Antiischemic Effects of SB203580 Are Mediated Through the Inhibition of p38α Mitogen-Activated Protein Kinase

Evidence From Ectopic Expression of an Inhibition-Resistant Kinase

Jody L. Martin, Metin Avkiran, Roy A. Quinlan, Philip Cohen, Michael S. Marber

The aim of the present study was to determine whether the attenuation of myocardial ischemic injury by SB203580 is due to the inhibition of p38 mitogen-activated protein kinase (MAPK) or to other documented nonspecific effects of the drug. We made adenoviral vectors encoding the α isofrom of p38 MAPK with or without site-directed mutations to prevent SB203580 binding and inhibition. In embryonal rat heart–derived cells and adult rat cardiocytes expressing wild-type p38α MAPK, injury was reduced significantly by SB203580 present during simulated ischemia. In contrast, SB203580 did not protect cells expressing the SB203580-resistant form of p38α MAPK. These observations suggest that SB203580-mediated protection depends on the inhibition of p38α MAPK.

Many studies have used SB203580 and related pyridinyl imidazoles inhibitors during lethal ischemia to infer that activation of p38 mitogen-activated protein kinase (p38 MAPK or SAPK2) is either beneficial or detrimental.1,2 Unfortunately, the actions of SB203580 are not confined to inhibition of p38 MAPK, because it has also been reported to inhibit thromboxane synthase,3 cyclooxygenases 1 and 2,4 PDK 1,4 and JNKs5 and to activate Raf-1.6 It is thus entirely possible that all studies, including our own,7 that have used SB203580 to implicate p38 MAPK in myocardial ischemic injury are flawed. This uncertain action of SB203580 is thought to be one of the reasons for investigators reaching contradictory mechanistic conclusions regarding the role of p38 MAPK in ischemia.1

The cocrystallization of p38α MAPK complexed to pyridinyl imidazoles has identified Thr106 as a key residue responsible for the interaction with the ATP-binding pocket.8 As predicted, mutations of this residue to amino acids with larger side chains render p38α MAPK resistant to SB203580.9 An SB203580-resistant mutant of p38α MAPK, where Thr106, His107, and Leu108 have been changed to Met, Pro, and Phe, respectively (T106M, H107P, L108F), has been constructed, studied, and shown to have effects in vivo and in vitro that are indistinguishable from wild-type p38α.10 However, in contrast to wild-type p38α MAPK, the triple mutant is inherently resistant to SB203580 in the same manner as the p38β and p38γ isoforms, which share the 106M, 107P, 108F consensus. In a previous study, in transfected cardiocytes, we have shown that p38α is the predominant SB-sensitive isofrom activated by ischemia.7 We therefore mounted the cDNAs for the parent wild-type p38α MAPK and the SB-resistant triple-mutant p38α MAPK in an adenoviral backbone to determine whether the effect of SB203580 on myocardial ischemia was p38α MAPK dependent.

Materials and Methods

Adult and neonatal rat cardiomyocytes were isolated, plated, and maintained in culture as we described previously.7,11,12 H9c2(2-1) cells (CRL-1446, ATCC) were grown under standard conditions.11 The wild-type and SB203580-resistant p38α cDNAs were inserted into the E1 region of the adenoviral genome by homologous recombination.11 Standard viral amplification and CsCl purification methods were used to purify adenoviruses encoding wild-type (WT) and SB203580-resistant (DR) p38α MAPK.11 All infections were at a multiplicity of infection of 10. This results in a transfection efficiency of greater than 90% in all cell types examined.

