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Cell-permeant peptide inhibitors of vasospasm and intimal hyperplasia

Michael J Osgood*, Charles R Flynn*, Padmini Komalavilas*,†, and Colleen Brophy*,†

*Department of Surgery, Vanderbilt University Medical Center

†VA Tennessee Valley Healthcare System, Nashville, TN 37232, USA

Abstract

Outcomes from vein graft bypass are limited by graft failure, leading causes of which include intimal hyperplasia and vasospasm. Intimal hyperplasia remains the most common cause of graft failure, but no therapeutic modalities have been shown to prevent intimal hyperplasia in humans. The small heat shock proteins are a class of naturally occurring proteins in vascular smooth muscle. These proteins have an integral role in maintenance of vascular tone and in cellular defense against various stressors. Transduction domains have enabled intracellular therapeutic delivery of peptide analogs of heat shock proteins, as well as peptide inhibitors of the kinases that phosphorylate these proteins. These cell-permeant peptides have been shown to prevent vasospasm and intimal hyperplasia *in vitro*. Since vascular bypass using vein grafts is analogous to autologous organ transplantation, *ex vivo* treatment of the vein graft with cell-permeant peptide inhibitors of vasospasm and intimal hyperplasia prior to implantation provides a unique opportunity for targeted treatment of the graft to improve patency.

Keywords

intimal hyperplasia; vasospasm; cell-permeant peptide; saphenous vein; heat shock protein; MAPKAP kinase 2

Introduction

Vascular and cardiac surgeons continue to use the saphenous vein more than all other conduits for peripheral and coronary revascularization procedures. However, the rate of vein

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Correspondence to: Michael J Osgood, 1161, 21st Avenue South, D-4314 Medical Center North, Nashville, TN 37232-0011, USA; michael.j.osgood@vanderbilt.edu.

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graft failure has remained unchanged over the past 50 years, and 30–45% of autogenous grafts will fail or develop flow-limiting stenosis requiring revision at 4–12 months.¹ Intimal hyperplasia remains the leading cause of graft failure.² While incompletely understood, intimal hyperplasia results from a cascade of molecular and cellular events. The robust occurrence of intimal hyperplasia near anastomotic areas of the graft, and therefore near areas of vascular injury, suggests that intimal hyperplasia may represent a response to the injury that occurs during surgical harvest, the degree of which may be proportional to the degree of injury.^{3,4} The cumulative effects of tissue injury during vein preparation and surgical bypass lead to impaired endothelial function,^{5,6} de-endothelialization,⁷ impaired smooth muscle vasomotor function,^{8,9} vasospasm¹⁰ and intimal hyperplasia.¹¹ Despite significant work in the area, no therapeutics have been successful in ameliorating intimal hyperplasia in humans. Novel peptide therapeutics have been recently developed which are beginning to show promise in prevention of vasospasm and inhibition of intimal hyperplasia in experimental models. However, because their target signaling pathways are intracellular, delivery of these synthetic peptides into the cell initially posed a challenge. Just as vascular surgeons have never regarded the skin as a significant barrier to the surgical management of vascular pathology, the cell membrane may be regarded as an insignificant obstacle to the intracellular delivery of peptide-based therapeutics. Instead, the ‘knife’ used to gain entry to the cell is actually a peptide borrowed from the human immunodeficiency virus (HIV).

