Chapter 3

Cell-Based Assays to Probe the ERK MAP Kinase Pathway in Endothelial Cells

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Summary

To understand signaling pathways in mammalian cells, cell-based assays are relatively new and extremely powerful tools. We have developed a battery of phenotypic assays to study signaling; two of them are described in detail in this chapter. A subset of these assays monitors mitogen-activated protein (MAP) kinase pathways. MAP kinases are principal regulators of fundamental processes in mammalian cells, including growth, cell division, differentiation, stress responses, and neoplastic transformation. Here we describe two cell-based assays querying the function of ERK (extracellular signal regulated kinase), one of the three principal MAP kinases in mammalian cells. We selected human umbilical vein endothelial cells (HUVECs), a primary cell type, because they show a very dynamic response to various activators. Both assays are phenotypic assays and use well-established phosphorylation-specific primary antibodies to study activation. Fluorochrome-coupled secondary antibodies were used to label phosphorylated target proteins; images were captured with the INCell Analyzer 3000 and analyzed with the INCell Analyzer 3000 software. The first of these two assays monitors phosphorylation of ERK1/2, while the second assay monitors activation of the transcription factor CREB (cAMP response element-binding protein). The assays described in this chapter cover major checkpoints of the ERK signaling pathway: (1) MAP kinase activation and (2) subsequent transcription factor activation. Both assays exhibit robust performance and can easily be used for high-throughput screening.

Key words: Automated microscopy, Cell-based assays, HUVEC, Phosphorylation-specific antibodies, Primary cells, Signal transduction.

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1. Introduction

Defects in the complex cellular signaling network that regulates cell proliferation and survival can result in neoplastic transformation of the cell. These defects have been used as models for the complex collection of diseases known as cancer. A major part of the signaling network relevant to proliferation and survival comprises three mitogen-activated protein (MAP) kinase pathways, termed the extracellular signal regulated kinase (ERK), JNK, and p38 pathways, so named for the principal kinases *(1–3)*. The significance of these regulatory pathways is underlined by recent and ongoing development of a number of MAP kinase inhibitors as cancer therapeutics *(4–6)*. High-throughput cell-based assays have proven to be powerful tools to deconvolute complex signaling pathways, including the MAP kinase pathways. For many decades, in vitro culture of mammalian cells has been harnessed to study basic biological questions. Diverse transformed and primary mammalian cells have been exploited to study diseases, including diabetes, cancer, and neurodegenerative disease *(7, 8)*. However, many breakthroughs have depended on the serendipitous discovery of a small-molecule inhibitor with specificity for a particular biological process. Recent technological advances in high-throughput screening have reduced the dependence of progress on serendipity. Existing and newly developed hardware, such as automated microscopes, imaging platforms, and robotic accessories, as well as software, such as automated image analysis programs, have enabled the probing of larger segments of chemical space for new biological activities. Here, we describe the application of cell-based assays to investigate the ERK pathway.

Many features of cell growth, development, and survival are controlled by MAP kinase pathways. Although the focus here is on the ERK pathway, many of the techniques can easily be adapted to the other two MAP kinase pathways. The ERK pathway is generally activated by a mitogen binding to a receptor tyrosine kinase, but this pathway can also be activated by ion channels, G-protein-coupled receptors, and integrins *(9, 10)*. An activated receptor is linked to a cascade of MAP kinases by a specific adaptor molecule, such as protein kinase C (PKC), son of sevenless (SOS), or the small GTPases of the ras/rap family. For the ERK pathway the cascade begins with phosphorylation of raf (a MAP kinase kinase kinase), followed by MEK1/2 (MAP kinase kinase) and ERK1/2 (MAP kinase). Phosphorylated ERK translocates to the nucleus where its role is to effect transcriptional activation and repression.

