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Modulation of HSP70 and HSP90 Expression by Sodium Salicylate and Aspirin in Fish Cell Line CHSE-214

Hyun Na Koo[1], Su Young Oh[1], Kwang Il Kang[1], Dae Yeon Moon[2], Han Do Kim[1] and Ho Sung Kang[1]*

[1]*Department of Molecular Biology, College of Natural Sciences, Pusan National University, Pusan 609-735,*
[2]*Deep-Sea Fisheries Division, National Fisheries Research and Developmet Institute, Kijang-gun, Pusan 619-900, Republic of Korea*

ABSTRACT—Nonsteroidal anti-inflammatory drugs (NSAIDs) such as sodium salicylate, aspirin and indomethacin have been reported to activate the heat shock transcription factor (HSF) without enhancing the expression of the heat shock proteins (HSPs). We investigated the effects of NSAIDs on the heat shock-induced HSP expression in fish cell line CHSE-214. Here we reveal that in CHSE-214 cells, co-exposure to sodium salicylate/aspirin and heat shock (24°C) enhances and prolongs the heat shock-induced HSP70 expression presumably through activation of the HSF. In contrast, the heat shock-induced HSP90 expression was inhibited by these NSAIDs. Thus, sodium salicylate and aspirin are likely to exert different effects on the heat shock-induced HSP70 and HSP90 expression. Indomethacin, another cyclooxygenase inhibitor, had no stimulatory or inhibitory effects on the heat shock-induced HSP70 and 90 expression, thereby indicating that sodium salicylate and aspirin may modulate the heat shock response via pathways not involving cyclooxygenase. Since anti-oxidants could inhibit the heat shock-induced HSP70 expression, the stimulatory effects of sodium salicylate and aspirin on the HSP70 expression are not likely due to their ability to act as anti-oxidants. Additionally, sodium salicylate and aspirin could exert synergistic effects on the HSP70 expression at lower temperatures (20–22°C) that did not induce the HSP70 expression.

INTRODUCTION

When cells are confronted with a variety of environmental stresses, e.g., heat shock, heavy metals and oxidative stress, they exhibit a rather common and ubiquitous defensive mechanism, referred to as the heat shock response (Morimoto *et al.*, 1994; Welch *et al.*, 1991). Upon exposure to stresses, heat shock gene transcription and heat shock protein (HSP) synthesis are rapidly enhanced (Morimoto *et al.*, 1994; Welch *et al.*, 1991). Induction of heat shock genes in higher eucaryotes is mediated by the activation of a preexisting heat shock transcription factor (HSF), which binds to a target sequence, the heat shock element (HSE), located in the promoter of heat shock-inducible genes (Morimoto, 1993; Sorger, 1991). The activation of HSF and subsequent induction of heat shock genes occur only during the initial phase of heat shock, followed by deactivation of HSF and attenuation of HSP transcription (Abravaya *et al.*, 1991; Morimoto, 1993; Sorger, 1991).

The HSPs can be divided into several families depending on their molecular weights; HSP90, HSP70, HSP60 and small heat shock proteins (Morimoto *et al.*, 1994; Welch *et al.*, 1991). The HSPs have special functions and act as molecular chaperone in physiological and stress conditions (Hartl, 1996). The heat shock response has also been suggested to play multiple cytoprotective roles against acute lethal exposure to stresses. Mammalian cells pretreated with a moderately high temperature can be protected from subsequent exposure to lethal temperature, which is called acquired thermotolerance. Induced synthesis of the HSPs during mild heat shock pretreatment has been shown to be important for acquired thermotolerance (Li *et al.*, 1991; Sanchez and Lindquist, 1990; Welch *et al.*, 1991).

The heat shock response and the HSPs are very conservative from prokaryotes to eukaryotes. Accumulated data have been reported that exposure of fishes to elevated temperatures, hypoxia and heavy metals can induce the heat shock response in a similar manner to other vertebrates (Bols *et al.*, 1992; Krone *et al.*, 1997; Misra *et al.*, 1989; Schmidt *et al.*, 1998). The protective roles of the HSPs and the heat shock

* Corresponding author: Tel. +82-51-510-2275;
FAX. +82-51-513-9258.
E-mail: hspkang@hyowon.pusan.ac.kr

response in thermotolerance and pollutant-induced cytotoxicity have also been suggested in fishes. It was recently demonstrated that thermoresistance is increased by the transfection of fish cell lines leading to the constitutive overexpression of HSP-encoding genes (diIorio *et al.*, 1996).