Phosphopeptide analogs of the small heat shock proteins, as well as a group of kinase inhibitors that regulate heat shock protein phosphorylation, are emerging as a promising group of peptide-based therapeutics capable of inhibiting vasospasm and intimal hyperplasia. The heat shock proteins regulate intracellular signaling pathways in vascular smooth muscle that modulate vascular tone and cytoskeletal dynamics and coordinate the cellular response to stress. HSP27 and HSP20 are members of the family of small heat shock proteins. Phosphorylation of HSP27 is associated with inhibition of smooth muscle relaxation, stabilization of the actin cytoskeleton and enhanced smooth muscle migration.^{12–15} In contrast, phosphorylation of HSP20 is associated with smooth muscle relaxation, disruption of actin stress fibers and focal adhesion complexes and inhibition of smooth muscle migration.^{16–20} Thus, HSP27 and HSP20 appear to have opposing functions relative to cytokinetic processes in smooth muscle. Therapeutic delivery of synthetic phosphopeptide analogs of the small heat shock proteins is attractive in that endogenous intra-cellular protective responses can be recapitulated. Peptide sequences originally derived from HIV have been adapted to facilitate intracellular transduction when linked to a phosphopeptide sequence of therapeutic interest. These agents may become powerful adjuncts with the potential to improve the success of surgical bypass using saphenous vein grafts.

In this review, we address the role of the small heat shock proteins both in normal smooth muscle physiology and in the pathophysiological states of intimal hyperplasia and vasospasm, we describe the creation of cell-permeant peptides, and we summarize the experimental data that support the therapeutic application of cell-permeant peptides in the prevention of vasospasm and vein graft intimal hyperplasia.

Role of the small heat shock proteins in vascular smooth muscle function

Vascular smooth muscle is becoming increasingly recognized as a dynamic entity capable of rapid structural and functional changes in response to external stimuli.²¹ Functional and structural adaptation appears to be mediated through cytoskeletal reorganization and modulation of contractile pathways. These responses are facilitated by integrins and focal adhesion proteins, FAK/Src family tyrosine kinases, paxillin, Rho family GTPases and small heat shock proteins.²¹ Heat shock proteins have emerged as a class of proteins that are expressed in response to physiological stressors. The expression of heat shock proteins was first demonstrated in *Drosophila* by exposure to high temperatures,²² and the expression of these proteins has since been demonstrated to occur secondary to vessel wall injury, oxidative stress, circulating catecholamines, hypoxia, osmotic stress, ischemia/reperfusion, heavy metals and aging.^{23,24}

Small heat shock proteins, a subclass of heat shock proteins, are so-named due to their molecular mass (20–30 kDa). This class includes HSP27 and HSP20, which are highly constitutively expressed in smooth muscle.²⁵ HSP27 (also termed HSPB1 and HSP25) is a central mediator of actin cytoskeletal organization, and its effects are dependent on its phosphorylation status. When phosphorylated by a kinase cascade involving p38 mitogen-activated protein kinase (MAPK) and MAPK-activated protein kinase 2 (MAPKAP kinase 2), HSP27 is associated with inhibition of smooth muscle relaxation, stabilization of the actin cyto-skeleton, and enhanced smooth muscle migration, effects associated with the phenotypic changes in smooth muscle cells in hyperplastic lesions (Figure 1).^{12–15} Aside from its effects on actin cytoskeletal organization, HSP27 exerts multiple cellular protective effects. HSP27 acts as a molecular chaperone to facilitate refolding of denatured proteins,²⁶ and facilitates enzymatic reactions which maintain adequate intracellular levels of reduced glutathione, thereby preventing oxidative stress.^{27,28} Moreover, HSP27 appears to confer resistance to inflammatory mediators, including interleukins and tumor necrosis factor.^{29,30} Many of these protective properties appear to be inhibited by phosphorylation of HSP27. Preventing its phosphorylation, therefore, is an attractive therapeutic option.