Successful development of a high-throughput cell-based assay poses several challenges. In general, owing to the large scale of the enterprise, considerations of biological authenticity

must be balanced against cost and practicality. For example, regarding choice of cell type, it is more practical to use an established cell line, although primary cells may be more biologically relevant. For the assays described here, we have chosen human umbilical vein endothelial cells (HUVEC), which are neither transformed nor genetically manipulated but can be cultivated for at least 16 cell doublings in culture *(11)*. Furthermore, they are commercially available in lots from pooled individuals, reducing genetic variability.

Another critical parameter of every assay is the type of readout that is used. For a cell-based assay focused on a signaling pathway, there is often considerable flexibility in the choice of readout. The most accessible readouts for interrogation of a signaling pathway can be any step in which a protein is modified (e.g., phosphorylated), translocated (e.g., moves from cytoplasm to nucleus), or changed in its abundance, provided the appropriate antibody reagents are available *(12)*. An alternate approach is to generate a reporter molecule that can monitor a given step of the pathway. This could be a GFP-tagged protein to monitor translocation, or a promoter/enhancer driving the expression of luciferase to monitor transcriptional activation *(13, 14)*. The use of reporter constructs involves extensive manipulation of cells that may affect the pathway to be studied.

We describe here two assays that monitor different stages of the ERK1/2 signaling pathway. In both assays, the pathway is activated using a phorbol ester, TPA (phorbol-12-myristate-13-acetate). TPA treatment is a standard procedure used to directly activate PKC, thereby bypassing the cell-surface receptor. This treatment produces a more robust and reproducible response than activation via a cell-surface receptor. The first assay described here detects phosphorylation and nuclear translocation of ERK1/2 using antibodies specific for the activated form of phospho-ERK. The second assay monitors a downstream event, namely the phosphorylation of the transcription factor CREB at Ser 133.

2. Materials

2.1. Cell Culture

1. Activators/inhibitors: Phorbol 12-myristate 13-acetate (TPA/PMA) (Calbiochem; San Diego, CA) and U0126 (Calbiochem).
2. Cells: HUVECs obtained as frozen stocks (Lonza Biosciences; Basel, Switzerland).
3. Medium: EGM-2 medium with supplements (Lonza Biosciences).

4. Phosphate buffered saline (PBS) for tissue culture (Lonza Biosciences).
5. DMEM containing 10% fetal calf serum (Lonza Biosciences).
6. T175 flasks (Corning; Lowell, MA).
7. View plates (PerkinElmer; Waltham, MA).
8. 0.05% Trypsin/EDTA (HyQ, Hyclone; Logan, UT).
9. Anti-phospho-ERK1/2 (Thr202/Tyr204) (BDBiosciences; San Jose, CA).
10. Anti-phospho-CREB (Ser133) (Cell Signaling; Danvers, MA).
11. Goat anti-mouse coupled to Alexa 647 nm (Molecular Probes; Carlsbad, CA).

2.2. Fixing and Staining

1. Permeabilizing/blocking solution: 10% (v/v) newborn calf serum (Gibco/Invitrogen; Carlsbad, CA; *see* **Notes 1** and **2**), 0.2% (w/v) Triton X-100 in PBS.
2. Wash solution: 10% (v/v) newborn calf serum (Gibco/Invitrogen) in PBS.
3. Primary antibody solutions: 0.25 μg/mL mouse IgG against ERK1/2 in PBS containing 10% (w/v) newborn calf serum and 0.2% Triton X-100.
   - The anti-CREB (Ser 133) antibody stock solution (purified mouse IgG) was diluted 1:1,000 in PBS containing 10% (w/v) newborn calf serum and 0.2% Triton X-100.
4. Secondary antibody solution: 2 μg/mL anti-mouse antibody coupled to Alexa Fluor® 647 nm (Molecular Probes) and 1μg/mL Hoechst 33342 (Calbiochem) in PBS containing 10% (v/v) newborn calf serum and 0.2% Triton X-100.

2.3. Image Acquisition

2.3.1. Equipment

1. Automated Microscope: INCell Analyzer 3000 (GE Healthcare; USA).
2. Plate Hotel: Kendro Cytomat Hotel (Kendro; Germany).
3. Robot Arm: Mitsubishi MELFA RU-2AJ arm (Mitsubishi; Japan).

