Published in final edited form as:

Pharmacol Ther. 2008 July ; 119(1): 44–54. doi:10.1016/j.pharmthera.2008.04.005.

Small Heat Shock Proteins in Smooth Muscle

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Abstract

The small heat shock proteins (HSPs) HSP20, HSP27 and α B-crystallin are chaperone proteins that are abundantly expressed in smooth muscles are important modulators of muscle contraction, cell migration and cell survival. This review focuses on factors regulating expression of small HSPs in smooth muscle, signaling pathways that regulate macromolecular structure and the biochemical and cellular functions of small HSPs. Cellular processes regulated by small HSPs include chaperoning denatured proteins, maintaining cellular redox state and modifying filamentous actin polymerization. These processes influence smooth muscle proliferation, cell migration, cell survival, muscle contraction and synthesis of signaling proteins. Understanding functions of small heat shock proteins is relevant to mechanisms of disease in which dysfunctional smooth muscle causes symptoms, or is a target of drug therapy. One example is that secreted HSP27 may be a useful marker of inflammation during atherogenesis. Another is that phosphorylated HSP20 which relaxes smooth muscle may prove to be highly relevant to treatment of hypertension, vasospasm, asthma, premature labor and overactive bladder. Because small HSPs also modulate smooth muscle proliferation and cell migration they may prove to be targets for developing effective, novel treatments of clinical problems arising from remodeling of smooth muscle in vascular, respiratory and urogenital systems.

Keywords

α B-crystallin; actin; asthma; atherosclerosis; bronchodilator; chaperone; contraction; cytoskeleton; HSP20; HSP22; HSP27; relaxation; vasodilator; stress-response proteins

1. Introduction

1.1 Chaperones

The small heat shock proteins HSP20, HSP22, HSP27 and α B-crystallin are widely expressed chaperone proteins. Chaperones interact with other proteins to facilitate normal functions including maintaining cytoskeletal structure, maintaining normal redox conditions and regulating translation, each of which facilitates cell survival (Arigo et al., 2007). During exposure of cells to chemical stressors such as oxidative stress chaperones preserve protein function by preventing denaturation and promoting proper protein refolding once the stress is alleviated (Welsh and Gaestel, 1998; Bryantsev et al., 2007). Chaperones also participate in folding of newly synthesized polypeptides, and in formation of a variety of multi-subunit

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protein assemblies. Some HSPs maintain proteins in unfolded states suitable for translocation across membranes. They also stabilize inactive or damaged forms of proteins that are induced by cellular signaling. Chaperone proteins are divided into several families according to their size -HSP 100, 90, 70, 60 and the small heat shock/ α -crystallin proteins. The small HSPs are ATP-independent chaperones that exist in all organisms from bacteria to humans and are highly conserved (Jakob et al., 1993; Kappe et al., 2003). These proteins are essential for cellular viability following cellular stress, and their expression is frequently but not always increased by environmental stressors. One result of inducible expression is that organisms gain tolerance to changes in chemical and physical stimuli, in effect adapting to changes in the local environment of the cell. We suggest the small heat shock proteins contribute to smooth muscle function under normal conditions as well as enable smooth muscle to adapt to altered local environments in disease states. The review will focus on factors that alter small HSP expression, the macromolecular structure of small HSPs and the functions of small HSPs with an emphasis on the most frequently studied proteins - HSP27 and HSP20.

Rapid adaptation to the local environment is a major physiological role of smooth muscle cells. Force generated by smooth muscle cells dynamically stiffens the walls of the vasculature, airways and intestines to regulate flow of blood, air, food and waste products. In atherosclerosis, asthma and inflammatory bowel disease smooth muscle cells also undergo hyperplasia and hypertrophy as well as becoming secretory cells that exacerbate inflammation (reviewed by Johnson and Knox, 1997; Singer et al., 2004; Libby, 2003). In this review we summarize evidence that small HSPs are targets for signaling pathways that regulate major cellular processes that underlie the adaptive function of smooth muscle. Evidence is presented for a role of small HSPs in smooth muscle contraction, proliferation, cell migration and secretion of signaling proteins.

1.2 Functions of HSP27 and HSP20 in nonmuscle cells

Figure 1 illustrates some of the cellular processes influenced by HSP27 and HSP20. Expression of HSP27 in nonmuscle cells is inducible by heat and chemical stressors such as sodium arsenite or cytokines (Santell et al., 1992; Zhou et al., 1993). HSP27 acts as a chaperone by binding to denatured proteins to promote proper refolding in conjunction with higher molecular weight HSPs (Jakob et al., 1993). Human HSP27 also maintains intracellular levels of reduced glutathione (Mehlen et al., 1996; Preville et al., 1999; Salinthoné et al., 2007). The chaperone function of HSP27 and the maintenance of high glutathione levels confers tolerance to a variety of chemical stresses including heat, oxidative stress and proinflammatory signals (Landry et al., 1989; Mehlen et al., 1996).

In addition to protecting against cellular stress HSP27 has also been implicated in cell differentiation and apoptosis by associating with AKT, MK2 and p38 MAP kinase (O'Shaughnessy et al., 2007; Wu et al., 2007). This activity is apparently dispensable during embryonic development because mice in which the *hspb1* gene is replaced with LacZ develop normally (Huang et al., 2007). However, *Hspb1* gene disruption does reduce heat stress tolerance in the knockout animal compared to wild type animals consistent with previous studies of HSP27 in cellular stress tolerance.

Part of the mechanism by which HSP27 enhances stress tolerance is by phosphorylation-dependent stabilization of the actin cytoskeleton. Purified unphosphorylated HSP27 inhibits actin polymerization in vitro (Miron et al., 1991; Benndorf et al., 1994). Phosphorylation reverses the inhibition, presumably favoring formation of F-actin. This is supported by studies of cultured cells in which phosphorylation of HSP27 was necessary for growth-factor stimulation of F-actin formation (Lavoie et al., 1993b), stabilization of focal adhesions (Schneider et al., 1998), and promotion of endothelial cell migration (Rousseau et al., 1997).

