Eur J Vasc Endovasc Surg **25**, 40–47 (2003)
doi:10.1053/ejvs.2002.1793, available online at http://www.sciencedirect.com on SCIENCE DIRECT®

Pharmacological Induction of HSP27 Attenuates Intimal Hyperplasia *In Vivo*

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Objectives: *intimal hyperplasia (IH) is a major cause of re-stenosis post-vascular intervention. Induction of heat shock proteins (HSPs), by thermal pre-conditioning, reduces IH. Our aim was to investigate the effect of the pharmacological HSP inducer herbimycin A on IH in the rat carotid balloon injury model.*
Materials and Methods: *thirty male Sprague–Dawley rats were randomized into three groups. All groups underwent balloon injury to the left carotid artery. Stress proteins were induced 18 h pre-operatively by heat shock or herbimycin A. Two weeks post-operatively, animals were sacrificed and carotid intima/media area ratio (I/M ratio) calculated using computerized planimetry. Neo-intimal proliferation was assessed immunohistochemically with PCNA (proliferating cell nuclear antigen). Western blot and immunohistochemistry for arterial HSP70 and HSP27 were performed.*
Results: *heat stress and herbimycin significantly reduced the I/M ratio ($p < 0.05$ vs balloon injury alone). Neo-intimal proliferation was significantly reduced in the heat stress and herbimycin groups ($p < 0.05$ vs balloon injury alone). Heat stress induced arterial HSP70 and HSP27. Herbimycin A increased arterial HSP27.*
Conclusion: *herbimycin A significantly attenuates IH after balloon injury. HSP27 may be the HSP involved in mediating this response. Pharmacological inducers of HSPs may have a therapeutic role to play in preventing re-stenosis post-vascular intervention.*

Key Words: Intimal hyperplasia; Heat shock proteins; Herbimycin A.

Introduction

Re-stenoses secondary to intimal hyperplasia (IH) remains the major limiting factor after vascular intervention. Thirty to fifty percent of coronary and peripheral arteries develop re-stenosis after angioplasty, a rate that has changed little since the introduction of the technique over 20 years ago.[1] The recently reported patency rates obtained with sirolimus coated stents[2] are promising but there use is limited especially in multisegment disease or distal peripheral vascular disease.[3] Arterial medial vascular smooth muscle cell (VSMC) proliferation, resulting from angioplasty-associated mechanical stretch injury, has been shown to be an important contributory mechanism to re-stenosis.[4–6] Strategies aimed at reducing VSMC proliferation post-injury, have had little clinical success.[7–9] The most likely explanation for the poor efficacy of these agents is the abundance of inflammatory mediators and growth factors involved in promoting the hyperplastic response.[10–13] Consequently, much interest now focuses on pre-conditioning arterial smooth muscle cells prior to intervention, in order to prepare them for subsequent injury and thereby limit the proliferation response.

Pre-conditioning is a process whereby exposure of a cell to a sub-critical stress serves to protect it from subsequent injury.[14] Pre-conditioning is brought about by the production of intracellular proteins known as stress proteins or heat shock proteins (HSP). Stress proteins are classified according to their apparent molecular weight and respective inducers, e.g. HSP70, HSP90 and HSP27. Heat shock proteins can be induced in a number of ways the most studied of which being a brief period of hyperthermia, this is known as thermal pre-conditioning. Recent attention has focused on pharmacological induction of HSPs.[15] Herbimycin A, a benzoquinoid ansamycin antibiotic is a known heat shock protein inducer.[16–18]

This work was funded by a research grant from the Health Research Board, Dublin, Ireland.
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1078–5884/03/010040 + 08 $35.00/0

