protein kinase C (PKC) inhibitor chelerythrine or by PKC down-regulation. Heparin was ineffective in counteracting that part of the effect of serum that was resistant to PKC inhibition or down-regulation. In serum-free cultured HEC, heparin completely abolished the increase in DNA synthesis and ODC mRNA expression elicited by a number of PKC activators. Cell exposure to difluoromethylornithine, an irreversible inhibitor of ODC enzyme, dramatically antagonised both serum- and phorbol 12-myristate 13-acetate (PMA)-stimulated DNA synthesis. These results suggest that inhibition of PKC-mediated ODC gene expression by glycosaminoglycans may represent an important mechanism in the regulation of HEC proliferation.**


*Key words:* Heparin; Ornithine decarboxylase mRNA; Cell proliferation

## 1. Introduction

The endothelial cell (EC) plays a crucial role in the integration and modulation of signals within the blood vessel wall. EC-released growth factors, including basic fibroblast growth factor (bFGF), endothelial cell growth factor (ECGF), transforming growth factor (TGF), insulin like growth factor-1 (IGF-I), nitric oxide (NO) and endothelin [1–6], have been shown to control cellular migration and proliferation in the course of atherogenesis and inflammatory responses [8]. EC is also the target cell for tumour neovascularization [8], a process which closely associates with tumour growth and metastasis [9].

Glycosaminoglycans, including heparin (H) and heparan sulphate (HS), exert remarkable effects on angiogenesis [10,11] and have been shown either to inhibit or to promote EC proliferation [12,13]. Most of the glycosaminoglycan-induced effects have been attributed to their ability to affect the amount of growth factors available at the EC surface [14,15] or the interaction of growth factors with the EC [16,17]. In this regard, bFGF is bound to HS in the extracellular matrix (ECM) and is released in an active form when ECM-HS is degraded by cellular heparanase [15,18]. Therefore, restriction of growth factor availability by binding to ECM glycosaminoglycans and local regulation of growth factor release have been regarded as mechanisms accounting for the effects elicited by glycosaminoglycans on angiogenesis under normal or pathological conditions [19]. HS has also been shown to promote angiogenesis by increasing EC response to ECGF [20] through a mechanism involving ECGF receptor occupancy [21]. On the other hand, glycosaminoglycans have been shown to bind to cell-surface receptors on EC and to be internalised [22]. These findings raise the possibility that glycosaminoglycans may regulate angiogenesis by directly acting extracellularly and/or intracellularly on EC.

While the roles of glycosaminoglycans have been investigated in many cell types, there are surprisingly few reports detailing their effects on EC function. In contrast to our understanding on the effects of glycosaminoglycans on growth factor-modulated angiogenesis, comparatively little is known about the molecular sequelae of events resulting from glycosaminoglycan interaction with EC. In the present study, we provided details of the action of H and HS on EC, from their direct effects on DNA synthesis to the analysis of the possible signal transduction pathway(s) involved in polyelectrolyte action. The molecular dissection of the effects of glycosaminoglycans on EC function was further expanded by investigating whether these polyelectrolytes may influence the expression of a target gene in cell proliferation. In particular, we decided to assess the effects produced by H on the expression of the EC ornithine decarboxylase (ODC) gene, which encodes for ODC, the initial rate limiting enzyme in polyamine synthesis [23,24]. This was done because the ODC gene has been shown to be overexpressed in many growth processes and since proliferative events [25–27] including angiogenesis [28,29] have displayed an absolute requirement for both ODC activity and polyamine biosynthesis [30,31].

## 2. Materials and methods

### 2.1. Cell culture

HEC were isolated from human umbilical cords and cultured as previously described [32]. Briefly, HEC were detached from the interior of the umbilical vein of a 30-cm segment of cord by treatment for 10 min at 37°C with 0.05% (w/v) collagenase Type II from *Clostridium hystolyticum* (Gibco BRL, Paisley, UK) in medium M199 (Gibco

