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Geldanamycin attenuates NO-mediated dilation in human skin

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Shastry, Shubha, and Michael J. Joyner. Geldanamycin attenuates NO-mediated dilation in human skin. *Am J Physiol Heart Circ Physiol* 282: H232–H236, 2002.— The binding of heat shock protein 90 (HSP90) to endothelial nitric oxide (NO) synthase (eNOS) can enhance eNOS activation. Studies have shown that the HSP90-specific inhibitor geldanamycin (GA) can cause attenuation of NO-mediated processes. Twenty subjects participated in one of two protocols. In each protocol, one forearm of each subject was instrumented with two intradermal microdialysis probes for drug delivery. Laser Doppler flowmeters were used to measure cutaneous blood flow. Skin sites were either treated with the endothelial agonist acetylcholine or locally heated to 42°C, a maneuver that evokes NO-mediated dilation. Interventions were performed with and without GA. In the presence of GA, maximal cutaneous vascular conductance (CVC) to ACh was 20 ± 3% lower than with ACh alone ($P < 0.001$). During local heating, maximal CVC in the presence of GA was 22 ± 6% lower than during heating alone ($P < 0.01$). The results show that GA can attenuate NO-mediated dilation in human skin, suggesting a potential role for HSP90 in activation of eNOS in the microcirculation.

heat shock protein 90; microdialysis; endothelial nitric oxide synthases; endothelium

NITRIC OXIDE (NO) is an important regulator of the cardiovascular system in humans and animals. Our laboratory has shown that NO is also involved in the control of cutaneous blood flow (CBF) during passive whole body heating (15, 16). Recently, studies have also linked NO to the vasodilation that is seen during local heating (7, 10). However, unlike whole body heating, where only ~30% of vasodilation evoked is NO dependent, the majority (~60%) of the dilation evoked by local heating is NO dependent.

Recent ex vivo and in vitro studies have shown an association between endothelial NO synthase (eNOS) and heat shock protein 90 (HSP90) (5, 14). This protein has been shown to act as a molecular chaperone, assisting in stabilization and refolding of other proteins during stress. HSP90 has also been shown to participate in many signaling pathways, including those involving tyrosine kinase (4).

Garcia-Cardena et al. (5) demonstrated that the binding of HSP90 to eNOS enhances eNOS activation and that eNOS exists in a complex with HSP90 in isolated cells and in the endothelium lining of intact vessels. Also, these authors demonstrated that inhibition of HSP90 in rat aortic rings attenuated ACh-induced relaxations (5), which are evoked through calcium-dependent NO synthesis. Shah et al. (14) showed that HSP90 inhibition in rat mesenteric arteries caused attenuation of ACh-induced relaxations, but did not affect relaxations to sodium nitroprusside (SNP), an NO donor. Khurana et al. (9) demonstrated that inhibition of HSP90 in canine basilar arteries was endothelium dependent. Taken together, these results indicate that HSP90 is necessary for full eNOS activation, especially in large conducting arteries.

However, this phenomenon has not yet been studied in the microcirculation or in humans. Therefore, our aim in this study was to investigate whether the observations of Garcia-Cardena et al. (5) and also of Shah et al. (14) concerning attenuation of ACh-mediated relaxations could be duplicated in humans using the cutaneous circulation as a model. We also sought to investigate whether inhibition of HSP90 would affect the local heating response in human skin, which is a normal physiological response that is NO dependent (7, 10).

METHODS

Subjects. The study was approved by the Institutional Review Board, and written informed consent was obtained from the subjects. Twenty subjects (10 males, 10 females) between the ages of 18 and 28 yr each participated in one of two protocols. The female subjects had a negative serum pregnancy test within the 48-h time period before the study. None of the subjects were taking medications, with the exception of some females who were taking oral contraceptives. Smokers and individuals with chronic medical conditions were excluded.

Subject monitoring. Mean arterial pressure (MAP) was monitored using the Finapres device (Ohmeda; Englewood, CO). Subjects were instrumented with two intradermal microdialysis probes (Bioanalytical Systems; W. Lafayette, IN) placed at different sites on the ventral portion of one forearm (7). Probes were placed ~5 cm apart so that perfusion at one

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site did not affect the other. Entry and exit points of each probe were ~2–3 cm apart. The probes used had a molecular weight cutoff of 20. The experimental site was referred to as *site 1*. The control site was referred to as *site 2*. Local heaters were placed on the sites, and CBF was measured with laser Doppler flowmeters (PF 5010, Perimed; Stockholm, Sweden) placed directly over the microdialysis sites in the middle of the local heaters. A period of 60–80 min was then waited to allow insertion trauma to subside.