Like HSP27 there is evidence HSP20 regulates actin cytoskeleton dynamics (Dreiza et al., 2005), but the full set of processes influenced by HSP20 is less well defined. One aspect of Hsp20 that distinguishes it from other members of the small HSP family is less efficient chaperone activity (van de Klundert et al., 1998a). However, Bukach et al., (2004) found recombinant HSP20 was as effective as alpha B crystallin in preventing insulin or alcohol dehydrogenase aggregation. HSP20 also acts extracellularly to inhibit platelet activation (Niwa et al., 2000), possibly by inhibiting calcium influx. In the brain HSP20 and HSP22 are both localized to various forms of plaque in Alzheimer's patients (Wilhelmus et al., 2006b; Wilhelmus et al., 2006a). The presence of HSP20 in nonfibrillar β -amyloid protein of senile plaques is thought to prevent protein denaturation (Wilhelmus et al., 2006b).

α B-crystallin is a major protein component of the mammalian lens (reviewed by Horwitz, 2003). Like other heat shock protein family members, α B-crystallin has chaperone properties that prevent precipitation of denatured proteins and increases cellular tolerance to stress (Dillmann, 1999; Head and Goldman, 2000; Liu and Steinacker, 2001). These are important functions that maintain lens transparency and prevent cataracts (Horwitz, 2003). α B-crystallin is expressed in a wide variety of cells where it forms multimeric complexes with other small HSPs and binds to many proteins with wide ranging functional effects. Details of the roles of α B-crystallin in the eye, muscle and brain are available in several excellent reviews (Dillmann, 1999; Head and Goldman, 2000; Liu and Steinacker, 2001; Horwitz, 2003).

2. Structure of HSP27 and HSP20

2.1 Domain features of small HSPs

Human HSP27 is a protein of 205 amino acids with the defining feature of a small HSP, an α crystallin domain at residues Glu⁸⁷ – Pro¹⁶⁸ (Figure 2) (Genbank No. NP 001531; Hickey et al., 1986). The α crystallin domain mediates dimerization, which is a common biochemical characteristic of small HSPs in solution (Lambert et al., 1999). An N-terminal hydrophobic domain containing a WDPF motif at Trp¹⁶ – Phe¹⁹ is immediately adjacent to a MAPKAP kinase (MK2) phosphorylation site at Ser¹⁵ (Lambert et al., 1999b). The WDPF motif is essential for chaperone activity and thermal tolerance conferred by HSP27 (Theriacault et al., 2004). The WDPF motif is linked to the α -crystallin domain by a region that varies in length depending on species. The linker contains two additional MK2 phosphorylation sites at Ser⁷⁸ and Ser⁸² in the human sequence. Phosphorylation of Ser⁷⁸ and Ser⁸² regulates polymer assembly and actin binding. The C-terminal 18–20 amino acids vary among species and are thought to form a flexible structure important in chaperone function. In a search for motifs common to actin binding proteins we noted the presence of the motif LSPEGTLTVEA in human HSP27 at amino acids 157–167. This motif is homologous with amino acid sequences in α -actinin, tensin, cofilin and focal adhesion kinase. In chicken tensin the LSPE motif is adjacent to the actin capping domain at Arg¹⁰³⁷-Val¹¹⁷² (Genbank No. CAA79215; Chuang et al., 1995). Whether the LSPE domain is important in interaction of HSP27 and actin in smooth muscle is unknown but it is consistent with the known effects of HSP27 to inhibit actin polymerization in vitro.

Human HSP20 is a 160 amino acid protein that also includes an α crystallin domain at residues Asp⁷⁰ – Ile¹⁴⁴ (Figure 2) (Genbank No. NP 653218; Kato et al., 1994). Two phosphorylation sites are present in the amino acid sequence. HSP20 is phosphorylated in vivo at Ser¹⁶ by cAMP-dependent protein kinase (PKA) and phosphorylation is increased by vasodilators (Beall et al., 1997; Beall et al., 1999). There is also a putative protein kinase C phosphorylation at Ser¹⁵⁷ in the rat sequence that appears to be stably phosphorylated and not well correlated with muscle contraction or relaxation (Meeks et al., 2005). Whether this second site modulates other functions of HSP20 such as multimer formation or chaperone activities is undefined, but is potentially significant given the functional importance of PKC in smooth muscle. Another

prominent feature of HSP20 is a sequence ¹¹¹GFVAREFHRRYRL¹²³ that is homologous to the inhibitory sequence of striated muscle troponin I (GKFKRPPLRRVRM) (Rembold et al., 2000). This motif has been proposed to inhibit smooth muscle contraction, possibly by interfering with binding of actin to myosin as troponin I does in striated muscles. A summary of the evidence addressing the function of HSP20 in regulating contraction is presented below (Section 4).

2.2 Macromolecular structures of small HSPs

Small HSPs exist in smooth muscle in macromolecular complexes ranging from dimers to multimers of 500–700 kDa. Multimeric complexes can contain more than one HSP as well as other binding partners (Brophy et al., 2000). Small HSP multimeric complexes are dynamically regulated by protein kinase signaling cascades that phosphorylate one or more sites on HSP27 and HSP20. The best defined pathways are cyclic nucleotide-dependent protein kinases that phosphorylate Ser¹⁶ of human HSP20 and the p38 MAP kinase/MK2 pathway that phosphorylates Serines 15, 78 and 82 of human HSP27. HSP22 contains PKC phosphorylation sites, is a substrate for PKC in vitro (Molloy and Andrews, 2001), but phosphorylation and regulation of multimers has not been described yet in smooth muscle cells. α B-crystallin is also phosphorylated in a variety of cells including cardiac muscle where phosphorylation is associated with translocation to the Z line (Golenhofen et al., 1998). However, regulation of the macromolecular structure by phosphorylation in smooth muscles is unexplored.