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BRL, Paisley, UK) containing 100 units/ml of penicillin G sodium salt and 100 μg/ml streptomycin sulphate (Sigma Chemical Co., St. Louis, MO, USA). HEC were harvested at 1000×*g* for 10 min and finally resuspended in 5 ml of medium M199 supplemented with 10% (v/v) FCS, 10% (v/v) newborn-calf serum (Gibco BRL, Paisley, UK), 2 mM glutamine and antibiotics as above. Cells were then plated in 25 $cm^2$ tissue culture flasks (Falcon, Oxnard, CA, USA) and cultured in an atmosphere of 5% $CO_2$/95% air. When confluent, HEC were subcultured at a split ratio of 1:2 by a brief treatment with 0.1% trypsin plus 0.02% EDTA in phosphate buffered saline (PBS) (120 mM NaCl, 2.5 mM KCl, 8.5 mM $NaH_2PO_4$, 1.5 mM $KH_2PO_4$), pH 7.3. Cultured cells were identified as endothelial by their typical cobblestone appearance and production of von Willebrand factor as measured by enzyme-linked immunosorbent assay [33]. Cell viability was checked by the trypan blue-exclusion method and used within three passages. In each experiment, HEC were plated at a density of 70 000 cells/ml in 24-well plates (Falcon, Oxnard, CA, USA). After 48 h, the medium was replaced and cells were synchronised following a 48-h incubation in serum-free medium M199 containing 0.2% (w/v) bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA).

### 2.2. Determination of cell cycle progression and radioactivity counting

Synchronised HEC were stimulated to growth in 24-well plates in the presence of medium M199, containing 5% (v/v) FCS. For determination of entry into S phase, cells were pulse-labelled with 1 μCi/ml [$^3$H]thymidine (specific activity 5 Ci/mmol, Amersham International plc, Bucks, UK) for 1 h at 3-h intervals. At the indicated time points, the medium was removed and the cell monolayer in each well was washed twice with PBS (1 ml), exposed to 5% (v/v) trichloroacetic acid (500 μl) for 5 min, then fixed in methanol (500 μl). Finally, the cells were digested by the addition of 25 M formic acid (500 μl). Each formic acid digest was transferred with one rinse of PBS (1 ml) to a scintillation vial containing 3.5 ml of INSTA-GEL scintillation fluid (Packard Instruments Co., Meriden, CT, USA), and radioactivity was determined by liquid scintillation counting [34] using a Wallac 1215 RackBeta liquid scintillation counter (LKB Instrument Inc., Gaithersburg, MD, USA).

### 2.3. Drug treatment and determination of DNA synthesis

Synchronised HEC were stimulated to growth for 48 h in the presence of medium M199, containing 5% (v/v) FCS (*group A*) or in a serum-free medium M199 containing different PKC activators [35], including phorbol 12-myristate 13-acetate (PMA) or phorbol 12,13-dibutyrate (PDBu) (Sigma Chemical Co., St. Louis, MO, USA) (*group B*). During the first 24 h, cells from each experimental group were exposed to the indicated concentrations of H or HS (from porcine intestinal mucosa, Sigma Chemical Co., St. Louis, MO, USA). Then, HEC were washed once in serum-free medium M199 (1 ml/well) and either reincubated with 5% (v/v) FCS-supplemented medium (*group A*) or cultured in serum-free medium M199 (*group B*) for the remaining 24 h. During this period HEC were added with 1 μCi/ml [$^3$H]thymidine. At the end of each experiment, cells were washed twice with ice-cold PBS (1 ml/well) and processed for liquid scintillation counting as above.

### 2.4. RNA extraction and determination of ODC mRNA

Synchronised subconfluent HEC grown in 25-$cm^2$ culture flasks (Falcon, Oxnard, CA, USA) were treated as indicated in the figure legends. Total RNA extraction [36] was performed at the indicated time points and the levels of ODC mRNA were assessed by using a sensitive solution hybridisation RNase protection assay [37,38]. Briefly, a 290-bp fragment purified from the full length cDNA coding for rat ODC was cloned in the transcription vector pGEM7z. Transcription of the plasmid linearized with *Apa*I (Boehringer Mannheim, Mannheim, Germany) generated a sense strand of ODC mRNA used to construct a standard curve of ODC mRNA, whereas transcription of the plasmid linearized with *Bam*HI (Boehringer Mannheim, Mannheim, Germany) in the presence of [$^{32}$P]CTP (specific activity 800 Ci/mmol, Amersham International plc, Bucks, UK) gave an antisense strand used to hybridise cellular ODC mRNA. The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide non-denaturing gel. The individual bands were counted for radioactivity by liquid scintillation spectrometry (LKB Instrument Inc., Gaithersburg, MD, USA), and cpm values were translated to pg values on a correlated standard curve. Data were expressed as pg of specific mRNA/μg of total mRNA.

