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Sab mediates mitochondrial dysfunction involved in imatinib mesylate-induced cardiotoxicity

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ABSTRACT

Imatinib mesylate is an effective treatment for chronic myelogenous leukemia and gastrointestinal stromal tumors. Although imatinib mesylate is highly tolerable, it has been implicated in severe congestive heart failure in mouse models and patients. A hallmark of imatinib mesylate-induced cardiotoxicity is mitochondrial dysfunction. The mitochondrial scaffold Sab has been implicated in facilitating signaling responsible for mitochondrial dysfunction in a c-Jun N-terminal Kinase (JNK)-dependent manner. We examined the impact of Sab-mediated signaling on imatinib mesylate cardiotoxicity in H9c2 rat cardiomyocyte-like cells. Silencing Sab increased the LD_{50} of imatinib mesylate 4-fold in H9c2 cells. Disrupting Sab-mediated signaling prevented imatinib mesylate-induced apoptosis as well. Knockdown of Sab or inhibition with a small peptide prevented oxidative stress, which was indicated by decreased reactive oxygen species production, lipid peroxidation, and protein carbonylation. Further, inhibition of Sab-related signaling partially rescued deficits in mitochondrial respiration, ATP production, and membrane potential in imatinib mesylate-treated H9c2 cells. Conversely, over-expression of Sab in H9c2 cells increased the cardiotoxicity of imatinib mesylate *in vitro* decreasing the LD_{50} over 4-fold. Sab expression was induced in H9c2 cells following cardiovascular-like stress in an AP-1 dependent manner. These data demonstrate that imatinib mesylate influences mitochondrial signaling leading to mitochondrial dysfunction and cardiotoxicity.

1. Introduction

Imatinib mesylate (Gleevec) is suggested to be a small molecule inhibitor targeting the kinase activity of the Bcr-Abl fusion protein commonly found in chronic myelogenous leukemia (CML) (Deininger et al., 2005). Administration of imatinib mesylate has been shown to be effective against chronic phase CML with over 70% of patients demonstrating cytogenic remission (Deininger et al., 2005; Van Etten, 2004). Additionally, imatinib mesylate has been shown to be efficacious against gastrointestinal stromal tumor (GIST) by proposed inhibition of KIT proteins (Miettinen et al., 1999). Although imatinib mesylate treatment has been linked to common toxic side effects such as neutropenia, thrombocytopenia, anemia, nausea, vomiting/diarrhea, fluid retention, and skin irritations, the drug is tolerated well by patients (Thanopoulou and Judson, 2012). However, imatinib mesylate-induced cardiotoxicity remains a topic of some controversy, specifically in older individuals where susceptibility to toxic substances is of concern. During an early clinical trial for imatinib mesylate, there was a report of left ventricular dysfunction and congestive heart failure (CHF) in patients without a prior history (Cohen et al., 2002). This cardiotoxicity was investigated by Kerkelä and colleagues, and they found that imatinib mesylate may induce endoplasmic reticulum (ER) stress leading to mitochondrial dysfunction and cell death (Kerkela et al., 2006). This toxicity has since been linked to inhibition of Abelson murine leukemia viral oncogene homolog-1 (ABL) (Kerkela et al., 2006), indirect effects on protein kinase B

Abbreviations: CHF, congestive heart failure; KIM, kinase interacting motif; JNK, c-Jun N-terminal Kinase; ATP, adenosine triphosphate; FRET, fluorescence resonance energy transfer; OCR, oxygen consumption rate; TMRM, tetramethylrhodamine methyl ester; eIF2α, elongation initiation factor 2α; XBP-1, X-box binding protein 1.

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(AKT) (Tarn et al., 2006), and activation of stress responses by mitogen-activated protein kinases (MAPKs) (Kerkela et al., 2006; Trent et al., 2010).

While these early studies demonstrated the potential for cardiotoxic effects by imatinib mesylate, recent studies have refuted this specific type of toxicity (Ribeiro et al., 2008; Will et al., 2008; Wolf et al., 2010). Specifically, Wolf and colleagues demonstrated that concentrations well in excess (10–50 μM) of clinically relevant concentrations (5 μM) for imatinib were required to induce cardiotoxicity in neonatal rat ventricular myocytes and cardiac fibroblasts *in vitro* (Wolf et al., 2010). However, the authors did report that imatinib contributed to hypertrophic responses in rat hearts (Wolf et al., 2010). Using a chemical approach, Will et al. examined the cardiotoxicity associated with imatinib, dasatinib, sunitinib, and sorafenib in H9c2 rat cardiomyocyte-like cells. It was found that only sorafenib impacted mitochondrial function and viability at clinically relevant doses. These studies raise questions regarding the potential cardiotoxicity of imatinib.

Despite these studies, continued examination of the imatinib mesylate cardiotoxic mechanism revealed that cell death was induced by activation of pro-apoptotic signaling by MAPKs, namely c-Jun N-terminal Kinase (JNK) and p38 (Kerkela et al., 2006). Indeed, imatinib mesylate has been shown to induce JNK activation and inhibition of JNK was shown to be cardioprotective following imatinib mesylate treatment (Kerkela et al., 2006; Sarszegi et al., 2012). Our previous research has demonstrated that cytotoxic JNK signaling occurs through the kinase's mitochondrial signaling events (Chambers et al., 2011). JNK associates with the mitochondrial scaffold, Sab, *via* an interaction between the JNK-interacting protein (JIP) binding site on JNK and the kinase interaction motif 1 (KIM1) on Sab (Wiltshire et al., 2002). Sab has two such KIM motifs capable of binding MAPKs (Wiltshire et al., 2002); however, only JNK (Hanawa et al., 2008) and p38γ (Court et al., 2004) have been shown to interact with Sab. To selectively investigate mitochondrial JNK signaling, we used a cell-permeable peptide containing the KIM1 motif of Sab (Tat-Sab_{KIM1}) to block JNK translocation to the mitochondria in response to anisomycin-induced stress (Chambers et al., 2011). While inhibition of mitochondrial JNK signaling prevented superoxide generation, loss of mitochondrial membrane potential, and cell death, the peptide had no impact on the nuclear translocation or functions of JNK *in vitro* (Chambers et al., 2011). Our studies demonstrate that mitochondrial JNK signaling on Sab may be crucial for toxic stress responses.

