Raf-1 is activated by the p38 mitogen-activated protein kinase inhibitor, SB203580

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Abstract SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole) is widely used as a specific inhibitor of p38 mitogen-activated protein kinase (MAPK). Here, we report that SB203580 activates the serine/threonine kinase Raf-1 in quiescent smooth muscle cells in a dose-dependent fashion. The concentrations of SB203580 required lie above those necessary to inhibit p38 MAPK and we were unable to detect basal levels of active p38 MAPK. SB203580 does not directly activate Raf-1 in vitro, and fails to activate Ras, MEK, and ERK in intact cells. In vitro, however, SB203580-stimulated Raf-1 activates MEK1 in a coupled assay. We conclude that activation of Raf-1 by SB203580 is not mediated by an inhibition of p38 MAPK, is Ras-independent, and is uncoupled from MEK/ERK signaling.

Key words: Ras; Raf; Mitogen-activated protein kinase; SB203580; Signal transduction

1. Introduction

Gene expression induced by extracellular factors regulates cellular functions including differentiation and proliferation. Signal transduction from the cell surface to the nucleus largely relies on activation of a family of cytoplasmic protein kinases, the mitogen-activated protein kinases (MAPKs), that, upon activation, translocate into the nucleus where they phosphorylate and regulate transcription factors. MAPKs consist of at least three groups: the extracellular signal-regulated kinases (ERK1 and ERK2), the N-terminal c-jun kinases (JNKs), and p38 (reviewed in [1,2]). A widely used strategy to explore the function of ERK and two members of the p38 group (p38α and p38β) is the use of specific inhibitors. PD098059 blocks ERK by preventing the activation of its immediate activator, MEK [3], whereas SB203580 directly inhibits p38α and p38β but not other p38 MAPKs [4–6]. A prerequisite for unambiguous interpretations of experiments involving these inhibitors is the knowledge of possible, additional effects. For instance, it has recently been suggested that the cyclooxygenase inhibitor indomethacin be used as a control for PD098059 and SB203580 because both MAPK kinase inhibitors also affect Cox1 and Cox2 activities [7].

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Abbreviations: ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; MEK, MAPK/ERK kinase; PDGF, platelet-derived growth factor; RBD, Ras-binding domain of Raf; SMC, smooth muscle cell

We started to utilize the SB203580 compound to explore a role of p38 in PDGF-BB signaling in baboon smooth muscle cells. Here, we report the unexpected finding that SB203580 strongly activates the serine/threonine kinase Raf-1. Raf-1 activation by growth factors is a complex process (reviewed in [8,9]). In quiescent cells, Raf-1 is located in the cytoplasm and is kept inactive by binding to 14-3-3 proteins. Activated, GTP-bound Ras binds to the RBD (Ras-binding domain) located in the N-terminus of Raf-1, thereby displacing 14-3-3 proteins [10,11] and translocating Raf-1 to the plasma membrane [12,13]. The molecular basis for activation of Raf-1 at the plasma membrane remains to be elucidated. It may include tyrosine phosphorylation and binding of phospholipids (reviewed in [8,9]).

Active Raf-1 phosphorylates MEK at two adjacent serine residues. This results in an activation of MEK that, in turn, phosphorylates and activates ERK.

In this study, we present evidence that SB203580 activates Raf-1 in a Ras-independent fashion and that SB203580-stimulated Raf phosphorylates MEK in vitro but not in intact cells.

2. Materials and methods

2.1. Materials

Monoclonal antibodies against MEK1 and Ras were purchased from Transduction Laboratories (Lexington, KY), polyclonal Raf-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and phospho-ERK specific antibodies from New England Biolabs Inc. (Beverly, MA). Tissue culture media and fetal calf serum were from Gibco-BRL (Gaithersburg, MD). SB203580 and SB202474 were from Calbiochem (La Jolla, CA). Protein A agarose was from Boehringer Mannheim (Indianapolis, IN) and [γ-^{32}P]ATP from Du Pont-New England Nuclear (Boston, MA). PDGF-BB was a kind gift from Zymogenetics (Seattle, WA). Catalytically inactive ERK2 (K52R) and MEK1 (K97M), and GST-RBD beads were prepared with minor modifications as described [14–16]. The ECL kit was purchased from Amersham (Arlington Heights, IL).

