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SB203580 Induces Prolonged B-Raf Activation and Promotes Neuronal Differentiation upon EGF Treatment of PC12 Cells

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Abstract—SB203580 is a p38 MAPK inhibitor that has been implicated in the activation of c-Raf. This study shows that the addition of SB203580 to PC12 cells causes the sustained activation of B-Raf but not of ERK. The addition of SB203580 prolonged the transient activation of both B-Raf and ERK by EGF alone. No significant change was detected in MAPKAPK-2 activity at low concentrations of SB203580, which induced neurite outgrowth in the EGF-stimulated PC12 cells. Therefore, these results indicate that SB203580 influences not only c-Raf as previously reported, but can also induce the activation of B-Raf, which in conjunction with EGF causes the sustained activation of ERK and differentiation in PC12 cells.

Key words: B-Raf, SB203580, ERK, neurite outgrowth, nuclear translocation, PC12 cells

Raf kinases comprise a group of signal transduction proteins that play a role in regulating proliferation and differentiation [1]. Mammalian cells contain three Raf isoforms: c-Raf (Raf-1), A-Raf, and B-Raf, which are products of three independent genes. The activity of the three Raf kinases is enhanced in response to various extracellular signals, mainly through their association with the small GTP binding proteins from the RAS family [2]. In addition, the maximum activation of c-Raf and A-Raf but not B-Raf requires phosphorylation at tyrosine residue 341 [3, 4]. The Raf kinases appear to be ubiquitously expressed, although B-Raf shows the highest expression levels in neuronal tissues and testis. PC12 cells, which are the subject of this study, contain all three Raf isoforms, which can be activated by both EGF and NGF. The activation of A- and B-Raf by NGF in PC12 cells is sustained, while c-Raf exhibits a transient activation by this stimulation. In contrast, only the transient stimulation of the Raf kinases was observed after EGF treatment [5]. The initial activation of the Raf kinases by NGF in PC12 cells requires the small G protein Ras that transmits its signal mainly to c-Raf. However, sustained activation appears to be mediated by the small G protein, Rap1, which transmits the signal mainly to its B-Raf, which is the main effector of Rap1 but not of Ras [6]. Interestingly, it has been demonstrated that the transient activation of the Raf kinases leads to the proliferation of PC12 cells while the sustained activation results in differentiation, which is manifested by neurite outgrowth in these cells [7, 8]. Along these lines, it has been shown that expression of oncogenic versions of the three Raf kinases mimics the sustained activation and leads to the differentiation of PC12 cells [3, 5]. Therefore, the three Raf kinases appear to be differentially regulated under similar stimulation conditions. The sum of the activity of the three Raf kinases is transmitted to activate the ERK cascade, which leads the signal into the nucleus to regulate all the transcriptions necessary for inducing either proliferation or differentiation in PC12 cells [9].

Another signaling pathway that participates in regulating neurite outgrowth in PC12 cells is the p38 MAPK cascade. Therefore, the constitutive activation of p38 MAPK was shown to induce neurite outgrowth when combined with EGF treatment [10]. This effect as well as

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the role of p38 MAPK in the NGF-induced proliferation is mediated through the phosphorylation of the transcription factor, CREB, which in turn activates a set of genes necessary for differentiation [11].

Pyridinylimidazole SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinyl-phenyl)-5-(4-pyridyl)-imidazole] is the main tool that is used to examine the downstream effects of the p38 MAPK cascade. It directly inhibits p38α (SAPK2a) and p38β (SAPK2b) with *in vitro* IC_{50} values of 0.3-0.6 μM [12], but does not inhibit the other p38 MAPKs (SAPK3 and SAPK4) [13]. SB203580 binds competitively with ATP [14], and the three dimensional structure of SAPK2a/p38α in a complex with the closely related pyridinyl imidazole has shown that these drugs are inserted into the ATP-binding pocket of SAPK2a/p38α [15-17]. Interestingly, in several cell types, SB203580 strongly activates c-Raf [18] as well as its downstream effectors ERK1/2, in a dose-dependent fashion [19]. However, in quiescent smooth muscle cells, c-Raf is activated by SB203580 without any detectible effect on the downstream ERK1/2, possibly because of enhancement in the phosphatase activity [20].

