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Heat induced HSP20 phosphorylation without increased cyclic nucleotide levels in swine carotid media

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Abstract

Background: Heat pretreatment of swine carotid artery has been shown to increase ser¹⁶-heat shock protein 20 (HSP20) phosphorylation and suppress force, i.e., reduce force with only minimal reduction in ser¹⁹-myosin regulatory light chain (MRLC) phosphorylation.

Results: We further investigated this response in intact histamine stimulated swine carotid artery rings. There was a heat threshold such that increased ser¹⁶-HSP20 phosphorylation and force suppression were observed between 43°C and 46°C. The increased ser¹⁶-HSP20 phosphorylation persisted up to 16 hours after 44.5°C heat treatment. Pretreatment of swine carotid media at 44.5°C increased ser¹⁶-HSP20 phosphorylation without increases in [cAMP] or [cGMP], suggesting an alternate mechanism, perhaps phosphatase inhibition, for the increase in ser¹⁶-HSP20 phosphorylation. Heat pretreatment at 47.5°C reduced force by decreasing MRLC phosphorylation rather than by large increases in ser¹⁶-HSP20 phosphorylation. HSP20 phosphorylation at the putative PKC site did not change with any treatment.

Conclusion: These results demonstrate that multiple mechanisms can induce force suppression that is correlated with ser¹⁶-HSP20 phosphorylation: 1) nitrovasodilators via cGMP, 2) forskolin via cAMP, and 2) thermal stress in a cyclic nucleotide independent manner.

Background

There are three general mechanisms whereby vascular smooth muscle force is regulated. 1) Activation mechanisms involve contractile pathways that increase ser¹⁹-myosin regulatory light chain (MRLC) phosphorylation, either by increases in myoplasmic calcium ([Ca²⁺]_i [1]) or by decreases in myosin light chain phosphatase activity [2]. The increased ser¹⁹-MRLC phosphorylation activates the myosin's ATPase producing crossbridge cycling and contraction. 2) Deactivation mechanisms are the reversal

of activation mechanisms: relaxation results from lower ser¹⁹-MRLC phosphorylation induced by either reductions in [Ca²⁺] [3,4] or increases in myosin light chain phosphatase activity [5]. Since dephosphorylated myosin does not cycle, force is reduced. 3) Force suppression occurs when force is reduced despite persistent elevation in ser¹⁹-MRLC phosphorylation levels. This is an exception to the above MRLC phosphorylation paradigm since phosphorylated myosin should cycle and produce force. Force suppression has been observed with elevations in

[cGMP] [6,7], [cAMP] [8], extracellular $[Mg^{2+}]$ [9], and with some pharmacologic treatments.

Cyclic nucleotide induced relaxation is associated with phosphorylation of heat shock protein 20 (also known as HSP20 or P20) on serine 16 [10–12]. More recently, ser¹⁶-HSP20 phosphorylation was shown to specifically and temporally correlate with "force suppression" rather than the "deactivation" form of relaxation [8,10]. A region of HSP20 (residues 110–121) has sequence homology with troponin I; peptides from this region bound thin filaments, reduced actin activated myosin S1 ATPase activity, and relaxed skinned swine carotid artery [10]. We hypothesized that binding of ser¹⁶-phosphorylated HSP20 to the thin filament may "turn off" thin filaments so that phosphorylated myosin does not interact with the thin filament (i.e. a model similar to skeletal muscle troponin I). This would explain low force with elevated MRLC phosphorylation. HSP20 has a second phosphorylation site, presumptively phosphorylated by PKC [12], of unclear significance.

HSP20 is a member of the heat shock protein superfamily and is known to provide resistance to heat treatment in cells [13]. Recently we found that heat pretreatment (4 hours at 44.5°C) of swine carotid artery increased ser¹⁶-HSP20 phosphorylation and suppressed force [14]. In this study, we addressed three questions: 1) What is the temperature threshold for increases in ser¹⁶-HSP20 phosphorylation and force suppression? 2) Does the heat pretreatment response diminish by prolonged recovery at 37°C after heat treatment, i.e. is the heat pretreatment reversible? And 3) Are increases in [cAMP] or [cGMP] responsible for the heat-induced increases in ser¹⁶-HSP20 phosphorylation?