Many drugs can modulate the heat shock response. Recently, nonsteroidal anti-inflammatory drugs (NSAIDs), originally known to inhibit the cyclooxygenase activity that mediates an inflammatory reaction, have been reported to modulate the heat shock response in yeast, *Drosophila* and mammals (Giardina and Lis, 1995; Jurivich *et al.*, 1995; Lee *et al.*, 1995; Winegarden *et al.*, 1996). For example, treatment of HeLa cells with sodium salicylate and aspirin, the widely used NSAIDs, activates DNA binding of the HSF without inducing the heat shock gene transcription (Jurivich *et al.*, 1995). In addition, indomethacin, another cyclooxygenase inhibitor, is also able to induce the HSF activation (Lee *et al.*, 1995). Furthermore, modulation of HSF1 by indomethacin results in protection against stress-induced cellular damage (Lee *et al.*, 1995).

In this study, to test whether these NSAIDs could modulate the heat shock response and could be used as drugs for enhancing thermotolerance in fishes, we examined the effects of sodium salicylate, aspirin and indomethacin on the HSPs expression using fish cell line CHSE-214. Here we reveal that sodium salicylate and aspirin stimulates the heat shock-induced HSP70 expression in CHSE-214 cells, whereas these NSAIDs inhibits the heat shock-induced HSP90 expression. Since indomethacin, another cyclooxygenase inhibitor, did not exert stimulatory or inhibitory effects on the heat shock-induced HSP70 and HSP90 expression, sodium salicylate and aspirin are likely to modulate the heat shock response via pathways not involving cyclooxygenase. Finally, we show that sodium salicylate and aspirin could lower temperature threshold for the heat shock response.

MATERIALS AND METHODS

Cell culture, heat shock and chemical treatment

The Chinook salmon embryonic cells (CHSE-214) were maintained at 18°C in Eagle's Minimum Essential Medium (EMEM) supplemented 10% fetal bovine serum (FBS) containing 1% penicillin/streptomycin (PS). HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum (CS) and 1% PS at 37°C in an incubator with 5% CO_2. To subconfluent cells, 50 mM sodium salicylate (SAL), 1 mM acetyl salicylic acid (ASA), 30 μM indomethacin (IDT), 20 μM 4-bromophenacylbromide (4-BPB) and 20 μM nordihydroguairetic acid (NDGA) were treated alone or in combination with heat shock (20, 22, and 24°C in CHSE-214; 40, 41 and 42°C in HeLa). Anti-oxidants such as ascorbic acid, butylated hydroxytoluene (BHT), and n-propylgallate, and an oxidant, pyrogallol, were added for 1 hr prior to exposure to heat shock.

Metabolic labeling and SDS-polyacrylamide gel electrophoresis

Newly synthesized proteins were labeled with L-[^{35}S]-methionine (30 μCi/ml; specific activity 1,000 Ci/mmol, Amersham) for final 1 hr before cell harvest. Protein labeling was terminated by washing cells twice with ice-cold 1×PBS. The labeled proteins were precipitated with 10% trichloroacetic acid. The pellets were lysed in 2×sodium dodecyl sulphate (SDS) lysis buffer and equal amounts of radioactivity (counts per minute) were applied to 10% SDS-polyacrylamide gel. Electrophoresis and fluorography were carried out as described previously (Kong *et al.*, 1996).

Nuclear extract preparation

Cells were plated with 4×10^6 cells on F75 flask (Nunc) and treated with heat shock and/or drugs for indicated times. Thereafter, the cells were harvested and the cell pellet rapidly frozen at –80°C. After thawing the pellet on ice, it was suspended in modified buffer A (10 mM Tris-HCl pH7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40). The nuclei were pelleted by centrifugation at 5,000 rpm for 10 min at 0°C. The nuclei were gently resuspended in buffer C (20 mM Tris-HCl pH7.9, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol, 420 mM NaCl) and incubated at 4°C for 15 min. The lysates were centrifuged at 10,000 rpm for 10 min at 4°C and the supernatants were diluted to modified buffer D (20 mM Tris-HCl pH7.9, 50 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), aliquoted and stored at –80°C. Protein concentration was determined by the method of Bradford with bovine serum albumin as standard.