Several studies on effects of SB203580 that are mediated by c-Raf have been published [18]. However, the effects of the inhibitor on B-Raf are not known. This study examined the effects of SB203580 alone and together with EGF on PC12 cells. The results show that SB203580 slightly activated B-Raf but not ERK, and did not cause any neurite outgrowth under the conditions used in this study. On the other hand, when applied in combination with EGF, SB203580 not only further activated B-Raf but also caused the neuronal differentiation via the ERK cascade. These results suggest that SB203580 activates not only c-Raf but also B-Raf and can induce the B-Raf specific functions such as the prolonged ERK activation and neurite outgrowth in PC12 cells.

MATERIALS AND METHODS

Materials. The p38 MAPK inhibitor, SB203580, was obtained from Calbiochem (USA). NGF, EGF, the phospho-specific antibody against ERK, β-actin, and anti-neurofilament 68 (NF68) antibodies were purchased from Sigma (USA). Anti-MAPKAPK-2 and anti-B-Raf antibodies were from Santa Cruz Biotechnology (USA). Anti-ERK and anti-phospho-MAPKAPK-2 antibodies were acquired from Cell Signaling (USA). Dulbecco's Modified Eagle Medium (DMEM), LipofectAMINE 2000, Optimum Minimum Essential Medium (Opti-MEM), fetal bovine serum (FBS), horse serum, trypsin, penicillin G, and streptomycin solution were purchased from Gibco BRL (USA).

Cell culture and Western blotting. PC12 cells were cultured in complete DMEM containing 10% horse serum and 5% FBS in a T-75 cm^2 culture flask at 37°C in a humidified incubator with 5% CO_2 until needed for the experiments [21]. Before treatment, PC12 cells were serum starved (0.1% serum) for 16 h and then stimulated for the indicated time at 37°C with 100 ng/ml EGF or NGF with 10 μM SB203580 pretreated for 30 min. After activation, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested with the cell lysis buffer A (50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml pepstatin A, 10 μg/ml aprotinin, and 20 μM leupeptin), which was followed by sonication (2 × 7 sec, 40 W) and centrifugation (15,000 rpm, 15 min, 4°C). The supernatants were collected and kept frozen at −20°C until needed. For Western blotting analysis, the protein concentration was determined by the bicinchoninic acid assay (Pierce, USA), and equal amounts of the proteins were resolved using SDS-PAGE electrophoresis.

B-Raf kinase assay. PC12 cells were rinsed twice with ice-cold PBS and scraped into chilled lysis buffer. A 20-μl sample of protein A-Sepharose beads was incubated with 5 μl of anti-B-Raf antibodies for 24 h at 4°C. Protein samples containing 300 μg protein each were rotated at 4°C for 2 h with the antibodies conjugated to the protein A-Sepharose beads. The immune complexes were washed with ice-cold RIPA buffer (137 mM NaCl, 20 mM Tris, pH 7.4, 10% (w/v) SDS, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin), twice with ice-cold 0.5 M LiCl, and twice with ice-cold buffer A. The immune complex pellets were suspended in the kinase reaction buffer containing phosphorylation substrate and 10 μCi of [γ-^{32}P]ATP, which were incubated at 30°C for 10 min. Each of the total kinase reactions was separated on SDS-PAGE.

Neurite assay. PC12 cells were seeded on 12-well plates coated with a collagen solution. The cells possessing one or more neurites with a length more than twice the diameter of the cell body were scored as being positive [22].

Localization study. The cDNA of rat ERK2 (bases 22-1096) was ligated into the *Apa*I and *Xba*I sites downstream to the green fluorescent protein (GFP) gene, and wild type human MEK1 cDNA was ligated into *BamH*I and *EcoR*V sites of pCDNA3 plasmid (Invitrogen, USA). The GFP-ERK2 construct was transfected into PC12 cells using LipofectAMINE 2000 Reagent according to the manufacturer's instructions (Life Technologies, Inc, USA). When MEK1 was cotransfected with GFP-ERK2, the ratio of 2 : 1 in the amount of DNA transfected was kept, respectively. Visualization was performed as described previously [23]. The cells were stimulated with EGF (100 ng/ml), NGF (100 ng/ml), or 10 μM SB203580, fixed with 3% paraformaldehyde, and visualized using a fluorescence microscope.