Heat shock proteins play an essential role in protecting cells against oxidative stress.[19,20] Therefore it is not surprising that a variety of studies have demonstrated that HSP induction, by pre-conditioning, limit subsequent injuries. Animal studies have shown that the heat shock response is protective against ischaemia re-perfusion induced injury in the heart,[20–23] small intestine,[24] skeletal and diaphragmatic muscle[25,26] and kidney.[27] Also the addition of one type of stress may provide protection against other types of insults, this is known as cross-tolerance. As examples, stress proteins induced by thermotolerance may provide protection during a subsequent ischaemic injury, or exposure to a heavy metal may provide protection against heat or sepsis. This suggests a valuable survival strategy in the adage that a "little stress is good" We have previously shown that pharmacological preconditioning with herbimycin A increases HSP expression and attenuates ischaemia reperfusion induced pulmonary injury.[18]

Recently thermal pre-conditioning by whole body hyperthermia has been shown to reduce IH *in vivo*.[28] The aim of this study was to determine whether it was possible to pre-condition arteries by pharmacological means with herbimycin A and hence reduce intimal hyperplasia in the rat carotid balloon injury model.

Materials and Methods

Animal preparation

All animal procedures were carried out in an approved animal research facility, under license from the Department of Health, Republic of Ireland. Thirty male Sprague–Dawley rats weighing 250–320 g were maintained on a purified chow pellet diet and water. The rats were randomized into 3 groups: group 1: balloon injury alone, group 2: heat shock and balloon injury, and group 3: herbimycin A and balloon injury. All underwent carotid balloon artery injury as described below. Animals in group 2 were thermally pre-conditioned, 18 h prior to balloon injury they were anaesthetized with halothane and partially immersed in a water bath (Grant Instruments LTD, Cambridge, U.K.) and the core temperature gradually raised to 41 ± 0.5°C for 15 min.[18,26,27] In group 3 100 µg of herbimycin A (H-6649, Sigma Chemical Co.) was dissolved in 80 µl of dimethylsulfoxide (DMSO, D-5879, Sigma Chemical Co.) and diluted in 720 µl of normal saline and administered 18 h prior to balloon injury by tail vein injection.[18] The animals in the other groups received the drug vehicle by tail vein injection 18 h prior to balloon injury.

Western blot analysis for HSP70 and HSP27

In order to determine the time of maximum HSP expression carotid arteries and aorta from a separate group of animals were analyzed at varying time points after thermal pre-conditioning (0, 8, 18 and 24 h post heat shock) ($n = 2$ at each time point). Arteries were also harvested from non-heat stressed rats and from rats given herbimycin A 18 h earlier ($n = 2$ per group). Each experiment was repeated three times.

Harvested arteries were immediately immersed in liquid nitrogen, pulverized with a mortar and pestle, and suspended in a cell lysis solution. After centrifugation a portion of the supernatant was used for protein concentration determination using a BCA Protein Assay Kit (Pierce). Equal amounts of protein were then suspended in SDS-glycerol loading buffer (pH 6.8, 62.5 mmol/Tris, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.01% bromophenol blue) and boiled for 10 min. Equal amounts of total protein were loaded onto a 12% and 15% polyacylamide gel, electrophoresed and transferred to nitrocellulose. The blots were blocked with 5% dried-milk reagent dissolved in TBST (1× Tris buffered saline with 0.05% Tween-20). The blot from the 12% gel was then incubated with a murine monoclonal anti-HSP70 antibody, SPA-810 (Stressgen, Victoria, British Columbia), diluted 1/2000 in 5% dried-milk reagent in TBST. A horseradish peroxidase conjugated goat anti-mouse immunoglobulin (DAKO) at a dilution of 1/1000, was used as the secondary antibody. The blot was washed several times between blocking steps with TBST. The blot from the 15% gel was incubated with a rabbit monoclonal anti-HSP-27 antibody, SPA-801 (Stressgen, Victoria, British Columbia) diluted to 1/2000 and a horseradish peroxidase conjugated goat anti-rabbit immunoglobulin (DAKO) was used as the secondary antibody. The immunoblots were visualized using Supersignal west pico chemiluminescent substrate (Pierce) and autoradiographed.

