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Inhibition of HSP27 Phosphorylation by a Cell-permeant MAPKAP Kinase 2 Inhibitor

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Abstract

Heat shock protein 27 (HSP27) has been implicated in many intracellular signaling processes. Since the phosphorylation of HSP27 can modulate its activity, the ability to inhibit phosphorylation of HSP27 might have clinical relevance especially with regard to the treatment of fibrosis. We have developed a cell permeant peptide inhibitor of MAPKAP Kinase 2 (MK2), an enzyme that phosphorylates HSP27, by combining a previously described peptide substrate of MK2 with a cell penetrating peptide. This novel MK2 inhibitor (MK2i) reduced HSP27 phosphorylation by MK2 *in vitro*. At 10 μ M, MK2i inhibited TGF- β 1-induced HSP27 phosphorylation in serum-starved human keloid fibroblasts. In addition, 10 μ M MK2i decreased TGF- β 1-induced expression of connective tissue growth factor and collagen type I within serum-starved keloid fibroblasts. Thus, MK2i represents a potential therapeutic for the treatment of fibrotic disorders.

Keywords

Heat shock protein 27; HSP27 phosphorylation; MAPKAP Kinase 2; keloid fibroblast; TGF- β 1; CTGF; collagen type I; peptide inhibitor

Introduction

Heat shock protein 27 (HSP27) has many diverse functions including chaperone activity [1], mRNA stabilization [2] and [3], inhibition of apoptosis [4] and [5], and modulation of actin polymerization [6], [7] and [8]. The activity of HSP27 likely depends on the phosphorylation state of serine residues at positions 15, 78, and 82 [9]. Since the activities of HSP27 have potentially important clinical ramifications, the ability to alter the phosphorylated state of HSP27 is of particular interest. HSP27 has been identified as a substrate of MAPKAP Kinase 2 (MK2) [10], which is activated by p38 MAP kinase [11]. Several groups have used small molecules, such as SB203580, to indirectly inhibit MK2 by decreasing p38 MAP kinase activity [12], [6], [2], [13] and [14]. However, because p38 MAP kinase is involved in a variety

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of intracellular signal transduction pathways, SB203580 and related compounds are not specific inhibitors of downstream kinases and can have unintended consequences, such as adverse central nervous system effects or abnormal liver function [15] and [16].

In an effort to identify a peptide domain specifically phosphorylated by MK2, Stokoe et al. identified the consensus sequence HyXRXSXX, where X is any amino acid and Hy is any hydrophobic amino acid [17]. Building upon this work, Hayess and Benndorf showed that the peptide KKKALNRQLGVAA selectively inhibited MK2 relative to PKA, PKC, and ERK1 [18]. However, this peptide is not cell permeant. By linking a novel cell penetrating peptide [19] to a modification of the peptide described by Hayess and Benndorf, we have developed a cell permeant MK2 inhibitor peptide (MK2i). To test our hypothesis that MK2i can inhibit intracellular phosphorylation of HSP27, primary human keloid fibroblasts (KFs) treated with MK2i were exposed to transforming growth factor beta 1 (TGF- β 1), a canonical mediator of cellular behavior known not only to influence proliferation, differentiation, and motility but also to stimulate HSP27 phosphorylation in a variety of cell types [20], [12] and [21]. We demonstrate that MK2i can inhibit TGF- β 1-induced HSP27 phosphorylation. In addition, MK2i treatment leads to a decrease in TGF- β 1-induced connective tissue growth factor (CTGF) and collagen type I expression from KFs.

Materials and Methods

Materials

For peptide synthesis, reagents were purchased from Anaspec (San Jose, CA). Dimethylformamide, diethyl ether, and acetonitrile were obtained from Mallinckrodt Chemicals (Phillipsburg, NJ). Unless otherwise indicated, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were used as received.