RESULTS AND DISCUSSION

This study first investigated the effect of SB203580, which is a specific inhibitor of p38 MAPK, on B-Raf in PC12 cells. To measure the extent of activation of B-Raf, we used immune complex kinase assay of extracts of PC12 cells treated with SB203580 under various concentrations. As shown in Fig. 1, B-Raf was inactive the in quiescent cells, and was activated almost 3-fold more than the basal levels in the presence of 0.1-20 μM SB203580 for 30 min. We chose to continue with 10 μM SB203580 because this concentration gave the highest B-Raf activation, and the extent of Raf activation was examined as a function of time. This study found that the addition of SB203580 to PC12 cells resulted in activation of B-Raf within 5 min, and the activity was still elevated even after 360 min (Fig. 1b).

The *in vitro* kinase assay was used to determine the effect of a combination of SB203580 and EGF on the B-Raf activity. Therefore, when PC12 cells were exposed to SB203580 with EGF, the B-Raf activity was increased almost 2-fold between 5 and 360 min, indicating a sustained effect (Fig. 1c). A small increase in B-Raf activity was detected also with a combination of SB203580 plus NGF when compared to the activation of B-Raf by NGF alone in PC12 cells (data not shown). Therefore, these results clearly show that SB203580 is able to induce not only c-Raf activity, as previously reported [18, 20], but also B-Raf activity, either when the SB203580 was added alone or in combination with either EGF or NGF.

Since the main effect of B-Raf as opposed to c-Raf in PC12 cells is to induce neurite outgrowth, this study investigated the effect of SB203580 on either EGF or NGF induced cellular differentiation in PC12 cells. As mentioned above, it was shown that EGF treatment causes proliferation, whereas NGF treatment induces differentiation in PC12 cells [8, 9]. Furthermore, Raf isozymes showed differential regulation by growth versus differentiation inducing factors in PC12 cells [5]. These results show that as expected, NGF caused neurite outgrowth in PC12 cells while EGF alone and SB203580 alone did not cause differentiation. However, when combinations of SB203580 with the different factors were used, the effects were different from those obtained by each drug alone (Fig. 2a). Therefore, the treatment of PC12 cells with SB203580 and NGF together resulted in an increase in neurite outgrowth. NGF alone induced a long-branched neurite extension in 10% of the cells, while NGF plus SB203580 (10 μM) induced similar neurite extensions in up to 40% of the treated cells.

Induction was observed also with EGF, because PC12 cells treated with EGF plus 10 μM SB203580 led to neuronal differentiation at a level similar to that of the NGF treatment (100 ng/ml) alone within 3 days (Fig. 2b). It was further found the induced neurite outgrowth was dependent on the SB203580 concentration when