### 2.5. Other cell treatments

Protein kinase C (PKC) down-regulation was accomplished by exposing HEC to serum-free medium M199 containing 1 μM PMA (Sigma Chemical Co., St. Louis, MO, USA) during the last 24 h of cell synchronisation. The PKC inhibitors chelerythrine or calphostin C (Alexis Corp., San Diego, CA, USA) were added at a concentration of 2.5 μM and 1 μM respectively, during the last 4 h of cell synchronisation. Difluoromethylornithine (DFMO) experiments were performed as indicated in the figure legends.

### 2.6. Statistical analysis

The statistical analysis of the data was performed by using the unpaired Student's *t*-test, assuming a *P* value less than 0.05 as the limit of significance. Data are expressed as mean ± S.E.M.

## 3. Results

To assess the cell entry into the S phase, cell cycle progression was analysed in HEC by pulse labelling in the presence of [$^3$H]thymidine. HEC were made quiescent by incubation in serum-free medium M199 for 48 h. Fig. 1A shows the time-

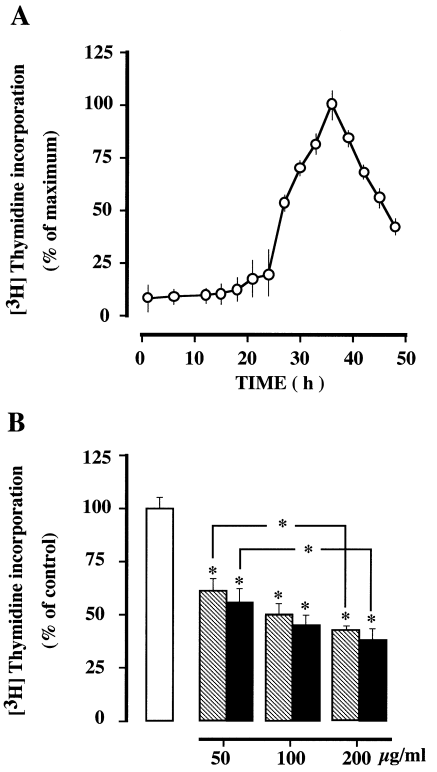


Fig. 1. A: Time-course of [$^3$H]thymidine incorporation in HEC exposed to 5% FCS. Synchronised HEC were stimulated to growth in the presence of 5% FCS. Cells were pulse-labeled with 1 μCi/ml [$^3$H]thymidine for 1 h at 3-h intervals for the determination of cell entry into the S phase. At the indicated time points, cells were washed twice with ice-cold PBS and processed for liquid scintillation counting as described in Section 2. Data represent the mean ± S.E.M. of three different experiments. B: Effect of heparin (H) and heparan sulphate (HS) on FCS-stimulated DNA synthesis. Cell exposure to the indicated concentrations of each polyelectrolyte was performed as described in Section 2 (white: control; diagonally hatched: H; black: HS). Cells were processed for liquid scintillation counting as described in Section 2. Data represent the mean ± S.E.M. of six different experiments. *: significantly different from the control value; – * –: significant difference between two groups.

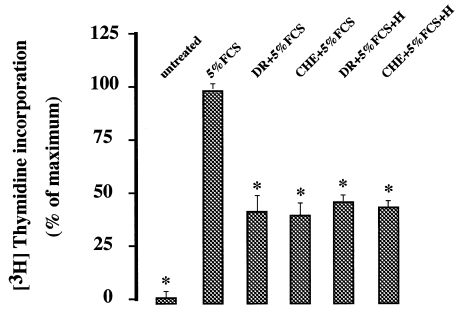


Fig. 2. Inhibition of the FCS-stimulated DNA synthesis in HEC by chelerythrine (CHE) (2.5 μM) or PKC down-regulation (DR). Untreated: growth-arrested HEC untreated; 5% FCS: HEC exposed to 5% FCS; DR+5% FCS: down-regulated HEC exposed to 5% FCS; CHE+5% FCS: chelerythrine-treated HEC exposed to 5% FCS; DR+5% FCS+H: down-regulated HEC exposed to 5% FCS plus 200 μg/ml H; CHE+5% FCS+H: chelerythrine-treated HEC exposed to 5% FCS plus 200 μg/ml H. Cells were processed for liquid scintillation counting as described in Section 2. Data represent the mean ± S.E.M. of six different experiments. *: significantly different from 5%.

