Specific Induction of the 70-kD Heat Stress Proteins by the Tyrosine Kinase Inhibitor Herbimycin-A Protects Rat Neonatal Cardiomyocytes

A New Pharmacological Route to Stress Protein Expression?

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Abstract

Heat shock protein (hsp) induction by stressful stimuli such as heat and ischemia is known to protect cardiac cells from severe stress. The ability to induce hsp's in the heart directly by "nonstressful" means would potentially have important clinical implications. In noncardiac cells, the tyrosine kinase inhibitor herbimycin-A has been shown to induce the 72-kD hsp. We therefore examined whether herbimycin-A and another tyrosine kinase inhibitor, genistein, could induce 70-kD hsp's in primary cultures of rat neonatal cardiomyocytes, and whether these treatments protect against severe stress. Primary cardiomyocytes were incubated with herbimycin-A or genistein. hsp induction was measured 16–20 h later by Western blotting. Cell survival after subsequent lethal heat stress or simulated ischemia was assessed using trypan blue exclusion and released lactate dehydrogenase activity. Our results indicate that, in cardiac cells, herbimycin-A induces 70-kD hsp's but not hsp90, -60, -25, or glucose-regulated protein 78, whereas genistein has no effect on hsp's. Moreover, hsp induction correlated with the ability of herbimycin-A to protect cells against severe stress, whereas genistein had no protective effects. This suggests that herbimycin-A may induce 70-kD hsp's via a tyrosine kinase–independent mechanism. These results indicate the possibility of a pharmacological approach to HSP70 induction and cardiac protection, which may ultimately be of clinical relevance. (*J. Clin. Invest.* 1996. 97:706–712.) Key words: myocardial protection • heat shock proteins • myocardial infarction • primary cardiomyocyte

Introduction

Over the past few years investigations have shown that part of the myocardium subjected to ischemic damage is potentially salvageable. Restoration of normal cardiac function would require removal of denatured cardiac protein and reestablishment of normal cardiac protein synthesis. The heat stress proteins, which are synthesized in all organisms in response to stresses such as heat and ischemia, may well play a pivotal role in providing protection from a subsequent stress (1, 2). The heat stress response is known to be initiated by activation of the heat shock factor (HSF)[1] 1, which is normally present in unstressed cells and rapidly trimerizes in response to metabolic stress. This enables HSF-1 to bind to the heat shock element located within the promoter of the genes encoding the heat shock proteins, resulting in a high level of transcription of these genes (1).

Previous investigators have found that the induction of heat shock proteins (hsp's) coincides with the appearance of cytoprotection. For example, exposure of cells to sublethal hyperthermia results in induction of hsp's and the acquisition of thermotolerance (3). Furthermore, the induction of hsp's by one insult, such as toxin exposure, may confer protection from a subsequent different insult, such as heat shock challenge (4). This potentially exploitable property of cells may allow a therapeutic approach to minimizing cellular injury during myocardial ischemia. In cardiac tissues, a wide variety of insults results in the synthesis of stress proteins, including myocardial ischemia (5, 6), trauma (7), and hyperthermia (8). Further studies have shown a direct correlation between the amount of the inducible 70-kD heat stress protein and the degree of myocardial protection. For example, we have examined the effects of whole body heat stress using an isolated rabbit papillary muscle model (9), concluding that the degree of contractile recovery after 30 min of hypoxia was related to the content of hsp72. In addition, Hutter et al. (10) were able to show that myocardial infarct size in the rat 24 h after different severities of whole body heat stress was inversely correlated with myocardial hsp72 content. Moreover, evidence that myocardial stress proteins are directly protective is provided by the observation that transfected myocyte lines overexpressing HSP70, but not hsp90, have enhanced resistance to hypoxic stress (11–13).

There is compelling evidence, therefore, that stress proteins may directly influence the resistance of the heart to ischemia, and hence any pharmacological intervention that would raise stress protein levels within the cell by a direct and "nonstressful" means would have important therapeutic implications. Interestingly, herbimycin-A, a benzoquinoid ansamycin antibiotic that inactivates p-60[v-src] tyrosine kinase, has been

1. *Abbreviations used in this paper:* BHK, baby hamster kidney; grp, glucose-regulated protein; HSF, heat shock factor; hsp, heat shock protein; hsp25, the 25-kD heat stress protein; hsp60, the 60-kD heat stress protein; hsp72, inducible member of the 70-kD heat stress protein family; hsp73, constitutive member of the 70-kD heat stress protein family; hsp90, the 90-kD heat stress protein family; LDH, lactate dehydrogenase.

shown to induce hsp72 in a range of cells, including A431 human epidermoid carcinoma cells, HeLa S3 cells, chick embryo fibroblasts, and N1H3T3 cells (14). As yet the exact mechanism of action of herbimycin-A in this regard is uncertain, but preliminary evidence suggests that hsp induction is not secondary to cellular damage (15).

