Inhibition of immediate-early-gene induction in renal mesangial cells by depletion of intracellular polyamines

Eckhard SCHULZE-LOHOFF,*†, Hans FEES,* Susann ZANNER,* Karl BRAND† and Ralf Bernd STERZEL*

*Medizinische Klinik IV and †Institut für Biochemie, Universität Erlangen-Nürnberg, D-91054 Erlangen, Germany

Mitogens have been shown to stimulate the activity of the rate-limiting enzyme for polyamine synthesis, ornithine decarboxylase (ODC), and ODC mRNA expression in cultured rat mesangial cells (MCs). In addition, inhibition of ODC by α -difluoromethylornithine (DFMO) results in growth arrest of MCs. To elucidate the mechanisms involved in the inhibition of MC proliferation due to polyamine depletion, we studied the effects of DFMO on the activation of phospholipase C and induction of the immediate early genes (IEGs), *c-fos*, *c-jun* and *Egr-1*, which are thought to regulate cell growth. Mitogenic 10 % fetal-calf serum (FCS) and 1 unit/ml thrombin activated phospholipase C in MCs within 30 s, as assessed by generation of [3 H]inositol phosphates. This activation was not affected by DFMO. mRNAs of the IEGs *c-fos*, *c-jun* and *Egr-1* were induced by FCS within 15 min. Expression of these genes reached a peak at 60 min and disappeared at 3 h. Treatment of MCs with a growth-suppressing dose of DFMO (5 mM) inhibited mRNAs of

all three IEGs by 52–87 % at 1 h. Total expression of *Egr-1* over 20–120 min was diminished by 41 %, and the time point of maximal expression was delayed by 40 min. This inhibitory effect was abolished in a time-dependent manner (1–3 days) by prior addition of 200 μ M putrescine, the reaction product of ODC. *Egr-1* mRNA expression was superinduced by the inhibitor of protein synthesis, cycloheximide. This effect was also blocked by DFMO. The results indicate that the DFMO-induced process of MC growth inhibition involves steps necessary for IEG activation. The signal-transduction step sensitive to polyamines occurs distal to the activation of phospholipase C. Since reconstitution of normal induction of IEGs requires 3 days, it seems likely that polyamine depletion affects the regulation of IEG expression in an indirect fashion. We conclude that activation of IEGs requires the presence of polyamines and plays a significant role in the induction of MC replication.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are low-molecular-mass cationic molecules, which are present in all prokaryotic and eukaryotic cells [1–3]. When cells are induced to proliferate, a rapid increase in polyamines occurs, due to increased activity of the rate-limiting enzyme for polyamine synthesis, ornithine decarboxylase (ODC). When cells are depleted of polyamines by use of highly specific inhibitors for ODC, such as DL- α -difluoromethylornithine (DFMO) [4], they cease to proliferate [2,5–7]. Conversely, when polyamine-depleted cells are supplemented with polyamines, they resume a normal growth rate. Recently it has been demonstrated that overexpression of ODC in NIH 3T3 cells results in transformation of these cells [8]. Those authors proposed that ODC should be considered as a proto-oncogene. Although the essential role of polyamines for cell proliferation is clear, the precise cellular events during the cell cycle which require polyamines are still unknown [2].

The cell cycle of proliferating cells is a sequence of events resulting in DNA replication and cell division [9]. Among the earliest events are rapid changes in activity of membrane-bound enzymes, such as activation of phospholipase C (PLC), which occurs within seconds [10]. The reaction products of PLC are inositol phosphates, which increase the level of intracellular Ca^{2+} , and diacylglycerol, which activates protein kinase C [11]. After further activation of a cascade of protein kinases and phosphatases, the first nuclear response is the activation of a

group of genes called the immediate early genes (IEGs) [12]. These genes code for transcriptional regulatory proteins and include the *fos* family (*c-fos*, *fos B*, *fra 1*), the *jun* family (*c-jun*, *jun B*, *jun D*) and the *Egr* family (*Egr 1–4*) [13,14]. These gene products are thought to regulate the expression of more distal genes, ultimately leading to DNA replication and mitosis [12].

Cultures of renal mesangial cells (MCs) offer a valuable tool to study growth-related cellular events [15,16]. Furthermore, better understanding of the growth regulation of MCs is needed for the elucidation of the pathogenesis of proliferative glomerulonephritis, an important disease of the kidney [17]. Previously we have examined the activation of ODC after mitogenic stimulation of cultured MCs, and the requirement of polyamines for MC proliferation [18]. In the present study, we report for the first time that the restriction point that is dependent on polyamines is distal to the activation of PLC and proximal to the expression of IEGs during the cell cycle of cultured rat MCs.

