The p38-MAPK inhibitor, SB203580, inhibits cardiac stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs)

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Abstract SB203580 is a recognised inhibitor of p38-MAPKs. Here, we investigated the effects of SB203580 on cardiac SAPKs/JNKs. The IC_{50} for inhibition of p38-MAPK stimulation of MAPKAPK2 was approximately 0.07 μM, whereas that for total SAPK/JNK activity was 3–10 μM. SB203580 did not inhibit immunoprecipitated JNK1 isoforms. Three peaks of SAPK/JNK activity were separated by anion exchange chromatography, eluting in the isocratic wash (44 kDa), and at 0.08 M (46 and 52 kDa) and 0.15 M NaCl (54 kDa). SB203580 (10 μM) completely inhibited the 0.15 M NaCl activity and partially inhibited the 0.08 M NaCl activity. Since JNK1 antibodies immunoprecipitate the 46 kDa activity, this indicates that SB203580 selectively inhibits 52 and 54 kDa SAPKs/JNKs.

Key words: Heart; Protein kinase inhibitor; SB203580; Stress-activated protein kinase/c-Jun N-terminal kinase; Mitogen-activated protein kinase-activated protein kinase 2

1. Introduction

The three well-characterised subfamilies of the mitogen-activated protein kinase (MAPK) superfamily are the extracellularly responsive kinases (ERKs), the c-Jun N-terminal kinases (JNKs) which are also known as stress-activated protein kinases (SAPKs), and the p38-MAPKs [1–3]. In perfused heart, p38-MAPK is activated by ischaemia and ischaemia/reperfusion, but the SAPKs/JNKs are activated only during the reperfusion phase following ischaemia [4]. SAPKs/JNKs are also powerfully activated in neonatal ventricular myocytes by cellular stresses [5]. Substrates for the MAPKs include MAPK-activated protein kinase 2 (MAPKAPK2) and the transcription factor c-Jun. MAPKAPK2 is preferentially phosphorylated by p38-MAPK [6] and c-Jun is a substrate for SAPKs/JNKs [7], although both are phosphorylated by ERKs in vitro [8,9]. SB203580 is a recognised inhibitor of p38-MAPK(α) and p38-MAPKβ [10–13]. It does not inhibit the other p38-MAPKs so far identified [12,13], and it was reported to have no effect on ERK or SAPK/JNK activities in vitro [11]. It has been proposed that SB203580 may be particularly useful in delineating the roles of p38-MAPK(α) and p38-MAPKβ in cellular responses [13]. We have re-examined the inhibitory effects of SB203580 on cardiac MAPKs. Here, we present evidence that two c-Jun N-terminal kinases (SAPKs/JNKs), activated in adult perfused hearts and neonatal ventricular myocytes, are inhibited by SB203580.

2. Materials and methods

2.1. Heart perfusions and primary culture of ventricular myocytes

Adult rat hearts were perfused and homogenates prepared as previously described [14]. Neonatal rat ventricular myocytes were cultured as described in [15]. Serum was withdrawn for 24 h, myocytes were exposed to 0.5 M sorbitol (30 min) in the absence or presence of up to 10 μM SB203580 and samples were prepared as described in [5].

2.2. Fast protein liquid chromatography of MAPKs and MAPKAPK2

Proteins (0.5 ml of extract) were separated by FPLC. SAPKs/JNKs were separated on a Mono Q HR5/5 column as previously described [5], except that the MAPKs were eluted using a 30 ml linear NaCl gradient (0–0.5 M NaCl). They were assayed by the direct method with myelin basic protein (MBP) or 0.5 mg/ml glutathione *S*-transferase-(GST-)c-Jun(1–135) as substrates, as previously described [5,16,17] except that the assay mix contained 0.1% (v/v) dimethyl sulphoxide or 10 μM SB203580 (final concentrations). Samples of fractions were taken for in-gel kinase assays as described in [5]. In some experiments, fractions were pooled and concentrated by ultrafiltration and prepared for immunoblot analysis [14]. For MAPKAPK2, proteins (0.5 ml) were applied to a Mono S HR5/5 column and MAPKAPK2 was purified and assayed as described [14].