We have therefore attempted to ascertain the ability of herbimycin-A to induce hsp's in primary cultured neonatal rat cardiomyocytes, using genistein, another tyrosine kinase inhibitor, for comparison. Furthermore, we investigated whether these drugs were able to afford any degree of protection against "lethal" heat stress or lethal simulated ischemia. In addition, we compared these agents to "thermal preconditioning," which is an established method of hsp induction (16, 17).

To clarify some of the terms used in this paper, the following definitions apply. (*a*) Nonstressful: does not induce a wide range of stress proteins: (*b*) mild stress: does not cause major lethality, i.e., known to be sufficient to induce stress proteins but not significant cell death; and (*c*) severe stress: kills a high proportion of cells.

Methods

Animals

This investigation was performed in accordance with the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986* published by HMSO 1986, London.

Cell culture

Isolated cardiac myocyte model. Ventricular myocytes from the hearts of neonatal Sprague Dawley rats < 2 d old were cultured using previously described methods (18–20). The cells were dispersed in a nominally calcium-free, Hepes-buffered salt solution containing 0.6 mg/ml pancreatin (GIBCO BRL, Gaithersburg, MD) and 0.5 mg/ml type II collagenase ($\sim$ 266 U/mg) (Worthington Biochemical Corp., Freehold, NJ) via a series of incubations at 37°C. The dispersed cells were preplated for at least 30 min to allow the fibroblasts to adhere to the plate, and the myocytes remained unattached. The myocyte-enriched unattached cells were replated on six-well gelatine coated plates at a density of 1.5–2 million cells per well. The cardiac myocytes were cultured at 37°C, 5–7% CO_2, in 4:1 DME/Medium 199 (GIBCO BRL) supplemented with 10% horse serum, 5% FCS, and 1% penicillin/streptomycin. After 24 h, the medium was replaced with serum-free medium to reduce fibroblast contamination. Cardiocyte cultures under these conditions start to beat synchronously within 72 h, and the percentage of beating cells exceeds 85% for the duration of the experiment.

Baby hamster kidney (BHK) cell culture. BHK (C13) cells were cultured at 37°C, 5–7% CO_2 in DME supplemented with 10% FCS (21).

Experimental protocols

Once the cultured cardiomyocytes were beating synchronously, the following protocols were carried out. Six-well plates were incubated for 4 h with herbimycin-A (1 μg/ml) or with genistein (50 μM), both agents having been dissolved in DMSO. Control plates were incubated with the vehicle DMSO or were simply left in medium. The cells were then washed with medium and returned to the incubator at 37°C. As a comparison, certain plates of cells were thermally preconditioned by incubation at 43°C for 30 min. The following day, cells were harvested for hsp levels or subjected to severe heat shock or "simulated" ischemia.

PAGE and Western blotting

20 h after the above treatments, the cells were harvested for analysis of HSP levels: $\sim 2 \times 10^6$ cells were harvested in 100 μl of 2× concentrated SDS-PAGE sample buffer, and proteins were separated by SDS-PAGE (10% acrylamide) (22). Proteins were transferred to nitrocellulose membranes (Hybond C; Amersham International, Little Chalfont, UK), and blots were probed with mAbs specific for the inducible isoform of hsp70 (hsp72) (C92F3A-5; Stressgen, Victoria B.C., Canada) or with mAbs specific for both constitutive and inducible isoforms (hsp73 and -72, respectively) (SPA-820; Stressgen) at 1:1,000 dilution. In addition, blots were probed with mAbs to hsp90 (AC88, a gift from Dr. D. Toft, Mayo Clinic, Rochester, NY) at a concentration of 12.15 μg/ml, to hsp60 (clone 4B9/89; Affinity Bioreagents, Neshanic Station, NJ) at 5 μg/ml, or to hsp25 (H0148; Sigma Chemical Co., Poole, UK) at a 1:500 dilution. Certain blots were also probed with an mAb specific for glucose-regulated protein (grp) 78 (SPA-827M; Bioquote, York, North Yorkshire, UK) at a dilution of 1:500. The blots were subsequently washed in PBS/0.5% Tween and then incubated with a peroxidase-conjugated rabbit anti–mouse IgG antibody (DAKOPATTS, Copenhagen, Denmark) at a dilution of 1:2,000, followed by detection using an enhanced chemiluminescence kit (Amersham International). The relative levels of hsp were determined using densitometry (Bio-Rad Laboratories, Richmond, CA), normalizing to the actin band on a duplicate Coomassie brilliant blue R250 (BDH Chemicals Ltd., Poole, UK)–stained gel. This procedure adjusts for slight variations in protein loading between samples. Initially, to determine the optimum time for further experimentation, the cells were harvested at intervals between 30 min and 48 h after treatment with tyrosine kinase inhibitor or thermal preconditioning to determine hsp levels. Maximal induction occurred at 20 h after either treatment; therefore, protection and further quantification of hsp levels were sought at this time point.