MATERIALS AND METHODS

Materials

[32 P]dCTP and [3 H]uridine were purchased from Amersham Buchler (Braunschweig, Germany). Dulbecco's modified Eagle medium (DMEM) was from Biochrom (Berlin, Germany), fetal-calf serum (FCS) from Boehringer (Mannheim, Germany), bovine insulin from Sigma (Deisenhofen, Germany) and all plastic articles were from Becton Dickinson (Heidelberg, Ger-

Abbreviations used: ODC, ornithine decarboxylase; DFMO, DL- α -difluoromethylornithine; PLC, phospholipase C; IEGs, immediate early genes; MCs, mesangial cells; FCS, fetal-calf serum; DMEM, Dulbecco's modified Eagle medium.

† To whom correspondence should be addressed.

many). DFMO was generously given by Merrell Dow Research Institute (Strasbourg, France).

Cell culture

Rat mesangial cells were isolated and cultured as described previously [19]. Briefly, kidneys were excised from male Sprague-Dawley rats (150–200 g), and the cortex was separated from the medulla and homogenized with razor blades. Glomeruli were isolated from the homogenate by sequential sieving and collected on a 75 μ m-pore sieve. After treatment with collagenase (500 units/ml; Sigma type IV) in PBS for 30 min at 37 °C, glomerular remnants, consisting predominantly of endothelial cells and MCs, were seeded in tissue-culture flasks (30 000/flask) containing DMEM supplemented with FCS (20%), bovine insulin (5 μ g/ml), penicillin (100 units/ml) and streptomycin (100 μ g/ml). After 3–4 passages by using trypsin (0.05%)/EDTA (0.02%), homogeneous cultures of MCs were obtained. Cells showed typical smooth-muscle-cell-like morphology and uniformly exhibited positive immunocytochemical staining for Thy 1.1, smooth-muscle-cell actin and myosin (Dako, Hamburg, Germany). The presence of macrophages, endothelial and epithelial cells was excluded by negative staining with anti-macrophage antibody ED-1 (Serotec, Kidlington, Oxford, U.K.), anti-factor VIII (Dako) and anti-cytokeratin (Boehringer Mannheim) [19]. The cells were used for experiments between passages 5 and 20.

Growth arrest of MCs and depletion of polyamines

MCs were seeded in culture flasks or microplates at low density in complete medium containing 10% FCS, which was dialysed against 0.15 M NaCl. Then 1 day after plating, 5 mM DFMO was added to the cells from 100-fold-concentrated stock solutions (in PBS). MCs were maintained in DFMO for 5–6 days to reach optimal polyamine depletion (Table 1). For the last 2 days of this preincubation period, medium was switched to 0.5% FCS to arrest cells in G₀ phase. Non-treated control cells were used at comparable density (60–70% confluency). At the end of the preincubation period, dialysed FCS or agonists were added from concentrated stock solutions. Putrescine, spermidine and spermine were measured after separation by h.p.l.c., by the method of Etter et al. [20]. Cell proliferation was assessed by counting MCs in triplicate wells in an automatic cell counter (Coulter Electronics, Luton, Beds., U.K.).

Measurement of RNA synthesis and cellular RNA content

For measurement of RNA synthesis, MCs were pulsed for 1 h with [³H]uridine (5 μ Ci/ml) in 96-well microplates at the time points indicated. The cells were washed twice with PBS, and the RNA was precipitated in ice-cold 10% trichloroacetic acid for 15 min. After three washes with 10% trichloroacetic acid, the precipitate was solubilized in 0.1 M NaOH and neutralized. Finally, radioactivity was determined in a Beckman liquid-scintillation counter. These assays were performed in six-fold replicates. The content of total cellular RNA was determined by the orcin method [21].

Determination of inositol phosphates

Confluent MCs were labelled in 24-well plates for 3 days with

5 μ Ci of [³H]inositol in inositol-free RPMI medium containing 10% FCS. FCS concentration was changed to 0.5% for the last 2 days of the labelling period. After preincubation with RPMI containing 20 mM LiCl for 30 min, this medium was removed and triplicate wells of MCs were stimulated with agonists. At given time points, the cells were extracted in ice-cold 10% trichloroacetic acid. The latter was removed by mixing with 0.5 M triethylamine in trichlorotrifluoroethane. The neutralized extracts were kept frozen until ion-exchange chromatography on Dowex 1X8. Inositol phosphates were eluted sequentially as described by Berridge et al. [22]. Each column was calibrated and standardized by applying authentic ³H-labelled inositol phosphates (InsP, InsP₂, InsP₃) obtained from New England Nuclear (Dreieich, Germany). To quantify incorporation of [³H]inositol into cellular phosphatidylinositol phosphates, an acidic chloroform/methanol extraction was performed as described [23]. The final organic phase was dried and counted for radioactivity in a Beckman scintillation counter.

