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Cyclic AMP inhibits c-Ha-ras protooncogene expression and DNA synthesis in rat aortic smooth muscle cells

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Abstract. Fetal bovine serum (10%) markedly increased ras transcript levels in growth-arrested (G_0-synchronized) smooth muscle cells by 8 h. This elevation was maintained for up to 18 h and returned to pre-stimulation levels within 24 h. Challenge of quiescent cells with serum in the presence of dibutyryl cyclic AMP (1–100 μM), a growth inhibitor for smooth muscle cells, attenuated serum-induced elevation of c-Ha-ras in a dose dependent fashion and prevented progression of the cells into S phase. These results demonstrate that expression of c-Ha-ras in rat aortic smooth muscle cells is cell-cycle dependent and that cAMP prevents the induction of this protooncogene by serum.
Key words. c-Ha-ras; aortic smooth muscle cells; cell cycle; cyclic AMP; proliferation

An increasing number of studies have implicated the products of cellular protooncogenes in the regulation of smooth muscle cell growth and differentiation. For instance, induction of the *sis* protooncogene which codes for the B chain of platelet-derived growth factor (PDGF) is enhanced in atherosclerotic vessels relative to the normal artery[1]. The proliferative response induced by PDGF is associated with activation of the PDGF receptor tyrosine kinase activity and phospholipase C-mediated hydrolysis of membrane polyphosphoinositides[2]. To date, little is known about the potential role of other growth-associated protooncogenes in normal and aberrant smooth muscle cell proliferation. Among these protooncogenes, ras genes may be particularly relevant since these genes code for guanine (G) nucleotide binding proteins which have been implicated in the regulation of signal transduction pathways for several growth factors, including PDGF[3].

Ras p21s can act through a protein kinase C pathway to induce transcription of genes containing 12-O-tetradecanoyl phorbol-13-acetate (TPA) responsive elements (TREs) in their promoter regions[4]. This activation is thought to be mediated by binding of the transcription factor complex AP-1/jun to its target site. Ras oncogenes have also been found to stimulate the promoter activity of genes containing cyclic AMP responsive elements (CREs)[5]. Previous studies in this laboratory have shown that both TPA and cyclic AMP are key modulators of smooth muscle cell growth and differentiation[6]. To examine further these relationships in vascular smooth muscle cells, the present studies have been designed to evaluate the cell cycle-related expression of c-Ha-ras in rat aortic smooth muscle cells and effects of cyclic AMP in this system. Our results show that induction of c-Ha-ras in response to serum is cell-cycle dependent and susceptible to interference by cyclic AMP.

Material and methods

Chemicals. ^{32}P-UTP was purchased from New England Nuclear; the SP6 polymerase RNA labeling system was obtained from Pharmacia. Nitrocellulose and nylon membranes were purchased from Bio-Rad laboratories. The X-ray film used for autoradiography was Kodak XAR-5. A 0.8 kb human c-Ha-ras cDNA containing exons I, II, III and IV, obtained from Oncogene Science, was used to generate radiolabeled complementary RNA using a SP6 RNA polymerase transprobe kit from Pharmacia. Rapid hybridization buffer was from Amersham. Dibutyryl cyclic AMP was purchased from Sigma Chemical Company.

Tissue culture. Aortic smooth muscle cells (SMCs) were cultured from naive rats and maintained under standard conditions as described previously[7]. The medium contained 0.1 or 10% fetal bovine serum (FBS), 100 units/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Cells were plated in 25 cm^2 flasks or 35 mm culture dishes and grown to near confluence. Synchronization in G_0 was accomplished by incubation of cells in 0.1% FBS for 72 h. Cells were released from G_0 by incubation in 10% FBS.

RNA analysis. The method of extraction of total RNA from the cells was essentially as described by Chomczynski & Sacchi[8]. Briefly, cells were rinsed twice with phosphate buffered saline, trypsinized and rinsed again in phosphate buffered saline before homogenization in solution D. Samples were extracted with phenol, chloroform, and isoamyl alcohol, precipitated twice with isopropanol and finally dissolved in 0.1% SDS. RNA samples were ethanol-precipitated until they could be readily dissolved in 0.1% SDS. The concentrations of RNA in the sample were determined by UV spectrophotometric analysis.