Tyrosine kinase inhibition

To demonstrate tyrosine kinase inhibition by herbimycin-A and genistein, at the doses used in these experiments, the following protocol was carried out. Herbimycin-A (1 μg/ml) or genistein (25, 50, 100, 150 μM) was added to wells of cardiocytes and incubated at 37°C, and controls were incubated with the vehicle DMSO or were left untreated. Initially, this experiment was performed with insulin-like growth factor 1 added to each well to stimulate tyrosine kinase activity, but there was sufficient intrinsic tyrosine kinase activity in control cells without growth factor for this experiment to be carried out on unstimulated cells. Samples were harvested with SDS-PAGE sample buffer at the following time intervals after the addition of drug: 10, 30, 60, 120, and 240 min. PAGE and Western blotting were carried out as above using a monoclonal antiphosphotyrosine antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at a dilution of 1:1,000. The secondary antibody was a peroxidase-conjugated rabbit anti–mouse IgG antibody (DAKOPATTS), diluted to 1:2,000, and enhanced chemiluminescence was used as above. Again, relative levels of phosphotyrosine were determined by densitometry normalizing to the actin band.

Lethal heat stress

20 h after incubation with herbimycin-A, genistein, or DMSO, or after thermal preconditioning, the cultures were subjected to a severe thermal stress by incubating them at 47°C for 90 min.

Lethal simulated ischemia

20 h after incubation with herbimycin-A, genistein, or DMSO, or after thermal preconditioning, the cardiocytes were transferred to an ischemic buffer adapted from Esumi et al. (23): (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$.2 H_2O, 4 mM Hepes) supplemented with 10 mM deoxyglucose, 0.75 mM sodium dithionate, 12 mM KCl, and 20 mM lactate, pH 6.5, and incubated for 90 min at 37°C. This buffer is designed to simulate the extracellular milieu of myocardial ischemia, with the approximate concentrations of potassium, hydrogen, and lactate ions occurring in vivo.

Determination of cardiocyte viability

Trypan blue exclusion. After lethal heat stress or simulated ischemia, the cells were washed with PBS, trypsinized for 2 min in 0.25 mg/ml trypsin in versine (GIBCO BRL), and then neutralized with neonatal calf serum. Cells were then centrifuged, the supernatant was aspirated, and the cardiocytes were resuspended in 300 μl of PBS. A 20-μl aliquot of cell suspension was then added to an equal volume of trypan blue, and the percentage of dead (blue) cells was determined using a hemocytometer. To establish that the administration of these drugs did not have a directly toxic effect upon the cardiocytes, trypan blue exclusion was also performed at 4 and 24 h after incubation with herbimycin-A or genistein, in comparison with controls, without lethal stress.

Lactate dehydrogenase (LDH) assay. LDH activity released from cardiomyocytes both before and after subjection to lethal stress, was determined using an LDH test kit (Sigma Chemical Co.). This test relies on the fact that LDH catalyzes the reduction of pyruvate to lactate, resulting in an equimolar amount of NADH oxidized to NAD. The oxidation of NADH results in a decrease in the absorbance at 340 nm. The rate of decrease in absorbance at 340 nm is directly proportional to LDH activity in the sample. Therefore, using a spectrophotometer measuring absorbance at 340 nm, the mean absorbance change per minute was determined in the media from each experimental group. This method of assessing cell damage was chosen because it is reliable even in the presence of ischemic buffer, unlike creatinine kinase activity (24).

Statistical analysis

All values are expressed as mean±SEM. The unpaired Student's *t* test was used to identify significant differences between experimental and control groups. Statistical significance was assumed at the $P < 0.05$ level.

Results

Incubation with herbimycin-A or genistein at all concentrations studied, or treatment by thermal preconditioning, did not result in significant cell death compared with controls as assessed by trypan blue exclusion (< 3%). In addition, light microscopic appearances were unchanged, and the cells continued to beat synchronously (data not shown). Furthermore, released LDH activity was negligible in treated cells and controls when measured immediately after addition of tyrosine kinase inhibitor or treatment with thermal preconditioning, and when measured at intervals until subjecting the cardiomyocytes to lethal stress (see below).