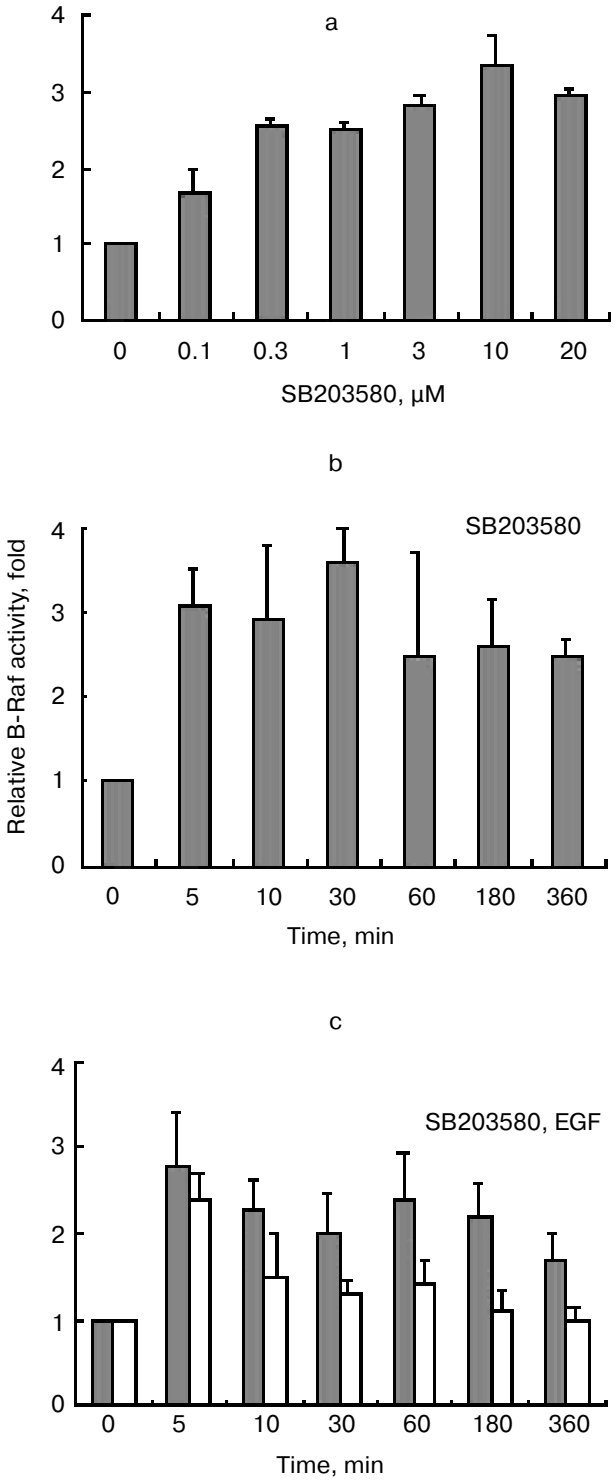

Fig. 1. B-Raf activity induced by SB203580. a) PC12 cells were stimulated for 30 min with different doses of SB203580. B-Raf activity was measured as described in "Materials and Methods". The kinase activities are presented as the fold activation relative to that obtained in the absence of SB203580. b) PC12 cells were stimulated with 10 μM SB203580 for different times. c) PC12 cells were treated with 100 ng/ml EGF for the indicated times after a pretreatment with 10 μM SB203580 for 30 min (gray columns) or without pretreatment (EGF alone) (white columns).

added to a constant concentration (100 ng/ml) of EGF (data not shown). Although these results clearly show an induction of differentiation, it should be noted that in H9c2 rat cardiac myoblasts, the addition of 5 μM SB203580 resulted in myogenic signals, and inhibited muscle differentiation [24]. Therefore, the effect of SB203580 appears to be dependent on the cell type.

Since these morphological changes are known to be associated with the expression of the neurofilament used as a biochemical indicator of PC12 cell differentiation, this study examined the induction of neurofilament from the differentiated cells using a Western blot analysis (Fig. 2c). This study found a good correlation between the percentages of cells with neurites and the profile of neurofilament induction with the different combinations of SB203580, EGF, or NGF. In particular, the amount of the expressed neurofilaments was higher following the SB203580 treatment with EGF. Several studies have reported that pro-differentiation effect of SB203580 in EGF-treated PC12 cells is independent of its function on the p38 MAPK but is mediated through an effect on the ERK pathway [18, 20, 25]. Therefore, these results further support the notion that a treatment of SB203580 with EGF can induce neurite outgrowth in PC12 cells.

Since it was shown that ERK cascade mediates the effects of B-Raf on neurite outgrowth, it was important to investigate this cascade in our system. This study examined the effect of 10 μM SB203580 on EGF-induced ERK phosphorylation using Western blot analysis. According to a previous study, ERK activation persisted for several hours following NGF but was short-lived after