Isolation and analysis of RNA

RNA was isolated from MCs by the guanidinium thiocyanate/phenol/chloroform extraction method, as described by Chomczynski and Sacchi [24]. Then 10 μ g of RNA per sample was separated by agarose-gel electrophoresis and stained with ethidium bromide to monitor the yield and quality of RNA isolation. RNA was transferred to a nylon membrane (Pall Biotek A, Dreieich, Germany) and fixed by baking for 2 h at 80 °C [25]. The fixed RNA was probed with the following ³²P-labelled cDNAs: *c-fos* (BamHI/BamHI fragment of *c-fos* cDNA, kindly provided by Dr. R. Bravo, Princeton), *c-jun* (EcoRI/EcoRI fragment of *c-jun* cDNA, also from Dr. R. Bravo), *Egr-1* (Xba/EcoRI fragment of *Egr-1* cDNA, kindly provided by Dr. V. P. Sukhatme, Chicago), 18 S rRNA (EcoRI/SalI fragment of 18 S rRNA cDNA, kindly provided by Dr. I. Grummt, Martinsried, Germany), γ -actin (PstI/Xba fragment of γ -actin cDNA, also from Dr. R. Bravo). For hybridization, inserts were labelled by random primer extension [26]. The filters were prehybridized for 2 h at 40 °C in a solution containing 50% formamide, 5 $\times$ SSC (3 M NaCl, 0.3 M trisodium citrate, pH 7.0), 5 $\times$ Denhardt's solution, 0.1% SDS and 200 μ g/ml sheared denatured salmon sperm DNA. The filters were then hybridized for 15–20 h at 40 °C in the same solution containing approx. 5 $\times$ 10⁶ c.p.m./ml ³²P-labelled cDNA probe. After hybridization, membranes were washed twice with 2 $\times$ SSC/0.1% SDS at 40 °C for 15 min, and twice with 0.2 $\times$ SSC/0.1% SDS at 40 °C for 15 min. The filters were then air-dried and autoradiographed. The size of the mRNA was estimated by comparison with 18 S and 28 S rRNA. The sizes of the mRNAs were: *c-fos* 2.2 kb, *c-jun* 2.7 kb and 3.2 kb, *Egr-1* 3.4 kb, 18 S rRNA 2.3 kb, γ -actin 2.1 kb. Quantification of the bands was performed by densitometry (Laser Scan Densitometer; LKB, Bromma, Sweden) and the intensity of IEG mRNA bands was related to the density of the control hybridization bands of γ -actin or 18 S rRNA.

Statistical analysis

Data were analysed by Student's *t* test or analysis of variance as appropriate. *P* values less than 0.05 were used as the criterion for statistical significance, and are indicated by asterisks.

RESULTS

By using DFMO, an irreversible inhibitor of ODC, the rate-limiting enzyme of polyamine synthesis, MCs were depleted by

Table 1 Polyamine contents of cultured MCs after treatment with 5 mM DFMO

MCs were seeded in 75 cm² culture flasks at low density (10⁵ cells/flask) in full DMEM containing 10% dialysed FCS. After 24 h (day 0), 5 mM DFMO was added. Cells were harvested every other day and cell number was measured by Coulter counter. Subsequently, the cells were suspended in 10% trichloroacetic acid and subjected to h.p.l.c. analysis as described in the text. The results of one analysis out of three independent experiments are shown; n.d., not detectable.

	Content (nmol/10 ⁶ cells)		
	Putrescine	Spermidine	Spermine
0 days untreated	0.17	7.02	2.93
4 days untreated	0.14	5.61	2.43
8 days untreated	0.29	6.05	3.94
2 days + DFMO	n.d.	0.37	2.76
4 days + DFMO	n.d.	0.19	1.63
6 days + DFMO	n.d.	0.23	1.60
8 days + DFMO	n.d.	0.23	1.90

Figure 1 DFMO-induced inhibition of cell replication and reversal by putrescine in serum-stimulated cultured MCs

MCs were depleted from polyamines by pretreatment with 5 mM DFMO for 5 days. During the last 2 days of the pretreatment period, the concentration of FCS was changed from 10% to 0.5%. Parallel dishes received 200 μM putrescine 3 days and 1 h before stimulation. After stimulation with 10% FCS, cell number was monitored by Coulter counter after 24, 48 and 72 h. Symbols: ■, 10% FCS; ○, 10% FCS/5 mM DFMO; ●, 10% FCS/5 mM DFMO/200 μM putrescine, 3 days; △, 10% FCS/5 mM DFMO/200 μM putrescine, 1 h. Data are expressed as increase in cell number given as N/N_0 (N_0 = cell number on day 0). Experimental values are means ± S.D. of 6-fold replicates (one out of three experiments): * $P < 0.05$, significant difference of experimental values versus cells treated with 10% FCS/5 mM DFMO.