2.3.2. Camera and Hardware Settings

1. Blue Camera: Neutral Density Filter 1, Emission filter 450–25 nm.
2. Red Camera: Neutral Density Filter 0, Emission filter 695–55 nm.
3. Exposure time of 1.7 ms.
4. Binning of 1.

3. Methods

Methods that are common to the assays described in this article are listed in the beginning of the Methods section. To simplify assay development and implementation we have developed standardized cell-culture and -staining procedures for our cell-based assays. These standardized procedures are described in this section. Although we routinely develop assays in a 96-well format, we have found that most assays can be easily transferred to a 384-well format if a higher-density format is needed.

3.1. Cell Culture

1. HUVECs were obtained as pooled, frozen stocks and grown in EGM-2 medium with supplements.
2. Cells were grown at 37°C, 5% CO_2, and 100% humidity (standard conditions) in T175 flasks in an Autoflow incubator (Nuaire).
3. To expand HUVECs, the medium was aspirated and the monolayer rinsed once with 20 mL PBS, followed by the addition of 5 mL of a trypsin/EDTA solution (0.05% trypsin/0.02% EDTA in PBS).
4. Cells were detached by incubation with 5 mL of trypsin/EDTA solution per T175 flask for 6–10 min, then resuspended in 20 mL of DMEM containing 10% fetal calf serum, and centrifuged for 5min at 1,000×g.
5. HUVECs were *see* ded at dilutions of 1:2–1:3 and grown to confluence. Experiments during the developmental phase were performed with cells kept in culture up to passage 9.

3.2. Cell Seeding and Stimulation

1. HUVECs were seeded using a 12-channel automatic pipettor at a density of 10,000 cells per well in 96-well plates ("ViewPlates") and grown for 16 h under standard conditions.
2. Specific signaling was initiated by removing the growth medium and replacing it with 100 µL prewarmed medium containing the activating agents.

3.3. Fixing and Staining

To facilitate easy transition from the assay development phase to different automated liquid-handling systems, we drastically reduced washing steps and incubation times for the postfixation procedures.

3.3.1. Fixation

1. Add 35 µL 16% paraformaldehyde to each well (*see* **Note 3**).
2. Fix for 20 min at room temperature.

3. Remove fixative solution and wash once with 100 µL PBS.
4. Store cells in 100 µL PBS at 4°C until ready to stain.

3.3.2. Staining (All Steps Are Performed at Room Temperature)

1. Remove PBS from cells.
2. Add 100 µL permeabilizing/blocking solution.
3. Incubate for 20 min.
4. Remove permeabilizing/blocking solution.
5. Add 50 µL primary antibody solution (*see* **Note 4**).
6. Incubate for 20 min.
7. Remove antibody solution and wash once with 175 µL wash solution.
8. Add 50 µL secondary antibody solution (*see* **Note 4**).
9. Incubate for 20 min.
10. Remove antibody solution and wash three times with 100 µL PBS.
11. Seal and store at 4°C in fresh 100 µL PBS until ready to scan.

3.4. Image Acquisition

1. Software settings ("Nuclear Traffic Module" of INCell Analyzer 3000 software).
   (a) Primary Measurement Parameters:
   - Nuc Marker: Blue
   - Cytoplasm: Red
   - Cyt Thresh: *T*=10
   - Clip Rings: N
   - Signal: Red
   - Sig Thresh: None
   - Erosion: 2
   - Dilation: 4

   (b) Object Definition Parameters:
   - Threshold: background+C
   - Constant C: 100.00
   - Discard Percentile: 10.00
   - Fill Holes: Y
   - Erosion: 0
2. Plates were imaged by sequential scanning using the red and blue channel.
3. To acquire the first image, samples were excited with the 647 nm laser line and images were recorded with the 695–55nm emission filter (red channel).
4. For the second image, the Hoechst dye was excited at 364 nm and images recorded with the 450–25nm emission filter (blue channel).