Results

Experimental protocol

Tissues were first equilibrated at 37°C [15]. This involved a "warm-up" 109 mM K⁺ contraction approximately 30 min after mounting, repeated stretching to $\sim 1 \times 10^5$ N/m² (~ 20 g for a 20 mg tissue), a release to $\sim 0.2 \times 10^5$ N/m² (~ 4 g), and a second 109 mM K⁺ contraction approximately 120 min after mounting. This protocol sets the muscle to the optimal length for force generation. The latter K⁺ contraction was used for force normalization. Tissues were then exposed to various temperatures for various times (solutions were replaced if evaporation was observed). Temperature was changed by adjusting the water bath set point. This procedure increased temperature within 5 min. Reducing temperature would have been slower but was accelerated by adding ice to the water bath. After the exposure to different temperatures, tissues were returned to 37°C for various times, contracted with 10 μ M histamine for 10 min, and then frozen for analysis.

Threshold for temperature dependent increase in ser¹⁶-HSP20 phosphorylation and force suppression

Equilibrated swine carotid medial rings were exposed to various temperatures for 4 hours, returned to 37°C for 1 hour, contracted with 10 μ M histamine for 10 min, and then frozen. Significant reduction in force and increases in ser¹⁶-HSP20 phosphorylation were observed with heat treatments between 43°C and 46°C (Fig. 1). Ser¹⁹-MRLC phosphorylation levels did not significantly differ in tissues treated at 44.5°C or 37°C, suggesting that the decreased force observed at 44.5°C was force suppression.

Histamine did not induce any contraction after pretreatment at 47.5°C, and histamine also did not significantly increase ser¹⁹-MRLC phosphorylation levels (MRLC phosphorylation was significantly less than that observed at 37°C). This result suggests that the decreased force observed at 47.5°C was caused by reduced ser¹⁹-MRLC phosphorylation. Ser¹⁶-HSP20 phosphorylation at 47.5°C was significantly lower than that observed at 44.5°C but higher than that observed at 37°C. These data suggest a biphasic response with ser¹⁶-HSP20 phosphorylation-associated force suppression with heat pretreatment between 43 and 46°C and deactivation by inhibition of ser¹⁹-MRLC phosphorylation after heat pretreatment at 47.5°C.

Pretreatment at 2°C induced a small, insignificant increase in ser¹⁶-HSP20 phosphorylation without significant effects on force. Putative PKC-HSP20 phosphorylation [12] was not significantly altered at any tested temperature.

Recovery after heat treatment

Swine carotid medial rings were exposed to 44.5°C for 4 hours, followed by 37°C for various times before stimulation with 10 μ M histamine for 10 min and freezing (Fig. 2). A set of control tissues were not exposed to 44.5°C and had low ser¹⁶-HSP20 phosphorylation (labeled "before"). Ser¹⁶-HSP20 phosphorylation levels were high both in tissues that were histamine stimulated at 44.5°C (0 time, i.e. no recovery at 37°C) and in tissues that were returned to 37°C for 15 min to 16 hours. Force was suppressed in all heat-treated tissues regardless of recovery time. There appeared to be a modest rebound in histamine-induced contraction 1 to 8 hours after return to 37°C.

Dependence of histamine-induced force on ser¹⁶-HSP20 phosphorylation

The relation between mean ser¹⁶-HSP20 phosphorylation and mean force from all the tissues that were stimulated with histamine is shown in the upper panel of Fig. 3. There is a clear relation between reduced histamine-induced force and increased ser¹⁶-HSP20 phosphorylation with all treatments except the 47.5°C heat pretreatment