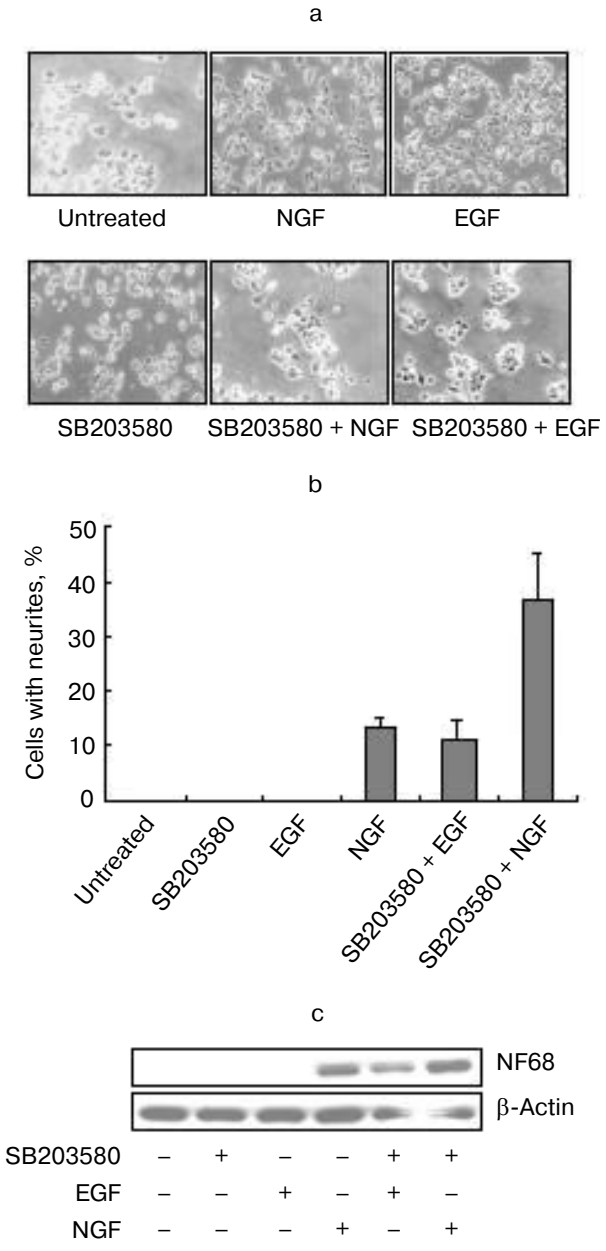

Fig. 2. Quantification of morphological changes induced by SB203580 with EGF in PC12 cells. The quantification of the percentage of cells with neurites in PC12 cells under different treatments as indicated was measured on day 3. The cells with one or more neurites that were more than twice as long as the cell body were counted as being positive for random selection under a microscope. The cell extracts were subjected to immunoblotting using anti-Neurofilament 68 antibodies.

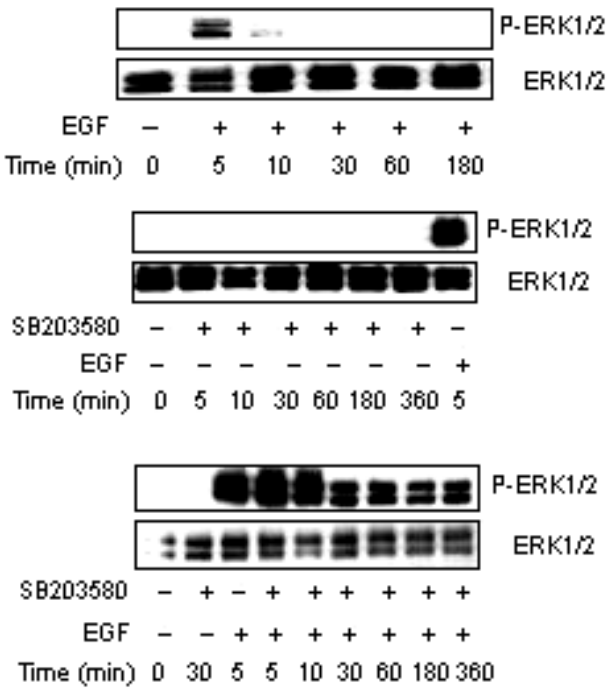

Fig. 3. Prolonged activation of ERK by SB203580 with EGF treatment. PC12 cells were treated with SB203580 (10 μM) alone or preincubated with SB203580 for 30 min, followed with by stimulation with EGF (100 ng/ml) for the indicated time points. ERK phosphorylation was detected by Western blotting using anti-phospho-ERK1/2 antibodies. The blots were then stripped and re-probed with anti-ERK1/2 antibodies.

EGF stimulation [7]. In our system, treatment of PC12 cells for 30 min with SB203580 alone did not cause any ERK activation, but it enhanced the EGF-induced activation of ERK throughout the time course investigated. Thus, these results suggest that the addition of SB203580 to EGF caused this stimulation to be sustained (Fig. 3). It is interesting to note that these results are similar to the results obtained in Swiss 3T3 cells, where exposure of the cells for 60 min with SB203580 activated the Raf kinases but failed to induce any activation of ERK2. This is despite the fact that it enhanced the EGF-dependent activation of c-Raf and ERK2 [18].