Peptide Synthesis and Purification

The MK2 inhibitor peptide, WLRRKAWLRRKALNRQLGVAA (MK2i), was synthesized at a 0.35 mmol scale (Rink amide resin) using Fmoc chemistry on an Apex 396 peptide synthesizer (Aapptec, Louisville, KY). Following synthesis, the peptide was cleaved with 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane, precipitated in cold diethyl ether, and collected by centrifugation. MK2i was purified and eluted using an acetonitrile gradient on an AKTA Explorer FPLC (GE Healthcare, Piscataway, NJ) equipped with a C18 reversed-phase column (Grace, Deerfield, IL). Fractions containing purified MK2i, as indicated by MALDI-TOF mass spectroscopy and analytical HPLC analysis, were collected, lyophilized, and stored at -80 °C.

Cell Culture

KFs were obtained as a gift from Dr. M. T. Longaker (Department of Surgery, Stanford University, Palo Alto, CA). The cells were isolated from three different patients as previously described [22], in accordance with the Helsinki Declaration of 1975 and with protocols approved by the Human Subjects IRB at Stanford University. Cells were maintained at 37 °C and 10% CO₂ atmosphere in Dulbecco's modification of Eagle's medium (DMEM, Mediatech, Harndon, VA) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and additional penicillin and streptomycin (1%) in 10-cm² dishes.

In Vitro Inhibition of MK2

An *in vitro* MK2 activity assay was performed using commercially available MK2 (Millipore, Billerica, MA), recombinant human HSP27 (Assay Designs, Ann Arbor, MI), and assay dilution buffer (ADB; final concentration: 20 mM MOPS, pH 7.2, 25 mM glycerol phosphate,

5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol; Millipore). On ice, 50 ng MK2 was added to 1.4 μ g recombinant human HSP27 in ADB with or without either 200 μ M of the cell permeable MK2 inhibitor peptide, MK2i, or 200 μ M of the cell impermeant MK2 inhibitor peptide KKKALNRQLGVAA (EMD Chemicals Inc., La Jolla, CA). Phosphorylation was initiated by adding ATP/Magnesium (Millipore; final concentration: 15 mM MgCl_2 and 100 μ M ATP) followed by incubation at 30°C for 30 minutes. The reactions were stopped with the addition of Laemmli buffer and subsequent heating of the samples at 100°C for 5 minutes. The proteins were separated on 15% polyacrylamide gels and then electrophoretically transferred to Immobilon PVDF membranes (Millipore) at 4 °C. The membranes were blocked with Odyssey blocking buffer (Li-Cor, Lincoln, NE) for one hour at room temperature and subsequently incubated with rabbit anti-phosphoserine 78/82 HSP27 antibodies [23] overnight at 4 °C. Total HSP27 expression was determined by incubating the blot with mouse anti-HSP27 antibodies (a gift from Dr. M. Welsh, University of Michigan, Ann Arbor, MI) followed by incubation with IRDye680 goat anti-mouse IgG (Li-Cor) and IRDye800 goat anti-rabbit (Rockland Immunochemicals, Inc., Gilbertsville, PA) secondary antibodies. Membranes were scanned and analyzed using an Odyssey Infrared Imaging System (Li-Cor).

Intracellular Inhibition of TGF- β 1 Induced HSP27 Phosphorylation

KFs were grown on cover slips (for microscopy) or in dishes (for western blot analysis) to 70% confluence and then serum starved for 48 hours by reducing the amount of FBS in the medium from 10% to 0.5%. After adding fresh medium (containing 0.5% FBS), the cells were subsequently stimulated with nothing (control), with 1.25 ng/ml TGF- β 1 (R&D systems, Minneapolis, MN) for 24 hours, or with MK2i for two hours followed by the addition of 1.25 ng/ml TGF- β 1 for 24 hours. The cells were then processed for immunocytochemical visualization or western blot analysis. These experiments were conducted with cells from two different patients and repeated three times with cells from each patient to verify that results were consistent between individual experiments.