2.3. Immunoblotting and in-gel kinase assays

These methods are described in [14].

2.4. 'Pull-down' SAPK/JNK assays

GST-c-Jun(1–135) was used to 'pull down' total SAPKs/JNKs from myocyte extracts as described in [5]. Pellets were washed in kinase assay buffer (20 mM HEPES pH 7.7, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM β-glycerophosphate) containing the final concentrations of SB203580. The pellets were resuspended in 15 μl kinase assay buffer containing twice the final concentrations of SB203580 and phosphorylation was initiated with 15 μl of kinase assay Buffer containing 20 μM ATP and 2 μCi [γ-^{32}P]ATP. The assay was completed as described in [5].

2.5. JNK1 immunokinase assays

JNK1 isoforms were immunoprecipitated from myocyte extracts as described in [14] using antibodies from Santa Cruz. The pellets were washed in kinase assay buffer containing the final concentrations of SB203580. GST-c-Jun(1–135) in 15 μl kinase assay buffer containing twice the final concentrations of SB203580 was added and phosphorylation was initiated with 15 μl of kinase assay buffer containing 20 μM ATP and 2 μCi [γ-^{32}P]ATP. The assay was completed as described in [5].

3. Results

3.1. Inhibition of MAPKAPK2 activation by SB203580

We first assessed the effects of SB203580 on p38-MAPK activation of MAPKAPK2 in neonatal myocytes. 0.5 M sorbitol (30 min) induced a large (approximately 16-fold) increase

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Abbreviations: ERK, extracellularly responsive kinase; FPLC, fast protein liquid chromatography; GST, glutathione *S*-transferase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAPK2, MAPK-activated protein kinase 2; SAPK, stress-activated protein kinase

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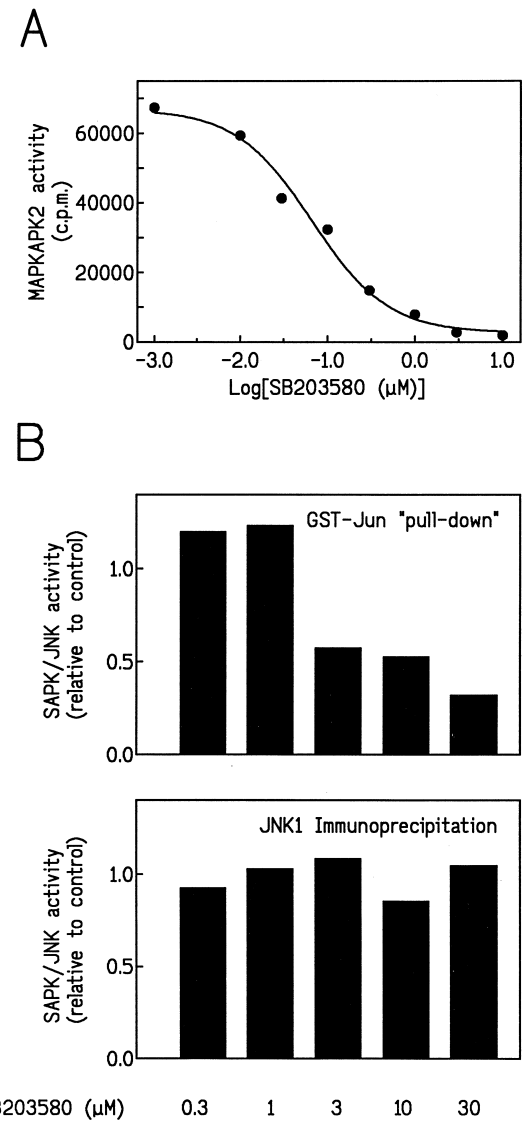