course of [$^3$H]thymidine incorporation into DNA in HEC exposed to 5% FCS, after release from a quiescent, growth-arrested state ($G_0$) which was confirmed by the low level of thymidine incorporation during the first 12 h (5% FCS proved to be the most effective concentration in stimulating DNA synthesis over a 0.01–20% range; data not shown). After a 24-h lag period corresponding to $G_1$ phase, the rate of DNA synthesis progressively increased up to 36–38 h (S phase), then declined during the following 10 h (Fig. 1A). Therefore, in all the experiments [$^3$H]thymidine was administered between 24 and 48 h.

To determine the effect of different polyelectrolytes on DNA synthesis, quiescent HEC were stimulated to leave the growth-arrested state by incubation for 48 h in medium M199, supplemented with 5% (v/v) FCS, and treated with H or HS (50–200 μg/ml range) for the first 24 h of this period. Fig. 1B shows that both H and HS significantly inhibited serum-induced DNA synthesis. Such an effect was evident at a concentration of 50 μg/ml and peaked at a concentration of 200 μg/ml. Both the selective PKC inhibitor chelerythrine [39,40] and a long-term exposure to PMA, an intervention leading to PKC down-regulation [41], elicited more than 50% reduction in the serum-stimulated DNA synthesis (Fig. 2). However, these treatments were ineffective in completely abolishing the effect of FCS (Fig. 2). The addition of H to chelerythrine-treated or to PKC-down-regulated HEC failed to produce any additive inhibition of the effect of FCS (Fig. 2). In the absence of serum, the PKC activators PDBu or PMA elicited a marked and dose-dependent increase in [$^3$H]thymidine incorporation (Fig. 3). In addition, each activator did not affect DNA synthesis in the presence of chelerythrine or in PKC down-regulated cells (Fig. 3). When HEC were exposed for 24 h to the above mentioned PKC activators in the presence of either H or HS, each polyelectrolyte was able to completely antagonise the stimulatory effect induced by each phorbol ester on DNA synthesis (Fig. 4).

The exposure of HEC to 5% FCS elicited a time-dependent increase in ODC mRNA expression (Fig. 5A). This stimulatory effect was evident after 1 h of treatment and reached a maximum at 2 h. Thereafter, ODC mRNA levels progressively declined and returned to the control value at 12 h. Fig. 5B shows that the addition of 200 μg/ml H to the incubation medium significantly, although not completely, inhibited serum-stimulated ODC gene expression. A partial significant inhibition of serum-induced ODC gene expression was also observed in chelerythrine-treated or in PKC-down-regulated cells (Fig. 5C). Under these experimental conditions, no further inhibition of the effect of serum was observed following the administration of H (200 μg/ml) (Fig. 5C).

A time-dependent increase in ODC mRNA was also found in serum-free HEC that had been exposed to PMA (Fig. 6A). The phorbol ester-mediated event peaked at 2 h. However, at this time point ODC mRNA levels were significantly lower than those observed following a 2-h exposure to FCS. The

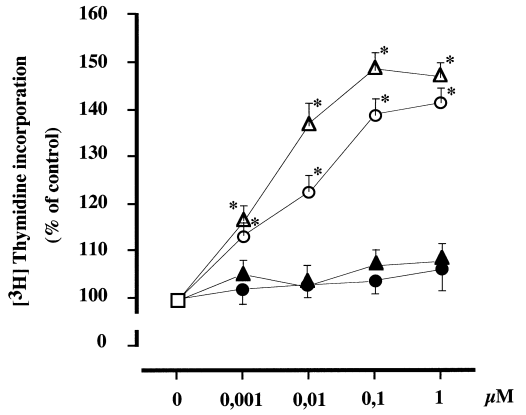


Fig. 3. Effect of different PKC activators on DNA synthesis in HEC. Normal HEC, down-regulated cells (DR) or chelerythrine (CHE)-treated cells were stimulated to growth for 48 h in the presence of serum-free medium M199. During the first 24 h, HEC were incubated in the absence (□, control) or in the presence of the indicated concentrations of PKC activators (○, PMA; △, PDBu; ▲, DR+PMA; ●, CHE+PMA). At the end of this period, the medium was removed, cells were washed once with medium M199 and reincubated for the last 24 h in the presence of serum-free medium M199, containing 1 μCi/ml [$^3$H]thymidine. Cells were processed for liquid scintillation counting as described in Section 2. Data represent the mean ± S.E.M. of six different experiments. *, significantly different from the control value.