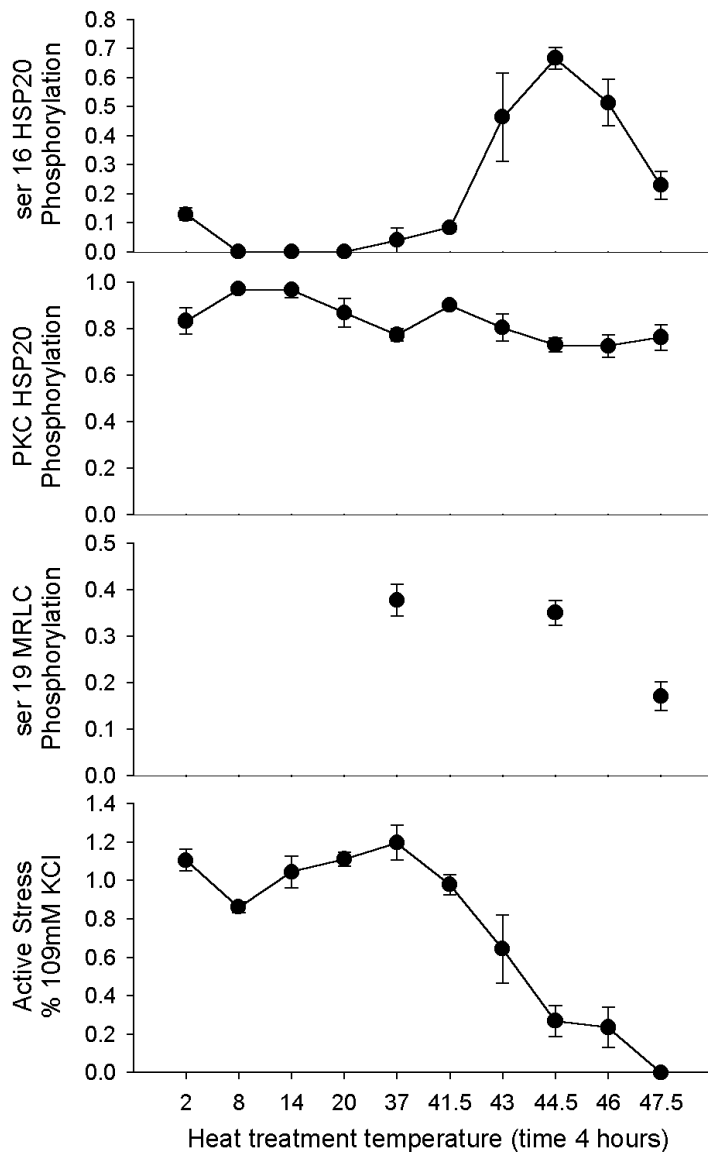


Figure 1

Threshold for temperature dependent increase in ser¹⁶-HSP20 phosphorylation and force suppression. Swine carotid artery tissues were exposed to various temperatures between 2°C and 47.5°C (shown on abscissa) for 4 hours, then returned to 37°C for 1 hour, and stimulated with 10 μM histamine for 10 min before freezing. Histamine induced ser¹⁶-HSP20 phosphorylation (mol P_i at ser 16 / mol HSP20) is shown in the top panel, putative PKC-HSP20 phosphorylation (mol P_i at the PKC site / mol HSP20) in the second panel, ser¹⁹-MRLC phosphorylation (mol P_i at ser 19 / mol MRLC) in the third panel, and force in the bottom panel (n = 4–8, except n = 3 at 14 & 20°C and n = 2 at 8°C, data presented as mean ± 1 SEM). Ser¹⁹-MRLC phosphorylation measurements were limited to tissues treated at 37°C, 44.5°C, and 47.5°C. Force was significantly reduced in tissues treated at 44.5°C or higher compared to 41.5°C or lower. Ser¹⁶-HSP20 phosphorylation was significantly higher in tissues treated between 43 and 46°C compared to tissues treated at less than 43°C or at 47.5°C. PKC-HSP20 phosphorylation did not significantly change. At temperatures between 43 and 46°C, low force was associated with elevated ser¹⁶-HSP20 phosphorylation levels despite elevated ser¹⁹-MRLC phosphorylation levels. At 47.5°C, reduced force was associated with significantly reduced ser¹⁹-MRLC phosphorylation (compared to tissues treated at 37°C or 44.5°C, i.e. there was deactivation).

