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INCREASED LEVELS OF C-FOS AND C-MYC mRNA IN ATP-STIMULATED ENDOTHELIAL CELLS

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In bovine aortic endothelial cells, ATP induced a transient and sequential accumulation of c-fos and c-myc mRNA, which was detected after 1 hour and 3 hours, respectively. The effect of ATP on c-fos mRNA was stronger than that of TNF and bFGF. Both ATP and bFGF increased c-myc mRNA after a 3 hour treatment, whereas TNF did not. If none of the 3 agonists tested induced a selective expression of c-fos or c-myc, each of them was associated with a different quantitative combination of the 2 signals, which might be related to the distinct responses that they trigger in endothelial cells.

ATP induces acute responses in vascular endothelial cells: a rapid and transient release of prostacyclin (PGI_2), a potent inhibitor of platelet aggregation, and a more sustained release of nitric oxide (NO), which relaxes the vascular smooth muscle and synergizes with PGI_2 to inhibit platelets (1, 2). These responses, which are mediated by P_{2y}-receptors, are likely to play an important role in the interaction between platelets, a rich source of adenine nucleotides, and the blood vessel wall (2). In endothelial cells, as elsewhere, P_{2y} receptors are coupled to a phospholipase C which hydrolyzes phosphatidyl-inositol bisphosphate (3). The effect of ATP on PGI_2 and NO

ABBREVIATIONS

BAEC : bovine aortic endothelial cells; aFGF : acidic fibroblast growth factor; bFGF : basic fibroblast growth factor; TNF : tumor necrosis factor.

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release is mediated by a rapid rise of cytosolic Ca^{2+} (4, 5). So far little is known about the late consequences of the interaction between ATP and its receptors on endothelial cells. Several biochemical responses classically associated with the action of mitogens have been detected in ATP-stimulated endothelial cells: activation of Na^+/H^+ antiport (6), phosphorylation of 28 kD heat shock proteins (7, 8) and stimulation of phosphatidylcholine metabolism (9, 10). It was shown recently that, in aortic endothelial cells, ATP induces a mitogenic response, measured by an increase in the number of [^{3}H] thymidine-labeled nuclei (11). In order to understand the link between the acute responses of endothelial cells to ATP and their late mitogenic response, we have studied the effect of ATP on the expression of c-fos and c-myc protooncogenes.

MATERIALS AND METHODS

Cell culture

Bovine aortic endothelial cells (BAEC) were obtained by collagenase digestion of a bovine aorta, as previously described (3). The cells were seeded on 100 mm Petri dishes and incubated at 37°C in a 5% CO_2 humidified incubator, in the following medium: MEM-D-valine (80% v/v), fetal calf serum (FCS: 20% v/v), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B. MEM-D-valine was used to prevent the proliferation of any contaminating smooth muscle cells. The medium was changed the following day and renewed twice a week. After 4 or 5 days, the primary cultures formed confluent monolayers and could be subcultured. The cells were detached by a 5 min incubation in a Ca^{++} - and Mg^+ - free Hanks buffer containing trypsin (10 mg/ml) and EDTA (1 mM). Thereafter, the cells were maintained and subcultured in the following medium: DMEM (60% v/v), Ham's F_{12} (20% v/v), FCS (20% v/v), with the same concentrations of penicillin, streptomycin and amphotericin B, as mentioned above.

Cell stimulation

BAEC (3rd - 5th passage) were seeded on 35 mm Petri dishes, at a density of ± 15.000 cells/cm^2, in culture medium containing 2.5% FCS. After 4 hours, this medium was removed and replaced by a serum-free medium. 24 hours later, ATP, Tumor Necrosis Factor-α (TNF) and basic Fibroblast Growth Factor (bFGF) were added and the incubation was continued for 30 min up to 6 hours. In most experiments, cycloheximide (10 µg/ml) was added for the last hour of the incubation.

RNA purification

At the time of harvest, the cells were rapidly scraped from the dish in 4M guanidinium monothyocyanate (1.9 ml for three 100 mm dishes) (12). After the addition of 840 mg $CsCl_2$ to the homogenate, each sample was layered on top of a 1.2 ml cushion of 5.7 M $CsCl_2$ in 0.1 M EDTA. Separation of total RNA was performed by centrifugation at 15°C for 17 h at 36,000 rpm in a Beckman SW56

rotor. The RNA was precipitated in 2 M LiCl and then treated with proteinase K (60µg/ml) in NaCL 0.3 M-SDS 0.1%. After classical phenol chloroform extractions, the amount of total RNA was determined spectrophotometrically.