Buffer HEB: 25 mM HEPES-NaOH, pH 7.5, 10% glycerol, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 100 mM Na-pyrophosphate, 50 mM NaF, 1 mM Na-vanadate, 1 mM benzamidine, 0.1% 2-mercaptoethanol, 1% Triton X-100, 1 μM pepstatin A, 2 μg/ml leupeptin and 20 kallikrein inhibitor units/ml aprotinin; buffer MLB: 25 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM Na-vanadate, 25 mM NaF, 0.1% 2-mercaptoethanol, 1 μM pepstatin A, 2 μg/ml leupeptin and 20 kallikrein inhibitor units/ml aprotinin; kinase buffer: 20 mM HEPES-NaOH, pH 7.5, 20 mM $MgCl_2$, 0.1% 2-mercaptoethanol; TTBS: 25 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 0.1% 2-mercaptoethanol.

2.2. Smooth muscle cell (SMC) culture and extraction

Baboon aortic SMCs were prepared as described previously [17]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 200 U/ml penicillin and 0.2 mg/ml streptomycin. SMCs were starved in serum-free medium for 2–3 days. After stimulation as indicated, cells were washed twice with phosphate-buffered saline and lysed in 1 ml/10 cm plate HEB (for

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kinase assays) and MLB (for ras assays). Lysates were incubated for 20 min on ice and cleared by centrifugation in a microfuge for 10 min at 10 000×*g*. When SB203580 was used, controls contained the same concentrations of DMSO (≤0.25%).

2.3. Kinase assays

For Raf-1 and MEK1 assays, 1 μg of antibody and 20 μl of protein A agarose slurry (1 mg/ml protein A) were added to 1 ml of extract containing approximately 500 μg protein. The samples were stirred overnight at 4°C. The beads were washed in buffer HEB followed by kinase buffer. Kinase reaction was performed on the beads in kinase buffer containing 1 μg of protein substrate and 0.1 mM ATP (5000 cpm/pmol). The assay mix was incubated for 30 min at 30°C. The reaction was terminated by adding 10 μl 4×Laemmli buffer. The samples were subjected to SDS-PAGE. The gels were stained with Coomassie blue to monitor equal loading, dried, and the extent of substrate phosphorylation was determined by phosphorimaging (facility at the Fred Hutchinson Cancer Research Center, Seattle, WA) or densitometric analysis of autoradiographs.

ERK activities were analyzed by determining the phosphorylation status of ERK1 and ERK2. 20 μl of extract was subjected to SDS-PAGE followed by Western blotting. The blots were incubated with phospho-ERK specific antibodies (1:1000) and the bands visualized by ECL using a protocol supplied by the manufacturer.

2.4. Ras assay

Twenty micoliters of packed GST-RBD beads were added to 1 ml cell extract. The mixture was incubated on a rocker for 30 min at 4°C. The beads were washed three times with 1 ml MLB before being subjected to SDS-PAGE. The affinity-precipitated Ras protein was visualized by Western blotting using a Ras antibody (0.5 μg/ml) and an ECL kit.

3. Results and discussion

3.1. SB203580 activates Raf-1

The treatment of quiescent SMCs with the p38 MAPK inhibitor, SB203580, results in a dose- and time-dependent activation of Raf-1 (Fig. 1). SB203580 only activates Raf-1 when added to intact cells. When cell lysates of quiescent cells were incubated with SB203580 before and during the immunoprecipitation, Raf-1 remained inactive. It is also important to note that an inactive derivative of the p38 MAPK inhibitor, SB202474 (25 μM), had no effect on Raf-1 activity (not shown).

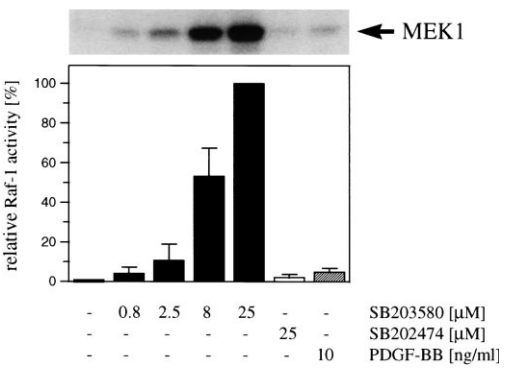

Fig. 1. Dose-dependent activation of Raf-1 by SB203580. Quiescent SMCs were stimulated for 30 min with different doses of SB203580 and 25 μM SB202474, and for 15 min with 10 ng/ml PDGF-BB, respectively. Raf-1 activity was determined as described in Section 2. The upper panel shows an autoradiograph of a representative experiment; the arrow indicates position of substrate. Lower panel: Relative kinase activities were calculated as percent of the highest activity obtained (at 25 μM SB203580). All data points are mean ± S.D. of three independent experiments.