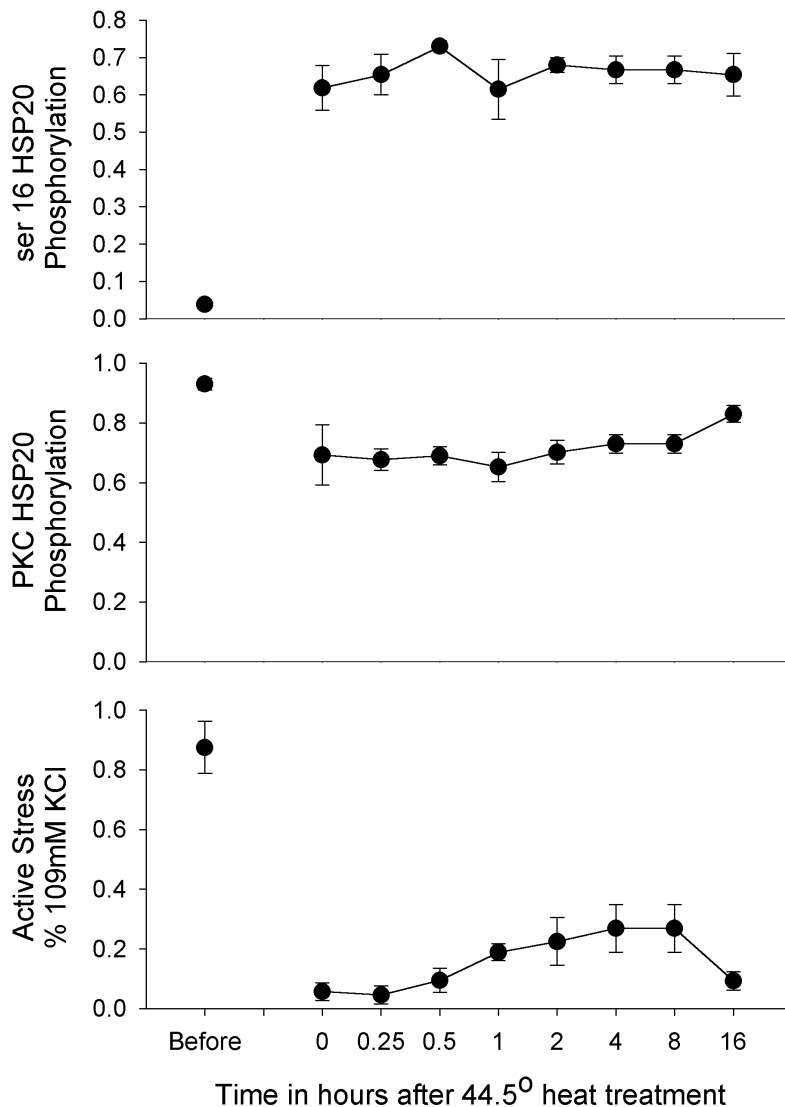


Figure 2

Recovery after heat treatment. Swine carotid artery tissues were exposed to 44.5°C for 4 hours and then either 1) stimulated with 10 μM histamine for 10 min before freezing (0 recovery time) or returned to 37°C for various times and then stimulated with 10 μM histamine for 10 min before freezing (positive recovery times). A unheated control set of tissues was stimulated with 10 μM histamine for 10 min and frozen without 44.5°C heat treatment (labeled "before"). Histamine induced ser¹⁶-HSP20 phosphorylation (mol P_i at ser 16 / mol HSP20) is shown in the top panel, putative PKC-HSP20 phosphorylation (mol P_i at the PKC site / mol HSP20) in the center panel, and force in the bottom panel (n = 4–6). Force was significantly reduced in all heat treated tissues regardless of recovery time compared to the unheated control. Ser¹⁶-HSP20 phosphorylation was significantly higher in all heat treated tissues regardless of recovery time compared to unheated control.