> 90% of putrescine and spermidine, and by > 35% of spermine over a period of 4–8 days (Table 1). This treatment resulted in a significant decrease in the growth rate of MCs maintained in FCS, as previously shown ([18]; Figure 1). Polyamine depletion did not result in a complete block of cell replication, since a considerable fraction of MCs was able to divide after acute stimulation with polyamine-free 10% FCS (Figure 1). Addition of putrescine (200 μM) 3 days before stimulation almost completely reversed the growth-inhibitory effect of DFMO. When putrescine was given only 1 h before stimulation, there was no reversal of the DFMO block at 1 and 2 days after stimulation. At

Table 2 Effects of DFMO on PLC activity induced by thrombin and FCS in cultured MCs

MCs were grown in medium supplemented with 5 mM DFMO or in growth medium alone for 5 days. FCS concentration was 10% during the first 3 days and 0.5% during the last 2 days of the pretreatment period. Labelling of phosphatidylinositol phosphates was performed by addition of 5 μCi/ml [³H]inositol for the last 3 days of the pretreatment period. To control for altered incorporation of [³H]inositol into phosphatidylinositol phosphates, this fraction was isolated by acidic chloroform/methanol extraction. Incorporation of [³H]inositol into phosphatidylinositol phosphates was increased 1.4-fold in the presence of DFMO. Therefore formation of inositol phosphates is expressed as c.p.m./10⁶ c.p.m. of [³H]inositol incorporated. Generation of $InsP_2$ and $InsP_3$ after stimulation for 30 s with thrombin (1 unit/ml) or 10% FCS was measured as described in the Materials and methods section. Data are expressed as means ± S.D. of triplicates (one of five experiments): * $P < 0.05$ for thrombin- and FCS-stimulated cells versus unstimulated corresponding controls; no significant difference was found between DFMO-treated cells and untreated controls.

	PLC activity (c.p.m./10 ⁶ c.p.m. of [³ H]inositol incorporated)	
	$InsP_2$	$InsP_3$
Control	1251 ± 240	382 ± 71
DFMO (5 mM) alone	1305 ± 196	312 ± 85
Thrombin (1 unit/ml)	22142 ± 2043*	1349 ± 197*
Thrombin (1 unit/ml) + DFMO	20457 ± 3166*	1223 ± 132*
FCS (10%)	3893 ± 991*	733 ± 112*
FCS (10%) + DFMO	4911 ± 1203*	640 ± 119*

day 3, putrescine started to raise the growth rate of DFMO-treated MCs.

To investigate the mechanism of DFMO-mediated growth inhibition, we first examined possible changes in activation of PLC after stimulation with thrombin and FCS. To correct for different labelling of phosphatidylinositol phosphates by [³H]inositol in the presence of DFMO, phosphatidylinositol phosphates were isolated by acidic chloroform/methanol extraction. Incorporation of [³H]inositol into phosphatidylinositol phosphates was increased 1.4-fold in the presence of DFMO (results not shown). Therefore, formation of inositol phosphates is expressed as c.p.m./10⁶ c.p.m. of incorporated [³H]inositol. After stimulation with agonists for 30 s, the increases in inositol phosphates were as follows: thrombin (1 U/ml), $InsP_2$ 17.7-fold, $InsP_3$ 3.5-fold; FCS (10%), $InsP_2$ 3.1-fold, $InsP_3$ 1.9-fold. In polyamine-depleted cells, the increases were not significantly different after correction for [³H]inositol incorporation (Table 2).

To elucidate the mechanism of DFMO-mediated growth inhibition in relationship to the expression of the IEGs, *c-fos*, *c-jun* and *Egr-1*, we first examined the normal course of IEG expression during the cell cycle of MCs induced by 10% FCS. MCs were stimulated with 10% FCS for 15 min–6 h. Under these conditions, DNA synthesis is induced at 18–36 h and cell division occurs between 24–48 h [18]. Transcripts of the IEGs were present at very low levels in resting cells (0 h) (Figure 2). After stimulation with FCS, enhanced expression of IEGs was observed after 15–60 min, and maximal amounts of all IEG transcripts were found at 1 h. At 3 h after stimulation with FCS, IEG expression returned towards the levels of resting cells (Figure 2).