Carotid artery balloon injury model

Anesthesia was induced and maintained with halothane. An incision was made in the anterior neck. The method of balloon denudation was adapted from the technique established by Clowes *et al*.[29] The bifurcation of the left common carotid artery was exposed and the left common, internal and external carotid arteries were controlled with surgical ligatures. A 2Ch Fogarty catheter (Baxter Edwards) was introduced through an arteriotomy in the external carotid artery and advanced to the level of

the omohyoid muscle. The balloon was inflated with saline and drawn three times up and down the common carotid artery, the balloon was then deflated and withdrawn and the external carotid artery ligated. A single trained operator preformed all procedures.

The animals were sacrificed two weeks post-operatively and the carotid arteries harvested. This timepoint is used as Clowes *et al*.[29] demonstrated that intimal SMC number increases to a maximum 14 days after injury and remains constant thereafter.

Evaluation of intimal hyperplasia

Fourteen days after balloon injury, the carotid arteries were harvested, each carotid artery was excised from the proximal edge of the omohyoid muscle to the carotid bifurcation. The middle third of the segment was then immersed in 10% unbuffered formalin for fixation for 24 h. Four μm cross sections were stained with haematoxylin and eosin. The intimal area and medial area of each artery were obtained by computerized planimetry (Samba software; Alcatel, Grenoble, France). Neointimal thickening was quantified as the ratio of intima to media areas (I/M ratio) from transverse arterial sections. Two to four sections were examined from each artery by an analyst blind to the treatment regimen and the average I/M ratio calculated.

Four μm sections of the paraffin embedded carotids, harvested from animals, two weeks after injury, were cut and mounted on vectabond (Vector laboratories) coated slides and air dried at 37 °C overnight. Sections were dewaxed and rehydrated before blocking endogenous peroxidase in 0.3% hydrogen peroxide. Sections were then microwaved at high power in a trisodium citrate buffer (pH 6.0) for 20 min before incubation in mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (DAKO) at 1/800 dilution in tris buffered saline (TBS) at room temperature for 40 min. Sections were washed in TBS and biotinylated goat anti mouse/rabbit immunoglobulins was added followed by treatment with streptABComplex/HRP (DAKO Duet). The antibody-antigen reaction was visualized with diaminobenzidine (Sigma Chemical Co.) and sections were lightly counterstained with Harris haematoxylin. Tonsil was used as a positive control. The PCNA positive and PCNA negative nuclei were counted in the neointima and the proliferation index was calculated by the following formula (positive nuclei stained by PCNA/total number of nuclei stained by haematoxylin).

Immunohistochemical localization of heat shock proteins

Carotid arteries were harvested from rats 18 h after heat shock, from untreated controls and from rats pre-treated with herbimycin A ($n = 4$ per group). Four μm sections of paraffin embedded sections were de-waxed, re-hydrated and endogenous peroxidase activity blocked with hydrogen peroxide. Sections were then incubated with goat serum diluted in TBS (1/10 dilution) for 30 min. Sections were incubated for one hour with a mouse monoclonal antibody against HSP70, SPA-810 (Stressgen, Victoria, British Columbia). After washing in TBS biotinylated goat anti mouse/rabbit immunoglobulin was added followed by treatment with streptABComplex/HRP (DAKO). The antibody-antigen reaction was visualized with diaminobenzidine (Sigma Chemical Co.) and sections were lightly counterstained with Harris haematoxylin. The same staining procedure was used for staining for HSP27 using a rabbit monoclonal antibody against HSP27, SPA-801 (Stressgen, Victoria, British Columbia).

Statistical analysis

Results are reported as mean ± SEM, to test homogeneity of variance Bartlett's test was performed and data was subsequently analyzed using a one way Analysis of Variance with Tukey–Kramer multiple comparison post hoc tests. Non parametric data was analyzed using Kruskal–Wallis Analysis of Variance and Dunnes multiple comparison post hoc tests.