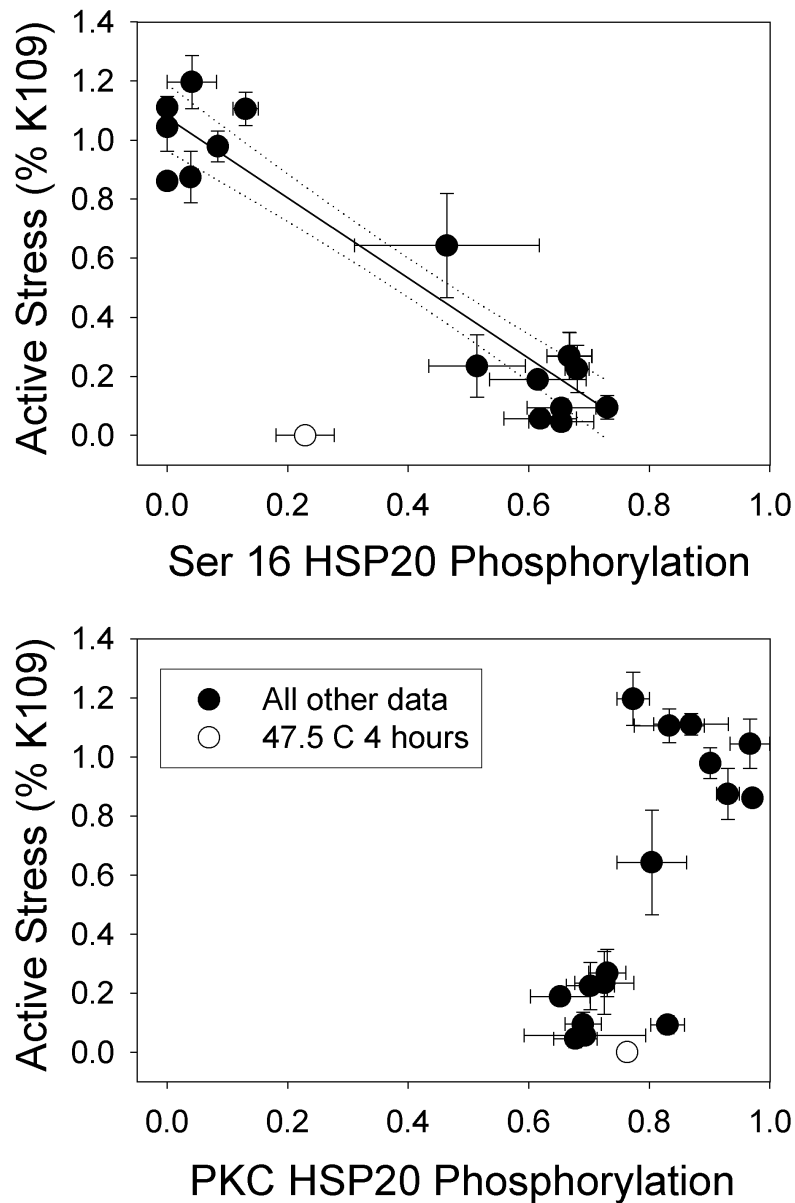


Figure 3

Dependence of contractile force on ser¹⁶-HSP20 and putative PKC-HSP20 phosphorylation in tissues maximally stimulated with histamine. Upper panel: Mean data demonstrate the relation between ser¹⁶-HSP20 phosphorylation (mol P_i at ser 16 / mol HSP20) and contractile stress for all tissues presented in Figs. 1 and 2. All tissues were stimulated with 10 μM histamine for 10 min. The tissues treated for 4 hours at 47.5°C are shown as an open symbol and is not included in the linear regression. All other tissues are shown as filled circles. Overall there was a good inverse correlation between ser¹⁶-HSP20 phosphorylation and contractile force with these heat pretreatment protocols ($r^2 = 0.90$, solid line is regression line, dotted line are 95% confidence intervals). **Lower panel.** Mean data demonstrate the relation between putative PKC-HSP20 phosphorylation (mol P_i at the PKC site / mol HSP20) and contractile stress. There was little relation between putative PKC-HSP20 phosphorylation and contractile force with these heat pretreatment protocols.

protocol. The lack of correlation at 47.5°C is expected given the lower ser¹⁹-MRLC phosphorylation (i.e. deactivation) observed after 47.5°C pretreatment. There was no correlation between putative PKC-HSP20 phosphorylation and force (lower panel of Fig. 3).

Cyclic nucleotide levels did not correlate with heat induced ser¹⁶-HSP20 phosphorylation levels

Swine carotid medial rings were stimulated with 10 µM histamine for 10 min either before (control) or at the end of a 4 hour 44.5°C treatment. Heat pretreatment did not significantly alter [cAMP] and [cGMP] levels despite significantly increased ser¹⁶-HSP20 phosphorylation and suppressed force. These data suggest that the increases in ser¹⁶-HSP20 phosphorylation cannot be explained by elevated [cAMP] and [cGMP] levels after 4 hours of heat pretreatment.

Discussion

Our prior study [14] found that 1) 44.5°C heat pretreatment of swine carotid artery increased ser¹⁶-HSP20 phosphorylation and suppressed force, i.e., a reduction in force without a significant reduction in MRLC phosphorylation. 2) The effects of heat pretreatment and nitroglycerin were additive, both on increasing ser¹⁶-HSP20 phosphorylation and suppressing force [14]. And 3) the relation between ser¹⁶-HSP20 phosphorylation and force was similar with heat pretreatment and nitroglycerin, suggesting a similar mechanism of action to suppress force [14].

The goal of this study was to extend our observations on heat treatment. We found that temperatures between 43 and 46°C increased ser¹⁶-HSP20 phosphorylation and suppressed force, i.e. reduced force without reductions in ser¹⁹-MRLC phosphorylation (Fig. 1). Excepting the 47.5°C response, the relation between mean ser¹⁶-HSP20 phosphorylation and mean force was similar with all treatments in these histamine stimulated tissues (top panel, Fig. 3), consistent with the hypothesis that ser¹⁶-HSP20 phosphorylation was mediating force suppression.

No contraction was observed after pretreatment at 47.5°C, however, this was not force suppression since MRLC phosphorylation was significantly reduced to near basal levels for the swine carotid. This may explain why the response observed after 47.5°C was not on the ser¹⁶-HSP20 phosphorylation/force relation shown in Fig. 3. This finding is consistent with the hypothesis that different temperatures reduce force by different mechanisms. For example, whole body hyperthermia at 41.5°C induced apoptosis in rats [16] while exposure to 48°C reportedly induces necrosis in cultured cells [17]. The similar temperature sensitivity of apoptosis and ser¹⁶-HSP20 phosphorylation suggests the possibility of a role

for HSP20 in the promotion of or protection from apoptosis. It is possible that HSP20 induced force suppression may be part of a protective response to moderate heat exposure. In contrast, the absence of a contractile and MRLC phosphorylation response after more extreme heat exposure (47.5°C) may represent tissue necrosis.