Heat shock protein induction. After 4 h of incubation with herbimycin-A, there was a significant increase in hsp72 from almost negligible levels to $> 100\times$ that found in control cells ($P < 0.0001$) when samples were normalized to the actin band from a Coomassie-stained gel (Fig. 1, *A* and *B*). This level of hsp72 increase was of a similar magnitude to that induced by thermal preconditioning, and there was no statistically significant difference between amounts of hsp72 induced by herbimycin-A or thermal preconditioning. Genistein treatment at a wide range of doses did not lead to any significant hsp induction. A study of the time course of hsp72 induction over 48 h showed maximal induction between 19 and 24 h after herbimycin-A treatment or thermal preconditioning, but again there was no evidence of hsp induction by genistein up to 48 h (Fig. 2).

To examine the effect of herbimycin-A on the constitutive 70-kD hsp (hsp73), we used the antibody that recognizes hsp72 and -73, since there is no antibody specific for hsp73 alone. In this experiment, herbimycin-A induced both constitutive and
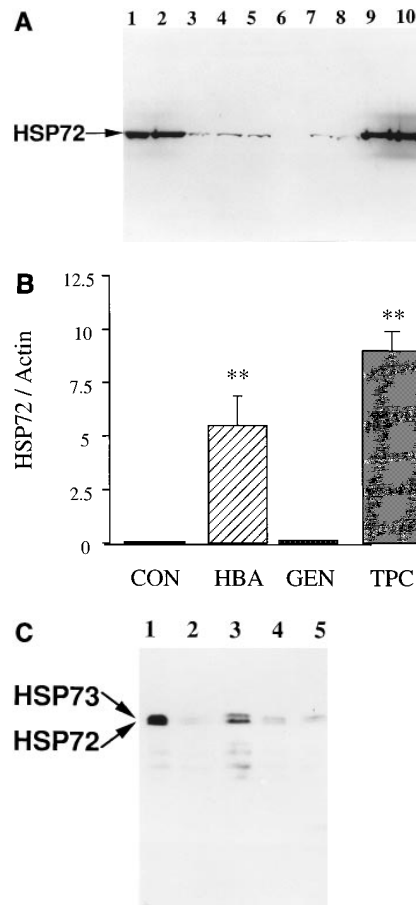
Figure 1. (*A*) Western blot of samples harvested from primary cardiomyocyte cultures 20 h after treatment with tyrosine kinase inhibitor. Six-well plates were incubated for 4 h with herbimycin-A or genistein dissolved in DMSO. For comparison, certain plates were thermally preconditioned by incubating them at 43°C for 30 min. Control plates were incubated with the vehicle DMSO or simply left in medium. The primary mAb recognizes the inducible form (hsp72) of the 70-kD heat shock protein. Lanes from left to right: *1*, herbimycin-A; *2*, herbimycin-A; *3*, control, no intervention; *4*, control, no intervention; *5*, control, DMSO; *6*, blank lane; *7*, genistein; *8*, genistein; *9*, thermal preconditioning; *10*, thermal preconditioning. The protein band representing hsp72 is indicated (*arrow*). (*B*) Relative levels of hsp72 as assessed by densitometry (Bio-Rad Laboratories) normalizing to the actin band on a duplicate Coomassie brilliant blue R250–stained gel. Bars represent 1 SEM. Data are presented as mean values (±SE) from duplicate determinations in five independent experiments. *CON*, control; *HBA*, herbimycin-A; *GEN*, genistein; *TPC*, thermal preconditioning. **$P < 0.0001$ (unpaired Student's *t* test). (*C*) Western blot of samples harvested from primary cardiomyocyte cultures 20 h after treatment with tyrosine kinase inhibitor. Six-well plates were incubated for 4 h with herbimycin-A (1 μg/ml) or genistein (50 μM) dissolved in DMSO. For comparison, certain plates were thermally preconditioned by incubating them at 43°C for 30 min. Control plates were incubated with the vehicle DMSO or simply left in medium. The primary mAb recognizes both the inducible (hsp72) and constitutive (hsp73) isoforms of the 70-kD heat shock protein. Lanes from left to right: *1*, herbimycin-A; *2*, genistein; *3*, thermal preconditioning; *4*, control, no intervention; *5*, control, DMSO. The protein bands representing hsp72 and -73 are indicated (*arrow*).
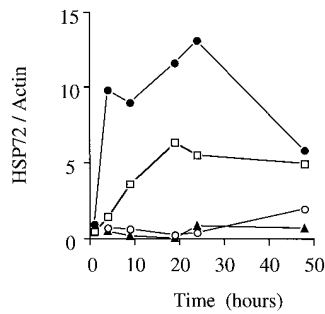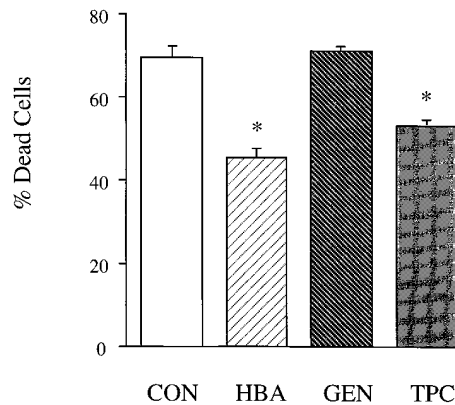
Figure 2. The time course of hsp72 induction. Primary cardiocytes were incubated with herbimycin-A or genistein for 4 h, and samples were harvested for Western blotting at intervals between 30 min and 48 h. Control plates were incubated with DMSO or simply left in medium. The primary mAb recognizes the inducible form of the 70-kD heat shock protein (hsp72). ——□——, herbimycin-A; ——●——, thermal preconditioning; ——○——, genistein; ——▲——, control.