5. As standard procedure, we recorded a single square field (0.561 mm^2) from each well. Using an exposure time of 1.7 ms and a binning factor of 1, we averaged 200–300 cells per image.
6. After scanning the plates, data files including images and descriptive metadata files ("run files") were transferred from the internal hard drive to a file server.
7. Analysis of the images was performed on personal computers (operating system Microsoft XP Professional, Microsoft Corporation) using the INCell Analyzer software (GE Healthcare). Analysis parameters and analysis modules used for the assays described in this chapter are listed below.
8. Files containing the metadata were imported into Microsoft Excel to facilitate graphic representation of the results.

3.5. Two Assays to Monitor the ERK1/2 Pathway

3.5.1. ERK1/2 Phosphorylation Assay

After activation with the phorbol ester TPA, the MAP kinase ERK1/2 is phosphorylated at positions threonine 202 and tyrosine 204, respectively. Phosphorylated ERK forms a dimer that is translocated to the nucleus (**Fig. 1A**). Using phosphorylation-specific primary antibodies and secondary antibodies coupled to the fluorochrome Alexa 647, only the phosphorylated/activated form of ERK is detected/labeled (**Fig. 1B**). The nucleus is labeled by Hoechst 33342. As the readout we determine the average intensity per pixel arising from the phosphorylated ERK in

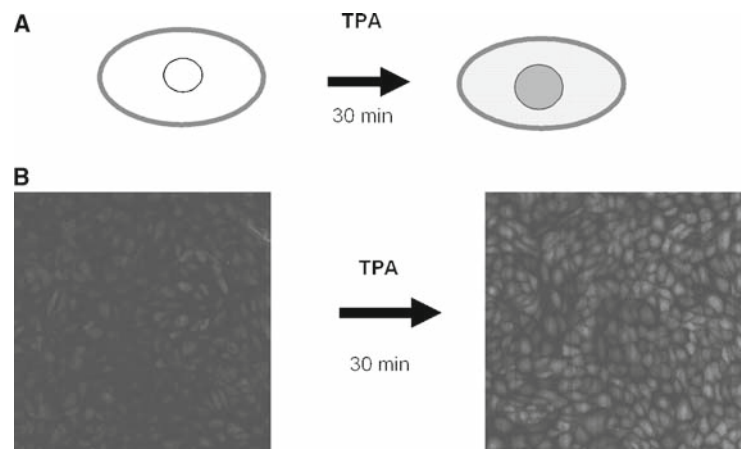

Fig. 1. Activation of ERK1/2. (A) Activation and translocation of phosphorylated ERK1/2 after TPA treatment. (B) HUVECs were stimulated with TPA as described in the text. To detect distribution of the MAP kinase ERK1/2, cells were fixed and permeabilized, and then incubated initially with an antibody against the phosphorylated form (Thr202/Tyr 204) of ERK1/2 and subsequently with secondary antibodies coupled to Alexa Fluor® 647. The left panel shows images of nonstimulated cells and the right panel shows images of TPA-stimulated cells.

the nucleus of the HUVEC. To analyze the images, the "Nuclear Traffic Module" of the INCell Analyzer 3000 software was used.

1. Cells were seeded at 10,000 cells per well and incubated for 16 h in EGM-2 medium using standard conditions. We found that 16 h incubation resulted in a constant and reproducibly low baseline of background ERK phosphorylation.
2. To activate the pathway, the medium was removed and 100 µL of EGM2 medium containing 1µM TPA was added.
3. After 30 min incubation at standard conditions, cells were fixed by adding 35 µL of 16% paraformaldehyde (*see* **Note 3**). We have observed that 30 min incubation with TPA works very well for most assay analysis requiring phosphorylation and/or nuclear translocation events. This includes assays for p38 and JNK MAP kinase activation, NFκB nuclear translocation, and the CREB phosphorylation/nuclear translocation assay described later in this chapter. **Fig. 1B** shows images of HUVECs treated with (**Fig. 1B**, right panel) or without TPA (**Fig. 1B**, left panel).
4. Pictures were taken with the INCell Analyzer 3000. Cells were immunostained with the phosphorylation-specific antibodies as described above. **Fig. 2** shows quantitative data obtained from analyses of images from the INCell 3000 software..
5. Increasing concentrations of TPA result in an increased phosphorylation and nuclear accumulation, and thus activation of ERK1/2 (**Fig. 2**). The curve shows a strong correlation between the TPA concentration and nuclear intensity of phosphorylated ERK, and plateaus at 60 nM with an AC_{50}