Neonatal cardiomyocytes were coinfected with adenovirus encoding constitutively active MKK6(b)13 and either the WT or the DR variant of p38α MAPK. After 36 hours, cardiomyocytes were exposed to new medium with or without 10 μmol/L SB203580 for 1 hour before harvesting. Equal amounts of protein were examined by Western blot analysis and probed with rabbit polyclonal anti-phospho-p38 MAPK and mouse monoclonal anti-phospho-Ser322332 as control.10 Thirty-six hours before simulated ischemia (SI) H9c2 cells were infected with the WT:p38α MAPK or DR:p38α MAPK adenoviruses. Thirty minutes before SI, where appropriate, cells were exposed to 1 μmol/L SB203580, which was then maintained during the 7 hours of SI. Ischemia was simulated using the hypoxic crystalloid buffers previously described.14,15 After 30 minutes of simulated reperfusion, viability was assessed by MTT bioconversion using standard methods. The readings were normalized to those of WT:p38α MAPK expressing cells in the absence of SB203580.

Adult cardiomyocytes were infected 1 hour after plating on laminin-coated dishes, and the experiments commenced 24 hours later. Samples were treated as above except that SI was for 4 hours, lactate dehydrogenase (LDH) release was used as the endpoint because lower plating density precluded measurements of MTT bioreduction, and SB203580 was used at both 1 and 10 μmol/L. The percentage of LDH release was calculated by assaying LDH activity in the supernatant and the corresponding lysed cell extract using the TOX-7 kit (Sigma Chemical Co) and normalized to release in WT:p38 MAPK–expressing cells in the absence of SB203580.

Autoradiographic images of the Western blots were scanned and then quantified using NIH Image analysis software. The densitometry reading obtained in SB203580-treated cells was normalized to the corresponding nontreated sample on each immunoblot.

Results and Discussion

Initial experiments were performed in isolated neonatal ventricular cardiomyocytes in which others, and we, have established that p38 MAPK is activated during ischemia and that SB203580 reduces injury. Given that ischemia only activates...
p38 MAPK by ∼3-fold, an effect little altered in p38α MAPK–overexpressing cells,7 we thought it best to characterize the WT and DR adenoviral vectors under conditions of maximal p38 MAPK activation. Cells were cotransfected with the upstream activator of p38 MAPK, constitutively active MKK6(b)E, and either WT.p38α MAPK or DR.p38α MAPK. Recently, a phosphospecific antibody to Ser82 of hsp27 has been developed that has been shown to be exquisitely responsive to activation of MAPKAPK2 by p38 MAPK.10 Figure 1 shows the extensive and equal activation of the p38s by MKK6(b)E and the ability of DR.p38α MAPK to maintain the phosphorylation of hsp27 despite the presence of a high concentration of SB203580 (10 μmol/L SB203580 is >100 times the IC50 for p38 MAPK inhibition). Neither WT.p38α nor DR.p38α MAPK altered the content of hsp27 in any of the cell types examined.

We then determined the action of SB203580 in H9c2 cells expressing WT.p38α MAPK or DR.p38α MAPK. The top panel of Figure 2 shows the post-SI survival of the H9c2 cells based on bioreductive capacity. Post-SI survival of H9c2 cells expressing WT.p38α MAPK was identical to that of cells infected with an E1-deleted virus containing only viral DNA (100 ± 2.1 versus 101.8 ± 4.3, n = 10; P = NS). SB203580 (1 μmol/L) during SI increased survival in cells expressing WT.p38α MAPK but not DR.p38α MAPK. A representative phospho-hsp27 immunoblot appears in the middle panel, and the bottom panel represents densitometric quantification of this and similar blots from protein harvested from 4 independent experiments. The effect of SB203580 on protection is associated with the most complete inhibition of the p38 MAPK pathway.