Drug preparation. Geldanamycin (GA) was obtained from Calbiochem (San Diego, CA). ACh was obtained from OMJ Pharmaceuticals (San German, PR). SNP was obtained from Abbott (N. Chicago, IL). GA was dissolved with 3% dimethyl sulfoxide (DMSO, Sigma; St.Louis, MO). ACh and SNP were dissolved in Ringer solution. GA was perfused at a concentration of 178 μM, whereas ACh was perfused at a concentration of 5.5 mM. SNP was infused at a concentration of 28 mM. All drugs were perfused into the skin with a mechanical syringe pump at a rate of 2 μl/min.

Protocol 1. Ten subjects (5 males, 5 females) participated in *protocol 1*. After subjects had been instrumented and insertion trauma had subsided, Ringer solution was perfused through both *sites 1* and *2* while baseline flows were measured. ACh was perfused through both sites until maximal dilation had been reached. ACh was then replaced with GA in *site 1* and replaced with vehicle in *site 2*. After CBF had returned to baseline values, GA and vehicle were then replaced with ACh + GA in *site 1* and ACh + vehicle in *site 2*. Four additional studies were performed in which sites were treated with SNP, once after the first ACh perfusion, without GA, and again after the second ACh perfusion in the presence of GA. This was to determine whether dilation to SNP was similar with and without GA. Only four of these studies were performed due to the long washout period of SNP.

Protocol 2. Ten subjects (5 males, 5 females) participated in *protocol 2*. As in *protocol 1*, after subjects had been instrumented and insertion trauma had subsided, Ringer solution was perfused through both *sites 1* and *2* while baseline flows were measured. A 3% solution of DMSO (vehicle) was then perfused through *site 2* for 10 min, whereas Ringer solution was perfused through *site 1*. Both sites were then heated to 42°C. During heating, Ringer solution was perfused through both sites. When CBF values had reached a plateau, local heaters were turned off, and CBF values in both sites were allowed to return to baseline (~1 h). Perfusion of GA in *site 1* and vehicle in *site 2* began ~30 min after local heaters were turned off. With GA and vehicle still perfusing through *site 1* and *site 2*, respectively, the second local heating to 42°C commenced. After a plateau in CBF, GA and vehicle were removed, and SNP was perfused through both sites for ~15–20 min until a plateau was reached.

Data collection and analysis. Data were collected on computer, digitized at 100 Hz, and then analyzed offline using signal-processing software (WinDaq, Dataq Instruments; Akron, OH). CBF was derived from the laser Doppler signal. MAP was derived from the Finapres signal. Cutaneous vascular conductance (CVC) calculations were made by dividing the laser Doppler flow signals by MAP.

Statistics. In both protocols, the effects of GA were compared with the responses in the same site before GA perfusion. In *protocol 1*, maximal CVC response to the first ACh perfusion was used as a 100% marker. In *protocol 2*, maximal CVC response to the first heating was used as a 100% marker. Repeated-measures one-way analysis of variance tests and paired *t*-tests were used to compare CVC and MAP responses during each protocol. *t*-Tests were used to compare CVC responses between *site 1* and *site 2*. Significance was set at $P < 0.05$ level for all comparisons. Significant effects were further analyzed by using Tukey's test. Data are reported as means ± SE.

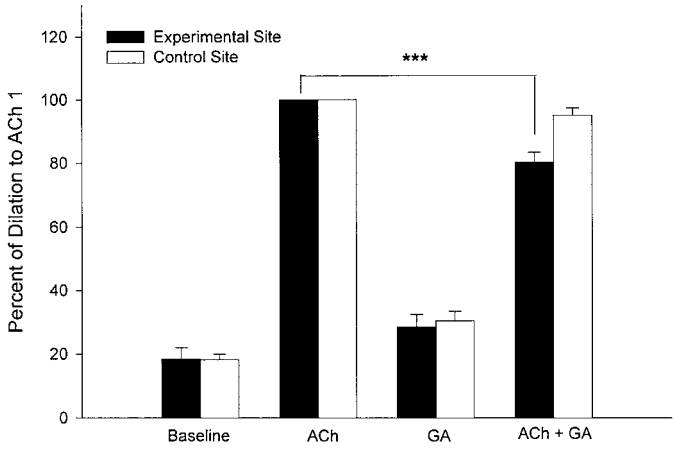

Fig. 1. Cutaneous vascular conductance (CVC) responses at baseline and with ACh, geldanamycin (GA)/vehicle, and ACh + GA/vehicle (*protocol 1*). In the experimental site, ACh + GA was significantly lower than ACh (***$P < 0.001$), whereas in the control site, ACh + vehicle was similar to ACh alone ($P > 0.05$).