Activation of cyclic nucleotide-dependent signaling pathways in vascular smooth muscle (Figure 1) converge at the phosphorylation of another small heat shock protein, HSP20 (also termed HSPB6).³¹ Vascular smooth muscle relaxation in response to nitric oxide (NO) and nitrovasodilators is mediated through activation of guanylate cyclase, which causes increased cyclic guanosine monophosphate (cGMP) and activation of cGMP-dependent protein kinase G (PKG).³² Alternatively, vascular smooth muscle relaxation occurs in response to prostaglandins and forskolin and this response is mediated by activation of adenylate cyclase, which causes increased cyclic adenosine monophosphate (cAMP) and activation of cAMP-dependent PKA.³³ Activation of these pathways mediates phosphorylation of HSP20, which is associated with smooth muscle relaxation, disruption of actin stress fibers and focal adhesion complexes, and inhibition of smooth muscle migration.^{16–20}

Role of the small heat shock proteins in the pathophysiology of vasospasm

Vasospasm remains a multifactorial and incompletely understood response to vein graft manipulation and surgical preparation. Inciting events may include pharmacological mechanisms of vasoconstriction, physical stimuli such as mechanical stretch or distention, and temperature changes.³⁴ Vasospasm may result from impaired relaxation of vascular smooth muscle, or alternatively may represent an exaggerated form of vasoconstriction. In support of the latter hypothesis, multiple triggers have been suggested. Potential contributing factors may include a combination of pharmacological and physical stimuli.³⁴ Pharmacological stimuli may include endogenous vasoconstrictive agents released during surgical graft preparation such as endothelin-1, thromboxane A₂, prostaglandin F₂, 5-hydroxytryptamine, histamine, acetylcholine (which has vasoconstrictive effects when endothelium is dysfunctional or denuded), norepinephrine, phenylephrine, potassium ions and angiotensin II.^{34,35}

Several lines of experimental data support the notion that vasospasm is more likely to result from impaired relaxation of vascular smooth muscle rather than from exaggerated vasoconstriction. Endothelial dysfunction and/or denudation caused by vein graft preparation and handling may induce loss of endothelial-dependent vasorelaxation.³⁶ Smooth muscle-dependent relaxation also appears to be blunted by surgical vein graft preparation, and these effects may be mediated in part by heat shock proteins. Smooth muscle-dependent vasorelaxation is mediated by cyclic nucleotide-dependent signaling pathways. Heat shock proteins have been implicated in the pathophysiology of vasospasm based on data from experimental models. Activation of cAMP- and cGMP-dependent signaling pathways converge on HSP20 and cause its phosphorylation.^{25,31} Moreover, endothelial-dependent relaxation of vascular smooth muscle has been demonstrated to correlate with increased phosphorylation of HSP20.³⁷ Stress-induced hypertension has been demonstrated to cause increased expression of HSP27 in vascular tissues of rats,³⁸ and this same stimulus decreases relaxation of aortic rings from borderline hypertensive rats.²⁴

Impaired vascular relaxation has been linked to altered phosphorylation of heat shock proteins. HSP27 phosphorylation has been demonstrated to increase in response to stressors and this response correlates with increased vascular smooth muscle tone; phosphorylated HSP27 then inhibits phosphorylation of HSP20, and this correlates with impaired vasorelaxation.^{24,39} The exact mechanism linking HSP27 and HSP20 with vascular smooth muscle contractility remains unknown. The fact that HSP27 and HSP20 coexist in macromolecular aggregates,²⁵ and are associated with the myosin light chains^{40,41} and with actin,^{40,42,43} suggests that these proteins may directly modulate the contractile elements of the cell. It remains to be determined whether these proteins directly modulate the contractile machinery or whether they modulate other elements or signaling pathways affecting the contractile machinery of smooth muscle.⁴⁴

Role of the small heat shock proteins in the pathophysiology of intimal hyperplasia