Figure 4. Percentage of cell death after lethal heat stress as assessed by trypan blue exclusion. Six-well plates of primary cardiocyte cultures were incubated with herbimycin-A, genistein, or DMSO (control) for 4 h. For comparison, certain plates were thermally preconditioned by incubating them at 43°C for 30 min. 24 h later, cells were exposed to 47°C for 90 min. After this lethal heat stress, cells were exposed to an equal volume of trypan blue, and the percentage of dead (blue) cells is shown. Data are presented as mean values (±SE) from duplicate determinations in five independent experiments. CON, control; HBA, herbimycin-A; GEN, genistein; TPC, thermal preconditioning. *$P < 0.005$ (unpaired Student's t test).

inducible isoforms of hsp70 (hsp73 and -72, respectively), whereas, in the thermal preconditioning group, the induction is mainly observed in the hsp72 band, as expected (Fig. 1 *C*). Because of the magnitude of induction of 70-kD stress proteins by herbimycin-A, the bands are almost confluent, resolution limited by the fact that there is no antibody available that recognizes hsp73 alone. In addition, there was no evidence of increases in hsp90 (Fig. 3 *A*), hsp60 (Fig. 3 *B*), or hsp25 (Fig. 3 *C*) after herbimycin-A or genistein treatment. Furthermore, these treatments did not induce the glucose-regulated protein grp78 (Fig. 3 *C*). However, as expected, the hsp's were induced by thermal preconditioning, as was grp78, which has also been shown to be induced by heat stress (25).

To compare these results in noncardiac cells, we followed the same protocol with BHK cells. After 4 h of incubation with herbimycin-A, there was a significant increase in hsp72 levels as well as a slight increase in hsp73 (Fig. 3 *D*). In contrast to our findings in cardiomyocytes, there was also a modest induction of other hsp's when these noncardiac cells were treated with herbimycin-A.

Survival after lethal heat stress. Treatment of cardiomyocytes with herbimycin-A led to a significant increase in survival after lethal heat stress in comparison with control cells as assessed using trypan blue exclusion. Each experiment was performed five times using five different preparations of cells, with at least three wells per treatment group. As seen in Fig. 4,

cell death in herbimycin-A–treated cells was 45.0% (±3.6), whereas cell death in control groups was 69.4% (±3.4) ($P < 0.005$, $n = 10$). Thus, herbimycin-A–treated cells have a statistically significant 30% improvement in survival after lethal heat stress. A similar degree of protection was obtained by thermal preconditioning with 52.9% (±1.8) dead; the difference in improved survival between the herbimycin-A–treated and the thermal preconditioning groups was not statistically significant. However, genistein (50 μM) treatment led to no significant improvement in survival (70.9% [±2.0]).

Comparable results were obtained by analysis of released LDH activity. None of the pretreatments resulted in significant LDH release, indicating that they did not produce significant cell damage. However, after subsequent lethal heat stress,
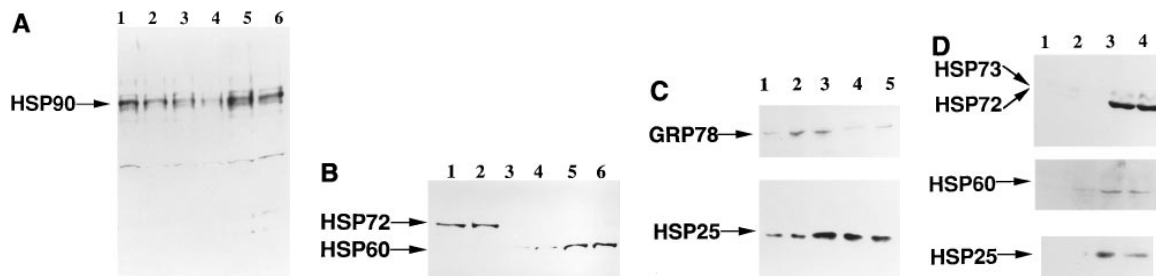