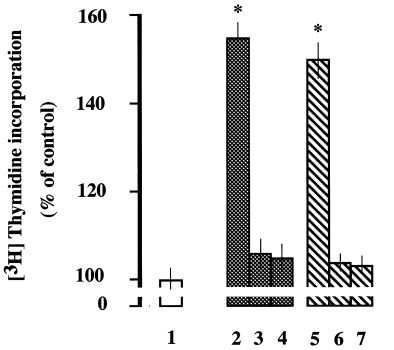


Fig. 4. Effect of H and HS on PKC-mediated DNA synthesis in HEC. Cell exposure to H or HS (200 μg/ml) or to each PKC activator (100 nM) was performed as described in Section 2. 1: Untreated cells; 2: PDBu; 3: PDBu+H; 4: PDBu+HS; 5: PMA; 6: PMA+H; 7: PMA+HS. Cells were processed for liquid scintillation counting as described in Section 2. Data represent the mean ± S.E.M. of six different experiments. *: significantly different from 1.

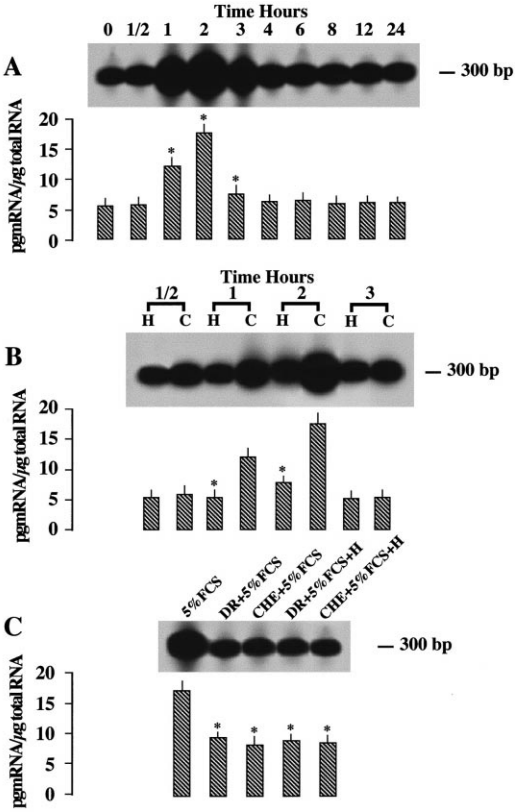


Fig. 5. A: Time course of the effect of 5% FCS on the ODC mRNA expression in HEC. Synchronised subconfluent HEC were stimulated with 5% FCS. At the indicated time points, total mRNA was extracted and processed for isolation and quantification. Data represent the mean ± S.E.M. of four different experiments. *: significantly different from time 0. Representative autoradiograms of the ribonuclease protection analysis of ODC mRNA are shown in the inset. B: Effect of heparin (H) on FCS-stimulated mRNA expression. Synchronised subconfluent HEC were stimulated with 5% FCS (C), or stimulated with 5% FCS plus 200 μg/ml H (H). At the indicated time points, total mRNA was extracted and processed for isolation and quantification. Data represent the mean ± S.E.M. of four different experiments. *: significantly different from C. Representative autoradiograms of the ribonuclease protection analysis of ODC mRNA are shown in the inset. C: Inhibition of the FCS-stimulated mRNA expression by chelerythrine (CHE) (2.5 μM) or PKC down-regulation (DR). 5% FCS: HEC stimulated for 2 h with 5% FCS; DR+5% FCS: down-regulated HEC stimulated for 2 h with 5% FCS; CHE+5% FCS: chelerythrine-treated HEC stimulated for 2 h with 5% FCS; DR+5% FCS+H: down-regulated HEC stimulated for 2 h with 5% FCS plus 200 μg/ml H; CHE+5% FCS+H: chelerythrine-treated HEC stimulated for 2 h with 5% FCS plus 200 μg/ml H. Data represent the mean ± S.E.M. of four different experiments. *: significantly different from 5% FCS. Representative autoradiograms of the ribonuclease protection analysis of ODC mRNA are shown in the inset.

