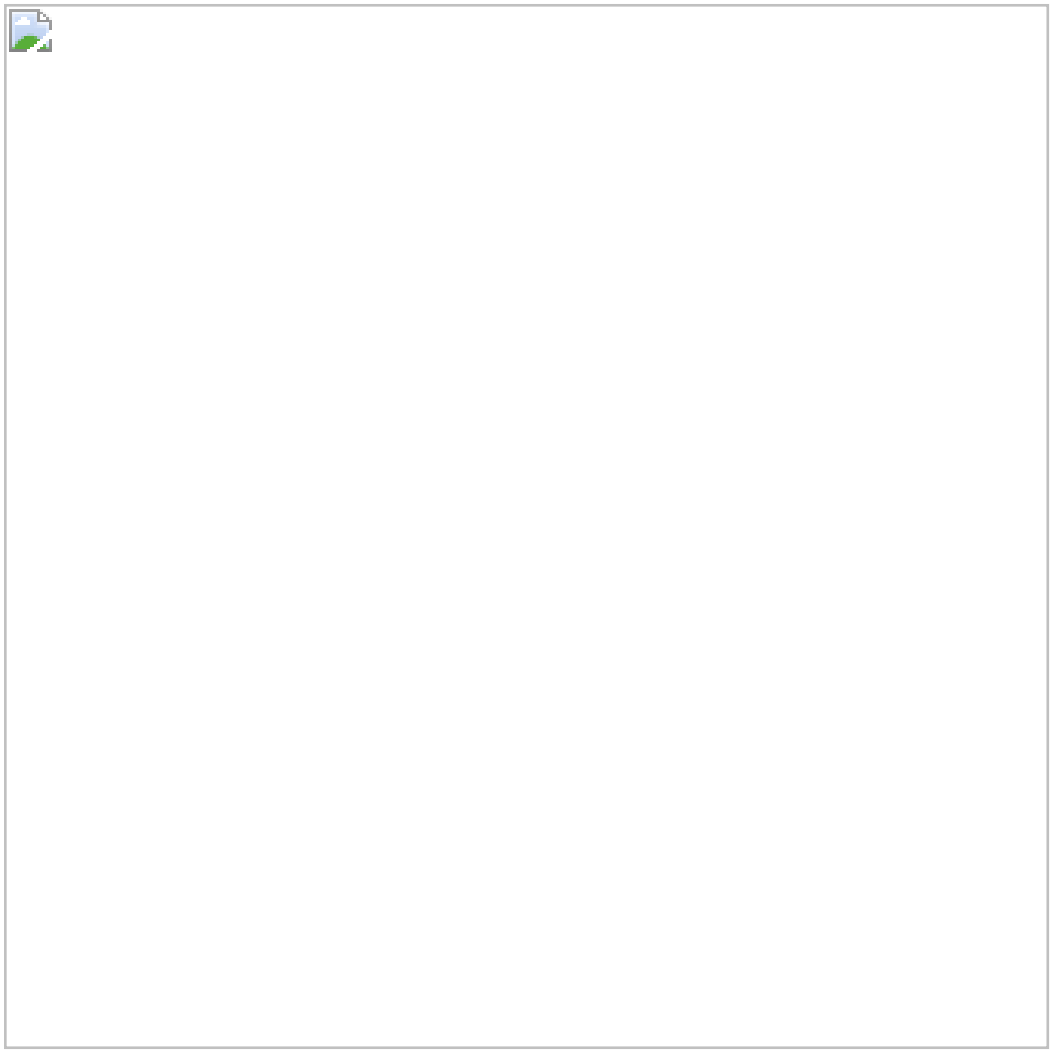

A Drastic Reduction in the Basal Level of Heat-shock Protein 90 in the Brain of Goldfish (Carassius auratus) after Administration of Geldanamycin

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Source: Zoological Science, 21(11) : 1085-1089

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.21.1085

ZOOLOGICAL SCIENCE **21**: 1085–1089 (2004)