Results

HSP70 and HSP27 protein expression in arterial tissue

In order to determine the timing of maximal HSP expression western blot was performed on protein extracted from the aorta and carotid arteries at the indicated time points after heat shock and from controls (Fig. 1). HSP70 expression was maximum at 18–24 h post heat shock, with a steady decrease in protein levels thereafter. Levels of HSP27 protein were maximum between 8 and 18 h post heat shock (Fig. 1). Western blot analysis was also preformed on carotid tissue from herbimycin A pre-treated rats. HSP27 was increased by pre-treatment with herbimycin A (Fig. 2). HSP70 expression did not alter significantly with herbimycin A treatment.

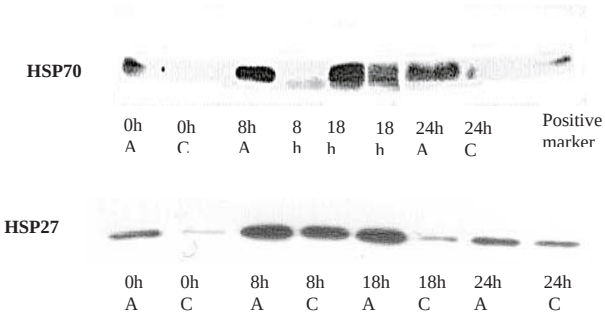

Fig. 1. Western blot time course of HSP70 and HSP27 induction in Aorta (A) and Carotid (C) at varying time points (0–24 h) post thermal preconditioning. Both heat shock proteins were maximally induced at 8–18 h post heat shock.

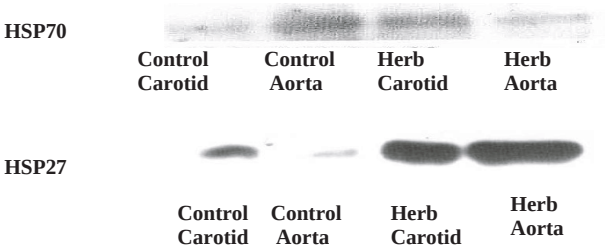

Fig. 2. Western blot of HSP27 and HSP70 expression in rat carotid artery in unstressed rat (control) and in rat 18 h post herbimycin A administration.

Immunohistochemical confirmation of HSP expression

Immunohistochemistry was preformed to confirm results obtained by western blot and to localize protein expression within the arterial wall. HSP70 and HSP27 were detected in the endothelium and medial smooth muscle cells of the carotids of thermally preconditioned animals (Figs 3 and 4). No significant staining for HSP70 was detected in untreated control carotids. Low levels of HSP27 were detected in untreated rats. Similar to the findings with western blot, HSP27 was present in increased amounts in the herbimycin A pre-treated animals relative to untreated controls (Fig. 4). HSP70 expression was not altered significantly by herbimycin A pre-treatment.

Effect of pre-conditioning on I/M ratio two weeks after balloon injury

Animals were harvested 14 days after balloon injury as described above. The intimal area, medial area and I/M ratio were obtained from each artery (mean ± SEM). The I/M ratio is used to analyze effects on