effect elicited by PMA on ODC mRNA levels was dose-dependent and was not observed in chelerythrine (CHE) or in calphostin C (CAL)-treated cells. No increase in ODC mRNA levels was detected following the addition of PMA to PKC down-regulated cells or following HEC treatment in the presence of the inactive phorbol ester 4α-phorbol 12-myristate 13-acetate (4α-PMA) (Fig. 6B). Cell exposure to 200 μg/ml H caused a complete suppression of the stimulatory effect induced by PMA or PDBu on ODC mRNA expression (Fig. 6C).

Fig. 7 shows that HEC treatment for 24 h in the presence of 5 mM difluoromethylornithine (DFMO), an enzyme activated irreversible inhibitor of ODC [42], markedly antagonised both serum- and PMA-stimulated DNA synthesis.

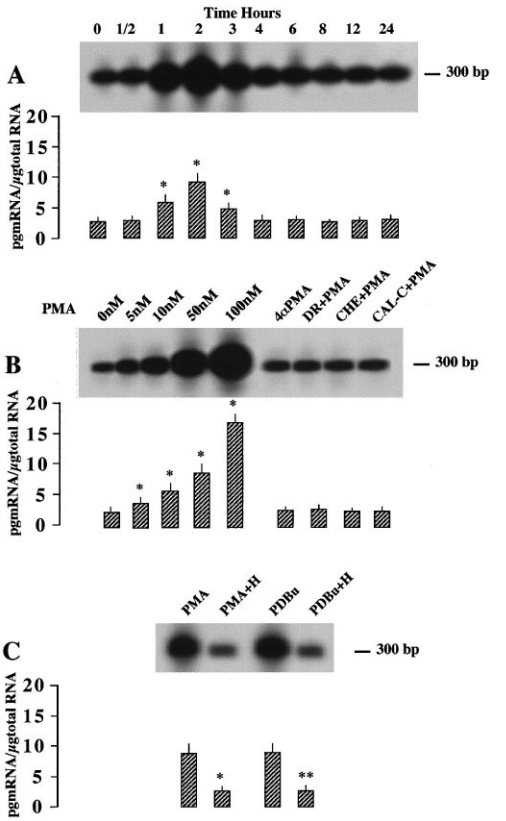


Fig. 6. A: Time course of the effect of PMA on the ODC mRNA expression in HEC. Synchronised subconfluent HEC were stimulated with 100 nM PMA. At the indicated time points, total mRNA was extracted and processed for isolation and quantification. Data represent the mean ± S.E.M. of four different experiments. *: significantly different from time 0. Representative autoradiograms of the ribonuclease protection analysis of ODC mRNA are shown in the inset. B: Dose-response of PMA effect on the expression of ODC mRNA. Synchronised subconfluent HEC were incubated in the presence of each indicated concentration of PMA for a period of 2 h. Data represent the mean ± S.E.M. of four different experiments. *: significantly different from 0 nM PMA. 4α-PMA: synchronised subconfluent HEC stimulated for 2 h with 100 nM of the inactive phorbol ester 4α-phorbol 12-myristate 13-acetate; DR+PMA: down-regulated HEC stimulated for 2 h with 100 nM PMA; CHE+PMA: chelerythrine-treated HEC stimulated for 2 h with 100 nM PMA; CAL C+PMA: calphostin C-treated HEC stimulated for 2 h with 100 nM PMA. Data represent the mean ± S.E.M. of four different experiments. Representative autoradiograms of the ribonuclease protection analysis of ODC mRNA are shown in the inset. C: Effect of 200 μg/ml H on PKC-mediated ODC mRNA expression in HEC. PMA: synchronised subconfluent HEC were stimulated for 2 h with 100 nM PMA; PMA+H: synchronised subconfluent HEC were stimulated for 2 h with 100 nM PMA plus 200 μg/ml H; PDBu: synchronised subconfluent HEC were stimulated for 2 h with 100 nM PDBu; PDBu+H: synchronised subconfluent HEC were stimulated for 2 h with 100 nM PDBu plus 200 μg/ml H. Data represent the mean ± S.E.M. of four different experiments. *: significantly different from PMA; **: significantly different from PDBu. Representative autoradiograms of the ribonuclease protection analysis of ODC mRNA are shown in the inset.