Intact endothelium produces NO, which has antithrombotic, anti-inflammatory, antiproliferative and vasodilatory effects. These properties may cumulatively inhibit intimal hyperplasia. NO additionally activates guanylate cyclase and it has been suggested that this property may confer additional resistance to intimal hyperplasia. As described earlier, activation of cGMP signaling pathways by NO causes phosphorylation of HSP20, and this response is correlated with smooth muscle relaxation, disruption of actin stress fibers and focal adhesion complexes, and inhibition of smooth muscle migration. Similarly, activation of guanylate cyclase by NO inhibits vascular smooth muscle proliferation and migration.^{45–47} Increased levels of cyclic nucleotides prevent conversion of vascular smooth muscle to a proliferative phenotype.^{48–51} Rat aortic smooth muscle cells undergo phenotypic modification from contractile to proliferative phenotype in culture, resembling the phenotypic modification occurring in smooth muscle cells in hyperplastic lesions *in vivo*. This change is associated with reduced expression of both PKG and HSP20. After transfection with PKG, it has been shown that these cells revert to a contractile phenotype and subsequently express both PKG and HSP20.^{31,49,52} Activation of cyclic nucleotide-dependent pathways with a cyclic nucleotide phosphodiesterase 3 inhibitor has been shown to inhibit intimal hyperplasia *in vivo* in a rat balloon double-injury model.⁵³ Furthermore, reduced levels of cGMP have been demonstrated in hyperplastic lesions in vascular tissue from rabbits⁵⁴ and humans.⁵⁵ Therefore, multiple lines of data support a central role of deficiencies in both cGMP signaling and HSP20 phosphorylation in the pathogenesis of intimal hyperplasia.

The cellular effects of phosphorylated HSP20 are opposed by phosphorylation of HSP27. In its dephosphorylated form, HSP27 exerts multiple cellular protective effects: it acts as a molecular chaperone to facilitate refolding of denatured proteins, maintains adequate intracellular levels of reduced glutathione, reduces oxidative stress and confers resistance to inflammatory mediators. However, phosphorylation of HSP27 has been correlated with enhanced smooth muscle cell migration,^{15,56} stress fiber formation,^{14,57} and extracellular matrix deposition,^{58–60} all of which are seminal events in the pathogenesis of intimal hyperplasia. Stress fiber formation has been correlated with conversion of vascular smooth muscle cells from the contractile to the synthetic phenotype.⁶¹

Origin and creation of cell-permeant peptides

One of the fundamental problems associated with protein and peptide-based therapeutics is that peptides do not cross the cell membrane. Gene therapy has been advocated as an approach to introduce molecules into cells. This approach involves transfecting cells with a gene that produces a specified protein. For example, transfection of inducible NO synthase has been demonstrated to reduce intimal hyperplasia in porcine vein graft models.⁶²

However, in many cases, the effectiveness of gene therapy has been limited by immunogenicity of the viral vectors, targeting the vectors to the appropriate cells, and the regulation of expression of the gene so that the correct amount of protein is produced by the cell. Finally, with gene therapy there is a delay in the expression of the target protein. Many

of these problems can be obviated by directly introducing the target protein into the cell using cell-permeant peptides, a process which relies on fewer steps for intracellular delivery of the therapeutic agent of interest (Figure 2).

There are a variety of natural peptides that are cell-permeant. These peptides act as a 'carrier' domain to carry the attached 'cargo' into the cell. Transduction of proteins into cells was first described in 1988 independently by Green and Loewenstein⁶³ and Frankel and Pabo,⁶⁴ with the discovery that an HIV protein, transactivator of transcription (TAT), can cross cell membranes. Subsequent studies demonstrated that an 11-amino-acid domain of TAT (YGRKKRRQRR) could be used to efficiently transfect the enzyme lacZ into cells in animal models without apparent toxicity.⁶⁵ This peptide has been optimized (YARAAARQARA) to deliver a variety of therapeutics.⁶⁶ There are additional naturally occurring cell-permeant peptides including the antennapedia motif (RQIKIWFQNRRMKWKK) derived from a family of *Drosophila* homeoproteins. The herpes simplex virus (HSV) VP22 protein is a small basic protein that is also directly taken up by cells. A common feature of these peptides is a high arginine and lysine content. In fact, hepta arginine (RRRRRRR) functions as a cell-permeant peptide. Discovery of the TAT protein has enabled synthesis of peptides containing the TAT domain to enable intracellular peptide transduction. These cell-permeant peptide domains have been engineered to deliver peptides that have properties that prevent vasospasm and intimal hyperplasia.