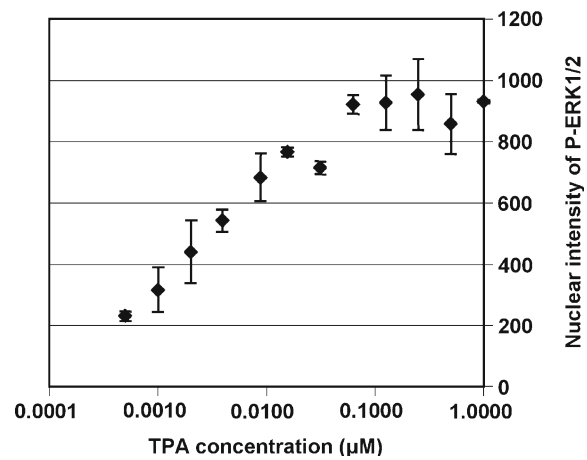

Fig. 2. TPA inducing phosphorylaton of ERK1/2 in a dose-dependent manner. HUVECs were stimulated for 30 min with the indicated concentrations of TPA. Cells were treated as described for Fig. 1. ERK1/2 activation is represented by nuclear fluorescence intensity (in arbitrary units) as a function of TPA concentration.

of 4nM. We have also successfully used H_2O_2 and sorbitol to stimulate phosphorylation and nuclear translocation of ERK1/2 (data not shown). These assays measure the response of the HUVECs toward oxidative and osmotic shock, respectively.

6. Activation of ERK strongly depends on MAP kinase kinase MEK1 activity as demonstrated with specific inhibitor U0126 *(15)*. U0126 inhibits the accumulation of phosphorylated ERK1/2 with an IC_{50} of 80 nM (**Fig. 3**). The results shown above validate our assay: ERK1/2 activation is dependent on TPA and is mediated by MEK1.

3.5.2. CREB: Nuclear Accumulation Assay

Once ERK1/2 is activated, a cascade of events is initiated, one chain of which results in activation of a set of kinases that includes p90 RSK and MSK kinases. These kinases phosphorylate the transcription factor CREB. The assay we describe here monitors the accumulation of phosphorylated CREB (on serine 133) in the nucleus. Using phosphorylation-specific primary antibodies and secondary antibodies coupled to the fluorochrome Alexa 647, only the phosphorylated form of CREB is detected/labeled (**Fig. 4B**). The nucleus is counterstained with Hoechst 33342. As readout, the ratio of intensity calculated from the phosphorylated CREB in the nucleus and in the cytoplasm of the cells was used. To analyze the images, we use the "Nuclear Traffic Module" of the INCell Analyzer 3000 software. The hardware and software settings are as described above.