To further analyze the effect of SB203580 on ERK, its subcellular distribution in response to SB203580 was examined. To this end, PC12 cells were transfected with a GFP-ERK2 construct where the cDNA of ERK2 was ligated to the GFP. GFP-ERK2 overexpression in PC12 cells resulted in the nuclear accumulation of the protein both before and after stimulation (data not shown). The coexpression with MEK1, which serves as a cytosolic

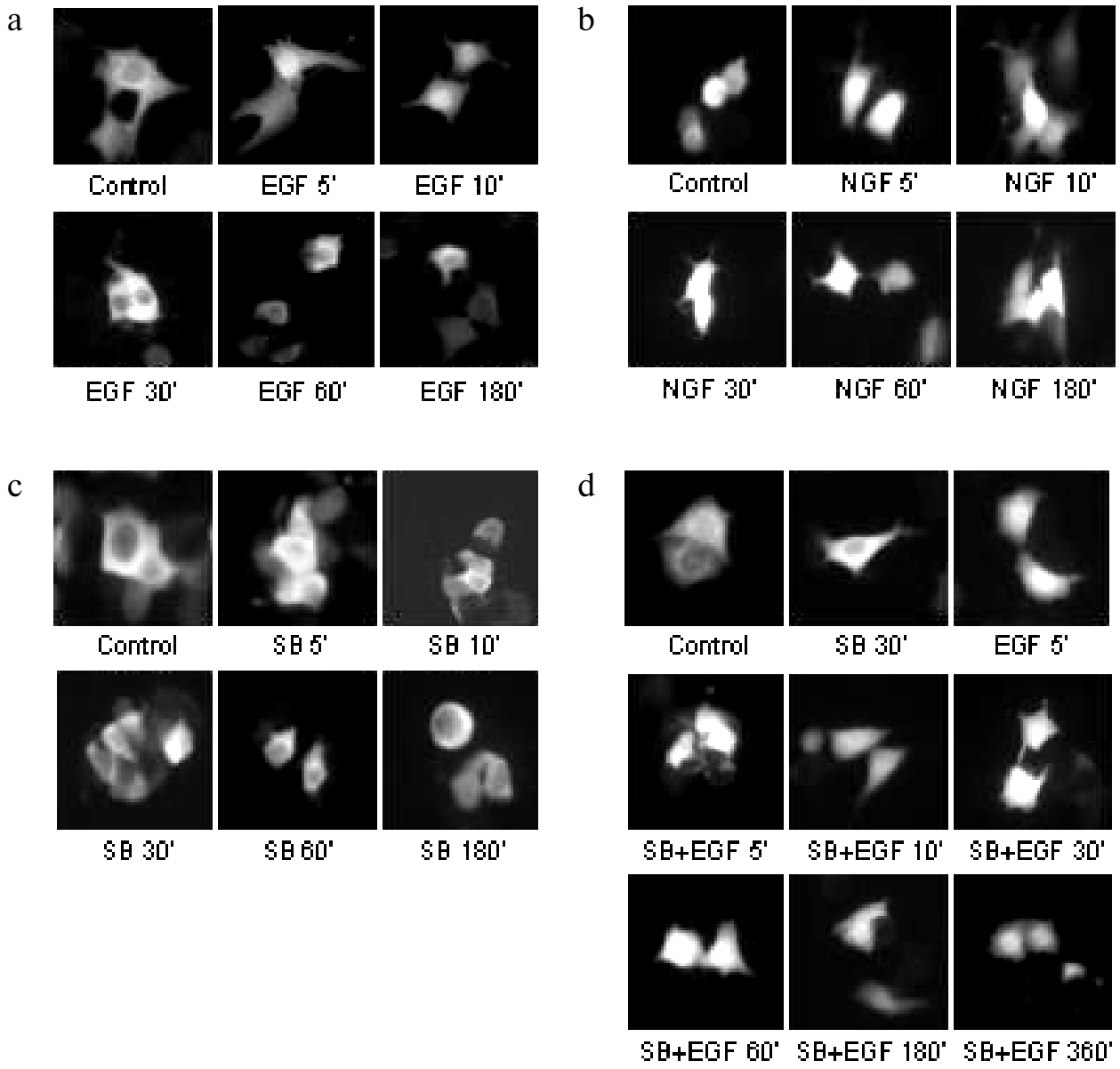

Fig. 4. Different localization of ERK by growth versus differentiation inducing factors with SB203580 (SB) in PC12 cells. PC12 cells were transfected with GFP-ERK2 and MEK1. After transfection, the cells were treated with or without 100 ng/ml EGF for the indicated times (a), with or without 100 ng/ml NGF for indicated time points (b), with 10 μM SB203580 for different times (c), or after a 10-μM SB203580 pretreatment, the cells were treated with 100 ng/ml EGF (d). Representative images of the transfected cells were identified by the fluorescence of GFP.