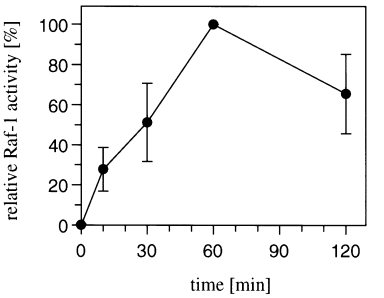

Fig. 2. Time course of Raf-1 activity induced by SB203580. Quiescent SMCs were stimulated with 25 μM SB203580 for different time points. Raf-1 activity was measured as described in Section 2. Kinase activities are presented as percent of the highest activity obtained (at 60 min). All data points are mean ± S.D. of three independent experiments.

Raf-1 was inactive in quiescent cells and became stimulated more than 100-fold in the presence of 8–25 μM SB203580 (Fig. 1). When the extents of Raf-1 activation induced by PDGF-BB and SB203580 were compared, 25 μM SB203580 was 30 times stronger than 10 ng/ml PDGF-BB which induced the same level of Raf-1 activity as 0.8 μM SB203580. In two independent experiments, 1 h incubation of 8 μM SB203580 activated Raf-1 approximately 30-fold in NIH 3T3 mouse fibroblasts demonstrating that this effect of the p38 MAPK inhibitor is not limited to SMCs (not shown).

The obvious question was whether the SB203580-induced activation of Raf-1 is mediated by an inhibition of p38 MAPK. We have recently reported that, in baboon SMCs, MAPKAP kinase-2 activation by pervanadate is completely abolished in the presence of 0.8 μM SB203580 [18]. This observation is consistent with published IC_{50} values for the SB203580 sensitive p38 MAPK isoforms, p38α and p38β [5,6]. Raf-1 activation by SB203580, however, did not reach a maximum at concentrations of SB203580 as high as 25 μM (Fig. 1). These data suggest to us that inhibition of p38 MAPK is probably not responsible for the stimulation of Raf-1 by SB203580. In addition, we did not detect significant levels of p38α or p38β activities in quiescent cells using either an immunoprecipitation assay with ATF-2 as substrate, or an immunodetection assay with an antibody specific for the phosphorylated form of p38 MAPK; both assays gave positive results when SMCs were stimulated with pervanadate (not shown). In the light of the recent observation that SB203580 blocks cyclooxygenases (Cox1 and Cox2 [7]), we tested whether the general Cox inhibitor, indomethacin, can mimic the activatory effect of SB203580 on Raf-1. The result was negative; indomethacin in concentrations up to 10 μM had no effect on Raf-1 activity (not shown; 1–10 μM indomethacin is typically used to block cyclooxygenases).

Time course experiments demonstrated that a 10 min incubation with SB203580 was sufficient to elicit approximately 25% of the maximal response that was measured at 60 min; after that time point, kinase activity declined (Fig. 2).

3.2. SB203580 does not stimulate Ras, MEK, or ERK

To investigate whether SB203580 mediates Raf activation by activating Ras, we treated quiescent SMCs with 25 μM

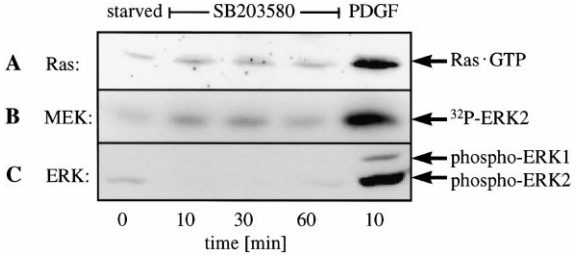

Fig. 3. Effects of SB203580 on Ras, MEK1, and ERK1,2. Quiescent SMCs were stimulated for the times indicated with 25 μM SB203580 and 10 ng/ml PDGF for 10 min, respectively. The activities of Ras, MEK1, and ERK1,2 were determined as described in Section 2. All experiments were performed at least three times with similar results. A: Western blot screened for Ras. B: Autoradiograph of MEK assay. C: Western blot screened for phospho-ERK.