Electromobility shift assay (EMSA) and Electromobility supershift assay (EMSSA)

Electromobility shift assays were performed according to the methods as described previously (Abravaya *et al.*, 1991), with modification. Double stranded oligonucleotides containing the HSE consensus sequence (5'-GATCCTCGAATGTTCGCGAAAAG- 3') were labeled using Klenow polymerase (Promega) and [α-^{32}P]-dCTP (Amersham, 3,000 Ci/mmol, 10 mCi/ml). 20 μg of nuclear protein was preincubated for 15 min at 0°C in 17 μl of a mixture containing binding buffer (20 mM Tris-HCl pH7.5, 5% glycerol, 40 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.05 mg/ml BSA), 1 μg poly(dI-dC) and protease inhibitors (cocktail solution; Sigma). Thereafter, binding reactions were performed for 40 min at room temperature with 200 nCi of radiolabeled oligonucleotide in a final volume of 20 μl. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis on a 4% acrylamide gel (acrylamide/bisacrylamide, 29:1) at 30 mA for 1 hr. Prior to sample loading, the gel was run for 30 min at 20 mA. After electrophoresis, the gel was exposed to an Fuji X-ray film for 12–24 hr at –80°C.

RESULTS

Sodium salicylate and aspirin, but not indomethacin, prolong the heat shock-induced HSP70 synthesis, while they inhibit the heat shock-induced HSP90 synthesis

Sodium salicylate, aspirin, and indomethacin have been reported to trigger the heat shock response in *Drosophila* and mammals (Jurivich *et al.*, 1995; Lee *et al.*, 1995; Winegarden *et al.*, 1996). To test whether these NSAIDs could modulate the heat shock response in fishes, we examined the influence of these NSAIDs on the HSPs expression in fish cell line CHSE-214. As shown in Fig. 1, and as described previously (Bols *et al.*, 1992; Kong *et al.*, 1996), the major heat-inducible polypeptide in CHSE-214 cells was HSP70. A high induction of HSP70 synthesis was observed during the initial phase (3 hr) of heat shock but decreased returning to control level at later phases (6–9 hr), which is thought to be refractive of attenuation in the HSF activation. Similarly, HSP90 level was slightly induced at 3–6 hr after heat shock and then gradually decreased. As shown in Fig. 1, co-treatment of 50 mM sodium salicylate and heat shock enhanced and prolonged the heat shock-induced

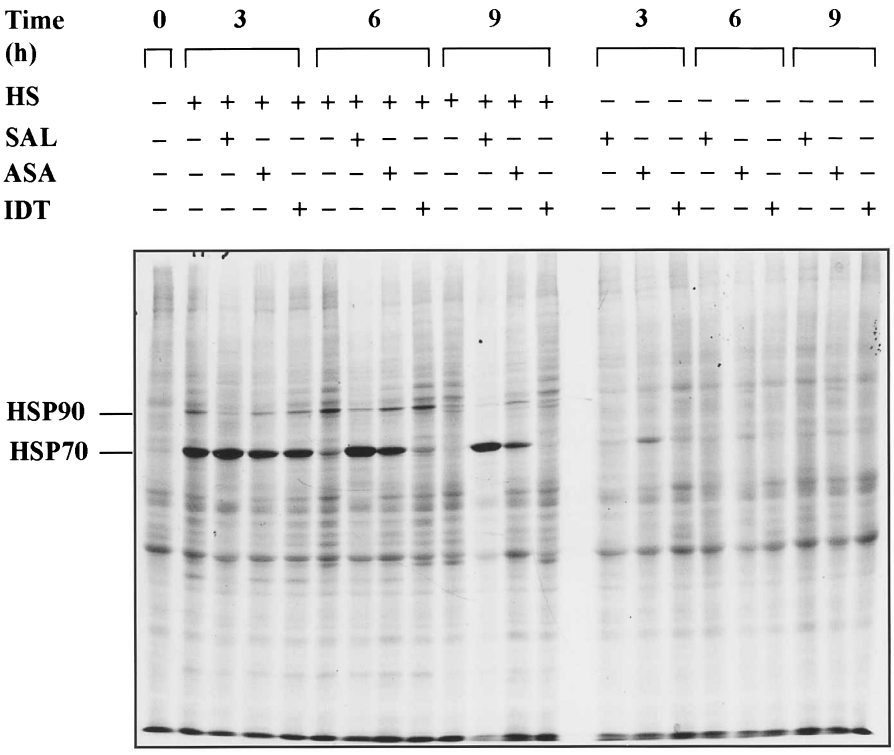

Fig. 1. Effects of nonsteroidal anti-inflammatory drugs on the synthesis of the HSPs. CHSE-214 cells were exposed to 24°C heat shock and/or 50 mM sodium salicylate (SAL), 1 mM acetyl salicylic acid (ASA) and 30 μM indomethacin (IDT) for 3, 6 and 9 hr. The cells were labeled with [^{35}S]-methionine for the last 1 hr and the labeled polypeptides were then analyzed by SDS-PAGE and fluorography.