RESULTS

Protocol 1. MAP increased gradually throughout the protocol, although these increases were not statistically significant. In *site 1*, CVC at baseline was 18 ± 4% of the maximal CVC response to ACh (Fig. 1). With ACh infusion, CVC increased significantly compared with baseline ($P < 0.001$, Fig. 1). With GA perfusion, CVC dropped to 28 ± 4% ($P < 0.01$ vs. ACh, $P > 0.05$ vs. baseline, Fig. 1). After perfusion of ACh + GA, maximal CVC response was blunted to 80 ± 3% ($P < 0.01$ vs. first ACh, $P < 0.001$ vs. GA, Fig. 1).

In *site 2*, CVC was 18 ± 2% at baseline (Fig. 1). With perfusion of ACh, CVC increased significantly ($P < 0.001$ vs. baseline, Fig. 1). Maximal CVC responses to ACh did not differ between *sites 1* and *2* ($P > 0.05$). With vehicle treatment, CVC in *site 2* decreased to 30 ± 3% ($P < 0.001$ vs. ACh, $P > 0.05$ vs. baseline, $P > 0.05$ vs. *site 1*, Fig. 1). After the perfusion of ACh + vehicle, CVC increased to 95 ± 2% ($P > 0.05$ vs. first ACh, $P < 0.001$ vs. vehicle, $P < 0.01$ vs. *site 1*, Fig. 1).

In the four additional studies performed, where SNP + GA/vehicle was compared with SNP alone, the first SNP perfusion was similar to the second in both sites (96 ± 5% of SNP alone in *site 1*, $P > 0.05$, 98 ± 3% in *site 2*, $P > 0.05$, data not shown).

Protocol 2. MAP gradually increased during the latter phases of this protocol. The increases attained significance compared with baseline only during the SNP perfusion period of the study ($P < 0.05$, 82 ± 3 vs. 93 ± 5 mmHg, Table 1). However, this increase was most likely not due to any effect of SNP on MAP but due to the length of the study (~6 h) and the fact that the increased values were measured in the afternoon compared with baseline values, which were measured in the morning.

Table 1. *MAP responses throughout study*

	Baseline	DMSO	Heat/ACh	GA	Heat/ACh	SNP
Protocol 1	72 ± 4		74 ± 4	79 ± 4	80 ± 4	
Protocol 2	82 ± 3	81 ± 4	83 ± 4	86 ± 3	90 ± 6	93 ± 5*

Values are means ± SE. During *protocol 2,* mean arterial pressure (MAP) values measured (in mmHg) during the sodium nitroprusside (SNP) phase were significantly higher than baseline values (*$P < 0.05$). DMSO, dimethylsulfoxide; ACh, acetylcholine.

Figure 2 is an individual record of a subject from this protocol. In this subject, there was a blunting of the local heating response in the experimental site, whereas the control site remained constant. Figure 3 indicates the responses for all subjects during different phases of this protocol. In *site 1*, CVC was 13 ± 1% of the first maximal heating at baseline. During the DMSO perfusion in *site 2* (Ringer solution in *site 1*), CVC in *site 1* remained constant at 14 ± 1% ($P > 0.05$ vs. baseline, Fig. 3). During heating, CVC increased significantly ($P < 0.05$, heating vs. baseline, Fig. 3). After heaters were turned off and the site was treated with GA, CVC dropped to 17 ± 2% ($P < 0.05$ vs. heating, $P > 0.05$ vs. baseline, Fig. 3). During heating + GA, maximal CVC to heating was significantly blunted to 78 ± 6% ($P < 0.01$ vs. first heating, $P < 0.001$ vs. GA, Fig. 3). Perfusion with SNP caused CVC to increase to 99 ± 7% ($P < 0.001$ vs. second heating, Fig. 2).

In *site 2*, CVC at baseline was 16 ± 2% of the CVC obtained with the first heating ($P > 0.05$ vs. *site 1*, Fig. 3). With DMSO treatment, *site 2* dilated slightly to 21 ± 3% ($P < 0.05$ vs. baseline, $P < 0.05$ vs. *site 1*, Fig. 3). After heating, CVC increased significantly ($P < 0.001$ vs. baseline and DMSO, Fig. 3). Maximal CVC values between *site 1* and *site 2* before normalization to 100% did not differ ($P > 0.05$). After heaters were turned off and the site was treated with vehicle, CVC in *site 2* dropped to 21 ± 3% ($P < 0.001$ vs. heating, $P > 0.05$ vs. baseline, $P > 0.05$ vs. *site 1*, Fig. 3). During the second heating (with vehicle), CVC was 94 ± 7% ($P > 0.05$ vs. first heating, $P > 0.05$ vs. *site 1*, Fig. 3). After perfusion with SNP, CVC increased to 108 ± 7% ($P < 0.05$ vs. second heating, $P > 0.05$ vs. *site 1*, Fig. 3).