Cell-permeant peptides inhibit vasospasm

The observation that phosphorylation of HSP20 correlates with improved relaxation of vascular smooth muscle led to efforts to deliver synthetic phosphopeptide analogs of HSP20 to vascular smooth muscle for therapeutic purposes. HSP20 is activated by phosphorylation of serine 16.³¹ *De novo* synthesis of a peptide containing optimized TAT linked to the phosphopeptide actin-binding domain of HSP20 (TAT-pHSP20) resulted in a cell-permeant peptide that relaxes vascular smooth muscle.^{16,67} This peptide is efficiently transduced by vascular smooth muscle (Figure 3). Preincubation of HSV rings with TAT-pHSP20 has been shown to prevent norepinephrine-induced contraction in a muscle bath suspension.⁶⁷ Moreover, TAT-pHSP20 induces relaxation of HSV tissue precontracted with norepinephrine, and this response is dose-dependent.⁶⁷ Similar findings have been replicated in porcine coronary artery,¹⁶ rabbit aorta,⁶⁷ and human umbilical artery smooth muscle.¹⁷ The *ex vivo* preparation of the saphenous vein graft for surgical bypass provides an ideal opportunity for administration and delivery of TAT-pHSP20 in a preservative solution prior to surgical implantation. In addition, TAT-pHSP20 has been shown to inhibit platelet aggregation in citrated human whole blood by an unknown mechanism.⁶⁷ Therefore, this agent has numerous potential benefits for vein graft preparation and has promising applications for prevention of early vein graft failure from vasospasm and thrombosis.

Cell-permeant peptides prevent intimal hyperplasia

Organ culture experiments using human saphenous vein have shown that the TAT-pHSP20 peptide inhibits intimal hyperplasia after 14 days in culture.²⁰ The intimal thickness and

intimal-to-medial ratio of HSV rings treated with TAT-pHSP20 (10 μ mol/L) at every culture medium exchange (every 2–3 days) was similar to that of the pre-culture rings, while untreated HSV rings grown in fetal bovine serum alone developed a significantly greater degree of intimal and medial thickening. These authors demonstrated efficiency of transduction of the TAT-pHSP20 peptide of greater than 90%. Rat embryonal aortic A10 smooth muscle cells demonstrated significantly reduced migration when treated with TAT-pHSP20 compared with untreated control cells, suggesting that inhibition of smooth muscle migration or inhibition of phenotypic conversion may account for the protective effects of this compound on intimal hyperplasia.²⁰

Inhibition of HSP27 phosphorylation is an attractive therapeutic modality because phosphorylation inhibits the cellular protective effects of this molecule, prevents vascular smooth muscle relaxation and additionally prevents phosphorylation of HSP20. HSP27 is phosphorylated by MAPKAP kinase 2. MK2i, a cell-permeant peptide inhibitor of MAPKAP kinase 2, has been demonstrated to inhibit intimal hyperplasia in an organ culture model of human saphenous vein.⁶⁸ MK2i (also known as MMI-0100) has additionally been demonstrated to inhibit intimal hyperplasia *in vivo* in a murine aortic bypass model.⁶⁹ Human saphenous vein rings treated with MK2i (10 μ mol/L) at every medium exchange developed 48% less intimal thickening in organ culture compared with control rings.⁶⁸ These authors also demonstrated that two-hour pretreatment of A7R5 rat aortic smooth muscle cells with MK2i inhibited migration in culture. Proteomic analysis of human dermal keloid fibroblasts treated with transforming growth factor (TGF)- β alone (which stimulates HSP27 expression) or TGF- β + MK2i demonstrated reduced expression of the extracellular matrix components fibronectin and collagen when MK2i was administered with TGF- β . Treatment of HSV segments with MK2i in organ culture was additionally associated with reduced expression of connective tissue growth factor. Therefore, inhibition of phosphorylation of HSP27 by MK2i confers inhibitory effects on smooth muscle cell migration and extracellular matrix production, both of which are key inciting events in the development of intimal hyperplasia.