There was no significant change in putative PKC-HSP20 phosphorylation [12] with any protocol. As a result there was no correlation between putative PKC-HSP20 phosphorylation and force (lower panel of Fig. 3).

We also investigated whether the heat pretreatment response was reversible. Ser¹⁶-HSP20 phosphorylation levels remained elevated and overall force remained low up to 16 hours after return of heat treated tissues to 37°C. This suggests that the process that increases ser¹⁶-HSP20 phosphorylation is not reversible, at least in vitro. This may indicate substantial tissue damage during the 4 hour exposure at 44.5°C.

Finally, we found that the increases in ser¹⁶-HSP20 phosphorylation were not explained by increases in [cAMP] or [cGMP] at the end of heat treatment (Fig. 4). This suggests that some other mechanism keeps ser¹⁶-HSP20 phosphorylation at high levels. Considering that the increased ser¹⁶-HSP20 phosphorylation remains elevated for 16 hours after return to 37°C, it is possible that heat treatment is reducing HSP20 phosphatase activity.

Our experiments suggest that temperatures of 43°C and above can alter arterial contractility. Such temperatures can occur physiologically. Brooks, et. al. [18] exhaustively exercised rats for 90 min on treadmills. Immediately after exercise (not during), thermocouples were inserted into thigh skeletal muscles and the rectum. They found an initial mean temperature of 44.1°C in skeletal muscle and 42.1°C rectally. Both temperatures fell exponentially with rest after exercise. It is likely that muscle temperature exceeded 44.1°C for at least part of the 90 min exercise. This suggests that profound exercise can increase regional temperature to levels that could potentially induce ser¹⁶-HSP20 phosphorylation and force suppression. Thermal stress may bring about a resistance to contractile agonists, which could have clinical significance in conditions such as hyperthermia, and/or sepsis with vasodilatory shock.

Heat treatment may have effects on smooth muscle beyond contractility. Orihara et al. [19] reported that exposure of vascular smooth muscle cells to 43°C for 2 hours inhibited cell proliferation. Further work is required to assess whether HSP20 is involved in this response.

These results also demonstrate that multiple mechanisms can induce force suppression that is correlated with ser¹⁶-