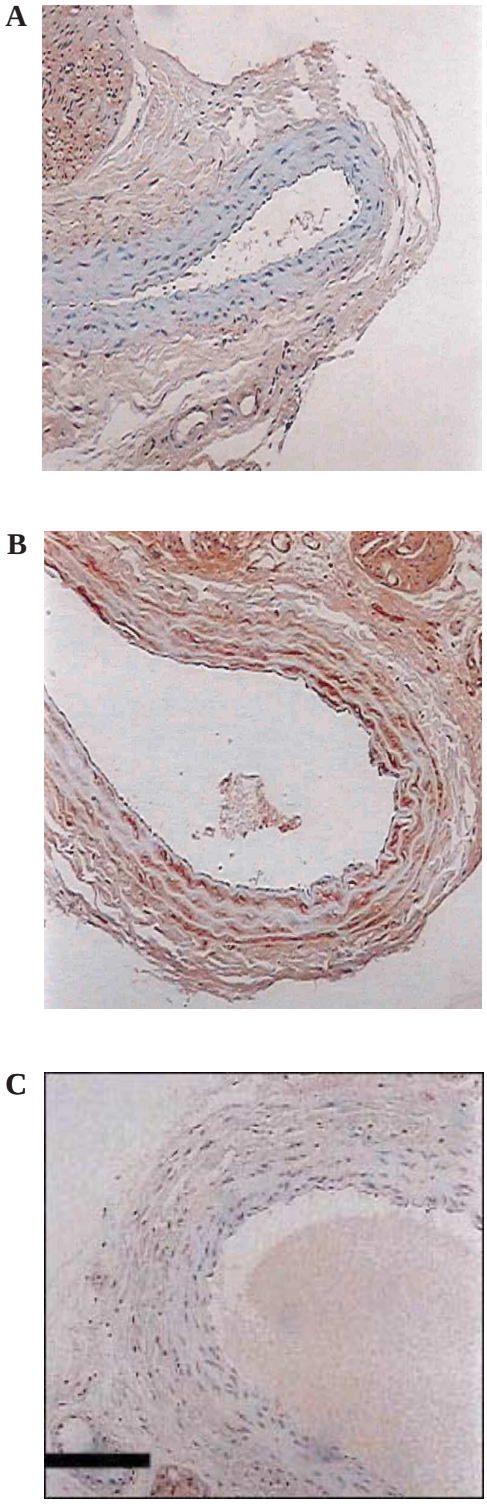

Fig. 3. Carotid arteries from rats given A, drug vehicle B, 18 h post heat shock and C, from rats given herbimycin A 18 h earlier were harvested, fixed in formalin, sectioned, and stained with a monoclonal anti-HSP70 antibody. The antibody was detected with an enzyme-conjugated detection system that yields a brown colour in positive cells. (Original magnification × 400) (scale bar = 50 μm).

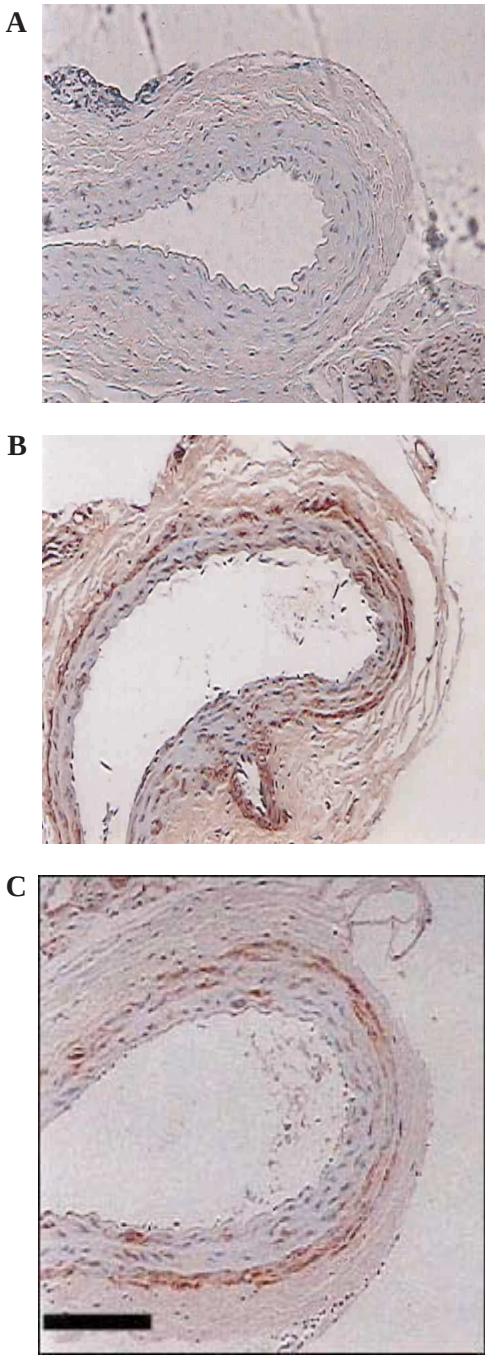

Fig. 4. Carotid arteries from rats given A, drug vehicle B, 18 h post heat shock and C, from rats given herbimycin A 18 h earlier were harvested, fixed in formalin, sectioned, and stained with a monoclonal anti-HSP27 antibody. The antibody was detected with an enzyme-conjugated detection system that yields a brown colour in positive cells. (Original magnification × 400) (scale bar = 50 μm).