A Drastic Reduction in the Basal Level of Heat-shock Protein 90 in the Brain of Goldfish (*Carassius auratus*) after Administration of Geldanamycin

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ABSTRACT—Geldanamycin (GA), a specific inhibitor of the chaperoning function of heat-shock protein 90 (Hsp90), has been shown to mimic heat shock (HS) in inducing expression of Hsp90, Hsp72 and other Hsps in unstressed mammalian cells. In the present study, intra-cerebral treatment of goldfish with GA (at a dose of 0.1 μg/g-body weight) diminished basal Hsp90 level to a 30–40% level in the brain, without affecting the basal Hsp72 level, as assayed 28–48 h after treatment. Whole-body exposure to HS significantly increased Hsp90 level in GA-untreated fish but not in GA-treated fish, while it significantly increased Hsp72 level in both GA-untreated and -treated fish. In both GA-untreated and -treated fish, plasma cortisol (PC) levels increased considerably 4 h after HS and then decreased in a time-dependent manner to the control levels 24 h after HS, showing no evidence of a GA effect on the time course of PC level. These results suggest that in the brain of goldfish, Hsp90 may not be involved as a key factor either in regulating Hsp72 expression both before and after HS or in the feedback regulation of HS-increased PC level, and support the idea that GA can be used in fish brain as a tool in elucidating the role of Hsp90 in complicated, Hsp-mediated biological processes.

Key words: goldfish, geldanamycin, heat shock, brain, heat-shock protein

INTRODUCTION

Geldanamycin (GA, a benzoquinone ansamycin) is a specific inhibitor of the chaperoning function of heat-shock protein 90 (Hsp90) and has been widely used as a tool in genetic, physiological and biochemical studies. Its inhibitory effect on Hsp90's function is known as a consequence of binding to the N-terminal ATP-binding site of Hsp90 (Whitesell *et al*., 1994; Schulte *et al*., 1995; Whitesell and Cook, 1996; Stebbins *et al*., 1997). Various intracellular effects of GA have been recorded in association with the binding activity of GA to Hsp90. (Whitesell *et al*., 1994). Induction of Hsp expression, including Hsp72 and Hsp90, in unstressed mammalian cells after GA treatment in in-vitro situations, is of special interest to the present context (Conde *et al*., 1997; Bagatell *et al*., 2001; Kumar *et al*., 2001; Winklhofer *et al*., 2001; Sittler *et al*., 2001). Recently, a similar effect of GA treatment has been demonstrated for Hsp72 expression in rat brains (Lu *et al*., 2002). The genomic sequence of the *heat shock factor-1* (*hsf-1*) gene from zebrafish has a high homology with the mammalian *hsf-1* gene in the DNA-binding domain (Råbergh *et al*., 2000). Furthermore, Hsps are structurally and functionally conserved even across kingdoms (Parsell and Lindquist, 1993). Hence, the augmentative effect of GA treatment on Hsp expression may be ubiquitous. When such effect is confirmed at the protein levels, GA would provide unique opportunities for studying Hsp-mediated physiological phenomena occurring in non-mammalian systems. These considerations prompted the present experiments, in which GA was administrated to the brain of goldfish and the effect was monitored by observing changes in the levels of Hsp72 and Hsp90 in the brain both before and after heat-shock (HS) treatment.

The goldfish is a model system for studying stress responses of the brain in relation to Hsp expression and plasma cortisol (PC) level (Kagawa *et al*., 1999; Kagawa and Mugiya, 2000, 2002). An analysis of PC levels was included in the present study as an extension of a recent work in which a close link between brain Hsp72 expression and PC levels was demonstrated after exposure to a potential predator (bluegill fish) of goldfish pretreated with a cortisol agonist (such as corticotropin) or antagonist (such as metyrapone) (Kagawa and Mugiya, 2002). In mammalian cells, Hsp90 and Hsp72 have been shown to form a com-

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plex with the glucocorticoid receptor and transport it from the cytosol to the nucleus (Pratt, 1993; Hutchison *et al*., 1994); GA has been reported to disrupt glucocorticoid receptor function by binding to Hsp90 in intact mammalian cells (Whitesell and Cook, 1996). Thus, a second aspect of the present study was to examine effect of intra-cerebral GA administration on HS-increased PC levels.