HSP27 assembles into multimers of varying sizes ranging from 50 kDa to 700 kDa with large (>500 kDa) multimers predominating (Figure 3). Stable dimers are thought to form through interactions of the α crystallin domain (Lambert et al., 1999). Multimers form from further interaction of nonphosphorylated dimers. Phosphorylation of Ser⁸² promotes dissociation of multimers (Benndorf et al., 1994), and additional phosphorylation of Ser¹⁵ may regulate interaction of dimers with actin filaments (Lambert et al., 1999). A model of multimer formation is shown in Figure 3. HSP27 monomers and dimers inhibit actin polymerization reactions in vitro and in vivo. This was shown by reduction of low-shear viscosity of F-actin (Miron T. et al., 1988), inhibition of F-actin polymerization in vitro (Benndorf et al., 1994) and immunofluorescence studies of F-actin in cultured cells (Miron et al., 1991; Lavoie et al., 1993b). Recent work on the mechanism of anthrax lethal toxin suggests HSP27 also sequesters G actin, which appears to be necessary for actin-based motility of neutrophils (During et al., 2007).

The small HSPs are thought to form heteromultimers in muscle tissue based on several observations. Large molecular mass complexes (200–500 kDa) are isolated from striated and smooth muscle extracts that contain HSP20, HSP27 and α B-crystallin (Kato et al., 1994; Brophy et al., 1999a; Pipkin et al., 2003). Small HSPs copurify from cell extracts (Kato et al., 1994; Pipkin et al., 2003). The composition and location of heteromultimers is probably dynamic. A good example is that heat stress and hypoxia cause HSP27 and α B-crystallin to move between the Z-disc and the I-band in cardiac myocytes (Lutsch et al., 1997; van de Klundert et al., 1998b; Golenhofen et al., 1999). Analogous translocation events are observed in smooth muscle where agonists stimulate translocation of HSP27 to membranes and the contractile apparatus (Ibitayo et al., 1999; Patil et al., 2004; Srinivasan et al., 2007).

Signaling pathways that regulate HSP27 phosphorylation are well defined and a current model is illustrated in Figure 3. Phosphorylation of HSP27 by MK2 is necessary for formation of F-actin after growth factor stimulation of nonmuscle cells (Lavoie et al., 1993b; Rousseau, 1997). MK2 activity is increased by phosphorylation by p38 MAPK. P38 MAPKs are activated by upstream activators MAP kinase kinases (MKK) 3, 4 and 6. Coupling between the MKKs and surface receptors is less certain. There are several protein kinases that function as MKK kinases (MKKK) upstream of p38 MAPK. In airway smooth muscle p21-activated protein

kinases (PAKs) appear to be obligatory enzymes coupling cytokine receptors to p38 MAP kinase phosphorylation (Dechert et al., 2001). It remains to be determined whether other protein kinases such as transforming growth factor-beta-activated kinase 1 (TAK1), apoptosis signal-regulating kinase 1 (ASK1) or mixed lineage kinases (MLK) also contribute to activation of the p38 MAPK/MK2/HSP27 pathway in smooth muscle. In addition to the well known role of p38 MAPK it is also known that protein kinase G (PKG) phosphorylates HSP27 at Thr¹⁴³ (Butt et al., 2001) in platelets. A T143E mutant reduces the stimulation of actin polymerization by S15D, S78D, S82D mutant HSP27. There is no evidence yet for or against this phosphorylation event in smooth muscles, but this is an obvious point of interest because cGMP and PKG signaling is fundamentally important in smooth muscle relaxation, proliferation and phenotype determination (Boerth et al., 1997; Lincoln et al., 2001; Zhou et al., 2007).

3. Regulation of expression of small HSPs in smooth muscles

Ten small heat shock proteins have been described in mammalian cells (Kappe et al., 2003) with three of them being prominently expressed in mammalian muscles (Table 1). HSP27, HSP20 and α B-crystallin have each been found in arterial (Beall et al., 1997; Kaida et al., 1999), venous (Negre-Aminou et al., 2002), airway (Larsen et al., 1997; Komalavilas et al., 2007), intestinal (Bitar et al., 1991), bladder (Batts et al., 2005) and uterine (Ciocca et al., 1996) smooth muscles. Many, but not all, heat-inducible proteins contain heat shock response elements (HSE) in the 5' untranslated region of the coding sequence (reviewed by Pirkkala et al., 2001; Trinklein et al., 2004). Heat shock response elements are characterized by inverted repeats of nGAAn that are binding sites for transcription factors called heat shock factors (HSF). HSF-1 is the predominant mammalian HSF. It exists as a monomer in unstressed conditions where it is chaperoned by heat shock factor binding protein B1 (HSFB1) as well as other proteins. Sequestration of HSF-1 by binding partners inhibits transcription of HSPs in unstressed cells. Stress induces dephosphorylation of negative regulatory sites at S303 and S307 of HSF-1. Dephosphorylated HSF-1 oligomers then bind to heat shock elements and increase transcription of the HSPs. There are also other unknown factors that upregulate heat-response genes based on a survey of heat responsive genes in HSF-1 knockout fibroblasts (Trinklein et al., 2004). Furthermore, not all genes with HSE motifs bind HSF-1 or are induced by heat shock. HSP27 was identified as HSF-1 dependent, but HSP20 and HSP22 genes were not identified in the genome-wide screen of HSF-dependent genes by Trinklein et al. (2004). Regulation of HSF activity in smooth muscle is underexplored, but could be fruitful. There is increasing interest in dynamic expression of small HSPs in cardiovascular and respiratory diseases.