Fig. 1. A: Inhibition of MAPKAPK2 activation by SB203580 in primary cultures of rat ventricular myocytes. MAPKAPK2 activities were assayed from myocytes exposed to 0.5 M sorbitol (30 min) in the presence of 0.1% DMSO containing up to 10 μM SB203580. B: SAPKs/JNKs were assayed by the 'pull-down' method (upper panel) or by a JNK1 immunokinase assay (lower panel) in the presence of increasing concentrations of SB203580. These experiments were repeated two times with similar results.

in MAPKAPK2 activity (Fig. 1A), which was inhibited by SB203580 with an IC_{50} of 0.07 μM (Fig. 1A).

3.2. SB203580 inhibition of c-Jun N-terminal kinases

We studied the effects of SB203580 on SAPKs/JNKs activated in neonatal rat ventricular myocytes by 0.5 M sorbitol (30 min). Total SAPK/JNK activities, measured using 'pull-down' assays with GST-c-Jun(1–135) [5], were inhibited by SB203580 with an IC_{50} of 3–10 μM (Fig. 1B, upper panel). The SAPK/JNKs are derived from at least three genes (*JNK1–3*), each of which produces alternatively spliced transcripts encoding proteins of approximately 46 kDa and 54 kDa [18]. Antibodies to JNK1 isoforms immunoprecipitate almost all of the cardiac 46 kDa activity and a proportion of the 52/54 kDa activity [5]. SB203580 (at concentrations of up to 30 μM) did not inhibit these immunoprecipitated isoforms (Fig. 1B, lower panel). Antibodies to JNK2 isoforms have not proved suitable for immunoprecipitation (results not shown), and antibodies to JNK3 are not available. We therefore partially purified the different isoforms from adult rat hearts subjected to ischaemia/reperfusion (Fig. 2A), and from neonatal ventricular myocytes exposed to 0.5 M sorbitol (30 min) (Fig. 2B) by Mono Q FPLC. Three peaks of activity were detected, eluting in the isocratic wash (results not shown) and at approximately 0.08 M and 0.15 M NaCl (Fig. 2). As shown on in-gel kinase assays (GST-c-Jun(1–135) as substrate), the peak