MATERIALS AND METHODS

Fish

Immature goldfish (*Carassius auratus*), weighing about 10 g, were purchased from the Inter aquaculture shop (Higashiosaka, Japan) and acclimated for 2 weeks in a 25-*l* glass tank equipped with an aeration system. Throughout the acclimation period, fish were maintained at 22±1°C under LD 12:12 (light phase, 09:00-21:00) and fed food pellets (TetraMin Staple Food®, TetraWerke, Germany) once a day. After the onset of the experiments, the fish were starved, and all aspects of the experiments were conducted in the light phase.

Treatment

GA was purchased from Wako Pure Chemical Co. (Osaka, Japan). Immediately before use, GA was dissolved at a concentration of 1 mg/ml in a 1:4 mixture of dimethylsulfoxide (Wako Pure Chemical Co., Japan) and physiological saline solution (0.1 M NaCl and 1.8 mM Na_2CO_3). Using a 26S-needle connected to a 10-μl microsyringe (Hamilton, CA, USA) as described by De Pedro *et al.* (1993), the GA solution was injected into the third ventricle of the brain. Prior to all injections, fish were lightly anesthetized with 0.1% 2-phenoxyethanol. The volume of GA solution injected was adjusted so as to achieve a GA dose of 0.1 μg/g-body weight, the same as the dose used by Lu *et al.* (1995) to treat rat brains. Fish treated in the same way with only solvent served as GA-untreated controls. The accuracy of injection into the third ventricle was assessed in a preliminary experiment by administering trypan blue and confirmed by the position of the dye's color in the brain. Following injections, all fish were maintained in the acclimation tanks. Hereafter, GA-treated and -untreated conditions are referred to as GA(+) and GA(–), respectively.

Twenty-four hours after GA treatment, fish in GA(+) and GA(–) groups were heat-shocked for 2 h at of 32°C, about 10°C higher than the acclimated temperature. The time of the heat shock (HS) after GA treatment was suggested by the report conducted on rat brain, in which the chaperoning activity of Hsp90 decreased 24 h after GA treatment (Lu *et al*., 1995). Hereafter, HS-treated and -untreated conditions are referred to as HS(+) and HS(–), respectively.

Sampling of tissues

The brains and blood were removed from 5 fish from each experimental group 4, 8 and 24 h after HS. Sampled brains were placed in homogenizing tubes containing phosphate-buffered saline (PBS: 2.7 mM $NaH_2PO_4{\cdot}2H_2O$, 3.7 mM $NaHPO_4{\cdot}12H_2O$, 73 mM NaCl, pH 7.4). In order to sample blood, fish were bled by cutting off the caudal peduncle; sampled blood was collected in heparinized capillary tubes. Sampled brains and blood were subjected to the analyses of the Hsp abundance and measurements of the plasma cortisol level, respectively.

Dot blotting

The sampled brains were individually homogenized in PBS in homogenizing tubes at 3,500 rpm for 1 min. Immediately before homoginazation, the proteolitic inhibitors (leupeptin and phenylmethylsulfonyl fluoride, Sigma, NY, USA) were added to the PBS to reach a final concentration of 1 mM. The homogenate was centrifuged at 12,000×g at 4°C for 30 min. The supernatants were stored at –40°C until they were subjected to protein analyses.