3.1 HSP27

Expression of HSP27 in many cell types is dynamic. Expression can increase in response to physical and chemical stressors including heat, mechanical strain, oxidative stress and proinflammatory mediators. In contrast to nonmuscle cells, expression of small HSPs in striated and smooth muscle is frequently constitutive, but can also be modified by chemical or physical stressors. For example, several vasoactive hormones enhance expression of HSP27 in vascular smooth muscle. Vasopressin, thrombin and sphingosine 1-phosphate all induce HSP27 expression by a p38 MAPK-dependent mechanism (Kaida et al., 1999; Tanabe et al., 2001; Hirade et al., 2002). HSP27 expression is enhanced in vascular smooth muscle by physically restraining rats (Udelsman et al., 1993; Fuchs et al., 2000), probably via hormonal and neuronal stress-response signals. Upregulation of HSP27 in uterine smooth muscle occurs during pregnancy (White et al., 2005), which is consistent with hormonal signals controlling expression. However, in the uterus mechanical signals as well as changes in autonomic innervation might also influence expression. A role for mechanical stress in HSP27 expression is supported by a study of saphenous vein exposed to arterial pressure and flow in vitro

(McGregor et al., 2004). McGregor et al., (2004) found that increased mechanical stress induced HSP27 expression and decreased CapZ expression. The authors suggested that HSP27 induction might be part of the adaptation of the actin cytoskeleton of venous smooth muscle cells transplanted into an arterial bed during coronary bypass surgery.

Innervation of skeletal muscle, contractile activity and the extrinsic load on the muscle can all influence small HSP expression (Sakuma et al., 1998; Koh and Escobedo, 2004; Huey et al., 2004). In cardiac muscle ischemic preconditioning, chronic ischemia and chronic beta adrenergic overactivity during heart failure all induce expression of small HSPs (Gober et al., 2004; Fan et al., 2005). Whether denervation, muscle inactivity or oxidative stress produces similar changes in small HSP expression in smooth muscle is unknown. This question may be relevant to conditions such as pulmonary hypertension, paralytic ileus, preterm labor, and chronic drug treatments that impair smooth muscle contraction. It seems likely that chronically altered mechanical activity of smooth muscle cells will induce compensatory changes in small HSP expression that tends to reestablish normal muscle function.

3.2 HSP20

HSP20 expression in smooth muscles is also variable. Some vascular tissues, airway smooth muscle and uterine smooth muscles express HSP20 constitutively (Beall et al., 1997; Komalavilas et al., 2007; Cross et al., 2007), but other tissues including the urinary bladder of rabbits express little HSP20 (Batts et al., 2005). Dynamic changes in expression occur in cerebral vascular smooth muscle, rabbit bladder and rat myometrium under various conditions. The protein is expressed at measurable levels normally in rat cerebral arteries, but expression is inhibited and relaxation is impaired after subarachnoid hemorrhage (Macomson et al., 2002). In pregnant rats HSP20 expression in the uterus is higher early in gestation and diminishes substantially at term (Cross et al., 2007). Cross et al., (2007) suggested higher HSP20 levels might favor mechanical quiescence of the uterus early in pregnancy with increasing contractility late in pregnancy. The opposite pattern of expression emerges during remodeling of the obstructed urinary bladder. Low levels of HSP20 expression in the normal bladder are converted to substantial expression when the bladder is obstructed (Batts et al., 2006). Higher levels of HSP20 expression correlate with effective cyclic nucleotide-mediated relaxation. Low or no expression of HSP20 is associated with diminished cyclic nucleotide-induced relaxation, suggesting phosphorylation of HSP20 can be a dominant mechanism of smooth muscle relaxation. An interesting question is how consistent this correlation is among smooth muscle types? Another issue is whether HSP20 expression varies under different physiological and pathophysiological conditions such as hypertension, atherosclerosis, asthma, interstitial cystitis, postsurgical trauma or inflammatory bowel disease? Each of these states elicits chemical stress signals and/or mechanical stressors that could alter small HSP expression. Altered HSP20 expression then might alter physiological regulation of muscle relaxation or response to vasodilators and bronchodilators. Possible molecular mechanisms by which HSP20 effects contraction are being actively pursued as described below in Section 4.

3.3 Signal transduction

Small HSP expression levels are probably controlled in smooth muscles by a combination of transcriptional regulation via heat shock factors, control of mRNA stability and regulation of translation. In many cells expression and phosphorylation of HSF-1 is regulated by stress signals to allow trimerization, binding to the heat shock response elements in the 5' untranslated regions of HSP genes and transcription of HSP mRNAs (reviewed by Ankar and Sistonen, 2007). Little is known of HSF-1 in smooth muscle, although in one study of A10 vascular smooth muscle cells HSF-1 was knocked down using small interfering RNA, and expression of HSP27 was reduced (Chen and Currie, 2006). Upstream of HSF-1, p38 MAP kinases probably have important regulatory effects because HSP27 can be induced by vasoactive agents

that activate the p38 MAPK cascade including thrombin, vasopressin and sphingosine 1-phosphate (Ito et al., 2000; Kozawa et al., 1999; Hirade et al., 2005; Cao et al., 2006). Activation of p38 MAPK is known to activate HSF-1 as well as activate MK2, a protein kinase that enhances stability of mRNAs (Hung et al., 1998; Kotlyarov and Gaestel, 2002). Interestingly the p38 MAPK/MK2 signaling cascade is also the pathway that promotes phosphorylation of HSP27, dissociation of HSP27 multimers and increased F-actin formation in actin cytoskeletal remodeling (Figure 3). This suggests dual effects of p38 MAPK on both short term events promoting HSP27 phosphorylation and in longer term processes promoting HSP27 expression.