intimal accumulation as it controls for variability due to animal size or perfusion artifact. One-way analysis of variance showed that the I/M ratio was significantly lower in the heat stress (1.14 ± 0.165) and

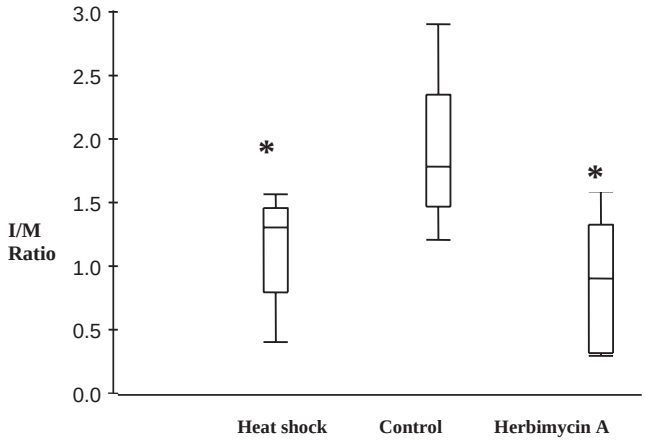

Fig. 5. The intimal area and medial area were measured 14 days after balloon injury using computerized planimetry and the I/M ratio was calculated. Graphic representation of I/M ratio 14 days post balloon injury in the heat shock group and balloon injury alone group (control) and herbimycin A group. $^{*} = p < 0.05$ vs control group.

herbimycin A (0.87 ± 0.19) group relative to the untreated controls (1.84 ± 0.2), ($p < 0.05$) (Figs 5 and 6).

Results of neointimal proliferation two weeks after balloon injury

The neointimal proliferation index, as determined by the percentage of neointimal cells staining positively for PCNA in rat carotids two weeks after balloon injury, was significantly ($p < 0.05$) lower in the heat stressed ($44.5\% \pm 4.07$) and herbimycin A ($43.8\% \pm 5.38$) treated animals than in the balloon injury alone group ($58.33\% \pm 5.13$).

Discussion

Our hypothesis was that induction of arterial heat shock proteins, with herbimycin A, *in vivo*, would decrease the proliferation response of the vessel wall to injury. This study confirmed that balloon injury of the rat carotid artery results in vascular smooth muscle cell proliferation and the formation of a thickened neo-intima, which can be reduced significantly by thermal pre-conditioning.[28] We have shown that, pretreatment with herbimycin A, significantly reduces the degree of intimal hyperplasia (IH) and neointimal proliferation two weeks after balloon injury. Preconditioning with herbimycin A was associated with increase expression of HSP27 in the endothelium and medial VSMCs.