SB203580 and determined the amount of GTP-bound Ras at different time points. Controls were stimulated with PDGF-BB for 10 min. Whereas the growth factor induced Ras activation, SB203580 failed to significantly increase the level of GTP-bound Ras (Fig. 3A). It is therefore unlikely that activation of Ras is required for SB203580 to stimulate Raf-1.

Similar results were obtained when we measured the activity of the Raf substrate MEK1 following incubation of SMCs with SB203580 and PDGF-BB, respectively. Whereas MEK1 was strongly activated by PDGF-BB, SB203580 failed to elicit a similar response; typically, a slight activation (1.5-fold) of MEK1 was observed by SB203580 (Fig. 3B). When the MEK1 substrates, ERK1 and ERK2, were measured, only PDGF-BB was stimulatory; 25 μM SB203580 rather decreased basal ERK activities (Fig. 3C). These data indicate that SB203580-induced Raf is not capable of engaging MEK/ERK signaling in intact cells. To rule out the possibility that SB203580-activated Raf-1 phosphorylated the recombinant MEK1 in vitro on sites other than those required for MEK activation, we performed a coupled assay. Immunoprecipitates of SB203580-activated Raf-1 were mixed with MEK-1 immunoprecipitates from quiescent SMCs, and phosphorylation of ERK was determined. Only assays containing both SB203580-stimulated Raf and MEK1 showed ERK phosphorylation; when Raf was immunoprecipitated from DMSO-treated cells, or in the absence of MEK, no ERK phosphorylation was measured (Fig. 4).

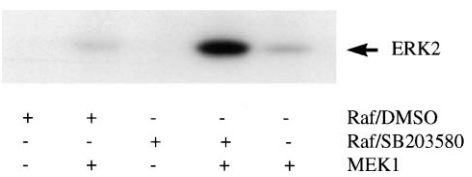

Fig. 4. SB203580-stimulated Raf-1 activates MEK1 in vitro. SMCs were incubated with 25 μM SB203580 or DMSO. Raf-1 was immunoprecipitated and combined with immunoprecipitates of MEK1 from quiescent SMCs as indicated. Samples were incubated for 30 min at 30°C in kinase buffer containing 1 mM ATP to allow phosphorylation of MEK1. The beads were washed to remove ATP, and MEK1 activities were determined towards ERK2 as described in Section 2. An autoradiograph from a representative experiment is shown. The arrow denotes the position of ERK2. The experiment was repeated twice with minor modifications yielding the same results.

3.3. Summary and conclusions

The mechanism of how SB203580 activates Raf-1 remains unclear: we suggest that this process is independent of an inhibition of p38 or cyclooxygenases, both targets for SB203580 [5–7]. At this point, we cannot distinguish between the two possibilities of SB203580 action: an inhibition of a Raf suppressor, or a stimulation of a Raf activator.

An interesting observation is that SB203580-activated Raf-1 failed to activate MEK in intact cells although it phosphorylates and activates MEK1 immunoprecipitated from the same cells in vitro.

This difference could be explained by an inhibitory effect of SB203580 on MEK1 in vivo. Such a mechanism may be responsible for the recently observed inhibition of ERK by SB203580 following arsenite treatment [19]. However, we have recently shown in pervanadate-stimulated SMCs that SB203580 prolongs the activation of MEK1 [18].

Alternatively, in intact cells, additional proteins may be required for the activation of MEK1 by Raf. Those may include additional MEK kinases or scaffold proteins. Examples for both have recently been described [20,21]. In in vitro assays, such requirements may be overcome by high substrate concentrations.

Our data suggest the possibility that effects of SB203580 that were attributed to p38 inhibition may, in part, be due to the activation of Raf-1. This is particularly interesting regarding recent reports that SB203580, and structurally related p38 MAPK inhibitors, can prevent apoptosis in certain cell systems [22–24] because Raf-1 has also been suggested in several studies to exert anti-apoptotic effects [25–27].

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