Constitutive expression of HSP27 in muscle is a notable difference between muscle and nonmuscle cells. There may be some fundamental cell-restricted differences in expression of HSF-1 and its control of HSP transcription. One possibility is that HSF-1 or HSF-2 oligomers are constitutively present in smooth muscle at low levels (Wilkerson et al., 2007). Another possibility is that chromatin remodeling machinery of smooth muscle allows chromatin in the HSE region to be relaxed allowing transcription to occur in the absence of stressors. There are no data in smooth muscles that address these issues yet, but the effect of histone deacetylase inhibition to enhance histone H3 acetylation and HSP26 expression in *Drosophila* is consistent with this hypothesis (Zhao et al., 2007). There is growing interest in using histone deacetylase inhibitors to treat cancer, inflammation and cardiac hypertrophy. It is possible some of the benefits of these agents are due to altering small HSP expression.

4. Functions of small HSPs in smooth muscles

In intact smooth muscle the small HSPs HSP27 and HSP20 probably regulates actin cytoskeleton structure and may modulate the interaction of actin and myosin. This is not surprising given the biochemical properties, the high levels of expression and cellular localization of these proteins in smooth muscle. HSP27 is constitutively expressed in smooth muscles at relatively high concentrations of 2 – 8 µg HSP27/mg total protein, (Miron T. et al., 1988; Hedges et al., 1999). HSP27 has been colocalized to contractile proteins in freshly dispersed intestinal smooth muscle cells stimulated with ceramide, and it coprecipitates with actin, tropomyosin and caldesmon suggesting some molecular association with contractile proteins (Ibitayo et al., 1999; Bitar et al., 2002; Somara and Bitar, 2004). Similarly, HSP20 is a relatively abundant protein that distributes broadly throughout smooth muscle cells and potentially interacts with both cytoskeletal proteins and with the actin and myosin crossbridges (Rembold and Zhang, 2001; Tessier et al., 2003).

4.1. HSP27 participates in contraction of smooth muscle

Bitar and colleagues provided the earliest evidence that HSP27 participates in smooth muscle contraction. They showed bombesin-induced contraction of permeabilized intestinal smooth muscle cells was inhibited by anti-HSP27 antibodies (Bitar et al., 1991). We subsequently showed anti-HSP27 antibodies also reduced endothelin-1 induced calcium-sensitization of chemically permeabilized canine pulmonary artery muscle (Yamboliev et al., 2000). Meloche et al. (Meloche et al., 2000) used SB203580 to block the p38 MAPK/MK2 pathway and block HSP27 phosphorylation in cultured rat aorta myocytes. We showed a similar effect of SB203580 in airway smooth muscle in blocking HSP27 phosphorylation during carbachol stimulation (Larsen et al., 1997). Brophy and coworkers have made similar observations correlating phosphorylation of HSP27 with contraction, or antagonism of relaxation in vascular smooth muscles (Beall et al., 1997; McLemore et al., 2005). Several groups have subsequently found that inhibiting the p38 MAPK/MK2/HSP27 signaling pathway reduces contraction in a variety of smooth muscles (Meier et al., 2001; Lee et al., 2007b; Macintyre et al., 2008). Overall, the data suggest HSP27 phosphorylation is sufficient to modulate contraction in many muscles but is not necessary for all agonists to elicit contraction. This is supported by an early study of rat aorta in which blocking HSP27 phosphorylation substantially inhibited angiotensin

II-induced contraction, but had no effect on phenylephrine-induced contraction (Meloche et al., 2000). While HSP27 phosphorylation appears to participate in contraction it is probably a parallel pathway that alters actin filament dynamics rather than a central control mechanism for altering actin-myosin crossbridge interaction. Effects on muscle contraction could be due to uncoupling crossbridges from actin attachment sites within the cell and at the cell membrane. There are no direct tests of this hypothesis, but the biochemical effects of HSP27 on actin and functional effects on cell migration and integrin-associated cytoskeletal remodeling described below suggest the idea has some merit.

4.2 HSP27 and cell migration

Contraction of smooth muscle depends on the presence of existing preformed F actin filaments. Contraction in response to agonists may also require G to F actin transformation. This is suggested by the fact that cytochalasin D partially, or completely blocks contraction in many smooth muscles (Wright and Hum, 1994; Saito et al., 1996; Mehta and Gunst, 1999; Jones et al., 1999; Rembold et al., 2007). Cytochalasin D acts at the plus end of uncapped, dynamic actin filaments to inhibit polymerization. However, G to F actin transition is not universally required under all conditions (Rembold, 2007). We have proposed that agonists that trigger actin remodeling employ HSP27 as one of the key modulators of this process (Gerthoffer and Gunst, 2001).

Migration of cells is another function that depends critically on G to F actin transition. Landry and coworkers first showed the p38 MAPK pathway was necessary in migration of vascular endothelial cells (Rousseau et al., 1997). We subsequently showed that activation of p38 MAP kinase leading to HSP27 phosphorylation is necessary for migration of tracheal smooth muscle cells (Hedges et al., 1999). Initial studies in airway smooth muscle showed the p38 MAP kinase signaling pathway mediates phosphorylation of HSP27 in response to serum, growth factors and cytokines (Larsen et al., 1997; Hedges et al., 1998). Blocking p38 MAPK activity with SB203580 inhibits activation of MK2, prevents phosphorylation of HSP27 and blocks smooth muscle cell migration (Hedges et al., 1999). Adenoviral overexpression of dominant negative p38 α MAP kinase and nonphosphorylatable HSP27 (S15A, S78A, S82A) inhibited cell migration completely. Expressing active MKK6 increased p38 MAP phosphorylation, HSP27 phosphorylation and cell migration. These studies established that activation of the p38 MAPK pathway by growth factors and proinflammatory cytokines promotes cell migration and that phosphorylation of HSP27 is necessary for migration. Subsequent investigations by other labs confirmed that the p38 MAPK/MK2/HSP27 pathway is necessary for migration of vascular smooth muscle cells (Iijima et al., 2002; Lee et al., 2007a), neutrophils (Jog et al., 2007), fibroblasts (Hirano et al., 2004) and breast epithelial cells (Kim and Kim, 2003). This pathway is also necessary for intracellular actin-based motility of *Listeria monocytogenes* (During et al., 2007), which emphasizes the broad significance of HSP27 phosphorylation in actin-mediated cell motility.