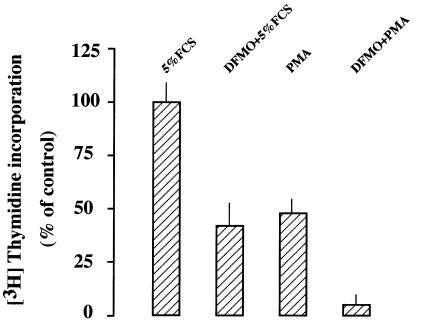


Fig. 7. Effects of difluoromethylornithine (DFMO) on FCS- and PKC-induced DNA synthesis in HEC. Synchronised HEC were cultured for 24 h in medium M199 supplemented with 5% FCS or in a serum-free medium containing 100 nM PMA. During this period, each group of cells received 5 mM DFMO. HEC were then washed once (1 ml/well) and reincubated for additional 24 h with 5% FCS or serum-free medium in the presence of 1 μCi/ml [$^3$H]thymidine. At the end of each experiment, cells were washed twice with ice-cold PBS (1 ml/well) and processed for liquid scintillation counting as described above. 5% FCS: HEC exposed for 48 h to 5% FCS; DFMO+5% FCS: HEC exposed to 5% FCS plus 5 mM DFMO during the first 24 h, and reincubated in 5% FCS after washing; PMA: serum-free HEC exposed for 24 h to 100 nM PMA; DFMO+PMA: serum-free HEC exposed to 100 nM PMA plus 5 mM DFMO during the first 24 h, and reincubated in serum-free medium M199 after washing. Data represent the mean ± S.E.M. of five different experiments. *: significantly different from 5% FCS; **: significantly different from PMA.

## 4. Discussion

The endothelium is of major importance to the maintenance of vascular homeostasis, due to its regulated secretion or surface expression of modulators of a wide variety of physiological processes [8]. Recently there has been much interest in the mechanism of action of antiproliferative agents, with a variety of studies showing that such agents may exert their effect on cell growth through the modulation of several genes [43]. Despite intense interest in the effects of glycosaminoglycans on cell proliferation, there are few reports describing such investigation using EC. Therefore we examined the effects of H and HS on serum- and phorbol ester-stimulated DNA synthesis in HEC. Furthermore, we analysed the effect of H on FCS- and PMA-stimulated ODC mRNA levels, since the ODC gene has been shown to be overexpressed in many growth processes and proliferative events, closely related to angiogenesis. The current experimental data show that both H and HS counteracted serum-stimulated DNA synthesis in HEC. In different cell types, including HEC, vascular smooth muscle cells and fibroblasts, PKC plays a crucial role in the modulation of cell growth, being implicated both in proliferative and antiproliferative responses [44,45]. Furthermore, phorbol ester sensitive PKC isoforms have been detected in HEC [46,47]. The present observation that both a PKC inhibitor and enzyme down-regulation significantly reduced [$^3$H]thymidine incorporation in serum-supplemented cells indicated that the effect of FCS on DNA synthesis may be at least in part mediated by PKC activation. However, failure of PKC inhibition or depletion of abolishing completely the FCS action indicates that PKC-independent mechanisms may also be conceivable. In this regard, both PKC-dependent and PKC-independent pathways have been implicated in the proliferative effect of serum in vascular smooth muscle cells [48]. The finding that H did not produce additive inhibition of the effect of FCS in chelerythrine-treated or PKC-depleted cells suggests that H might have acted by suppressing a serum-activated PKC-dependent pathway, being without effect on DNA synthesis stimulated by serum through PKC-unrelated events. The possibility that inhibition of a PKC-dependent pathway may be a major signalling mechanism in the antiproliferative action of H was further supported by the capability of this polyelectrolyte of abolishing completely the stimulatory effect elicited on DNA synthesis by a number of PKC activators in a serum-free medium. These results also suggest that the inhibitory effect of H on serum-induced [$^3$H]thymidine incorporation is unlikely to be attributable to interference of the polyelectrolyte with growth factors or other factors in the serum. Rather, the present observations support the possibility that H may have interacted directly with HEC to affect PKC-mediated events involved in the regulation of HEC proliferation. Here we show that both FCS and PMA were able to stimulate ODC gene expression in HEC. ODC plays a rate limiting role in polyamine biosynthesis and is intimately associated with cell proliferation and function [49,50]. Moreover, an increase in ODC gene expression has been observed in different cell types in response to a number of proliferative agents, including bFGF, serum and phorbol esters [27,49,51,52]. The present observation that only part of FCS-induced overexpression of ODC mRNA could be prevented by PKC inhibition or down-regulation suggests that, similar to the FCS-mediated increase in HEC DNA synthesis, the stimulation of ODC gene expression induced by serum might have occurred through different signal-transduction pathways, presumably involving both PKC-dependent and PKC-independent mechanisms. Such a hypothesis is further supported by the observation that, differently from the effect of serum, the stimulatory effect of PMA on ODC mRNA expression could be totally abolished in chelerythrine-treated or PKC-down-regulated cells and by the finding that the peak increase in ODC mRNA levels in the presence of PMA was significantly lower than that observed in serum-stimulated cells.