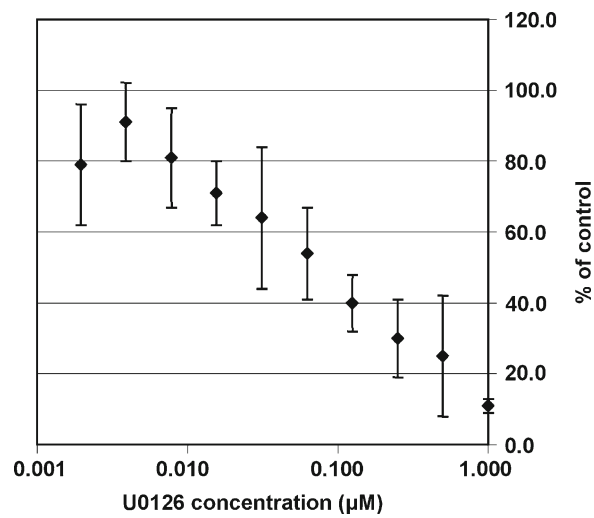

Fig. 3. TPA-induced phosphorylation of ERK dependent on MEK1. Cells were seeded as described above. After 16 h, increasing concentrations of the MEK1 inhibitor U0126 were added for 1 h. HUVECs were then stimulated with TPA for 30 min, and fixed and stained as indicated in the text. Phosphorylation and activation of ERK is expressed as nuclear intensity of phospho-ERK1/2 staining relative to staining in the absence of U0126 (% of control; 100% = 0 µM U0126), as a function of U0126 concentration.

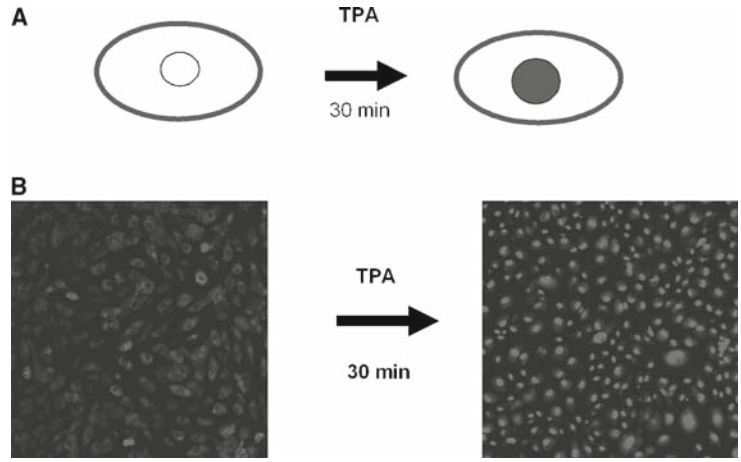

Fig. 4. Nuclear accumulation of phosphorylated CREB. (A) Phosphorylation and nuclear accumulation of phosphorylated CREB (Ser133) after TPA treatment. (B) HUVECs were stimulated with TPA as described in the main text. To detect distribution of transcription factor CREB, cells were fixed and permeabilized, and then initially incubated with a monoclonal antibody against the phosphorylated form (Ser133) of CREB and subsequently with secondary anti-mouse-IgG antibodies coupled to Alexa Fluor® 647. The left panel shows images of nonstimulated cells, and the right panel shows images of TPA-stimulated cells.

1. HUVECs were seeded at 10,000 cells per well (96-well Packard view plate, as described above) and incubated for 96 h in EGM2 medium at standard conditions.
2. A 96 h incubation will deplete growth factors from the medium and therefore greatly reduce the baseline amount of phosphorylated CREB (*see* **Note 5**).
3. As was done for the ERK1/2 assay, 1 μM TPA for 30min was used as an activator in the present assay (**Fig. 4**).
4. To initiate activation of the pathway, the medium was removed, and 100 μL of EGM-2 medium (not supplemented) containing 1 μM TPA was added.
5. After 30 min incubation at standard conditions, cells were fixed by adding 35 μL of 16% paraformaldehyde (*see* **Note 3**). **Fig. 4** shows images of HUVECs treated with (**Fig. 4B**, right panel) or without TPA (**Fig. 4B**, left panel). Images were taken with the INCell Analyzer 3000.
6. Cells were stained with the phosphorylation-specific antibodies against CREB as described above. As shown in **Fig. 5**, the nuclear accumulation of phosphorylated CREB shows a strong dependence on the concentration of TPA.
7. The plateau is reached at 250 nM of TPA and the AC_{50} is 15nM. TPA-induced nuclear accumulation of phosphorylated CREB is mediated by active MEK as shown in **Fig. 6**.