The exact mechanisms by which HSP27 regulates actin remodeling and cell migration is not clear. Because HSP27 interacts with a number of cytoskeletal and contractile proteins it is possible it has multiple molecular effects on cell structure. Miron et al. (1988) were the first to show interaction of HSP27 and actin. The authors found a homolog of HSP27 in chicken gizzard that inhibited new actin polymerization as well as promoted depolymerization of existing filaments. Later work on mammalian cell lines showed overexpressing HSP27 increased actin polymerization induced by fibroblast growth factor or thrombin (reviewed by Landry and Huot, 1999). HSP27 is commonly referred to as having actin capping activity that is relieved by phosphorylation. Where this occurs in the cell is unclear, but there is evidence of a functional association of HSP27 and integrin-mediated actin assemblies. A study by (An et al., 2004) investigated HSP27 interaction with the actin cytoskeleton using microbeads that

were anchored via RGD peptides to integrins and the cytoskeleton. Nanoscale (nm) motion of the beads was observed which are due to rearrangement of the microstructure of the actin cytoskeleton (Deng et al., 2004). An et al. (2004) showed bead motion increased when actin polymerization was blocked, suggesting untethering of actin and integrins. Induction of HSP27 phosphorylation by chemical stressors such as arsenite decreased bead motions which the authors interpreted as increased tethering of actin filaments and integrins. Furthermore, cells expressing constitutively active HSP27 exhibited reduced bead motions. This supports a model where phosphorylated HSP27 favors stable actin-integrin interactions and thus reduces RGD-microbead motion. Collectively, these data suggest HSP27 associates with integrins and the actin cytoskeleton in a phosphorylation-dependent manner as illustrated in Figure 3.

4.3 HSP27 and cell survival in smooth muscle

Cell proliferation depends on actin filament remodeling which allows detachment and rounding of cells. Cell survival also depends critically on the integrity of the actin cytoskeleton. Because HSP27 participates in actin remodeling it is not surprising it influences both proliferation and survival. HSP27 is anti-proliferative in nonmuscle cells and has antiapoptotic effects mediated by multiple aspects of apoptotic signaling including the Fas ligand/Daxx, caspase and mitochondrial pathways (Concannon et al., 2003; Beere, 2001). Champagne et al. (1999) studied apoptosis of vascular smooth muscle cells and found upregulation of HSP27 by heat shock did not prevent apoptosis, but did reduce necrotic cell death. This is in contrast to cultured human arterial smooth muscle cells in which HSP27 was knocked down with siRNA (Martin-Ventura et al., 2006). In the latter case, plasmin-induced apoptosis was significantly enhanced in the absence of HSP27. Recent work on human airway smooth muscle addressed the issue by surveying mitogenic and proapoptotic treatments and testing for effects of HSP27 overexpression on cell survival (Salinithone et al., 2007). Sodium nitroprusside, H₂O₂, and Fas antibody were effective inducers of smooth muscle cell death, but no evidence of apoptosis was observed suggesting cell death resulted from necrosis. Overexpression of wild type HSP27 decreased proliferation, increased cell survival and preserved glutathione levels in the face of high concentrations of H₂O₂. Blocking the p38 MAPK pathway to prevent HSP27 phosphorylation did not have any effect on ASM proliferation or death. The results support the notion that HSP27 may regulate airway smooth muscle cell proliferation and survival, but that p38 MAPK activation may not be necessary for the prosurvival effects. The literature to date suggests that like muscle contraction, the precise role of HSP27 in smooth muscle apoptosis and cell survival may depend on the tissue type and stimulus used to trigger cell death.

4.4 Functions of HSP20 in smooth muscle

The functions of HSP20 in vascular and airway smooth muscles have been described by recent work of Brophy and colleagues and Rembold and colleagues. The collective work from these groups shows phosphorylated HSP20 is a very effective inhibitor of smooth muscle contraction. Figure 4 is a model summarizing two proposed mechanisms – cyclic nucleotide-dependent depolymerization of F-actin and a direct inhibitory effect on actomyosin crossbridges. One of the more compelling features of HSP20 for smooth muscle physiology is that it is phosphorylated at Ser¹⁶ by either PKA or PKG, and phosphorylation is increased by vasodilators (Beall et al., 1997; Beall et al., 1999). HSP20 forms aggregates in carotid artery smooth muscle that coelute from gel filtration columns with HSP27 (Brophy et al., 1999a). Phosphorylated HSP27 inhibits phosphorylation of HSP20 by PKA in vitro (Fuchs et al., 2000). Binding of phosphorylated HSP20 to contractile proteins is thought to somehow inhibit force production. This was convincingly shown by transduction of a cell permeant HSP20 phosphopeptide mimic into intact vascular smooth muscle which induced relaxation (Woodrum et al., 2003; Flynn et al., 2003). Similar results have been obtained in a recent study of bovine tracheal smooth muscle (Komalavilas et al., 2007). Brophy and coworkers propose

that phosphorylated HSP20 inhibits smooth muscle contraction as part of the mechanism of action of nitrovasodilators and β -adrenergic agonists. It is well known that cyclic nucleotide-dependent relaxation is primarily a result of reducing intracellular calcium and dephosphorylation of 20 kDa myosin light chains. However, there are several reports of relaxation of smooth muscle that occurs independent of changes in myosin light chain phosphorylation (Gerthoffer et al., 1984; Gerthoffer, 1987; Rembold et al., 2000), suggesting some mechanism in addition to dephosphorylation inhibits or reverses force development. Phosphorylated HSP20 is a very likely candidate.