Protein concentrations in the supernatants were determined by the method established by Bradford (1976). Samples corresponding to 60 μg of protein were mixed with an equal volume of sample buffer (0.001% bromophenol blue, 2.3% SDS, 10% glycerol, 0.0625 M Tris, 5% 2-mercaptoethanol, pH 6.8) and boiled at 100°C for 3 min. The boiled mixtures were spotted on polyvinylidene difluoride (PVDF) membranes (Millipore, Japan) using a dot blotter (Advantec, Japan) connected to an aspirator. In an effort to block unspecific immuno-reactions, the treated PVDF membranes were incubated with Tris buffer solution (TBS: 20 mM Tris, 500 mM NaCl, pH 7.5) containing 5% skim milk for 1 h. Thereafter, the membranes were incubated with TBS containing the primary antibodies, polyclonal rabbit antibody cross-reactive with Hsp72 (Stressgen; SPA812, Canada) and monoclonal mouse antibody cross-reactive with Hsp90 (Stressgen; SPA830, Canada), for 24 h. After being washed twice in TTBS (TBS containing 0.05% polyoxyethylene(20)sorbitan monolaurate) and once in TBS, the membranes were incubated with TBS containing the secondary antibodies, horseradish peroxidase-conjugated anti-mouse immunoglobulin-G (Stressgen, Canada) and anti-rabbit immunoglobulin-G (Sigma, USA), for 2 h. Finally, the membranes were washed twice in TTBS and once in TBS and treated with TBS containing 3 mM 3,3-diaminobenzidine tetrahydrochloride (DAB: Wako Pure Chemical, Osaka, Japan), 0.015% H_2O_2, and 16.7% methanol for 20 min. Optical densities (ODs) of Hsp70 and Hsp90 in each spot on the DAB-stained PVDF membranes were measured using a Scion Image (Scion Co., USA).

In a preliminary experiment, the specific immno-reactivities of the antibodies used for Hsps 72 and 90 were subjected to the western blot analysis using protein sampled from brain of goldfish; the reactivities were clearly recognized by a single dot at positions corresponding to 72 and 90 kDa proteins, respectively (data not shown).

ELISA of cortisol

Plasma was separated by centrifugation of sampled blood at 700×g for 20 min. The PC level was measured using a cortisol-ELISA kit (Oxford Biomedical Research, USA).

Statistical analyses

Data from five fish were expressed as means±SE and necessary statistical tests were performed using the two-sided ANOVA.

RESULTS

Hsp level before and after HS

Typical immuno-reaction dots obtained for Hsp90 and Hsp72 sampled at a time point corresponding to 8 h after HS from the brains of GA(–)HS(–), GA(+)HS(–), GA(–)HS(+) and GA(+)HS(+) fish are shown in Fig. 1. The time-course data obtained for the OD values that reflect the density of dots are shown in Fig. 2 and abstracted in Table 1.

As can be seen in Fig. 1, the basal levels of Hsp72 and Hsp90 in the brain of intact GA(–)HS(–) fish, which reflect their constitutive expression, were comparable with each other. These levels were 0.33 and ca. 0.34 as OD values, respectively, over the experimental time course (Fig. 2A). When GA was administrated in the brain at a dose of 0.1 μg/kg, the basal Hsp90 level markedly diminished, as visualized by the immuno-reaction dot that was fainter than the

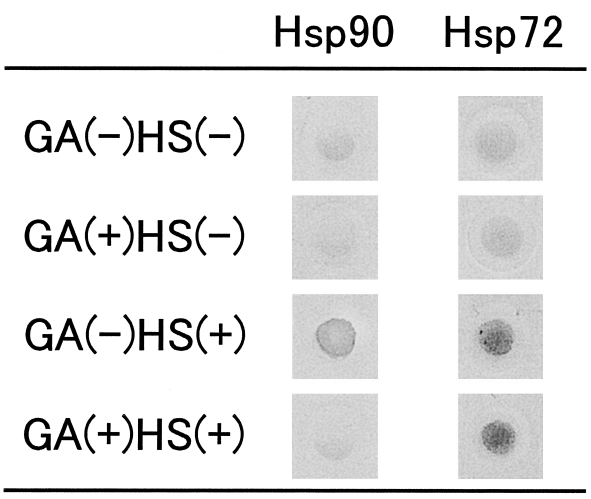

Fig. 1. Typical immuno-reaction dots obtained for Hsp90 and Hsp72 from the brains of goldfish treated with GA and/or HS at a time point corresponding to 32 h after GA and 8h after HS.

control dot (Fig. 1) and demonstrated by OD values (Fig. 2A). The ratios of the OD values in the GA(+)HS(–) fish to those in the control GA(–)HS(–) fish were 0.3-0.4 (Table 1). This means that the basal Hsp90 level was reduced by 60–70% after GA administration. In contrast, the basal Hsp72 levels did not change at all after the administration (Figs. 1 and 2A, Table 1).