To examine the effect of polyamines on IEG induction, MCs were pretreated for 5 days with 5 mM DFMO, to achieve depletion of polyamines (Table 1). In order to arrest the cells in G₀, FCS concentration was decreased from 10% to 0.5% for the final 2 days of the pretreatment period. Control MCs were also growth-arrested by serum depletion, but not treated with DFMO. IEG mRNA levels were determined by Northern-blot analysis at

Figure 2 Expression of IEG mRNAs during MC cell-cycle progression

After growth arrest of MCs in medium containing 0.5% FCS for 3 days (time point 0 h), MCs were stimulated by adjusting the medium to 10% FCS for various time periods (15 min–6 h). RNA was isolated and subjected to Northern-blot analysis. Specific hybridization with 32 P-labelled cDNAs is shown (one of three experiments). To control for loading and transfer efficiency, this filter was also probed for γ -actin.

the end of the pretreatment period (0 h) and after stimulation with 10% FCS for 1 h, when expression of all three IEGs was maximal. As illustrated in Figure 3, very low levels of mRNAs for the three IEGs were detectable in resting MCs. After stimulation with 10% dialysed FCS for 1 h, control cells exhibited a strong increase in IEG expression, whereas IEG induction was suppressed in polyamine-depleted cells by 52–87%. All lanes showed comparable bands for γ -actin, to ensure comparability of the hybridization (Figure 3).

Since it is possible that inhibition of IEG expression at 1 h was due to delayed expression, we studied the effect of DFMO on *Egr-1* expression at 20, 40, 60, 80, 100 and 120 min (Figure 4). Although in control cells *Egr-1* expression reached a maximum at 40 min, in polyamine-depleted cells maximal expression of *Egr-1* occurred at 80 min. The summation of the laser-densitometry values of the hybridization bands from DFMO-treated cells of all time points was decreased by 41% as compared with the values obtained for control cells. Similar results were found for *c-fos* hybridization (results not shown).

Next, we asked whether the inhibitory effect of polyamine depletion is due to inhibition of protein synthesis. We compared the effect of DFMO with the inhibitor of protein synthesis, cycloheximide (Figure 5). After pretreatment with cycloheximide (10 μ g/ml) for 10 h, *Egr-1* was superinduced by FCS, as described by Sukhatme and co-workers [14]. The superinduction was strongly inhibited by simultaneous pretreatment with DFMO

Figure 3 Effect of polyamine depletion by DFMO on expression of *c-fos*, *c-jun* and *Egr-1*

For depletion of polyamines, MCs were maintained for 3 days in normal growth medium (10% FCS) with (+) or without 5 mM DFMO. For growth arrest in G_0 , MCs were cultured for another 2 days in resting medium (0.5% FCS) with or without 5 mM DFMO. At this time point (0 h), the medium was adjusted to 10% FCS to induce expression of IEGs. mRNA levels were assessed by Northern-blot analysis in resting cells (0 h) and stimulated cells (1 h). One out of five independent experiments is illustrated. As control, this filter was also probed for γ -actin.

Figure 4 Effect of DFMO on time course of *Egr-1* expression

MCs were growth-arrested and pretreated with 5 mM DFMO as for Figure 3. DFMO-treated and control cells were stimulated with FCS for 20, 40, 60, 80, 100 and 120 min. RNA was isolated and subjected to Northern-blot analysis. Hybridization for *Egr-1* and γ -actin in one of three experiments is illustrated.

Figure 5 Opposite effects of DFMO and cycloheximide on serum-induced *Egr-1* expression

MCs were growth-arrested and pretreated with 5 mM DFMO as for Figure 3. In addition, two dishes (one DFMO-treated dish and one control dish) were treated with the protein-synthesis inhibitor cycloheximide (10 μ g/ml) for 10 h before stimulation. After addition of FCS for 1 h, RNA was isolated and hybridized for *Egr-1* and γ -actin as control. Three experiments with similar results were performed.

Figure 6 Reconstitution of DFMO-mediated *Egr-1* suppression by putrescine

MCs were growth-arrested and pretreated with DFMO as for Figure 3. To stimulate *Egr-1* expression, MCs were incubated with 10% FCS for 1 h or 6 h with (+) or without DFMO. MCs were treated with DFMO for 6 days, then stimulated with 10% FCS for 1 h and supplemented with 200 μ M putrescine for 3 days, 1 day or 1 h before the end of the FCS stimulation period. At the given time points, MCs were harvested and RNA was isolated. *Egr-1* transcripts were detected by hybridization with radioactive *Egr-1* cDNA. As control, this filter was also probed for 18 S rRNA. The Figure shows one out of three analogous experiments.

(Figure 5). Thus opposite effects of DFMO and cycloheximide on *Egr-1* induction were observed.