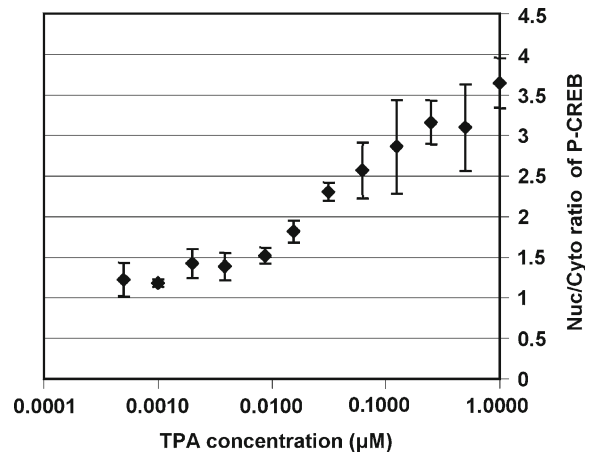

Fig. 5. TPA inducing phosphorylation of Ser133 of CREB in a dose-dependent manner. HUVECs were stimulated (96 h after seeding) for 30 min with the indicated concentrations of TPA. Cells were fixed and stained as described in Fig. 3. Activation is expressed as the nuclear-to-cytoplasmic ratio of the fluorescent signal as a function of TPA concentration.

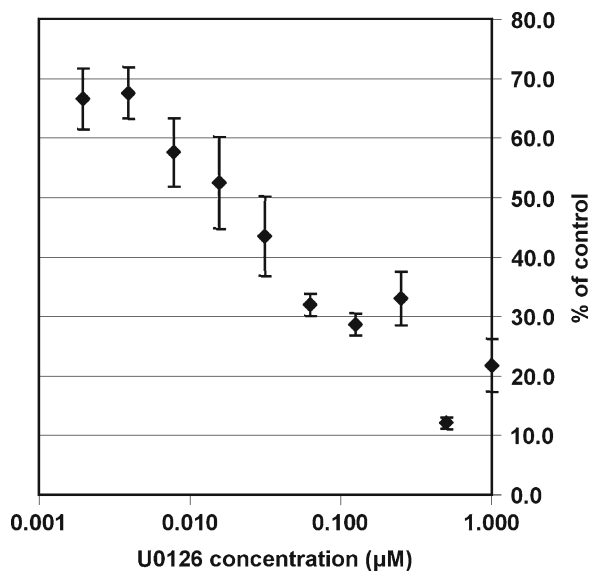

Fig. 6. Phosphorylation of CREB dependent on MEK1/ERK1/2 activation. Cells were seeded as described above. After 96 h, increasing concentrations of the MEK1 inhibitor U0126 were added for 1 h. HUVECs were then stimulated with TPA for 30 min and fixed and stained as indicated in the protocol section. Activation is expressed as the nuclear-to-cytoplasmic ratio of phospho-CREB staining relative to staining in the absence of U0126 (% of control; 100% = 0 µM U0126), as a function of U0126 concentration.

8. Ninety-six hours after seeding, cells were treated with increasing concentrations of U0126, a specific MEK1 inhibitor, in 90 µL of EGM-2 medium (not supplemented).
9. After 1 h, 10 µL of TPA (10 µM) was added to activate the pathway. Increasing concentrations of the MEK1 inhibitor U0126 resulted in a dose-dependent inhibition of nuclear accumulation of phosphorylated CREB. The IC_{50} for this process is 70nM.

4. Notes

1. Using 10% serum as blocking solution is much less likely to cause technical problems such as contamination or clogging than blocking reagents based on milk powder.
2. We routinely buy outdated frozen newborn calf serum to reduce costs.
3. In general, we find that it is not necessary to remove the medium or wash the cells before adding the fixative.
4. For most antibodies, 20-min incubation at room temperature is sufficient.
5. We find that the 96-h starvation period is an easy and reproducible way to induce quiescence in HUVECs.

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