For immunocytochemical analysis, cells on cover slips were washed with Tris-buffered saline (TBS), fixed with formalin, permeabilized with TBST (TBS with 0.05% Tween 20), and incubated with rabbit anti-phosphoserine 78/82 HSP27 antibodies. Prior to imaging with a Zeiss Axiovert microscope, the cells were washed and labeled with the following dyes: Cy2-conjugated affinity-purified goat anti-rabbit antibody (Rockland Immunochemicals) for phosphorylated HSP27 (ser 78/82; green fluorescence), Alexa 586-conjugated phalloidin (Invitrogen) to reveal the actin cytoskeleton (red), and 4',6-diamidino-2-phenylindole (DAPI) for nuclear visualization (blue).

For western blot analysis, adherent cells were rinsed with PBS and lysed using UDC buffer (8 M urea, 10 mM dithiothreitol, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). Lysates were centrifuged (6000 g, 20 min), and the supernatant was collected. Equal amounts of protein (20 μ g/lane), as determined by a BCA protein assay, were separated on 4-20% polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with Odyssey blocking buffer for one hour at room temperature and probed overnight at 4 °C with the following primary antibodies: rabbit anti-CTGF (Torrey Pines Biolabs, Houston, TX), rabbit anti-collagen type I (Cortex Biochem, San Leandro, CA), mouse anti-HSP27, rabbit anti-phosphoserine 78/82 HSP27 (developed in our laboratory), and rabbit anti- β -actin (Sigma-Aldrich). After washing, the membranes were incubated with IRDye680 goat anti-mouse IgG and IRDye800 goat anti-rabbit IgG secondary antibodies for one hour at room temperature. Protein-antibody complexes on washed membranes were visualized using an Odyssey Infrared Imaging System.

Statistical Analysis

All protein expression data are presented as means $\pm$ standard deviations. Western blot bands were quantified by densitometry, and protein expression normalized to the β -actin loading control. Using a significance level of $\alpha = 0.05$, one-way ANOVA followed by a Tukey post-hoc test was used to compare experimental groups.

Results

MK2i Inhibits MK2 Activity In Vitro

To determine the effect of MK2i peptide on MK2 activity, an *in vitro* kinase assay was performed using purified MK2 to phosphorylate recombinant HSP27. A comparison of phosphorylated HSP27 to total HSP27 revealed that in a representative blot (Fig. 1), the synthetic MK2i peptide inhibited the phosphorylation of HSP27 by 80% relative to a control receiving no inhibitor. The commercially available Hayess and Benndorf peptide inhibited HSP27 phosphorylation by 64%.

MK2i Inhibits TGF- β 1 Induced Intracellular HSP27 Phosphorylation

TGF- β 1 was used as an agonist to stimulate the HSP27 phosphorylation within KFs in a manner similar to that observed in other cell types [20], [12] and [21]. After 24 hours, serum starved KFs pretreated for two hours with MK2i followed by treatment with TGF- β 1 (Fig. 2C) remained viable and showed a marked decrease in the phosphorylation of HSP27 relative to cells treated only with TGF- β 1 (Fig. 2B). In addition, these images indicate that KFs treated with MK2i experienced both a loss of central actin and a reduction in stress fiber formation. Quantification of the ratio between phosphorylated HSP27 and total HSP27 revealed that MK2i used at 10 μ M decreased TGF- β 1 induced HSP27 phosphorylation in KFs (Fig. 3). When exposed to TGF- β 1 alone, HSP27 phosphorylation increased 2.3-fold relative to that in cells not treated with TGF- β 1. Compared with the TGF- β 1 condition, pretreatment with 10 μ M MK2i significantly diminished HSP27 phosphorylation by $39 \pm 22\%$ ($p < 0.05$) to levels similar to that of the untreated control. Pretreatment with 5 μ M MK2i did not mitigate TGF- β 1 induced HSP27 phosphorylation indicating a threshold for MK2i inhibitory activity.