Figure 3. (*A*) Western blot of samples harvested from primary cardiomyocyte cultures 20 h after a 4-h incubation with herbimycin-A (1 μg/ml) or genistein (50 μM). The primary mAb recognizes the 90-kD heat stress protein. Lanes from left to right: *1*, thermal preconditioning; *2*, herbimycin-A; *3*, genistein; *4*, genistein; *5*, control, DMSO; *6*, control, no intervention. The protein band representing hsp90 is indicated (*arrow*). (*B*) Western blot of samples harvested from primary cardiomyocyte cultures 20 h after a 4-h incubation with herbimycin-A (1 μg/ml) or genistein (50 μM). Two primary mAbs have been used, one recognizing hsp60, the other specific for the inducible isoform of the 70-kD heat stress protein (hsp72). Lanes from left to right: *1*, herbimycin-A; *2*, herbimycin-A; *3*, genistein; *4*, genistein; *5*, control, DMSO; *6*, control, no intervention. The protein bands representing hsp60 and -72 are indicated (*arrow*). (*C*) Western blot of samples harvested from primary cardiomyocyte cultures 20 h after a 4-h incubation with herbimycin-A (1 μg/ml) or genistein (50 μM). Two primary mAbs have been used, one recognizing grp78, the other specific for the 25-kD heat stress protein (hsp25). Lanes from left to right: *1*, herbimycin-A; *2*, genistein; *3*, thermal preconditioning; *4*, control, no intervention; *5*, control, DMSO. The protein bands representing grp78 and hsp25 are indicated (*arrow*). (*D*) Western blot of samples harvested from noncardiac BHK cells 20 h after a 4-h incubation with herbimycin-A (1 μg/ml). The mAbs used recognize hsp73/72, -60, and -25. Lanes from left to right: *1*, control; *2*, control; *3*, herbimycin-A; *4*, herbimycin-A.
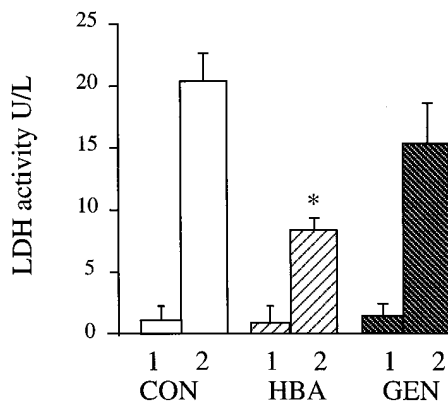
Figure 5. Cell damage after lethal heat stress as assessed by released LDH activity. 24 h after incubation with herbimycin-A, genistein, or control (either DMSO or no intervention), cells were exposed to 47°C for 90 min. Values before lethal stress are labeled 1, and values after lethal heat stress are labeled 2. LDH released into the media as a result of cell damage was determined with an LDH test kit. Data are presented as mean values (±SE). *CON*, control; *HBA*, herbimycin-A; *GEN*, genistein. Significant protection is observed in the herbimycin-A group. *$P < 0.05$.

significantly more LDH activity was observed in control cells, 20.2 U/liter (±2.6), compared with the herbimycin-A–pretreated group (8.25 U/liter [±0.25] [$P < 0.05$]). Genistein-pretreated cells gave levels of LDH activity that were similar to controls (15.25 U/liter [±3.55]) (Fig. 5) (thermal preconditioning data not available).