We then examined whether the DFMO-mediated suppression of *Egr-1* is due to polyamine depletion (Figure 6). DFMO-treated cells and controls were pretreated as described for Figure 3. Stimulation with 10% FCS was performed for 1 h or 6 h. In addition, 200 μ M putrescine was added for different time intervals (1 h, 1 day, 3 days) before harvesting the cells, which were stimulated with 10% FCS for 1 h. *Egr-1* mRNA was induced rapidly in control cells within 1 h and disappeared at 6 h. In

Figure 7 Effect of DFMO on total RNA synthesis (a) and cellular RNA content (b)

(a) RNA synthesis was measured by [3 H]uridine incorporation in MC cultures that were treated with or without 5 mM DFMO for 5 days and maintained in resting medium (0.5% FCS) for the last 2 days. After stimulation with 10% FCS or PBS as control, [3 H]uridine incorporation was measured at given time points as described in the Materials and methods section. Experimental values are means $\pm$ S.D. of 6-fold replicates (one out of three experiments). (b) In parallel dishes, total cellular RNA content was measured by the orcin method. Data represent means of duplicate values (one out of three experiments). Symbols: ■, 10% FCS; ●, 10% FCS/5 mM DFMO; □, PBS; ○, PBS/5 mM DFMO.

DFMO-treated cells, the *Egr-1* mRNA level at 1 h was inhibited by 72%, as judged by laser densitometry, and was not detectable at 6 h. When putrescine was added to DFMO-treated cells for 1 h, 1 day or 3 days, *Egr-1* expression increased in a time-dependent manner towards control values. The inhibition diminished after 1 h putrescine to 66%, after 1 day to 52% and after 3 days to 15%.

Finally, we compared the effects of DFMO treatment on the expression of single genes with general effects of polyamine depletion on RNA synthesis and cellular RNA content. MCs were pretreated with DFMO for 5 days and maintained in resting medium for 2 days, followed by stimulation with 10% FCS or PBS as control. [3 H]uridine incorporation in non-stimulated cells was slightly higher in the presence of DFMO than in untreated cells (Figure 7a). This result was paralleled by a higher cellular RNA content in polyamine-depleted cells than in untreated cells (Figure 7b). After stimulation with 10% FCS, untreated cells showed a greater increase in [3 H]uridine incorporation and cellular RNA content than did polyamine-depleted cells.

Non-specific effects and toxicity of DFMO treatment and polyamine depletion on MCs were excluded by the following criteria: 5-day treatment with DFMO (5 mM) did not induce

microscopic changes of MC morphology; also, MC viability was unaltered, as shown by lack of uptake of Trypan Blue and release of lactate dehydrogenase (results not shown).

DISCUSSION

It is well documented that polyamines are required for normal cell growth, but it is unclear which cell-cycle events require polyamines [1–3]. Previous studies have demonstrated that DNA synthesis, i.e. the S-phase of the cell cycle, is sensitive to polyamine depletion. These findings were supported by experiments *in vitro* indicating that DNA synthesis requires polyamines [3,27]. In addition, several investigations provided evidence for polyamine-dependent restriction points during the G_0 – G_1 transition and G_1 -phase in various cell types [28–31]. So far, the precise location of the block has not been determined.

Studies on the interaction of polyamines with polyphosphoinositol formation revealed both stimulatory and inhibitory effects. Sechi et al. [32] have reported that the hydrolytic activity of soluble phospholipase A_2 and PLC on phospholipid vesicles is inhibited by polyamines. However, more recent work has revealed stimulatory effects of polyamines on isolated isoforms of PLC [33,34]. In addition, it has been reported that polyamines stimulate GTPase activity of purified G-proteins (G_0/G_i) from calf brain, which are considered to function as regulatory molecules of PLC [35]. No information is available on the effect of polyamine depletion on PLC activity in intact cells. In the present study of renal MCs in culture, we did not find significant effects of depletion of polyamines on the formation of inositol phosphates. We found increased formation of [3H]inositol phosphates in polyamine-depleted cells. This increase occurred in parallel with the proportional increase in [3H]inositol incorporation into the phosphatidylinositol bisphosphate fraction. Assuming that the same compartment of phospholipids was labelled with [3H]inositol during treatment with DFMO, these results indicate that the activation of PLC by serum and thrombin is not altered in polyamine-depleted MCs as compared with control cells (Figure 1). We conclude that the reported effects of polyamines on isolated PLC do not play a predominant role in the net effect of inositol formation in intact polyamine-depleted cells. Therefore, it is unlikely that the observed growth-inhibitory action of DFMO is mediated through PLC.

In contrast, the subsequent and transient induction of IEGs by serum was inhibited by 52–87% at 1 h. For *Egr-1*, this suppression was characterized by a decrease in total mRNA level over time and a delay of the maximal expression. The inhibitory effect was reversible by addition of putrescine. For full reconstitution of normal IEG expression, putrescine had to be added for 3 days. This corresponded to a similar time course for reconstitution of cell replication upon addition of putrescine. These results suggest a primarily indirect effect of polyamine depletion on IEG expression and cell replication in our cell-culture system. It is conceivable that polyamine-depleted cells resynthesize a factor upon putrescine supplementation which is required for normal IEG induction. The time course of growth restoration with putrescine in DFMO-treated MCs is different from the reports by other groups [2,5] and may be attributed to differences in the specific cell types and culture conditions. Our data indicate that the inhibitory event which is caused directly or indirectly by polyamine depletion occurs very early during G_1 -phase, i.e. distal to activation of PLC and proximal to activation of IEGs. This block does not completely abolish transition of MCs through G_1 -phase, since a delayed expression of *Egr-1* is observed and a considerable fraction of cells reaches mitosis.