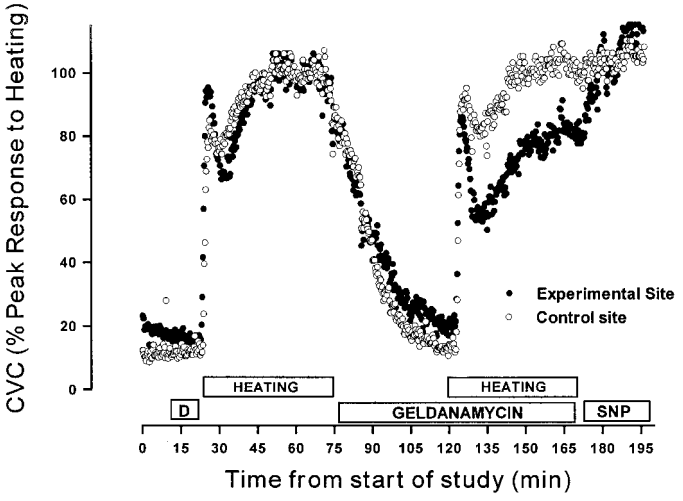

Fig. 2. Individual record of a subject in *protocol* 2. This subject showed a slight dilation with DMSO in the control site. Experimental site showed a ~20% blunting during the second heating, whereas the control site was similar to the first heating. Sodium nitroprusside (SNP) caused the experimental site to dilate further.

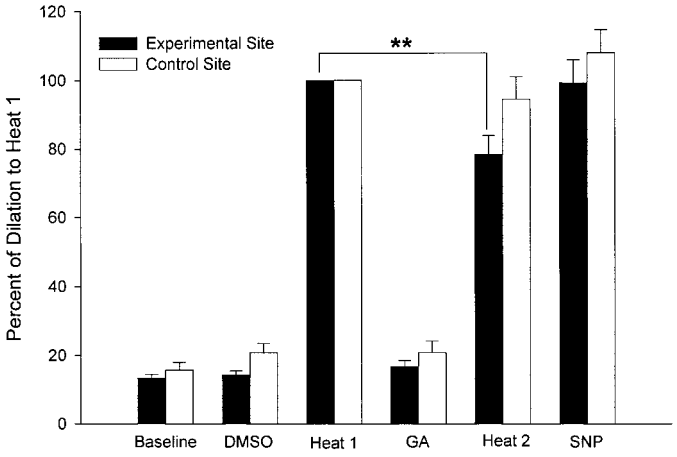

Fig. 3. CVC responses at baseline and with DMSO/Ringer, heating, GA/vehicle, heating + GA/vehicle, and SNP. DMSO caused a mild dilation in the control site ($P < 0.05$). In the experimental site, heating + GA was significantly lower than heating alone (**$P < 0.01$), whereas in the control site, heating + vehicle was similar to heating alone ($P > 0.05$). In the experimental site, SNP caused CVC to dilate significantly ($P < 0.05$).

DISCUSSION

Our results indicate that GA can attenuate dilations to ACh and also local heating by ~20% in human skin, indicating a potential role for HSP90 in these responses. The findings also suggest that dilation to SNP was not altered by GA, indicating that the vasodilatory properties of the vessels themselves in response to exogenous NO are not compromised by GA. These findings, the first to study the phenomenon of HSP90-eNOS interaction in vivo in humans, as well as in the microcirculation, suggest a potential role for HSP90 in eNOS activation in human skin vessels.

Our findings are supported by the observations of Garcia-Cardena et al. (5) and also of Shah et al. (14). Garcia-Cardena et al. showed that GA significantly attenuated the maximal response to ACh, but not SNP, in isolated rat aortic rings. Shah et al. demonstrated that relaxations to ACh in the presence of GA were significantly attenuated in rat mesenteric arteries; relaxations to SNP were not affected.

In both of the aforementioned studies, the degree of the attenuations seen with GA was greater than that seen in our study. In these studies, relaxation to ACh was attenuated by 60–70%, but only ~20% in our study. There are several potential reasons for this discrepancy. First, it is possible that a larger concentration of GA would have caused greater attenuation. However, it was our intention to use the lowest concentration of GA that would effect a noticeable atten-

uation, and several preliminary studies were carried out to determine this dose. In fact, to compensate for any loss of the drug due to equilibration and diffusion, we used a concentration of GA that was 10 times those used by Garcia-Cardena et al. (5) and also Shah et al. (14).