Transfer of RNA. RNA samples were prepared by heating RNA in 20X SSC (saline-sodium-citrate) and

deionized formaldehyde at 65 °C for 10 min. Three decreasing concentrations of each RNA sample (1.0, 0.5 and 0.25 µg) were loaded to ensure accurate loadings. The RNA was blotted onto a nylon or nitrocellulose membrane using a dot blot manifold from Bio-Rad. The blotted RNA was cross-linked to the membrane using a UV cross-linker (Stratagene) according to the manufacturers' specifications. Membranes were enclosed in seal-a-meal bags and hybridized to a c-Ha-ras cRNA probe in rapid hybridization buffer at 70 °C for 2 h after prehybridization for 30 min. Membranes were then washed twice for 10 min in 2X SSC and 0.1% SDS, twice for 10 min in 0.1X SSC and 0.1% SDS at room temperature, and twice for 30 min in 0.1X SSC and 0.1% SDS at 65 °C. Autoradiographs were developed by exposing the membranes to X-ray film for 2–6 h at room temperature. Autoradiographic signals were scanned using a Visage 110. The specificity of hybridization of the cRNA probe to c-Ha-ras was verified by cross hybridization with other cDNA templates including c-myc, c-fos and α-actin, where no detectable hybridization signals were seen.

DNA measurement by flow cytometry. For this assay, approximately 650 cells/mm^2 were growth-arrested for 72 h and then stimulated with 10% serum for 9, 20, 25, 26, 27 and 28 h. At the end of the desired time period, cells were rinsed twice in phosphate buffered saline, trypsinized and collected in conical tubes. Cells were resuspended in medium containing 0.1% serum and adjusted to a final concentration of 10^6 cells/ml. Nuclei were stained in a solution containing propidium iodide as described previously[9].

DNA synthesis. In order to correlate the results of flow cytometry with DNA synthesis, measurements of ^{3}H-thymidine incorporation at various time points were performed. Briefly, synchronized cells (approximately 80,000) were stimulated with 10% serum and incubated with 0.5 µCi/ml of ^{3}H-thymidine for various time periods. Cells were harvested at the end of each phase and the cell number was determined using a hemocytometer. DNA synthetic rates were determined using a liquid scintillation counter. The radioactive counts were normalized by protein content as determined by a micro-Biuret method[10].

cAMP treatment. Dibutyryl cAMP was dissolved in Medium 199 to obtain the desired final concentrations. Following growth-arrest for 72 h in 0.1% FBS media, cells were stimulated with 10% serum in the presence of dibutyryl cyclic AMP for up to 20 h.

Statistics. Values are shown as mean ± SEM. Analysis of variance was used to evaluate the significance of the differences observed. The 0.05 level was accepted as significant.

Results

Incubation of cultured SMCs in 0.1% serum for 72 h was associated with low levels of c-Ha-ras mRNA as compared to those observed following challenge with 10% serum (fig. 1). A sharp increase in c-Ha-ras mRNA levels was observed at 8 h following exposure of growth-arrested cells to 10% serum. Elevated c-Ha-ras mRNA levels were maintained for up to 18 h and then declined with 24 h. Flow cytometric studies revealed that incubation of SMCs in 0.1% serum for 72 h synchronized most of the cell population in G_0. This profile was comparable to the proportion of unstimulated rat peripheral blood lymphocytes used as a standard growth-arrested cell population (data not shown). Release of the cells from G_0 upon incubation in 10% showed that the highest proportion of SMCs in $G_0 + G_1$ occurred at 9 h, decreased as the cells progressed through the cycle, and rose again at 28 h. The proportion of SMCs in S phase was lowest at 9 h following stimulation with 10% serum, continued to increase at the 20 and 25 h time points, and declined at 28 h (fig. 2). Measurements of ^{3}H-thymidine incorporation into DNA in growth-arrested rat aortic SMCs revealed that DNA synthesis was initiated 19 h after serum stimulation and continued for up to 27 h (data not shown). Challenge of growth-arrested SMCs with 10% serum in the presence of dibutyryl cAMP (1–100 µM) for 20 h was associated with a dose-dependent decrease in ^{3}H-thymidine incorporation into DNA. The highest concentration of dibutyryl cAMP tested (100 µM) caused a 4-fold inhibition of ^{3}H-thymidine incorporation, which suggested that dibutyryl cAMP inhibited serum-induced DNA synthesis in aortic SMCs (fig. 3). Serum stimulation of growth-arrested SMCs in the presence of dibutyryl cAMP (10–100 µM) for 8 h also resulted in a significant decrease of the steady-state c-Ha-ras mRNA levels associated with serum stimulation (fig. 4). As seen with DNA synthesis, 100 µM