Survival after lethal simulated ischemia. Similarly, treatment of cardiomyocytes with herbimycin-A or with thermal preconditioning led to a significant increase in survival against a subsequent lethal ischemic insult in comparison with control cells as assessed by trypan blue exclusion. Duplicate determinations were made in five independent experiments using five different preparations of cells. As shown in Fig. 6, in the herbimycin-
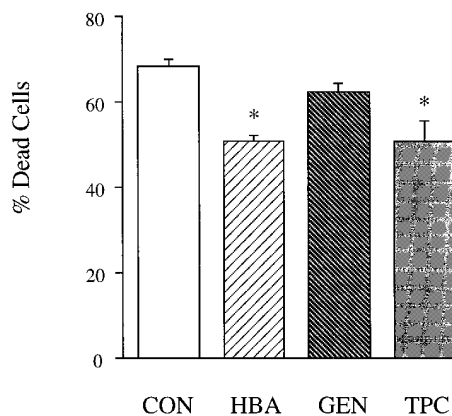
Figure 6. Cell damage as assessed by trypan blue after lethal simulated ischemia. Six-well plates were incubated with herbimycin-A, genistein, or DMSO (control) for 4 h. For comparison, certain plates were thermally preconditioned by incubating them at 43°C for 30 min. 24 h later, all groups of cells were transferred to an ischemic buffer for 90 min at 37°C. Cell viability was then determined by trypan blue exclusion. The graph illustrates percentage of cell death (blue cells) presented as mean values (±SE) from duplicate determinations in five independent experiments. *CON*, control; *HBA*, herbimycin-A; *GEN*, genistein; *TPC*, thermal preconditioning. *$P < 0.001$.
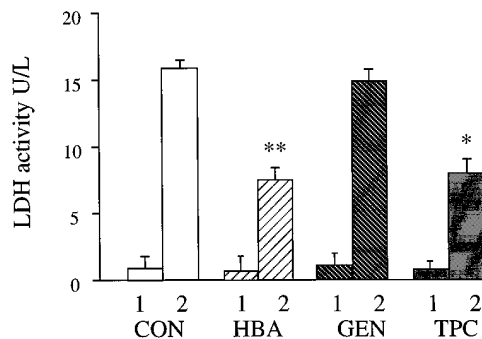

Figure 7. Cell damage after lethal simulated ischemia as assessed by released LDH activity. 24 h after incubation with drug or thermal preconditioning (43°C for 30 min), cells were transferred to an ischemic buffer for 90 min at 37°C. Values before lethal stress are labeled 1, and values after lethal simulated ischemia are labeled 2. LDH released into the media as a result of cell damage was determined with an LDH test kit. Data are presented as mean values (±SE). *CON*, control; *HBA*, herbimycin-A; *GEN,* genistein; *TPC*, thermal preconditioning. Significant protection is observed in the herbimycin-A group (**$P < 0.001$) and also in the thermal preconditioning group (**$P < 0.01$).

A–treated group, the percentage of dead cells was 50.6% (±5.6) ($P < 0.001$), 50.6% (±1.9) ($P < 0.001$) in the thermally preconditioned group, and 68.1% (±1.9) in control cells. Again, genistein treatment did not alter survival significantly from control values with 62.4% (±2.6) dead cells in this group. Thus, herbimycin-A–treated cells have a statistically significant 25% improvement in survival when exposed to a subsequent ischemic insult.

Results obtained with LDH assay as a marker of cell damage yielded comparable results (Fig. 7). None of the pretreatments resulted in significant LDH release, indicating that they did not produce significant cell damage. However, after subsequent lethal simulated ischemia, control cells released significantly more LDH, 15.9 U/liter (±0.8), than those pretreated with herbimycin-A (7.4 U/L [±1.1] [$P < 0.001$]), or thermal preconditioning (8.0 U/liter [±2.0] [$P < 0.01$]). Genistein (50 μM)–pretreated cells had similar levels of LDH activity to controls (14.8 U/liter [±1.1]).

Tyrosine kinase activity. In view of the different effects on hsp72 induction observed with the tyrosine kinase inhibitors herbimycin-A and genistein, we wished to confirm that both these inhibitors were having the expected effect on tyrosine kinase activity. Western blotting with monoclonal antiphosphotyrosine antibody showed reduced activity in both herbimycin-A–treated cells and genistein (concentrations 25, 50, 100, and 150 μM)–treated cells in comparison with controls (Fig. 8). Genistein appeared to have a more immediate effect, with inhibition of activity occurring after just 10 min for all concentra-
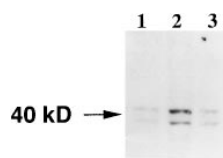
Figure 8. Western blot of samples prepared from primary cardiocyte cultures 30 min after incubation with herbimycin-A or genistein. Controls were incubated with the vehicle DMSO. The primary mAb is an antiphosphotyrosine. Lanes from left to right: *1*, herbimycin-A; *2*, control; *3*, genistein. Both herbimycin-A and genistein inhibit the phosphorylation of a prominent protein doublet at 40 kD.

tions of drug, whereas herbimycin-A inhibited tyrosine kinase after 30 min of incubation.

Discussion

Earlier studies have shown that expression of stress proteins from a variety of different environmental stimuli correlates with protection against subsequent adverse stress. We have been able to show that the benzoquinoid ansamycin antibiotic herbimycin-A is able to induce 70-kD hsp's in primary neonatal rat cardiomyocytes, as well as protecting these cells against lethal heat stress and simulated ischemia. Genistein, also an inhibitor of tyrosine kinase, neither induced 70-kD hsp's nor conferred any protection to these cells. We initially screened a wide range of concentrations of genistein to confirm that its lack of effect on hsp induction and protection was apparent at all concentrations.