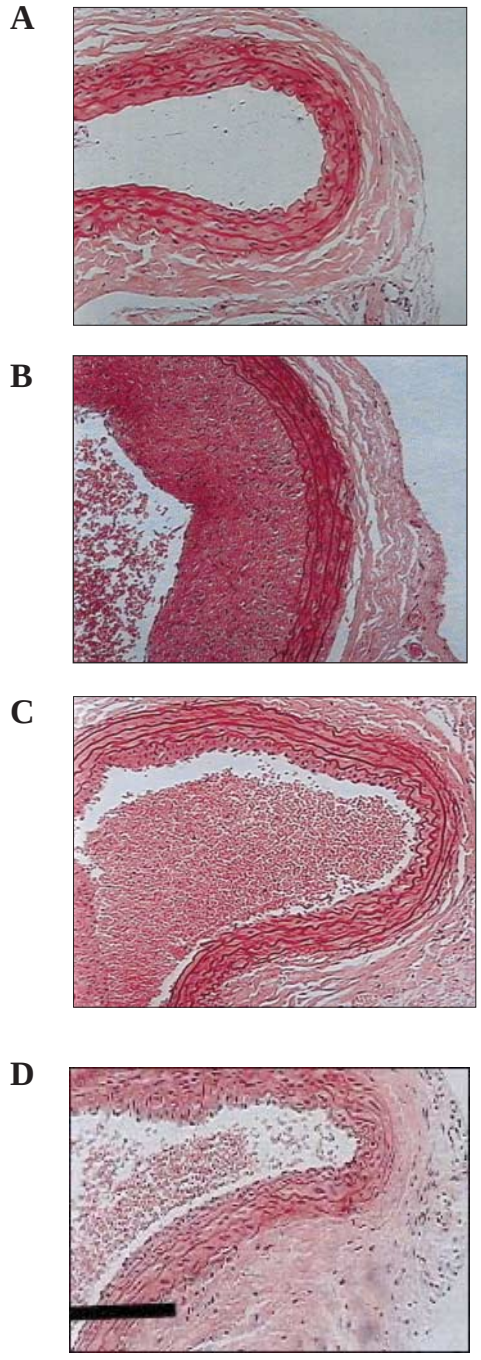

Fig. 6. Haematoxylin and eosin stained photomicrographs of rat carotid artery sections 2 weeks post balloon injury: (A) shows uninjured carotid artery; (B) shows injured control artery, (not pretreated); (C) shows injured carotid in heat pretreated rat; (D) shows injured carotid in herbimycin A pretreated rat. (Original magnification × 400) (scale bar = 50 μm).

Vascular injury results in the release of inflammatory mediators and growth factors, such as prostaglandins and thromboxane, tumor necrosis factor α (TNFα), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), which activate medial VSMCs, which in turn migrate and proliferate to form a thickened neointima.[10–13,30] Strategies aimed at reducing IH have largely focused on limiting VSMC proliferation post intervention, and have had little clinical success.[7–9] Currently there is interest in modifying the response of the vessel[28] to injury by pre-conditioning the vasculature prior to the injury. Thermal pre-conditioning has been shown to diminish the severity of tissue injury upon subsequent exposure to a more severe, noxious or injurious stimulus.[21–23,31] Induction of the heat shock response in arterial VSMCs, *in vitro*, limits proliferation following mechanical injury.[32] Neischis *et al*. found that thermal preconditioning prior to vascular injury reduces intimal hyperplasia.[28]

Despite the abundance of experimental evidence demonstrating the benefits of thermal pre-conditioning, clinicians are understandably reluctant to employ "heat shocking" as a treatment for their patients. There is currently widespread interest in pharmacological induction of stress proteins.[15] Herbimycin A is a benzoquinoid ansamycin antibiotic and a known tyrosine kinase inhibitor. Hedge *et al.* demonstrated that treatment of cells with herbimycin A results in increased expression of all the constitutively expressed stress proteins and confers a thermotolerant like phenotype.[16] The heat shock protein inducing effect of herbimycin A has been shown to be independent of its tyrosine kinase inhibiting property.[16,17] Hedge *et al.* demonstrated that herbimycin A unlike heat shock, or other metabolic stressors, did not induce any adverse cellular effects.[16] We investigated if herbimycin A would induce HSPs in arterial tissue and whether this induction would confer a protective effect against the proliferation response of VSMCs to injury.

We found that HSP70 and HSP27 where maximally induced 8–18 h post heat shock. Previous work has demonstrated that the time course for induction of stress proteins, and the magnitude of this response, is similar in thermal pre-conditioned and herbimycin A treated cells.[16,17] We have shown that pretreatment with herbimycin A induces HSP27 in the VSMCs and endothelium of the arterial wall, but in contrast to heat shock, herbimycin A did not increase arterial HSP70. Heat shock has previously been shown to non-specifically increase an array of HSPs[32] whereas pharmacological induction is more specific.[16] Herbimycin A has been suggested to have a cell type specific effect on some HSPs.[16,17] HSP27, a small stress protein (sHSP), is increased in the myocardium and VSMCs of spontaneously hypertensive rats.[33,34]