Furthermore, it is not clear whether polyamine depletion affects the signalling cascade leading to transcription of IEGs or stability of the IEG mRNAs.

When we analysed the effects of polyamine depletion on total RNA synthesis and cellular RNA content in resting MCs, we found a slight increase in these parameters compared with control cells. This largely excludes a general inhibition of RNA synthesis in polyamine-depleted MCs. The greater increase in RNA synthesis and cellular RNA content in untreated MCs may be due to a higher division rate of these cells (Figure 1).

The role of polyamines in the regulation of protein kinase activity is at present unclear. Auvinen et al. [8] reported increased tyrosine phosphorylation in NIH 3T3 cells that had been transformed by over-expression of ODC; this effect could be reversed by DFMO. It has been reported that polyamines inhibit protein kinase C by competing for the Ca^{2+} -binding site at the molecule [36]. However, the precise effects of polyamine depletion on mechanisms involving protein kinase C and other kinases during mitogenesis remain to be determined.

In recent years much interest has focused on the role of polyamines in the regulation of gene expression. Polyamines bind to DNA and RNA because of their charge properties [1]. Spermine strongly binds to the two phosphate groups in each strand of the DNA double helix, spanning the minor and the major groove. Polyamines can also cause distortions of the usual B-form DNA, including transition to A- and Z-forms [37]. Recent data indicate that most of the cellular polyamines exist as a polyamine–RNA complex [38]. Double-helical RNA structures, such as stems and loops in rRNA and mRNA, are stabilized by polyamines [3]. Therefore it is an interesting concept that polyamines might be involved in regulation of gene transcription and translation of mRNAs, as has been shown for the key enzymes of polyamine synthesis, ODC and S-adenosylmethionine decarboxylase [3]. Furthermore, treatment of cultured colon carcinoma cells with DFMO resulted in decreased mRNA levels of the proto-oncogene *c-myc* [39]. Using nuclear run-on assays, it was demonstrated that the down-regulation of *c-myc* after depletion of polyamines is due to decreased transcription [40]. These experiments also showed that addition of spermidine caused a marked enhancement of gene transcription not only of *c-myc* but also of *c-fos* and histone 2A, whereas transcription of *c-myc*, ODC and β -actin was unaffected [40]. These data strongly support the concept that polyamines act as transcriptional regulators *in vivo*.

In summary, our results demonstrate a restriction point for cell replication in polyamine-depleted MCs located distally to activation of receptor-coupled enzymes, such as PLC, and proximal to activation of IEGs. Since the early polyamine-dependent phase described above precedes the increased activity of ODC at 6–9 h, these events do not seem to be related to each other. Clearly, more precise information is needed on the role of polyamines in the normal function of the kinase cascade and transcription of IEGs. Even though the exact mechanism of DFMO interference remains uncertain, our findings support a permissive role of polyamines at a very early phase of the cell cycle. Disturbance of this function contributes to growth arrest caused by polyamine depletion. The relevance *in vivo* of this effect of polyamine depletion *in vitro* on MC proliferation is uncertain. It is tempting to speculate, however, that inhibition of growth-associated genes and MC proliferation by inhibitors of polyamine synthesis may be a valuable approach to develop therapeutic strategies in proliferative diseases of the glomerulus. A recent report by Gunnia et al. [41] on the successful treatment of mice with lupus glomerulonephritis by using DFMO in the drinking water is compatible with this concept.

This study was supported by the Wilhelm Sander-Stiftung, München, and the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 263, Bonn, Germany. We thank U. Quotschalla for determination of polyamine concentration. The expert technical assistance of E. Pausch and S. Reichel is gratefully acknowledged.