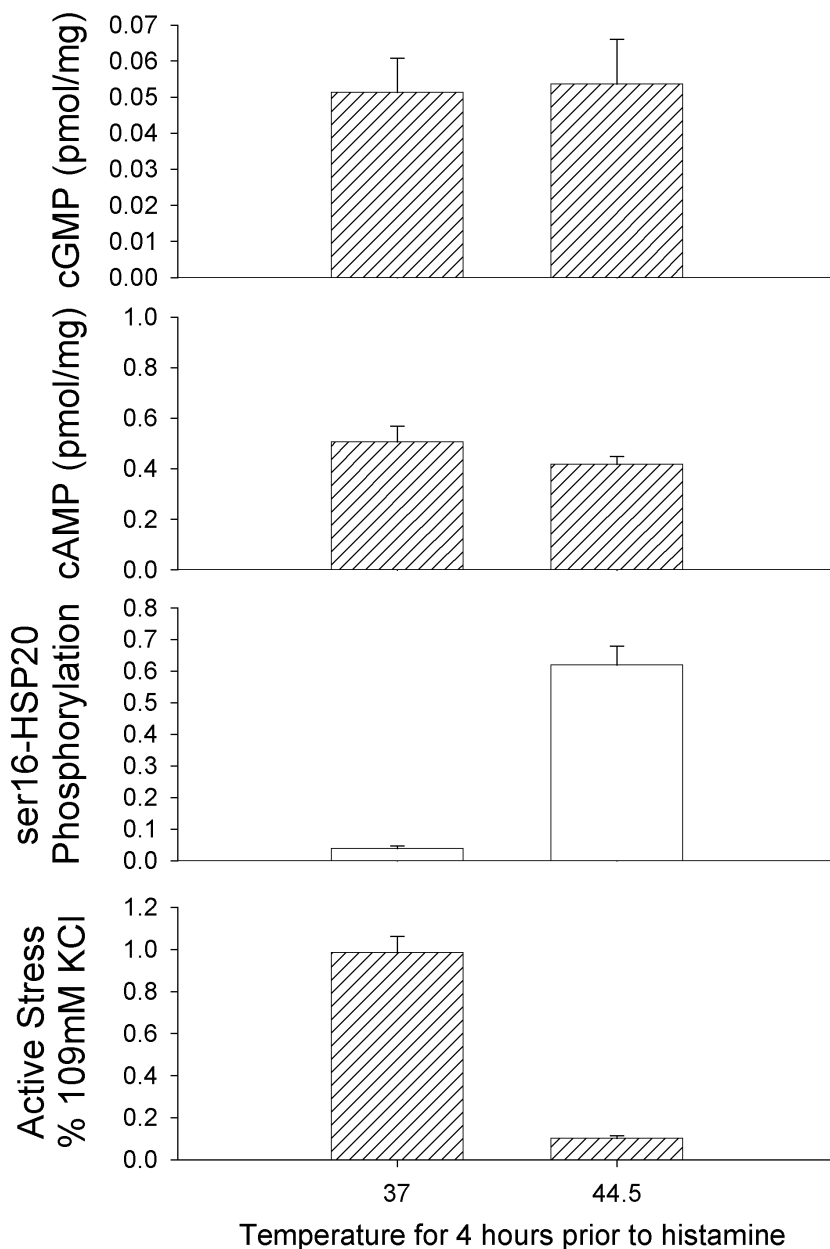


Figure 4

Cyclic nucleotide levels did not correlate with heat induced ser¹⁶-HSP20 phosphorylation levels. Swine carotid artery tissues were either 1) stimulated with 10 μM histamine for 10 min at 37°C or 2) 44.5°C for 4 hours and then stimulated with 10 μM histamine for 10 min at 44.5°C prior to freezing. Histamine induced [cGMP] is shown in the top panel, [cAMP] in the center panel, ser¹⁶-HSP20 phosphorylation (mol P_i at ser 16 / mol HSP20) in the third panel, and force in the bottom panel (n = 4, data presented as mean ± 1 SEM). The ser¹⁶-HSP20 phosphorylation data is from a second set of tissues treated identically (the "before" group from Fig. 2). Treatment at 44.5°C did not significantly alter [cAMP] and [cGMP] levels despite significantly increasing ser¹⁶-HSP20 phosphorylation and suppressing histamine induced force.

HSP20 phosphorylation. Our prior studies showed that increases in cGMP by nitrovasodilators and cAMP by forskolin increase ser¹⁶-HSP20 phosphorylation and induce force suppression [8]. Such results suggest that the correlation between ser¹⁶-HSP20 phosphorylation and force suppression is robust in the swine carotid stimulated with 10 μ M histamine. However, it must be stressed that different contractile conditions may be associated with different relationships between ser¹⁶-HSP20 phosphorylation and force given the multiple mechanisms whereby agents can cause relaxation [3–5]. For example, smooth muscle relaxation can occur by agents that reduce myoplasmic Ca²⁺ by inducing hyperpolarization, reducing Ca²⁺ influx without changes in membrane potential, increasing Ca²⁺ efflux, or by reducing Ca²⁺ release from the internal store. The response can depend on the form of contractile activation, e.g. nitroglycerin reduces Ca²⁺ better after agonist activation compared with K⁺ depolarization [20]. Comparison of ser¹⁶-HSP20 phosphorylation/force relationships require careful analysis in comparison with ser¹⁹-MRLC phosphorylation levels to clearly show that the change in force is force suppression rather than deactivation.

Methods

Tissues

Swine common carotid arteries were obtained from a slaughterhouse and transported at 0°C in physiological salt solution (PSS). PSS contained (mM): NaCl, 140; KCl, 4.7; 3-[N-morpholino] propane sulfonic acid (MOPS) 5; Na₂HPO₄, 1.2; CaCl₂, 1.6; MgSO₄, 1.2; D-glucose, 5.6; pH adjusted to 7.4 at 37°C. Dissection of medial rings, mounting and determination of the optimum length for stress development at 37°C was performed as described [21]. The carotid "ring" preparation differs in the method of mounting from our prior heat study which employed carotid "strips" [14]. The mounting procedure, rings vs. strips does not alter the behavior of the carotid media in tissue baths. The intimal surface was mechanically rubbed to remove the endothelium.

Antibodies

Rabbit anti-HSP20 antibody was made commercially via repeated injection of gel purified recombinant HSP20 (sequence confirmed by mass spectroscopy). After confirmation of an antigenic response, serum was collected and frozen for future use. Specificity was verified as described [8].