When HS was given to GA(–) fish, both Hsp90 and Hsp72 significantly increased over the control levels, as the post-HS time increased from 4 to 8 h (Fig. 2B, see also Fig. 1). The increased levels were maintained up until 24 h after HS. The ratios of the OD values in GA(–)HS(+) fish to the control values in the GA(–)HS(–) fish for Hsp90 and Hsp72 at the time point 8 h after HS were 1.2 and 1.4, respectively. When HS was given to GA(+) fish, Hsp72 increased over the control level as the post-HS time increased from 4 to 8 h, and the increased level was constant up until 24 h after HS, as it was the case in the GA(-)HS(+) fish (Fig. 2B, see also Fig. 1). The ratio of the OD value in GA(+)HS(+) to the control value in GA(+)HS(–) fish for Hsp72 at the time point 8 h after HS was 1.5, a ratio similar to that recorded for the same protein in the GA(-)HS(+) fish. In contrast, the Hsp90 levels in the GA(+)HS(+) fish remained virtually unchanged and overlapped those in the GA(+)HS(–) fish over the experimental time course (Fig. 2, Table 1).

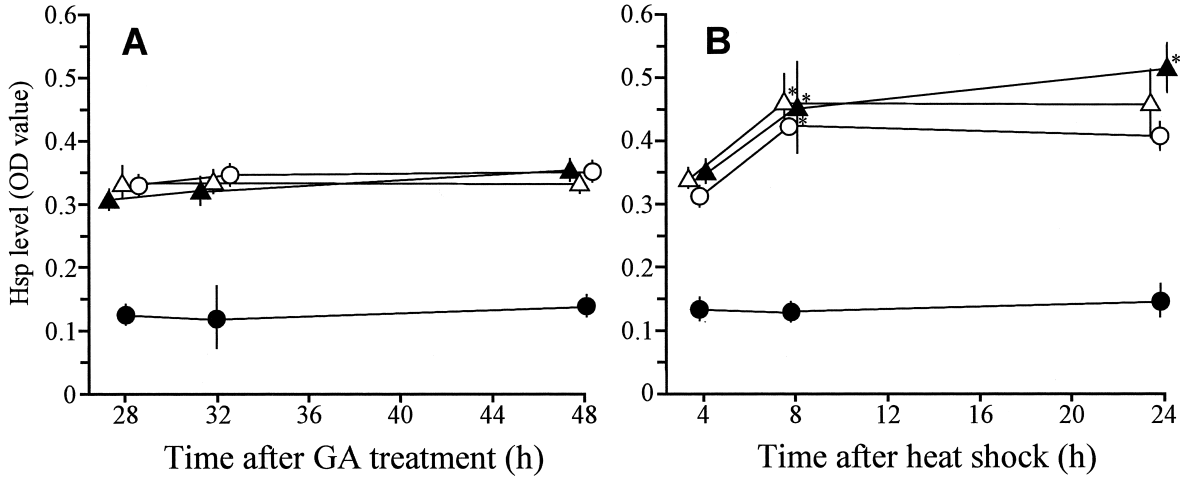

Fig. 2. Variations in Hsp levels in the brains of goldfish with time after GA or HS. Panels A and B show the time course data from HS(–) and HS(+) fish, respectively. The time point 28h after GA in panel A corresponds to the time point 4h after HS in panel B. The symbols ● and ▲ represent mean OD values of Hsp90 and Hsp72, respectively, in GA(+) fish. The symbols ○ and △ represent mean OD values of Hsp90 and Hsp72, respectively, in GA(–) fish. Errors are SE (N=5). *, Significant at P=0.05 in comparison with the respective HS(–) fish.