Northern blot analysis

After denaturation using glyoxal according to the procedure of MacMaster and Carmichael (13) equal aliquots (4 to 10 µg per lane) were fractionated by electrophoresis on a 1% agarose gel in 10 mM phosphate pH 7.0. Acridine staining of independent lanes revealed that the amounts of RNA were equal in all samples. As size markers, phage lambda cleaved by EcoRI and HindIII, was treated similarly. Glyoxylated RNA were transferred by diffusion blotting to a nylon membrane (Pall Biodyne A) using 20 x SSC (1 x SSC = 0.15 M NaCl/0.015 M Na Citrate) as described (14). After baking, the blots were prehybridized overnight at 42°C in a buffer consisting of 50% formamide (v/v), 5 x Denhardt solution (100 x solution: 2% Ficoll, 2% polyvinylpyrrolidone), 5 x SSPE (20 x solution: 3.6 M NaCl, 0.2 M Na phosphate pH 8.3, 20 mM EDTA), 0.3% SDS, 250 µg/ml denaturated salmon testes DNA, 200 µg/ml BSA. Essentially the third exon of the human c-myc gene (1.398 bp ClaI fragment of PKH47 human c-myc obtained from Dr Saule), and v-fos (1.400 bp Bgl II fragment of pFBJ-neo obtained from Dr R. Muller EMBL) DNA probes were [α^{32}P] labeled by random priming extension to a specific activity of approximately 2 x 10^8 cpm/µg (15). Hybridizations were carried out for 48 h at 42°C in the same buffer as for prehybridization, but containing in addition 10% sulfate (W/V) and the heat denatured probe. Filters were washed in 2 x SSC - 0.1% SDS at room temperature, four times ten minutes, and then in 0.1 x SSC - 0.1% SDS at 60-65°C, four times thirty minutes.

Filters were autoradiographed at -70°C using Kodak XAR-5 films (Eastman Kodak, Rochester N.Y.) in the presence of intensifying screens. The same filter was probed successively with c-myc and v-fos. Probe was stripped from the paper by incubating for 1 h at 65°C in 50% formamide, 10 mM Na phosphate pH 6.5, and then by washing for 15 minutes at room temperature in 2 x SSC, 0.1% SDS. As this study was not carried out with a cell line, the levels of induction of mRNA was different from one culture to another, but the experiments have been repeated 2 or 3 times with similar data being obtained.

Materials

Collagenase type IA was purchased from Cooper Biochemical (Worthington Biochemical Corporation). DMEM, Ham's F12, MEM-D-Val, FCS, penicillin, streptomycin, amphotericin B and glutamine were from Gibco laboratories. Trypsin was obtained from Flow Laboratories. ATP and cycloheximide were obtained from Sigma Chem. Co. Recombinant human TNF was a gift of Dr W. Fiers. Recombinant bovine bFGF was purchased from Amersham.

RESULTS

ATP induced the expression of c-fos mRNA in BAEC (Fig. 1 and 2). Although this effect could be detected in the absence of cyclo-

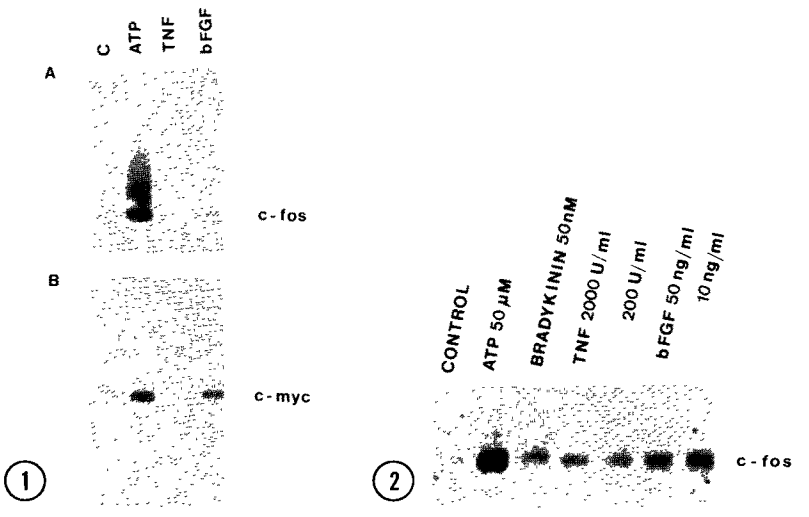

Figure 1

Effects of ATP, TNF and bFGF on the levels of c-fos and c-myc mRNA in BAEC. BAEC, cultured as described in Methods, were incubated with ATP (50 µM), TNF (200 U/ml) or bFGF (20 ng/ml) for 1 hour (panel A: c-fos) or 3 hours (panel B: c-myc). Cycloheximide (10 µg/ml) was present throughout the incubation (A) or during the last hour (B). C: control.