Mitochondrial JNK signaling was previously linked to oxidative stress induced cardiotoxicity in adult ventricular cardiomyocytes (Aoki et al., 2002). Oxidative stress from hydrogen peroxide/ferric sulfate production of superoxide initiated JNK translocation to mitochondria and apoptosis *in vitro* (Aoki et al., 2002). We found that mitochondrial JNK signaling contributed to cardiotoxicity associated with ischemia/reperfusion injury in adult rats (Chambers et al., 2013a,b) Five minutes before reperfusion (25 min of ischemia), Tat-Sab_{KIM1} was randomly injected into the left ventricle. Following 24 h of reperfusion, inhibition of mitochondrial JNK signaling reduced oxidative stress, mitochondrial dysfunction, cell death, and infarct size associated with ischemia/reperfusion injury (Chambers et al., 2013b). These studies suggest that signaling on Sab could be associated with cytotoxic stress responses in the heart.

Given the involvement of mitochondrial JNK and Sab in cardiotoxic responses, we decided to examine whether signaling on Sab and the mitochondrial levels of Sab, contributed to imatinib mesylate-induced cardiotoxicity. Treatment of rat cardiomyocyte-like H9c2 cells with imatinib mesylate resulted in oxidative stress, mitochondrial dysfunction, and induction of apoptosis in a Sab-dependent manner. Gene silencing of Sab expression or administration of the Tat-Sab_{KIM1} peptide prior to imatinib mesylate treatment reduced oxidative stress, mitochondrial dysfunction, and apoptosis. Over-expression of Sab enhanced the cardiotoxicity of imatinib in H9c2 cells. The results demonstrate that signaling on Sab may contribute to imatinib mesylate cardiotoxicity.

2. Experimental procedures

2.1. Cell culture, imatinib mesylate treatment, and gene manipulation

Undifferentiated rat H9c2 cardiomyocyte-like cells (ATCC CRL-1446) were maintained between passages 5 and 20 for all experimental protocols in Dulbecco's Modified Essential Media (DMEM – Invitrogen, 10566) supplemented with 10% fetal bovine serum (Invitrogen, 10082147) and 100 U/mL penicillin/streptomycin (Invitrogen, 15140) at 37 °C and under 5% CO_2 as described previously (Chambers et al., 2013b). Imatinib mesylate (LC Laboratories, I-5508) was reconstituted in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL. Administration of imatinib mesylate was conducted by adding drug to a final concentration of 50 μM (this concentration was reproducibly determined to be approximately twice the LD_{50} of imatinib cardiotoxicity in H9c2 cells at 24 h) or in a dose-dependent manner required to calculate LD_{50} values. To reduce Sab expression in H9c2 cells, we chose to use small interfering RNA (siRNA)-mediated gene silencing. To adequately survey the progression of imatinib-induced cardiotoxicity, we used multiple time points as indicated in the following procedures. Specifically, twelve-hour time points were used to assess events that occurred early (decreased oxygen consumption for example); we also used twenty-four hour time points for measurements involving intermediate events, such as oxidative stress, while forty-eight hour or seventy-two hour time points were reserved for measurements related to endoplasmic reticulum (ER) stress and cell death. Treatment with 10 μM thapsigargin for 24 h was used as a positive control for ER stress. Succinctly, H9c2 cells were plated in a 96-well plate at a density of 1.0×10^4 cells per well and incubated overnight. siRNAs specific for Sab, c-Jun, or luciferase-targeting siRNAs (Luc siRNA) were purchased from Qiagen and prepared as a 2 μM solution in 60 mM KCl, 6 mM HEPES (pH 7.5), 0.2 mM $MgCl_2$. The siRNAs were combined with transfection reagent DharmaFECT® 1 (Thermo Scientific) in serum free DMEM and added to the cells at a final concentration of 100 nM. The cells were incubated for 72 h, and silencing was confirmed by monitoring Sab protein levels using an in-cell western and quantitative western analysis. For over-expression, the pLOC:RFP and pLOC:Sab plasmids were purchased from Thermo Scientific. Plasmids were transfected separately into cells using the Fugene®HD (Promega) at a 3.5:1 ratio of transfection reagent to plasmid DNA. Briefly, plasmid DNA was diluted in serum-free DMEM and combined with Fugene® HD. The mixture was incubated at room temperature for 15 min, and then it was added to cell cultures in a drop-wise manner. Following 8 h of incubation, the cells were placed in fresh media, and protein expression was monitored by western blot analysis at 72 h. Transfection efficiency was determined by co-expression of green fluorescent protein on both pLOC plasmids. For experiments, only cultures with greater than 70% transfection efficiencies were used in our studies.

2.2. In-cell western blot analysis

Specific protein levels were analyzed using in-cell western technology (Egorina et al., 2006). Following gene silencing or drug administration, H9c2 cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 25 min at room temperature.