REFERENCES

- Tabor, C. W. and Tabor, H. (1984) *Annu. Rev. Biochem.* **53**, 749–790
- Pegg, A. E. (1988) *Cancer Res.* **48**, 759–774
- Heby, O. and Persson, L. (1990) *Trends Biochem. Sci.* **15**, 153–158
- Metcalfe, B., Bey, P., Danzin, C., Jung, M., Casara, P. and Vever, J. (1978) *J. Am. Chem. Soc.* **100**, 2551–2553
- Mamont, P. S., Duchesne, M.-C., Grove, J. and Bey, P. (1978) *Biochem. Biophys. Res. Commun.* **81**, 58–66
- Brand, K. (1987) *J. Biol. Chem.* **262**, 15232–15235
- Netzker, R., Greiner, E., Eigenbrodt, E., Noguchi, T., Tanaka, T. and Brand, K. (1992) *J. Biol. Chem.* **267**, 6421–6424
- Auvinen, M., Paasinen, A., Andersson, L. C. and Holta, E. (1992) *Nature (London)* **360**, 355–358
- Rozengurt, E. (1986) *Science* **234**, 161–166
- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–194
- Nishizuka, Y. (1986) *Science* **223**, 305–312
- Sukhatme, V. P. (1990) *J. Am. Soc. Nephrol.* **1**, 859–866
- Bravo, R. (1990) in *Growth Factors, Differentiation Factors and Cytokines* (Habenicht, A., ed.), pp. 324–343. Springer, Heidelberg
- Sukhatme, V. P., Cao, X., Chang, L. C., Tsai-Morris, C. H., Stamenkovich, D., Ferreira, P. C. P., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T., Le Beau, M. M. and Adamson, E. D. (1988) *Cell* **53**, 37–43
- Mene, P., Simonson, M. S. and Dunn, M. J. (1989) *Physiol. Rev.* **69**, 1347–1424
- Schlöndorff, D. (1987) *FASEB J.* **1**, 272–281
- Sterzel, R. B. and Lovett, D. H. (1988) in *Immunopathology of Renal Disease* (Wilson, C., ed.), pp. 137–173. Churchill Livingstone, New York
- Schulze-Lohoff, E., Brand, K., Fees, H., Netzker, R. and Sterzel, R. B. (1991) *Kidney Int.* **40**, 684–690
- Lovett, D. H., Ryan, J. L. and Sterzel, R. B. (1983) *J. Immunol.* **130**, 1796–1801
- Etter, R., Dietrich, S. and Battaglia, R. (1990) *Mitt. Geb. Lebensmittelunters. Hyg.* **81**, 106–119
- Ashwell, G. (1957) *Methods Enzymol.* **3**, 73–107
- Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, I. P. and Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482
- Mayr, G. W. (1992) in *Tyrosine Phosphorylation/Dephosphorylation and Downstream Signalling* (Heilmayer, L. M. G. and Fischer, E., eds.), pp. 115–131. Springer, Heidelberg
- Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–157
- Maniatis, T. (1982) in *Molecular Cloning: A Laboratory Manual* (Maniatis, T., Fritsch, E. F. and Sambrook, J., eds.), pp. 187–210. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13
- Koza, R. A. and Herbst, E. J. (1992) *Biochem. J.* **281**, 87–93
- Seidenfeld, J., Block, A. L., Komar, K. A. and Naujokas, M. F. (1986) *Cancer Res.* **46**, 47–53
- Schaefer, E. L. and Seidenfeld, J. (1987) *J. Cell. Physiol.* **133**, 546–552
- Charollais, R. H. and Mester, J. (1988) *J. Cell. Physiol.* **137**, 559–564
- Kaminska, B., Kaczmarek, L. and Grzelakowska-Sztabert, B. (1990) *Exp. Cell. Res.* **191**, 239–245
- Sechi, A. M., Cabrini, L., Landi, L., Pasquali, P. and Lenaz, G. (1978) *Arch. Biochem. Biophys.* **186**, 248–254
- Haber, M. T., Fukui, T., Lebowitz, M. S. and Lowenstein, J. M. (1991) *Arch. Biochem. Biophys.* **288**, 243–249
- Hedekov, C. J., Thams, P., Gembal, M., Malik, T. and Capito, K. (1991) *Mol. Cell. Endocrinol.* **78**, 187–195
- Bueb, J. L., Da Silva, A., Mousli, M. and Landry, Y. (1992) *Biochem. J.* **282**, 545–550
- Schuber, F. (1989) *Biochem. J.* **260**, 1–10
- Thomas, T. J., Gunnia, U. B. and Thomas, T. (1991) *J. Biol. Chem.* **266**, 6137–6141
- Watanabe, S., Kusuma-Eguchi, K., Kobayashi, H. and Igarashi, K. (1991) *J. Biol. Chem.* **266**, 20803–20809
- Celano, P., Baylin, S. B., Giardiello, F. M., Nelkin, B. D. and Casero, R. A. (1988) *J. Biol. Chem.* **263**, 5491–5494
- Celano, P., Baylin, S. B. and Casero, R. A. (1989) *J. Biol. Chem.* **264**, 8922–8927
- Gunnia, U. B., Amenta, P. S., Seibold, J. R. and Thomas, T. J. (1991) *Kidney Int.* **39**, 882–890