In our experiments in cardiac cells, herbimycin-A induced only the 70-kD hsp's and not a wide range of other hsp's. However, in noncardiac BHK cells, we did observe a wider range of hsp induction, in agreement with the work of Hedge et al. (15), who found that herbimycin-A induces a range of hsp's in fibroblasts, indicating that herbimycin-A may have a cell type–specific effect on some hsp's. At least in cardiac cells, our results show a correlation between 70-kD hsp induction and tolerance against lethal heat stress and ischemia, with such protection occurring in the absence of induction of the other hsp's. However, we cannot assume from this a directly protective effect of hsp70, since herbimycin-A may induce other non-hsp proteins. For example, other candidates for protection include endogenous antioxidants such as manganese superoxide dismutase (26), which we did not measure in our experiment. However, hsp72 transfection studies in cell lines (11–13), taken together with a number of transgenic studies (27, 28) in which expression of an hsp72 transgene in the mouse heart protects against ischemia/reperfusion injury, as well as the known actions of hsp72 in protein folding and transport, provide strong evidence for a directly protective effect. It is also clear from our results that the induction of 70-kD hsp's by a pharmacological route can provide effective protection without any induction of the other hsp's.

The time course for induction of hsp72 was similar in both thermally preconditioned groups and those treated with herbimycin-A, and the magnitude of the hsp response was also comparable in these groups. It is particularly interesting to note that, in our experiments in cardiac cells, herbimycin-A did not induce hsp90, -60, or -25. In contrast, the heat shock response results in the synthesis of a number of stress proteins, and in our experiments thermal preconditioning induced the other hsp's as expected. This suggests that the mechanism of hsp72 induction by herbimycin-A may not occur via activation of the heat shock transcription factor, unlike the agents or mechanisms that induce the stress response (1), but, rather, that herbimycin-A may act via a distinct and possibly less "stressful" pathway for hsp72 induction. This is also supported by the fact that herbimycin-A appears to induce both isoforms of hsp70 (hsp73 and -72) strongly, and this contrasts with the pattern of induction evoked by the stress response.

We have been able to show that, although used at doses adequate for tyrosine kinase inhibition, genistein was unable to induce any hsp's and was similarly unable to protect cardiocytes from lethal stress. Hence, the tyrosine kinase–inhibitory activity of herbimycin-A is unlikely to be responsible for its action with regard to 70-kD hsp induction and enhanced tolerance against lethal stress. Although genistein and herbimycin-A are both tyrosine kinase inhibitors, their modes of action are quite dissimilar. Herbimycin-A has a benzoquinone moiety and is thought to modify thiol groups on its target kinase covalently (29), and it therefore may have other actions related to this thiol reactivity. Interestingly, recent reports have suggested herbimycin-A may directly modify the transcription factor NFκB (30). It has also been shown that NFκB activation is inhibited by sodium salicylate, which prevents the degradation of IκB (31). IκB is an associated protein that inhibits NFκB activity; on phosphorylation of IκB, the complex dissociates and NFκB moves to the nucleus, where it activates gene expression (32, 33). Furthermore, sodium salicylate induces the heat shock–responsive chromosomal puffs in *Drosophila* salivary glands and induces HSF DNA binding activity in cultured *Drosophila* cells as well as activating DNA binding by the HSF in cultured human cells (34). This suggests a possible link between NFκB and stress protein transcription, with herbimycin-A possibly inducing 70-kD hsp's via its inhibitory action on NFκB.

Further elucidation of the pathway by which herbimycin-A acts may not only enable us to understand the mechanisms of hsp72 induction but also lead us to strategies for targeted hsp72 induction and possibly protection against lethal stress, particularly ischemia, in a clinically relevant context.

Acute myocardial infarction remains the most common single cause of death in men in the Western world. Interventions such as thrombolytic therapy and aspirin have revolutionized the treatment of myocardial infarction. However, the mortality benefit of thrombolytic therapy is diminished if treatment is administered late (35, 36). Hence, any intervention that could delay the onset of myocardial necrosis would increase the time available for thrombolytic therapy. The ability to induce a prolonged state of resistance to ischemia in the myocardium would also be beneficial in situations such as unstable angina, high risk coronary angioplasty, surgery involving cardiopulmonary bypass, and in explanted hearts before transplantation— all conditions in which the heart is rendered transiently ischemic. Our results indicate that it is possible to specifically induce 70-kD hsp's in cardiomyocytes and thus protect them from injury. It may eventually be possible to induce hsp70 in vivo and protect the human myocardium from ischemia via pharmacological manipulation, thus exploiting the endogenous protective mechanisms of the heart.

Acknowledgments

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