Summary

Small heat shock proteins have an integral role in the maintenance of vascular tone and smooth muscle homeostasis. Aside from their role in regulation of vascular tone, these proteins serve a protective role in the response to multiple cellular stressors. The most extensively studied small heat shock-related proteins in vascular tissue are HSP20 and HSP27. These proteins have complementary protective roles in physiological conditions, but play opposing and detrimental roles in pathophysiological conditions. The cellular milieu in response to the mechanical, osmotic, ischemic and pro-oxidant stressors of surgical removal, ‘back table’ manipulation and storage, and surgical reimplantation favors exaggerated cellular responses including vasospasm and intimal hyperplasia. If a therapeutic agent is to be successful in preventing intimal hyperplasia, it must inhibit a central mediator of the multiple cellular stressors occurring at the time of surgical bypass.

The cell-permeant peptides reviewed in this article are promising agents for improving outcomes from vein graft bypass. Experimental data demonstrate that these agents induce

vasodilation and prevent vasospasm via direct effects on the smooth muscle. They have also been shown to prevent platelet aggregation. Moreover, these agents prevent phenotypic modulation and migration of vascular smooth muscle cells and inhibit production of extracellular matrix components, which are sentinel events in the formation of hyperplastic vascular lesions. The *ex vivo* manipulation of the saphenous vein conduit after surgical harvest provides a prime opportunity for therapeutic administration of these agents. While the experimental data summarized herein are promising, these agents lack human experimental data demonstrating an improvement in vein graft patency. Preclinical and clinical evaluation of these molecular entities is ongoing.

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Figure 1.

Cellular responses to relaxation and stress mediated by the small heat shock proteins, HSP20 and HSP27. Nitric oxide (NO) and nitrovasodilators activate guanylate cyclase (GC), leading to increased levels of cyclic guanosine monophosphate (cGMP), which activates cGMP-dependent protein kinase G (PKG). Prostaglandins (PGs) and forskolin (FSK) activate adenylate cyclase (AC), leading to increased levels of cyclic adenosine monophosphate (cAMP), which activates PKA. PKG and PKA both phosphorylate HSP20, which is associated with disruption of actin stress fibers and enhanced smooth muscle relaxation. Cellular stressors stimulate P38 mitogen-activated protein kinase (MAPK), which activates MAPK-activated protein kinase 2 (MAPKAP kinase 2), leading to phosphorylation of HSP27. Phosphorylated HSP27 is associated with inhibition of smooth muscle relaxation, stabilization of the actin cytoskeleton, and enhanced smooth muscle migration. Inset (a) is a representative figure illustrating the disruption of actin stress fibers (red staining) occurring with smooth muscle relaxation induced by activation of cyclic nucleotide-dependent pathways. Inset (b) is a representative figure illustrating the stabilization of the actin cytoskeleton (red staining) occurring with phosphorylation of HSP27 and subsequent smooth muscle contraction

Figure 2.

Protein transduction versus gene therapy. Both modalities initially require cloning the gene into an expression vector. In addition, gene therapy (black arrows, top) requires: (1) packaging the vector in a virus, (2) infecting the target cell, (3) viral escape from vesicles, (4) viral replication and nuclear localization, (5) integration of the gene in the nucleus, (6) transcription, (7) mRNA export, (8) translation and (9) post-translational modification to produce the final active protein. Protein transduction (red arrows, bottom), bypasses these steps as the DNA is translated into protein *in vitro* (1) and introduced directly into the cell (2–3)

Figure 3.
Transduction of fluorescein isothiocyanate-labeled transactivator of transcription-pHSP20 in
a rat carotid artery