Table 1. Effects of intra-cerebral GA administration on Hsp and PC levels in HS(–) and HS(+) goldfish

Post HS (h)	GA(+)/(GA–)[a]					
	Hsp90		Hsp72		PC	
	HS(–)	HS(+)	HS(–)	HS(+)	HS(–)	HS(+)
4	0.4±0.05	0.4±0.06	0.9±0.09	1.0±0.05	0.8±0.21	0.9±0.42
8	0.3±0.14	0.3±0.04	1.0±0.09	1.0±0.16	1.0±0.40	1.2±0.16
24	0.4±0.06	0.4±0.07	1.0±0.05	1.1±0.16	1.7±0.29	0.8±0.16

[a] Relative ratio (±SE) of mean OD value of Hsp or mean concentration of PC in GA(+) group to that in GA(–) group.

PC level

Results obtained for the PC are shown in Fig. 3 and abstracted in Table 1. As can be seen in Fig. 3, the basal PC levels were almost constant over the experimental time course, and GA administration did not cause a significant change in the basal PC levels. Following HS, the PC levels increased over the control levels 4 h after HS in both GA(+) and GA(–) fish to similar extent. PC levels in the GA(+)HS(+) and the GA(–)HS(+) fish relative to the control levels in the GA(+)HS(–) and the GA(–)HS(–) fish were 6.0 ±1.3 (142.4±25.7 to 23.7±2.8) and 5.2±2.2 (160.6±61.0 to 30.7±5.5), respectively. HS-induced increases in the PC levels were followed by time-dependent decreases to the control levels 24 after HS in both GA(+)HS(+) and GA(–)HS(+) fish, resulting in time-course curves that were almost indistinguishable from each other.

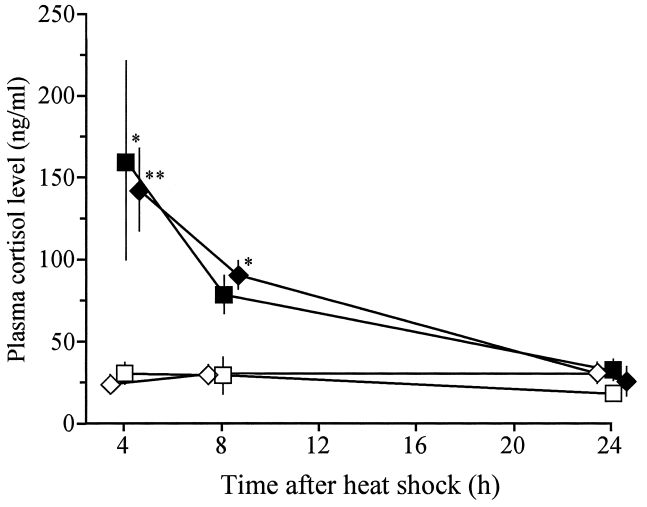

Fig. 3. Variations in PC levels with time after HS. The symbols ◆ and ■ represent mean PC levels in the GA(+)HS(+) and the GA(–)HS(+) fish, respectively. The symbols ◇ and □ represent the mean PC levels in the GA(+)HS(–) and the GA(–)HS(–) fish, respectively. Errors are SE (N=5). * and **, Significant at P=0.05 and P=0.01 in comparison with the respective HS(–) fish, respectively.

DISCUSSION

GA has been shown to mimic HS in mammalian cells, in inducing expression of Hsp90, Hsp72 and other Hsps in unstressed cells (Conde *et al*., 1997; Bagatell *et al*., 2001; Kumar *et al*., 2001; Winklhofer *et al*., 2001; Sittler *et al*., 2001; Lu *et al*., 2002). The HS-like effect supports the current model in mammalian cells, in which GA releases heat-shock factor-1 (HSF-1), which is normally bound to Hsp90 in unstressed cells, by binding to a specific site on Hsp90, and free HSF-1 trimerizes and activates transcription of Hsp genes (Zou *et al*., 1998; Knowlton and Sun, 2001). In the present study, HS increased significantly both Hsp72 and Hsp90 in brain of goldfish (Fig. 2B), but intra-cerebral administration of GA did not mimic HS. Instead, the treatment drastically decreased basal Hsp90 level in the brain, whereas it had no effect on basal Hsp72 level (Table 1), despite the high abundance of Hsps in intact brains (Fig. 2A). It appears from the present results as if Hsp90 is not involved in maintaining basal Hsp72 level in unstressed brain of goldfish.