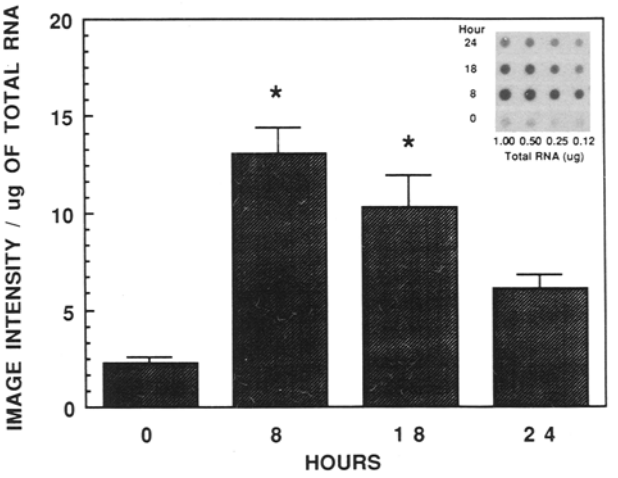

Figure 1. Steady state levels of c-Ha-ras mRNA in rat aortic SMCs. Cells cultured from naive rats were growth-arrested by incubation in low serum-medium (0.1%) for 72 h and stimulated with 10% serum for various times. Total RNA was extracted at the indicated time points and hybridized to labeled RNA probes generated from 0.8 kb ras cDNA as described in the methods section. *Significantly different from time 0 ($p < 0.05$).

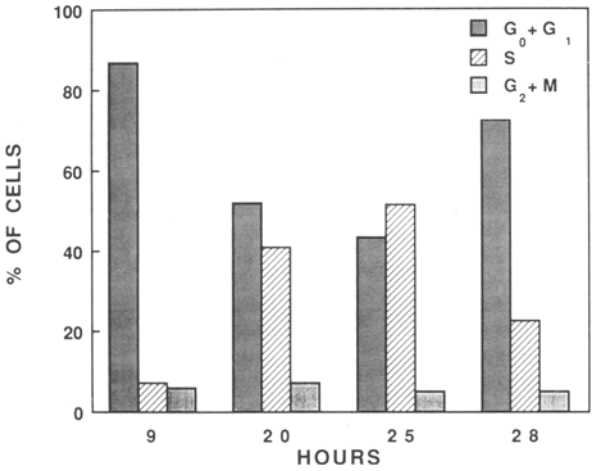

Figure 2. Proportion of rat aortic SMCs in different phases of the cell cycle at various times following serum stimulation. Cells were growth-arrested for 72 h by serum deprivation and stimulated with 10% serum for the time periods indicated.

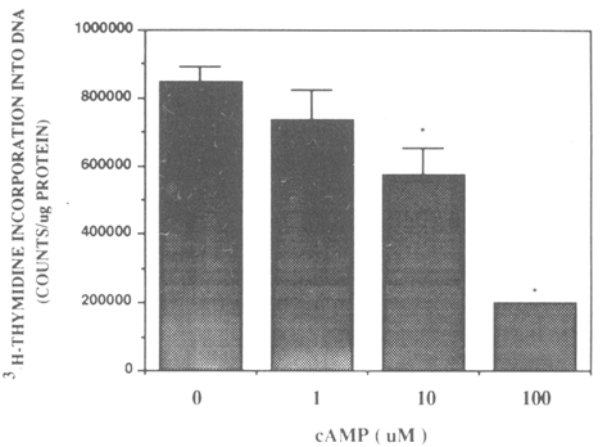

Figure 3. ^{3}H-thymidine incorporation into DNA in quiescent rat aortic SMCs stimulated with 10% serum in the presence or absence of dbcAMP. Cells were growth-arrested for 72 hr and stimulated with 10% FBS in the presence of 0.5 μCi/ml of ^{3}H-thymidine and dibutyryl cAMP (1–100 μM) for 20 h. Thymidine incorporation rates were measured using a liquid scintillation counter. The radioactive counts were normalized to protein content as determined by a Micro-Biuret method.

dibutyryl cAMP induced maximal reduction in the steady-state c-Ha-ras mRNA levels (fig. 4).