HSP27 is a molecular chaperone that acts as a regulator of the intracellular redox state and is a

potent inhibitor of apoptosis.[33] Like HSP70, HSP27 is involved in mediating the protection conferred by sub-lethal heat shock.[36] We have shown for the first time that arterial HSP27 is increased by both heat shock and herbimycin A and that both these treatments limit the degree of IH post balloon injury. Our results suggest that HSP27 may be the main stress protein involved in limiting the response of VSMC to injury. In order to further define the role of HSP70 and HSP27 in limiting VSMC proliferation, *in vitro* experiments, using anti-sense oligonucleotides aimed at blocking HSP70 and HSP27 are required.[37]

Herbimycin A as well as being a HSP inducer is also a tyrosine kinase inhibitor. Tyrosine kinase inhibitors applied topically to the vessel at the time of vascular intervention, or orally following vascular injury, have being shown to reduce intimal hyperplasia.[38,39] In our study herbimycin A was given intravenously 18 h prior to vascular injury and therefore the tyrosine kinase inhibiting effect of herbimycin A is unlikely to be responsible for the observed reduction in intimal hyperplasia.

At present the mechanisms whereby stress proteins protect the arterial wall from the deleterious effects of balloon injury can only be inferred. In this arterial balloon injury model intimal thickening is initiated by a combination of de-endothelialization, barotrauma and cell death followed by VSMC activation and proliferation. Indolfi *et al*. demonstrated that the proliferation response in this model appears to be directly proportional to the degree of balloon injury.[40] Perlman and others have recently shown that up to 70% of the vascular smooth muscle cells in the media undergo apoptosis at 30 min post balloon injury rat carotid balloon injury model).[41,42] It has been reported that severe medial VSMC loss occurs in vein grafts even when prepared without distension with apoptosis contributing to this loss.[43] Inhibition of HSP70 expression has been shown to stimulate apoptosis and intimal hyperplasia in vein segments *in vitro*.[44] HSP70 and HSP27 have been shown to enhance resistance to apoptosis in many tissues,[37,45–47] this may partially account for the observed reduction in intimal hyperplasia observed in the pre-conditioned groups.

Unfortunately herbimycin A is currently not licenced for use in humans. There is however increasing evidence to support the future use of herbimycin A as a pharmacological stress protein inducer in humans, for example, Hedge *et al*. in 1995, observed no deleterious effects of herbimycin A at the concentrations used to induce stress protein expression in cells in culture.[16] In this and previous *in vivo* experiments we have used a very low dose of herbimycin A as a pharmacological pre-conditioner, given as a single intravenous injection 18 h prior to injury to successfully attenuate the subsequent inflammatory response.[18] Also the dose used in these experiments (400 μg/kg) was well below the LD50 (19 mg/kg).[48] Currently there is much interest in using other tyrosine kinase inhibitors to treat chronic myeloid leukaemia.[49] Further toxicity studies looking at the effect of herbimycin A *in vivo* are required before considering the use of this powerful stress protein inducer in patients prior to vascular surgery. Currently we are also investigating the effect of other pharmacological HSP inducers, such as geranylgeranylacetone (teprenone), on intimal hyperplasia. Teprenone is licenced in Japan for treating peptic ulcer disease in humans and has been shown to induce HSPs in the gastric mucosa.[50]

In summary the current study supports the concept that thermal pre-conditioning of the vasculature reduces the subsequent response of the VSMCs to injury. This study also demonstrates that pharmacological induction of stress proteins in the vasculature is possible and results in a similar reduction in the VSMC proliferation. Our results also suggest that HSP27 may be the chief stress protein that mediates the effects of pre-conditioning in the vasculature. Finally pharmacological HSP inducers may have a therapeutic role to play in limiting the response of the vessel wall to injury and thereby reducing the incidence of re-stenosis post vascular intervention.

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Accepted 5 October 2002