Measurement of HSP20 and MRLC phosphorylation

Swine carotid arteries were first thermally and histamine stimulated, followed by freezing in an acetone-dry ice slurry [15]. After air drying, the tissues were homogenized in a buffer (20 mg wet weight tissue/ml buffer) containing 1% SDS, 10% glycerol, and 1 mM dithiothreitol. Full

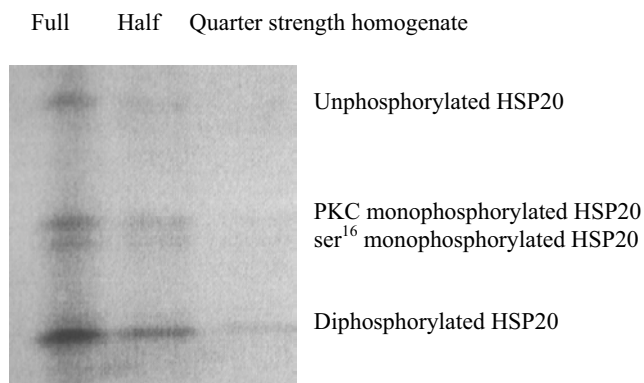


Figure 5

Representative blot of HSP20 immunostaining. The left column is full strength homogenate, the center column half strength homogenate, and the right column quarter strength homogenate. The top band is unphosphorylated, the center doublet is PKC monophosphorylated on top and ser¹⁶ monophosphorylated on bottom, and the bottom band is diphosphorylated HSP20. Full Half Quarter strength homogenate Unphosphorylated HSP20 PKC monophosphorylated HSP20 ser¹⁶ monophosphorylated HSP20 Diphosphorylated HSP20

strength, half strength, and quarter strength dilutions of samples were then separated on one-dimensional isoelectric focusing gels (ampholytes were a 50:50 mixture of pI 4–6.5 and pI 5–8 for HSP20 and a 50:50 mixture of pI 4–6.5 and pI 4.5–5.4 for MRLC), blotted to PVDF or nitrocellulose membranes, immunostained with our rabbit polyclonal anti-HSP20 antibody (1:5000) or rabbit polyclonal anti-MRLC antibody (1:4000 in 1% bovine serum albumin and 0.01% sodium azide), and detected with enhanced chemifluorescence (ECF) for HSP20 and enhanced chemiluminescence (ECL) for MRLC [22]. A representative blot is shown in Fig. 5. The dilutions ensured that the detection system was in the linear range. Immunoblots were scanned on a Molecular Dynamics fluorimager.

Phosphorylation was determined by change in the isoelectric point (pI) for each phosphorylation species. There are two HSP20 phosphorylation sites in the swine carotid, therefore, there are four immunoreactive species: unphosphorylated at pI 6.3 (top band in Fig. 5), monophosphorylated on a putative protein kinase C (PKC) site at pI 6.0 (second from the top band in Fig. 5), monophosphorylated on ser¹⁶ at pI 5.9 (second from the bottom band in Fig. 5), and diphosphorylated on both ser¹⁶ and the putative PKC site at pI 5.7 (bottom band in Fig. 5). Phosphorylation at ser¹⁶ was confirmed by mass

spectroscopy sequencing of the pI 5.7 isoform in our laboratory. Ser¹⁶-HSP20 phosphorylation was reported as the percent of ser¹⁶ monophosphorylated (pI 5.9) plus diphosphorylated HSP20 (pI 5.7) in relation to the sum of all four species.

Identification of the "PKC" site is putative and is best described by Beall, et al. [12]. They found that phorbol dibutyrate induced phosphorylation in the C terminal region of HSP20 based on tryptic digest analysis. We calculated putative PKC-HSP20 phosphorylation as the percent of putative PKC monophosphorylated HSP20 (pI 6.0) plus diphosphorylated HSP20 (pI 5.7) in relation to the sum of all species. For MRLC, phosphorylation was determined as the percent of phosphorylated smooth muscle MRLC in relation to total smooth muscle MRLC (i.e. non-muscle MRLC were ignored).

Cyclic nucleotide assays

[cAMP] and [cAMP] were determined by immunoassays were performed in a separate set of tissues treated identically except the homogenization buffer was 0.1 M HCl (detailed methods in [23]). Prior analyses showed the sensitivity of these assays in carotis smooth muscle homogenates [23]. The University of Virginia Diabetes Core lab performed the immunoassays with commercial reagents.

Statistics

Data are presented in the text and figures as mean \pm 1 SEM. Symbols without error bars reflect errors smaller than the size of the symbol. Data were compared by ANOVA and Student-Newman-Keuls if $n > 2$ and Student's unpaired t test if $n = 2$ with significance defined as $p < 0.05$.

Authors' Contributions

CR designed the study and wrote the manuscript. EK performed the experiments.

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