The different outcomes from the reported results may be ascribed to the facts that the protein levels of Hsp72 and Hsp90 were monitored 28 to 48 h after GA treatment in the present study, while the mRNA levels were monitored at appropriate times after GA treatment in the previous in-vitro studies (Conde *et al*., 1997; Bagatell *et al*., 2001; Kumar *et al*., 2001; Winklhofer *et al*., 2001; Sittler *et al*., 2001). That is, an increase of Hsp expression after GA treatment, if any, would not have been detected with the methods used in the present study, unless it was followed by an appreciable increase of Hsp level in the time range used. This might be the case. Nevertheless, the results from the present study are of interest, in suggesting that GA can be used in fish brain as a tool in elucidating Hsp90's role in complicated biological processes that seem to be mediated by Hsps. In a previous study in which Hsp90 was shown to buffer spontaneously arising morphological variations in *Drosophila melanogaster*, larval treatment of wild-type flies with GA produced visible abnormalities that were similar to those arose in flies with low Hsp90 function due to mutations in the Hsp90 gene (Rutherford and Lindquist, 1998). This fact supports the use of GA as a mimic of mutation that confers low Hsp90 level.

In our recent study, there was a close link between Hsp72 expression in brain and stress-increased PC levels, as assayed after exposure to bluegill fish of goldfish pretreated with a cortisol agonist or antagonist (Kagawa and Mugiya, 2002). This finding suggests possible involvement of Hsp72 in neuro-endocrinal control of stress-increased PC levels. In the present study, GA provided a unique opportunity to examine possible involvement of Hsp90 in the control of HS-increased PC levels. It was found that GA administration did not affect either the time-course of HS-increased PC level (Fig. 3) or HS-induced increase of Hsp72 (Table 1), despite it completely suppressed an increase of Hsp90 level after HS (Fig. 1). These results may be an indication that an increment of Hsp90 in brain of goldfish after HS is not essential either in regulating HS-inducible Hsp72 expression in the brain or in feedback regulation of HS-increased PC level. An important issue to be addressed in testing validity of this hypothesis would be whether GA administration can affect Hsp90 level and cortisol receptor function in the brain compartments involved in neuro-endocrinal control of environmentally increased PC level. It has been reported that in mammalian cells, Hsp90 and Hsp72 form a complex with cortisol receptor and transport it from the cytosol to the nucleus in mammalian cells (Pratt, 1993; Hutchison *et al*., 1994), and GA treatment disrupts cortisol receptor function (Whitesell and Cook, 1996).

In summary, the present study has shown that intracerebral treatment of goldfish with GA can diminish basal

Hsp90 level considerably and suppress HS-inducible increase of Hsp90 level completely in the brain, without affecting Hsp72 levels both before and after HS. Furthermore, it has shown that GA treatment is ineffective in causing any change in the time course of PC level after HS that reflects negative feedback regulation of HS-increased PC level. All theses results support the notion that GA can be used in fish brain as a tool in elucidating Hsp90's role in complicated, Hsp-mediated biological processes, such as the defense of brain tissues against the neurotoxicity of environmental factors. Before GA is used for this purpose, however, mechanistic studies on the apparent suppressive effect of GA on Hsp90 expression are needed.

ACKNOWLEDGEMENTS

I thank Dr. Kazuo Fujikawa for valuable suggestions and discussions during the preparation of this report, and Ms. Aiko Nagatsuka for technical support in the analyses of Hsp level and plasma cortisol concentration.

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(Received May 10, 2004 / Accepted September 13, 2004)