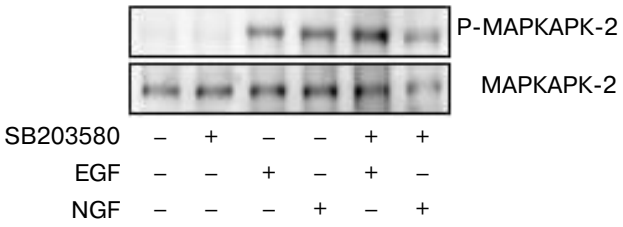

Fig. 5. Effect of SB203580 on the phosphorylation of MAPKAPK-2. PC12 cells were treated with SB203580 alone or preincubated with SB203580 for 30 min, followed with by stimulation with EGF (100 ng/ml) or NGF (100 ng/ml) for 30 min. After treatment, PC12 cells were harvested for immunoblotting with antibodies to phospho-MAPKAPK-2. The membrane was then stripped and re-probed with anti-MAPKAPK-2 antibodies.

anchoring protein for ERK, resulted in the cytosolic distribution of GFP-ERK2 in the resting cells, which reverted to a nuclear one upon stimulation. Therefore, treatment with EGF led to the rapid and transient translocation of GFP-ERK2 to the nucleus, which was almost complete within 10 min, and the GFP-ERK2 was relocalized to the cytosol within an additional 20 min (Fig. 4a).

In contrast to the EGF treatment, NGF induced the localization of ERK in the nucleus lasting for 180 min (Fig. 4b). As expected, SB203580 alone, which failed to activate ERK, did not cause the nuclear translocation of GFP-ERK2 (Fig. 4c). However, SB203580 with EGF, which caused a sustained activation of B-Raf and ERK, resulted in the nuclear localization of GFP-ERK2 that lasted 360 min (Fig. 4d). This finding suggests that the long-lasting localization of GFP-ERK2 is dependent on the prolonged B-Raf and ERK2 activation by SB203580 with EGF in PC12 cells.

The fact that the effect of SB203580, which is a p38 MAPK inhibitor, correlated with the activation of B-Raf and the ERK cascade was quite unexpected, because p38 MAPK itself has previously been shown to play a role in PC12 cell differentiation [10, 26].

Therefore, the inhibition of the p38 MAPK should have resulted in the inhibition of the neurite outgrowth in the PC12 cells, and not in their potentiation. Therefore, the effect of the SB203580 on the NGF and the EGF stimulated p38 MAPK cascade in PC12 cells was examined. For this purpose, the activation of MAPKAPK-2, a downstream effector of p38 MAPK, as a readout for the activation of the p38 MAPK cascade was measured. The results showed that EGF and NGF induced the phosphorylation of MAPKAPK-2. The addition of 10 μM SB203580 to the cells that were treated with growth factors did not inhibit, or even enhanced, MAPKAPK-2 activation under the condition used in this study (Fig. 5). Higher concentrations of the inhibitor (up to 80 μM) inhibited the activation of MAPKAPK-2 (data not shown). These results are consistent with the activation of MAPKAPK-2 in 293 cells being blocked by the interaction of SB203580 with SAPK2/p38, and with c-Raf activation being triggered probably by low affinity interaction of SB203580 with another protein [18]. The stimulation of EGF-induced neurite outgrowth by 10 μM SB203580 becomes plausible due to the activation of the B-Raf/ERK cascade, which is not accompanied by the inhibition of the p38 MAPK pathway.

In summary, SB203580 induced a prolonged B-Raf activation in PC12 cells, which is similar to the previously reported activation of c-Raf. Unlike c-Raf, this activation did not result in the activation of ERK, which indicates that the transmission of the signal from B-Raf to ERK needs to cross a higher threshold (probably phosphatases) than the c-Raf signal. In addition, SB203580 had a synergistic effect on B-Raf and ERK activation by EGF, resulting in the sustained activation of the two kinases. On the other hand, no inhibition of the p38 MAPK cascade was detected at the concentrations of SB203580 used. Therefore, a combination of SB203580 and EGF causes differentiation in the PC12 cells via the B-Raf/ERK pathway.

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