A number of experimental observations in the present study suggests the possibility that H might exert its antiproliferative action on HEC by inhibiting a PKC-dependent pathway controlling ODC gene expression. First, H markedly inhibited serum-induced ODC gene expression to the same extent as chelerythrine or PKC down-regulation. Second, the inhibitory effect of H was not additive to the down-regulation of ODC mRNA induced by PKC inhibition or depletion in serum-supplemented cells, indicating that each of these treatments and the polyelectrolyte might have inhibited ODC gene expression by following the same signal-transduction pathway. Third, similar to interventions aiming at inhibiting or down-regulating PKC, HEC exposure to H completely suppressed phorbol ester-induced ODC gene expression in serum-free HEC. The fourth experimental observation is that HEC exposure to the irreversible ODC inhibitor DFMO elicited a dramatic decrease in both serum- and PMA-stimulated [$^3$H]thymidine incorporation. This finding is in agreement with other studies showing that serum-stimulated endothelial cell proliferation could be nearly suppressed by inhibition of polyamine synthesis [42] and further supports the hypothesis that H, by inhibiting PKC-mediated ODC gene expression might have affected a crucial mechanism of regulation of HEC proliferation.

The possible implications of the present study remain to be elucidated. However, it is now evident that the endothelial cell plays a crucial role in angiogenesis [53] and that it might affect the growth and differentiation of neighbouring cells [7]. As H acts on endothelial cells that lie immediately adjacent to the myocytes within the intact myocardium, it is conceivable that the antiproliferative effect of H reported here would affect the availability of endothelial cell-released growth factors from the myocardial cell. In this regard, both endothelin and insulin-like growth factors have been reported to trigger the activation of an early gene program as well as that of fetal genes in cultured myocytes, thus switching myocyte growth from hyperplasia to hypertrophy [54,55]. Akin to the paracrine role of endothelial cell is the capability of nitric oxide to spread out of its site of production influencing many different tissue elements, including neuronal, glial and vascular elements, that are not necessarily in close anatomical juxtaposition [56].

The present findings, indicating that the antiproliferative effect of H may be largely dependent upon down-regulation of PKC-mediated induction of the ODC gene may be of particular relevance in consideration of the angiogenic role of polyamines in both normal and malignant tissues. In this regard, intimal hyperplasia, a process which has been implicated both in atherogenesis and inflammatory responses, has been shown to be drastically reduced by ODC inhibition [57]. Furthermore, a large body of experimental evidence indicates that tumour growth mainly depends on formation of new blood vessels [9,58] and it is increasingly becoming evident that inhibition of polyamine synthesis strongly counteracts tumour-induced angiogenesis [30,31]. Interestingly, the use of stable transfected cells with an expression construct containing ODC DNA has established a causative role for ODC overexpression in the acquisition of a transformation phenotype related to neoplastic development [59]. Considered together, these findings suggest the possibility that the inhibitory effect of H on HEC ODC gene expression observed in the present study may represent a prominent feature in the control of pathological processes of the blood vessel wall and in the modulation of angiogenic stimuli leading to neoplastic growth.

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