Second, in contrast to the animal studies, the primary vessels that dilated in response to either ACh or heating in our study were probably resistance vessels, where the role of NO might be less pronounced than in the large conducting vessels used in the animal studies (11). Another factor that could explain the modest attenuation we observed involves the contribution of NO to ACh-evoked cutaneous vasodilation. Warren (19) found that administration of a NOS inhibitor to skin in vivo significantly attenuated local vasodilation caused by ACh. However, both Noon et al. (8) and Khan et al. (12) demonstrated that CBF responses were more prostanoid dependent rather than NO dependent, because NOS inhibition appeared to have little effect on the ACh response in human skin, whereas aspirin had a much greater inhibitory effect. Moreover, results by Buus et al. (2) have shown that ACh relaxation in human subcutaneous small arteries is mainly dependent on a non-NO, nonprostanoid endothelium-dependent mechanism. Taken together, these results seem to indicate that the degree of NO contribution to the ACh response is uncertain and may vary in different types of vessels.

We also sought to investigate the involvement of HSP90 in eNOS activation using local heating, which evokes physiological vasodilation that is NO dependent (7, 10). Kellogg et al. (7) demonstrated that local heating to 42°C caused a maximal dilation in skin and that administration of NOS inhibitors caused the heating response to be blunted by almost 60%. In our study, administration of GA caused the heating response in the experimental site to be attenuated by ~20%, whereas the response in the control site did not change. While it could be argued that the minor vasodilating properties of DMSO may be responsible for increasing the second heating response in *site 2*, it should be pointed out that preliminary studies from our laboratory have shown that successive local heating responses in the same site tend to be similar. This is especially true if there is at least a 1-h period between heatings. In our protocol, there was at least a 1-h period between the first and second heating.

Recent data from our laboratory provide further evidence that the attenuation in the local heating response during GA administration may be NO linked. Minson et al. (10) demonstrated that two independent mechanisms mediate the biphasic response seen with local heating. The second, more sustained, rise in CVC appears to be NO dependent; however, the initial peak appears to be mediated by an axon-reflex-mediated, fast-responding vasodilator system that is NO independent. The data from the current study indicate that GA did not affect this initial peak, which is consistent with our hypothesis that GA-induced HSP90 inhibition affects eNOS activation.

There are some potential limitations to this study. Some of the female subjects in the study were using oral contraceptives. Results from Charkoudian et al. (3) indicate that women in the high hormone phase of oral contraceptives showed augmented cutaneous vasodilation to local warming. Also, a study conducted by Russell et al. (13) indicates that HSP90 may also be involved in estrogen-modulated eNOS activation. In that study, results indicated that the mechanism by which estrogen activates eNOS involves changes in the binding pattern of HSP90 to eNOS (13). We did not find any significant differences in CVC responses between women on oral contraceptives and the other women in the study. However, our study was an acute one, occurring on only 1 day.

Another limitation is the fact that we were not able to determine whether the attenuation of CVC responses with GA was due to inhibited binding of HSP90 to eNOS. Because there is a component of vasodilation to local heating that does not appear to be NO mediated, it is possible that other HSP90-mediated mechanisms relating to local vasodilation may have been impaired, causing the decreases in CVC seen with GA. However, this possibility seems unlikely, considering the evidence that NO-mediated responses can be attenuated through HSP90 inhibition (1, 5, 6, 9, 13, 17). Although GA was initially thought to be a tyrosine kinase inhibitor, it is now thought that the effect of GA on tyrosine kinase is actually accomplished through HSP90 inhibition (4). GA is now widely accepted as an HSP90-specific inhibitor (5, 18) and, therefore, it is assumed that the attenuation of CVC seen in the current study was related in some manner to impairment of HSP90.

What is the nature of this purported interaction between eNOS and HSP90? Gratton et al. (6) have evidence suggesting that the presence of HSP90 causes calmodulin to displace eNOS from a complex with caveolin-1. Caveolin-1 is a protein that maintains eNOS in an inactive state, whereas calmodulin causes eNOS stimulation. This evidence is supported by the fact that there is a calmodulin-binding site present on HSP90. These findings seem to indicate a role for calcium and calcium-dependent proteins in the activation of eNOS by HSP90.

In summary, our results indicate that HSP90 may be necessary for full activation of eNOS in human skin vessels. This suggests a potential relationship between these two proteins in humans, not only in skin, but also perhaps in other areas as well.

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