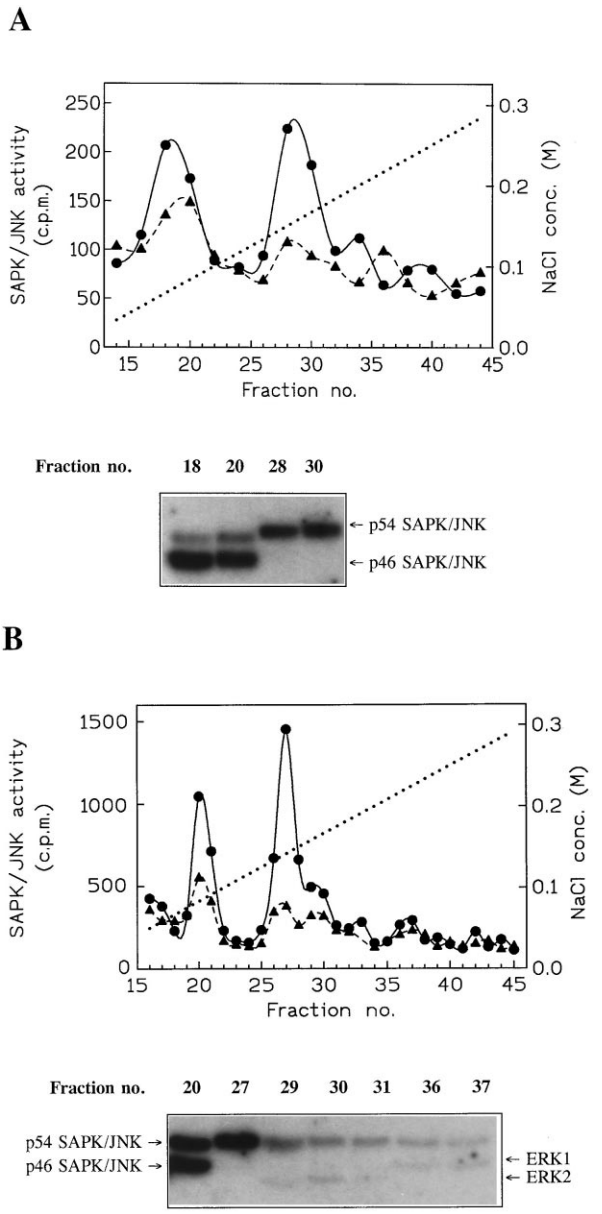

Fig. 2. Inhibition of partially purified SAPKs/JNKs by SB203580. A: Isolated hearts were subjected to 20 min ischaemia followed by 10 min reperfusion. B: Myocytes were exposed to 0.5 M sorbitol for 30 min. In the upper panels, MAPKs were separated by Mono Q FPLC and fractions were assayed for SAPK/JNK activity in the absence (●, solid line) or presence (▲, dashed line) of 10 μM SB203580. The NaCl gradient is shown by the dotted line. In the lower panels, fractions with SAPK/JNK activity were assayed by the in-gel kinase method with GST-c-Jun(1-135) as substrate. The experiment was repeated a further three times with similar results.

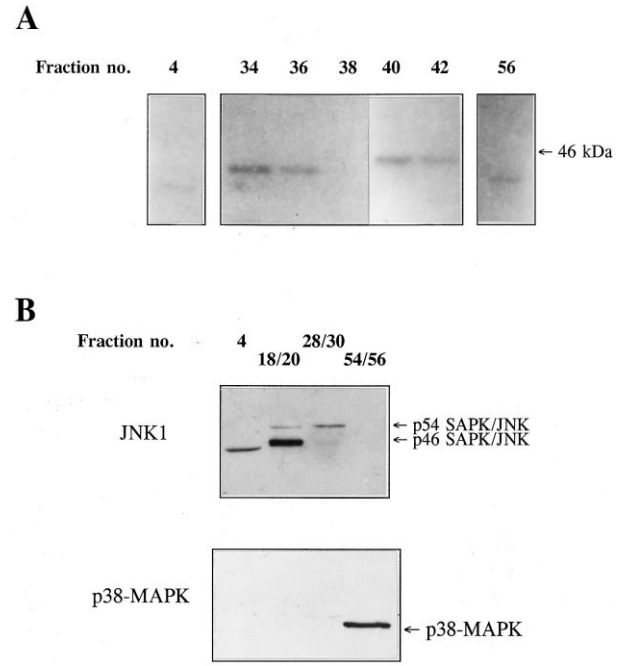

Fig. 3. p38-MAPK elutes from Mono Q FPLC at a higher NaCl concentration than SAPKs/JNKs. Adult perfused hearts were subjected to 20 min ischaemia followed by 10 min reperfusion and the MAPKs separated on Mono Q FPLC. A: All fractions were assayed for MAPK activity by the in-gel kinase method with GST-MAPKAPK2(46–400) as substrate. Only the fractions which contained kinase activity are shown. The arrow to the right of the panel shows the position of the 46 kDa molecular mass standard. B: Fractions from the Mono Q FPLC column containing SAPK/JNK or p38-MAPK activity were concentrated 7–15-fold by ultrafiltration and immunoblotted using antibodies to JNK1 (upper panel) or p38-MAPK (lower panel). The experiment was repeated a further two times with similar result.