In light of the above, we then performed similar experiments in freshly isolated adult cardiomyocytes. Figure 3

Figure 1. Characterization of adenoviral vectors encoding SB203580-sensitive p38α MAPK (WT.p38) and SB203580-resistant p38α MAPK (DR.p38). Neonatal cardiomyocytes were coinfected with either of these viruses together with a constitutively active upstream kinase MKK6(b)E. After 36 hours, coinfected cells were either left untreated or exposed to 10 μmol/L SB203580 for 1 hour before harvesting. Equal amounts of protein were examined by Western blot analysis and probed with antibodies recognizing dually phosphorylated p38 MAPK (pp38) and phospho-hsp27 (phsp27). Control samples were infected with an identical adenoviral vector containing only viral DNA (empty vector). Even at high concentration, SB203580 fails to inhibit DR.p38α MAPK as evidenced by sustained phosphorylation of hsp27 to a level similar to that seen in control-infected cells. Viral overexpression of p38 MAPKs causes a slight decrease in endogenous p38 as seen previously.7 As in Figures 2 and 3, the decrease in hsp27 phosphorylation that is caused by SB203580 in cells expressing DR.p38 MAPK is probably caused by inhibition of endogenous p38 MAPKs. The content of PKCδ has been included as an index of protein loading.

Figure 2. The cardioprotective effect of intras ischemic SB203580 on H9c2 cells is p38α MAPK dependent. H9c2 cell survival was assessed by MTT bioreduction, as an index of postischemic mitochondrial dehydrogenase activity. Cell viability data are from 3 independent experiments (each in triplicate or more). Only cells expressing the WT.p38 MAPK were protected by SB203580 (1 μmol/L). Parallel samples, from 4 independent experiments, were harvested for immunoblotting after 3 hours of simulated ischemia. The phospho-hsp27 signal was scanned and quantified with the NIH Image analysis software. The signal in the presence of SB203580 was normalized to that in the absence of SB203580. SB203580 failed to protect and substantially inhibit the p38α MAPK pathway in the cells expressing DR.p38α MAPK. The percentage reduction in phospho-hsp27 in the presence compared with the absence of SB203580 in WT.p38- vs DR.p38-expressing cells is 80.6 ± 2.8% vs 35.1 ± 12.9%, respectively. *P<0.001 by unpaired t test; **P<0.001 vs all other groups by ANOVA with post hoc Tukey.
shows results in a similar format as Figure 2. With intraischemic SB203580 (1 μmol/L), there is a significant reduction in LDH release in cells expressing WT.p38α MAPK. In contrast, protection is completely abrogated when cells express DR.p38α MAPK. Indeed, even when used at 10-fold higher concentration, SB203580 still protected cells expressing WT.p38α MAPK (70.9% ± 6.2) but not DR.p38α MAPK (101.0% ± 10.3). This differential effect on protection is accompanied by a reduced ability of SB203580 to inhibit the phosphorylation of hsp27 in DR.p38α MAPK–expressing cells (see bottom panel).

In 2 distinct cell-based models, we have shown that the ability of SB203580 to attenuate ischemic injury is abolished when cells express a mutant p38α MAPK that is insensitive to inhibition by this agent. These results strongly suggest that the cardioprotective action of SB203580 is the result of inhibition of p38α MAPK and not due to the documented inhibition or activation of other kinases. Our study also illustrates the utility of the complementary application of cell-based models and ectopic expression of drug-resistant kinases to verify the mechanism of action of kinase inhibitors.

Acknowledgments

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References


7. Saurin AT, Martin JL, Heads RJ, Foley C, Mockridge JW, Wright MJ, Wang Y, Marber MS. The role of differential activation of p38-MAPK–encoding adenoviral vector were protected by SB203580 whereas those expressing DR.p38α MAPK were not. A representative immunoblot is shown above quantified densitometric data for the phospho-hsp27 band from 4 separate experiments. In keeping with the findings in H9c2 cells (Figure 2), SB203580 was only protective when it was able to markedly inhibit p38α MAPK activation, and this only occurred in the cardiocytes expressing WT.p38α MAPK. The percentage reduction in phospho-hsp27 in the presence compared with the absence of SB203580 in WT.p38- vs DR.p38-expressing cells is 72.6% ± 3.9% vs 26.8% ± 10.4%, respectively. **P<0.001 by unpaired t test; *P<0.001 vs all other groups by ANOVA with post hoc Tukey.

Key Words: SB203580 • p38 mitogen-activated protein kinase • preconditioning • isolated cells • signaling •
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