Discussion

The elevation of c-Ha-ras mRNA levels during the G_1 phase of the cycle suggests that ras products are required for proliferative events preceding the onset of DNA synthesis in rat aortic smooth muscle cells. This interpretation is consistent with the observation that c-Ha-ras mRNA levels were lowest during the G_0 and G_2 phases of the cycle and elevated during mid to late G_1. Our results are in agreement with several studies implicating p21 ras in the regulation of DNA synthesis in other cell types. Campsi et al.[11] have reported that c-ras transcript

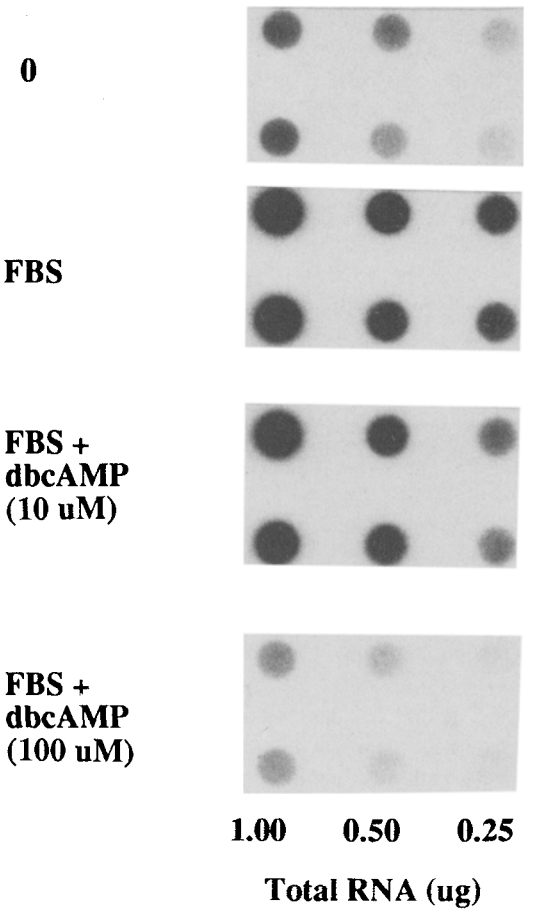

Figure 4. Steady state c-Ha-ras mRNA levels in quiescent SMCs stimulated with 10% serum in the presence or absence of db-cAMP. Total RNA from control and dibutyryl cAMP-treated cultures of rat aortic SMCs was hybridized to a ras cRNA probe. Two representative samples from each group of 4 are presented in the inset.

levels rose during late G_1 in mouse fibroblasts and continued to increase as cells entered the S phase. On the other hand, NIH 3T3 cells induced to divide by the addition of serum do not progress into S phase when treated with anti-p21 monoclonal antibody.[12]

Challenge of quiescent smooth muscle cells with serum in the presence of dibutyryl cyclic AMP significantly decreased serum-induction of c-Ha-ras transcript levels and DNA synthesis in a dose-dependent fashion. Previous studies have demonstrated that in addition to its ability to regulate several metabolic pathways, cyclic AMP modulates growth and differentiation in vascular smooth muscle cells[6]. Although the latter actions are poorly understood, our data suggests that the actions of cAMP may involve interference with cell cycle-related expression of protooncogenes. The mechanism by which cyclic AMP decreases steady state levels of the c-Ha-ras protooncogene in aortic smooth muscle cells is at present unknown. Recent studies have shown that cyclic AMP treatment is associated with transcriptional down-regulation of c-myc and changes in chromatin structure

near the c-myc promoter sites in HL-60 cells[13]. Furthermore, Cohr et al.[14] have shown that inhibition of cyclic AMP phosphodiesterase by 4(3-butoxy-4-methoxybenzyl)-2-imidazolidinone inhibits ras expression in neuroblastoma cells. The AP-1/jun family of transcriptional factors are known to bind CREs in DNA[15]. Interestingly, the AP-1/jun family also binds to TRE, which suggests that the observed interaction between c-Ha-ras and cyclic AMP may be mediated by modulation of transcriptional regulation in response to serum mitogens. Because CREs represent only one of the nuclear end-points of the cyclic AMP-dependent protein kinase A pathway, the possibility that other phosphorylation reactions participate in the growth inhibitory effects of cyclic AMP should also be considered.

The study of vascular growth and differentiation is of particular significance for the elucidation of mechanisms responsible for the initiation and progression of the atherogenic processes. Because the products of protooncogenes play an essential role in the transduction of mitotic signals by growth factors, the modulation of c-Ha-ras during the course of the cell cycle may be relevant to the deregulation of smooth muscle cell growth in atherogenesis. This hypothesis is supported by previous studies in our laboratory showing that G protein-mediated metabolism of membrane phospholipids is differentially regulated in smooth muscle cells of varying proliferative potential[6]. Our results show that expression of c-Ha-ras in aortic smooth muscle cells is cell cycle dependent and that interference with the serum-induced response by cyclic AMP may account for its antiproliferative actions.

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