MK2i Inhibits TGF- β 1 Induced Expression of CTGF and Collagen Type I

Since TGF- β 1 has been shown to stimulate CTGF and collagen expression in disorders associated with excessive scarring [24;25], the effect of MK2i on the expression of CTGF and type I collagen in TGF- β 1 stimulated KFs was evaluated. At 10 μ M, MK2i significantly decreased the TGF- β 1-induced expression of both CTGF and collagen by $68 \pm 7.1\%$ (Fig. 4) and $76 \pm 20\%$, respectively ($p < 0.05$). In both cases, KFs treated with 10 μ M, MK2i had CTGF and collagen expression levels similar to that of untreated control cells. As with HSP27 phosphorylation, lower concentrations of MK2i had no effect on TGF- β 1 induced CTGF or collagen type I expression.

Discussion

The phosphorylation state of HSP27 can influence cellular activity. Cellular processes such as contraction, cell adhesion, cytokinesis, cell motility, and migration, all require dynamic reorganization of the actin cytoskeleton. Consequently, HSP27 has attracted considerable interest as a modulator of actin filament dynamics [6], [7], [26] and [8]. For example, transfection of cells with dominant active phosphorylated mutants of HSP27 leads to abundant stress fiber formation [26], and increases in the phosphorylation of HSP27 are associated with smooth muscle migration [12]. In addition, HSP27 has been implicated in the stabilization of various mRNA [2] and [3] and the inhibition of apoptotic pathways [4] and [5]. Therefore,

molecular strategies that inhibit HSP27 phosphorylation could become a useful therapy to prevent or limit smooth muscle migration, myofibroblast phenotype modulation, extracellular matrix production, intimal hyperplasia, inflammation, fibrosis, and scar formation.

MK2i represents a novel, cell permeant biological inhibitor of MK2 activity. Although the initial KKKALNRQLGVAA peptide was more selective as an inhibitor of MK2 relative to PKA, PKC, and ERK1 [18], the selectivity of MK2i towards a variety of kinases remains the subject of current investigation. In this study, we show that MK2i can inhibit MK2 activity *in vitro* as evidenced by decreased phosphorylation of recombinant HSP27 (Fig. 1). Qualitatively, both the phosphorylation of HSP27 and the relative amount of stress fiber formation were reduced in KFs treated with both MK2i and TGF- β 1 (Fig. 2). This result supported the hypothesis that MK2i can function in an intracellular environment to inhibit MK2 activity.

To investigate the ability of MK2i to affect potential fibrotic responses, KFs were treated with MK2i and subsequently stimulated with TGF- β 1. As a multifunctional cytokine capable of regulating cell growth, differentiation, and extracellular matrix production, TGF- β 1 has been implicated in wound healing and fibrotic responses such as keloid formation [27] and [28]. TGF- β 1 can activate the mitogen-activated protein (MAP) kinase superfamily, including p38 MAP kinase, which can result in downstream phosphorylation of both MK2 and HSP27 [29]. At least some of the fibrotic effects of TGF- β 1 signaling are closely associated with the p38 MAP kinase pathway. For example, smooth muscle α -actin expression and myofibroblast differentiation induced by TGF- β 1 depend on MK2 activity [30]. In addition, many cell types have responded to TGF- β 1 treatment by increasing HSP27 phosphorylation [20], [12] and [21]. Similarly, we observed an increase in HSP27 phosphorylation within KFs stimulated with TGF- β 1. When treated with the higher dose of MK2i, relative HSP27 phosphorylation was significantly reduced (Fig. 3). However, the lower dose of MK2i showed no significant change in relative HSP27 phosphorylation indicating a threshold for MK2i activity.