eluting at 0.08 M NaCl contained two SAPK/JNK activities of approximately 46 and 52 kDa relative molecular mass, and the peak eluting at 0.15 M NaCl contained a 54 kDa SAPK/JNK (Fig. 2A,B, lower panels). The isocratic wash contained a 44 kDa SAPK/JNK activity (results not shown). No other SAPK/JNK activities were eluted by up to 0.5 M NaCl. No 38 kDa activity was detected in any of the three SAPK/JNK peaks. SB203580 (10 μM) completely inhibited the SAPK/JNK activity eluting at 0.15 M NaCl and partially inhibited (approximately 50%) the SAPK/JNK activity eluting at 0.08 M NaCl (Fig. 2A,B, upper panels), but had no effect on the SAPK/JNK activity eluting in the isocratic wash (results not shown). Hyperosmotic shock also activates the ERKs in ventricular myocytes [5] which eluted at approximately 0.20 M (fractions 29–31) and 0.25 M NaCl (fractions 36 and 37), and were detected as minor bands in the in-gel assays using GST-c-Jun(1–135) as substrate (Fig. 2B, lower panel). Neither peak of ERK activity was inhibited by 10 μM SB203580 (results not shown), consistent with published data [11].

3.3. Separation of p38-MAPK and c-Jun N-terminal kinases by Mono Q FPLC

We studied the elution profile of cardiac p38-MAPK on Mono Q FPLC using extracts of hearts subjected to ischaemia/reperfusion. Using in-gel kinase assays with GST-MAPKAPK2(46–400) as substrate, kinase activities were detected in fraction 4 (isocratic wash, 40 kDa), fractions 34–36 (0.25 M NaCl, 42 kDa), fractions 40–42 (0.29 M NaCl, 44 kDa) and fraction 56 (0.4 M NaCl, 38 kDa) (Fig. 3A). The elution of p38-MAPK at approximately 0.4 NaCl is consistent with published data [5,6,17]. Fractions 4, 18–20 (∼0.08 M NaCl), 28–30 (∼0.15 M NaCl) and fractions 54–56 (∼0.4 M NaCl) were concentrated by ultrafiltration and were analysed by immunoblotting with antibodies from Santa Cruz (Fig. 3B). Antibodies to JNK1 detected bands in fraction 4 (44 kDa), fractions 18–20 (46 and 52 kDa), and fractions 28–30 (54 kDa), but not fractions 54–46 (Fig. 3B, upper panel). In contrast, a p38-MAPK antibody detected a single 38 kDa band in fractions 54–56 (Fig. 3B, lower panel). These experiments show that p38-MAPK elutes from Mono Q FPLC at higher concentrations of NaCl than the SAPKs/JNKs and is not responsible for the c-Jun N-terminal kinase activities in fractions 18–20 and 28–30 which are inhibited by SB203580.

4. Discussion

Although SB203580 is widely accepted as a specific inhibitor of p38-MAPK(α) and p38-MAPKβ [10–13], we have shown here that it inhibits at least two kinases which phosphorylate the N-terminal transactivation domain of c-Jun (Fig. 1B, Fig. 2A,B). These kinases have a similar apparent relative molecular mass to established SAPK/JNK isoforms (approximately 52 and 54 kDa) (Fig. 2), and elute from Mono Q columns at substantially lower NaCl concentrations than p38-MAPK (Fig. 3A). Furthermore, no 38 kDa c-Jun N-terminal kinase activity was detectable. These data indicate that SB203580 is indeed inhibiting SAPK/JNK isoforms. Recently, SB203580 was shown to inhibit certain epitope-tagged human SAPK/JNK isoforms which were overexpressed and immunoprecipitated from UV-treated COS-7 cells [19]. JNK2 isoforms were more susceptible to inhibition than JNK1 isoforms and were inhibited by micromolar concentrations of SB203580 [19]. Our data are consistent with inhibition of JNK2-related isoforms. Importantly, although the IC_{50} for inhibition of p38-MAPKs is (in our hands) 0.07 μM (Fig. 1A), 10 μM SB203580 is frequently used in order to establish the involvement of p38-MAPK in cellular responses, a concentration which completely inhibits at least one other kinase in the heart (54 kDa SAPK/JNK). Since many interventions that activate p38-MAPK also activate SAPKs/JNKs [2], our data suggest that caution should be exercised in the use of SB203580 to implicate the participation of p38-MAPK in signalling pathways.

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