There are two nonexclusive mechanisms proposed for the relaxing effects of phosphorylated HSP20. It has been suggested to inhibit crossbridge formation directly via a troponin I like effect (Rembold et al., 2000; Yoshino et al., 2005). A troponin I like motif is clearly sufficient to relax smooth muscle, but there are no data testing the necessity of this mechanism. HSP20 might also inhibit transmission of force by decreasing actin polymerization (Dreiza et al., 2005). Inhibiting actin polymerization is sufficient to relax smooth muscles, but may not be necessary for relaxing all smooth muscles under all conditions. For example Meeks et al. (2005) showed forskolin relaxes swine carotid artery in the absence of detectable changes in F and G actin levels. HSP20 may well have both effects, acting in parallel as shown in Figure 4. Support for a cytoskeletal mechanism of relaxation comes from studies showing HSP20 interacts with actin, HSP27 and α -actinin (Brophy et al., 1999b; Tessier et al., 2003). Phosphorylated HSP20 also interacts with 14-3-3 proteins which are chaperones that bind phosphoproteins (Dreiza et al., 2005; Chernik et al., 2007). One of the binding partners of 14-3-3 proteins in smooth muscles is the slingshot phosphatase, which dephosphorylates and activates cofilin. Active cofilin promotes actin depolymerization, which should promote relaxation (Figure 4). An additional effect of HSP20 might be interaction with alpha actinin and disruption of critical actin crosslinks (Tessier et al., 2003). This might prevent proper actin attachment and transmission of force vectors from myosin motors to the extracellular matrix (Fig. 4). It is also possible that small heat shock proteins HSP27 and HSP20 simultaneously regulate actin filament dynamics, possibly by altering the number and characteristics of actin attachment sites at membrane dense plaques and cytoplasmic dense bodies in smooth muscles.

Effects of HSP20 on the cytoskeleton or on crossbridge function are not the only possible mechanisms of relaxation. HSP20 also alters calcium signaling in cardiac muscle and in platelets. In platelets HSP20 reduced agonist-induced calcium entry (Niwa et al., 2000), which if it occurred in a muscle cell should favor relaxation. However, in the myocardium HSP20 enhances rather than inhibits contraction (Fan et al., 2005; Islamovic et al., 2007). There are apparently cell-type differences in how HSP20 alters calcium handling, and whether it does so in smooth muscle is not known. Regardless of the exact mechanism(s) of action it is clear that phosphorylated HSP20 is an effective smooth muscle relaxant that might well prove to be a high value target for development of new vasodilators and bronchodilators.

5. Small HSPs in smooth muscle as therapeutic targets

5.1 HSP20 as a vasodilator and bronchodilator

Any drug that acts on the same target proteins as phosphorylated HSP20 (eg. F-actin and actomyosin) should have similar pharmacological profiles as drugs that increase cyclic nucleotides. Beta adrenergic agonists used to treat asthma work by increasing cAMP and activating PKA. Nitrovasodilators and phosphodiesterase inhibitors used to treat hypertension and erectile dysfunction respectively all act via cAMP or cGMP, which probably results in phosphorylation of HSP20 by PKA or PKG. Small molecules and phosphopeptides that mimic HSP20 should therefore be effective vasodilators and bronchodilators. Small peptide inhibitors that relax smooth muscle have been described (Flynn et al., 2003), but animal studies and human trials demonstrating vasodilation or bronchodilation in vivo have not yet been reported.

Several challenges arise during development of peptide vasodilators – one is a problem drug delivery of peptides to the sites of action, and another is manufacturing costs of peptide or protein drugs. It may be very useful to discover or design small molecular inhibitors that mimic phosphorylated HSP20 and have suitable pharmacokinetic and toxicity characteristics. Some progress has been made showing proof of principle of cell permeant peptide mimics (Flynn et al., 2003), but the cost and drug delivery issues are not fully addressed yet.

There is great theoretical appeal to adding a fundamentally new approach to elicit smooth muscle relaxation, particularly in clinical situations where vasospasm is unresponsive to existing vasodilators. Another potentially important clinical application would be developing mechanistically novel bronchodilators for use in pulmonary medicine. Bronchodilation by local delivery of drugs that mimic phosphorylated HSP20 to the airways would be useful in treating obstructive airways disease. However, an important caveat is that the role of HSP20 in epithelial and endothelial barrier function is undefined. Many of the same cytoskeletal and contractile proteins participate in both smooth muscle and nonmuscle cell contraction and migration, so the potential for off target effects is a reasonable concern. In addition to vascular and airway smooth muscle, bladder smooth muscle might be an interesting target. Drugs inhibiting overactive urinary bladder smooth muscle work primarily via altering neurotransmission. A more direct smooth muscle inhibitor would add to the very brief list of currently used drugs. Future translational work on developing direct acting smooth muscle relaxants will be welcome, and may be highly significant for clinical problems where inhibiting smooth muscle contraction is a therapeutic goal.