Figure 2

Increased level of c-fos mRNA in stimulated endothelial cells: comparison between ATP, bradykinin, TNF and bFGF. BAEC, cultured as described in Methods, were incubated for 1 hour in the presence of the various agonists and of cycloheximide (10 µg/ml). C: control.

heximide (not shown), the signal was much more intense in cycloheximide-treated cells. Accumulation of c-fos mRNA in response to ATP was a transient phenomenon, detectable after 1 hour but no longer after 3 hours (not shown). The effect of ATP on c-fos mRNA had a greater magnitude than the maximal effect of either TNF or bFGF (Fig. 1 and 2). At concentrations of agonists producing similar increases in inositol phosphates and PGI_2, ATP was also more effective than bradykinin in increasing c-fos mRNA (Fig. 2).

After 3 hours, ATP increased the level of c-myc mRNA (Fig. 1 and 3). bFGF was also stimulatory, whereas TNF had no effect. The stimulatory effect of ATP could be detected in the absence of cycloheximide (Fig. 3). The constitutive expression of c-myc in these experiments might be related to the fact that BAEC were not quiescent following a 24 hours deprivation of serum (11). The action of ATP had a characteristic time course (Fig. 3). After 1

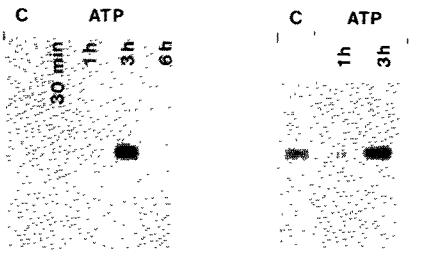

Figure 3

Time course of c-myc mRNA accumulation in ATP-stimulated BAEC. BAEC, cultured as described in Methods, were incubated with ATP (50 μM) for the indicated times. Left and right panels correspond to distinct experiments, which were performed respectively in presence and in absence of cycloheximide (10 μg/ml). C: control without ATP.

hour the level of c-myc mRNA was actually decreased. The later increase was transient: it was observed at 3 hours, but no longer after 6 hours.

DISCUSSION

Two studies of c-fos and c-myc expression in human umbilical vein endothelial cells have been reported previously (16, 17). Colotta et al. (16) observed that both interleukin-1 and TNF induced an accumulation of c-fos mRNA which peaked after 1 hour. A coordinated expression of c-fos and c-myc was induced by serum and aFGF or bFGF, separately or in combination: the accumulation of c-fos and c-myc mRNA was transient, with peak values at 1 and 2 hours respectively (17). In the present study, we have obtained similar results in bovine aortic endothelial cells. We have indeed detected c-fos mRNA in TNF-and bFGF-stimulated cells after 1 hour of treatment and c-myc mRNA in cells stimulated by bFGF for 3 hours. In addition, we have shown that ATP also produces a sequential accumulation of c-fos and c-myc mRNA and that it is a stronger inducer of c-fos than either TNF or bFGF.

ATP, TNF and bFGF induce distinct responses in endothelial cells. The release of PGI_2 and NO has been identified as a major acute effect of ATP, whereas late responses have not yet been characterized, except for the preliminary evidence of a mitogenic action (11). In the case of TNF and FGF, neither rapid functional responses nor the second messengers involved in the late actions have been identified so far. TNF induces a reprogramming of endothelial cells towards a procoagulant and proinflammatory

surface (18) and inhibits endothelial cell proliferation in vitro (19). FGF is mitogenic for the endothelial cells and produces some effects, such as the down-regulation of prostaglandin H synthase (20) and the secretion of plasminogen activator (21), which are opposite to those of TNF. Although the responses of endothelial cells to ATP, TNF and bFGF are so different, these 3 agonists all stimulated the expression of c-fos. This promiscuous induction was actually not unexpected in view of the bewildering array of cells responses in which c-fos is expressed (22). Expression of c-myc is an early response to mitogenic stimulation in many cell types, although a direct causal link between c-myc and cell proliferation has not been firmly established. In endothelial cells, bFGF and ATP, which increase cell proliferation, induced an accumulation of c-myc mRNA, whereas TNF, an inhibitor of proliferation, did not. If none of the agonists tested induced a selective expression of either c-fos or c-myc, each of them was associated with a different quantitative combination of the 2 signals, which might be related to the distinct responses of endothelial cells.

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