Previously, KFs have been shown to increase both CTGF and collagen type I expression following treatment with TGF- β 1 [24] and [31]. Data from this study parallel those findings as TGF- β 1 induced a 2.4-fold and 3.1-fold increase in expression of CTGF and collagen type I, respectively (Fig. 4). As with HSP27 phosphorylation, a dose threshold was observed since the lower dose of MK2i did not affect TGF- β 1 induced expression of either CTGF or collagen. However, the 10 μ M MK2i treatment significantly reduced the levels of both proteins to levels comparable to those in KFs that were not stimulated with TGF- β 1. Since TGF- β 1 induced CTGF expression results in concomitant collagen type I expression [32], the observed decrease in collagen levels in cells treated with MK2i likely followed changes in CTGF expression. Although the mechanism of CTGF expression has not been shown to be associated with the p38 MAP kinase pathway, the ability of MK2i to inhibit TGF- β 1 induced CTGF production might have occurred indirectly as a consequence of reduced HSP27 phosphorylation.

Recent work has identified an inverse relationship between intracellular levels of G-actin and CTGF [33] and [34]. This relationship suggests that cytoskeletal dynamics can regulate CTGF expression. It is known that cytoskeletal dynamics can be affected by HSP27. For example, overexpression of HSP27 resulted in decreased actin fragmentation when cells were exposed to oxidative stress [5]. In addition, the phosphorylation of HSP27 has been shown to stabilize actin stress fiber formation following heat shock [26]. Thus, the phosphorylation state of HSP27 can regulate actin dynamics. In the current study, we show that MK2i was able to inhibit TGF- β 1 induced HSP27 phosphorylation and attending actin stress fiber formation within KFs (Fig. 2). We speculate that the coincident decrease in CTGF and collagen type I expression resulted from HSP27-related changes in actin stress fiber formation and impairment of mechanotransduction important to TGF- β 1 signaling.

In conclusion, we have developed a novel, cell permeant inhibitor of MK2i capable of intracellular inhibition of HSP27, CTGF, and collagen type I. Although future studies will continue to investigate mechanism of action, MK2i specificity to MK2, and *in vivo* models, MK2i and related compounds could become useful therapeutics for inhibiting fibrotic responses such as scar formation, interstitial nephritis, cardiac fibrosis, pulmonary fibrosis, and intimal hyperplasia.

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Fig. 1. Effect of MK2i on the *in vitro* phosphorylation of recombinant HSP27 by MK2
 Recombinant HSP27 was phosphorylated with no enzyme (control) or with MK2 in the absence (no inhibitor) or presence of either an inhibitor peptide from [18] or MK2i. Panel A depicts a representative western blot showing levels of HSP27 and phosphorylated HSP27 (pHSP27). Panel B shows a plot of the relative intensities of the ratio of pHSP27 to HSP27 for each treatment.

Fig. 2. Effect of MK2i on TGF- β 1-induced phosphorylation of HSP27 in KFs

Serum-starved cells were treated with nothing (control; panel A), TGF- β 1 for 24 hours (panel B), or MK2i for two hours followed by TGF- β 1 for 24 hours (panel C). Following treatment, cells were labeled to detect phosphorylated HSP27 (green), actin (red), and the nucleus (blue).

Fig. 3. MK2i inhibits TGF- β 1-induced phosphorylation of HSP27 in KFs

Serum-starved cells were treated with nothing, TGF- β 1 for 24 hours, or MK2i for 2 hours followed by TGF- β 1 for 24 hours. Panel A shows a representative western blot of phosphorylated HSP27 (pHSP27), HSP27, and β -actin. HSP27 and pHSP27 levels were quantified relative to β -actin expression, and the ratio of pHSP27 to HSP27 was normalized to the untreated condition (panel B). Data are expressed as the mean $\pm$ standard deviation (n=3).

Fig. 4. MK2i inhibits TGF- β 1-induced expression of CTGF and collagen type I in KFs
 Serum-starved cells were treated with nothing, TGF- β 1 for 24 hours, or MK2i for 2 hours followed by TGF- β 1 for 24 hours. Panel A shows representative western blots of CTGF, collagen, and β -actin. CTGF and collagen levels were quantified relative to β -actin expression and normalized to the untreated condition (panel B). Data are expressed as the mean $\pm$ standard deviation (n=3); * and ** indicate $p < 0.05$.