5.2 HSPs and atherosclerosis

A recent review by Libby and colleagues suggests that biomarkers of inflammation are becoming indicators of atherosclerosis, and are as useful clinically as plasma lipid profiles (Packard and Libby, 2008). C-reactive protein is a well studied marker, but additional candidates are being touted, including HSP27 (Martin-Ventura et al., 2004; Duran et al., 2007). Soluble HSP27 is released from arterial tissue, and release is lower from plaque than from normal vessel wall (Martin-Ventura et al., 2004). Plasma levels of soluble HSP27 were also lower in patients with atherosclerosis compared to healthy controls. Park et al. (2006) reported lower HSP27 levels in plaques, lower levels of phosphorylation of HSP27 and higher plasma levels of secreted HSP27 in humans with acute coronary syndrome. The functional significance of these observations is unclear, but important questions are raised about the contribution of HSP27 in atherogenesis. Soluble small HSPs may act as auto-antigens that exacerbate the immune response (Mehta et al., 2005) in addition to acting intracellularly to allow smooth muscle cells to adapt to a change in local environment. Because HSP27 has anti-proliferative effects, reduced expression in the presence of growth factors and inflammatory mediators might favor smooth muscle growth and perhaps contribute to plaque formation. One approach to counter these effects would be to enhance or maintain normal levels of expression of small HSPs. A recent report showed resveratrol enhances HSP27 expression in cultured human aortic smooth muscle and reduces proliferation (Wang et al., 2006). Further studies of the predictive value of soluble HSP27 levels in atherosclerosis and the benefit of agents that modify small HSP expression are warranted. In addition, it will be useful to know if HSPs are secreted from other smooth muscle tissues and thereby contribute to inflammation in asthma, inflammatory bowel disease, interstitial cystitis and uterine dysfunction. Modifying heat shock protein expression and secretion may prove to be beneficial in a wide range of clinical problems in which inflammation occurs around smooth muscle cells.

Abbreviations

HSE, heat shock response element; HSF, heat shock factor; HSP, heat shock protein; MAP kinase, mitogen-activated protein kinase; MK2, MAP kinase-activated protein kinase 2; MKK, MAP kinase kinase; PAK, p21-activated protein kinase; PKA, cyclic AMP-dependent protein kinase; PKG, cyclic GMP-dependent protein kinase.

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Figure 1. Cellular processes modified by HSP20 and HSP27 in smooth muscles

Multiple extracellular signals impinge on smooth muscle cells to modify the macromolecular structure of small HSPs. Key protein kinases are shown that catalyze phosphorylation of these proteins which changes their molecular associations thus modifying a variety of downstream processes. HSP27 phosphorylation is catalyzed by MAPKAP kinase 2 (MK2) and HSP20 phosphorylation is catalyzed by cyclic nucleotide-dependent protein kinases (PKA and PKG). HSP27 has been shown to promote muscle contraction, increase cell migration and enhance cell survival under stress conditions. Activation of HSP20 causes smooth muscle relaxation and is cardioprotective.

A. HSP27

B. HSP20

Figure 2. Domain structure of human HSP27 and HSP20

A. The N terminus of HSP27 contains a WDPF motif (grey box) necessary for multimer formation and may influence actin binding. Multimers are reduced to smaller complexes and ultimately to dimers by phosphorylation of consensus MK2 phosphorylation sites at Serines 15, 78 and 82. HSP22 has been shown in cardiac muscle to bind phosphorylated HSP27. The C terminus includes an α -crystallin motif (black box) that is highly conserved among species. The crystallin domain is necessary for dimer formation. The extreme C-terminus is a flexible region of the sequence which varies with species. **B.** HSP20 contains an α -crystallin motif (black box) highly homologous to that in HSP27. Serine 16 is an important site for phosphorylation by cyclic nucleotide-dependent protein kinases (PKA and PKG). A motif similar to the minimal inhibitory region of cardiac troponin I is present at residues Gly¹¹¹-Leu¹²³. In rat HSP20 there is also a PKC phosphorylation site at Ser¹⁵⁷ that is not conserved in human HSP20.

Figure 3. Signaling pathways regulating HSP27 structure and function

HSP27 is phosphorylated in response to diverse stimuli in smooth muscles including neurotransmitters, cytokines and growth factors. These agents act via GPCR and tyrosine kinase receptors to activate the p38 MAP kinase cascade culminating in activation of MK2. MK2 and homologs MK3 and MK5 phosphorylate HSP27 thus promoting dissociation from large macromolecular assemblies. Only a 20 mer and dimer is shown in figure, but there is a range of complexes with Mr from 50 to >500 kDa. The complexes are not necessarily homomultimers, but can include several other small HSPs such as HSP20 and α B-crystallin. Phosphorylation of HSP27 favors stabilization of F-actin filaments by undefined molecular mechanisms. One notion is HSP27 acts as a capping protein and phosphorylation dissociates the cap thus allowing polymerization to proceed. HSP27 may also sequester G actin. F-actin formation is necessary to support contraction, cell migration and a variety of biochemical processes that control cell redox state, focal contact formation and cell survival.

Figure 4. Hypothesized mechanisms of smooth muscle relaxation by HSP20

Agents that inhibit contraction by cyclic nucleotides may do so in part by activating adenylate cyclase (AC) which in turn activates protein kinases A and G (PKA, PKG). HSP20 is phosphorylated on Ser16 by these kinases, and phosphorylated HSP20 is sufficient to relax smooth muscle, in some cases independent of changes in myosin light chain phosphorylation. Two proposed mechanisms for relaxation are: 1. Depolymerization of actin via an indirect activation of cofilin involving activation of slingshot phosphatase. Phosphorylated HSP20 is thought to compete with slingshot for binding to 14-3-3 proteins. Unbound slingshot keeps cofilin active and favors actin depolymerization. 2. HSP20 has also been proposed to interact with and inhibit actomyosin by virtue of a troponin I motif.

TABLE 1

Small heat shock proteins expressed in smooth muscles

HUGO Gene Name	Common name	Functions
HSPB1	HSP27	Chaperone, regulates F-actin formation, cell migration, anti-apoptotic
HSPB2	MKBP	Myotonic dystrophy kinase binding protein, function in smooth muscles is undefined
HSPB5	α B-crystallin	Forms multimers with other small HSPs
HSPB6	HSP20	Regulates F-actin, cytoskeletal structures, smooth muscle relaxation
HSPB8	HSP22, H11 kinase	Cardioprotective, cardiac hypertrophy, function in smooth muscle is undefined