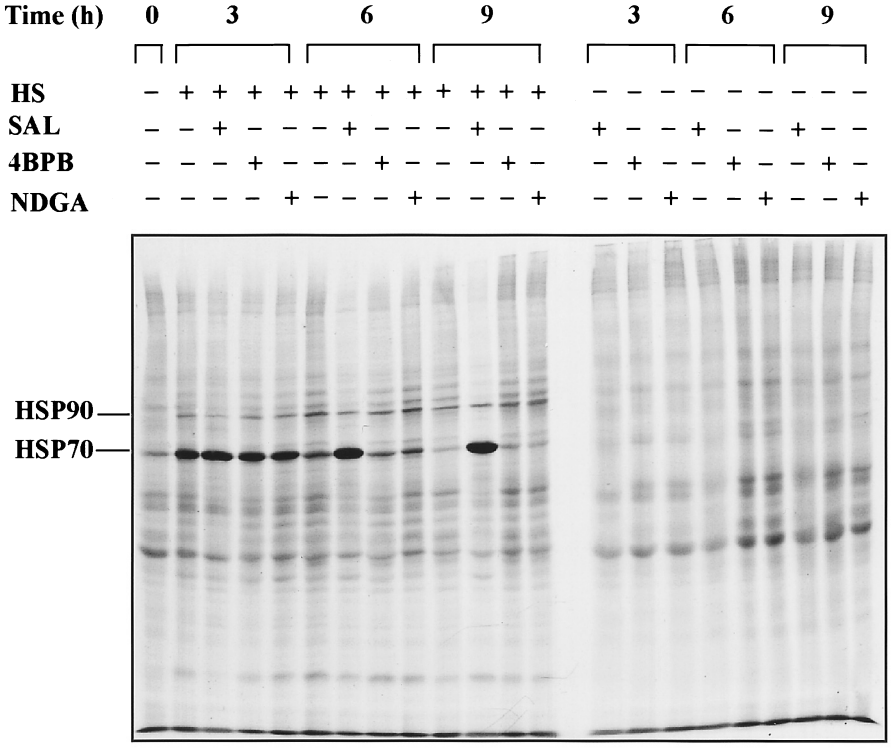

Fig. 2. Effects of inhibitors of arachidonic acid metabolism on the synthesis of the HSPs. CHSE-214 cells were exposed to 24°C heat shock and/or 50 mM sodium salicylate (SAL), 20 μM 4-bromophenacylbromide (4-BPB) and 20 μM nordihydroguairetic acid (NDGA) for 3, 6, and 9 hr. The cells were labeled with [^{35}S]-methionine and the labeled polypeptides were then analyzed by SDS-PAGE and fluorography.

HSP70 synthesis, the level of which was maintained at 9 hr of heat shock. Sodium salicylate alone did not induce the HSP70 synthesis. The heat shock-induced HSP90 expression, however, was decreased by co-exposure to sodium salicylate and heat shock. Similar to sodium salicylate, aspirin enhanced the heat shock-induced HSP70 synthesis, but its effect was less than that of sodium salicylate. In contrast to sodium salicylate and aspirin, indomethacin did not exhibit any stimulatory or inhibitory effects on the HSP70 and HSP90 expression in CHSE-214 cells.

NSAIDs could inhibit the cyclooxygenase activity, which is involved in the metabolism of eicosanoids. To investigate whether the heat shock response is linked to eicosanoid metabolism, we examined the effects of 4-BPB (a PLA2 inhibitor) and NDGA (a lipoxygenase inhibitor) on the HSPs expression. As shown in Fig. 2, neither 4-BPB nor NDGA did influence the heat shock-induced HSP70 and HSP90 expression.

Effects of anti-oxidants and oxidants on the heat shock response

It has been previously reported that besides inhibiting the cyclooxygenase activity, sodium salicylate and aspirin could act as anti-oxidants, which directly scavenge such reactive oxidants as hydroxyl radical and hypochlorous acid. We tested whether the effects of sodium salicylate on the heat shock response are due to their ability to act as antioxidants. CHSE-214 cells were heat-shocked after 1 hr pretreatment of anti-oxidants (ascorbic acid, BHT and n-propylgallate) and oxidant (pyrogallol). As shown in Fig. 3, the heat shock-induced HSP70 synthesis was inhibited by antioxidants, but not by sodium salicylate. In addition, oxidant alone induced the synthesis of HSP70. These results suggest that the effects of sodium salicylate on the heat shock response are not likely due to its antioxidant activity.

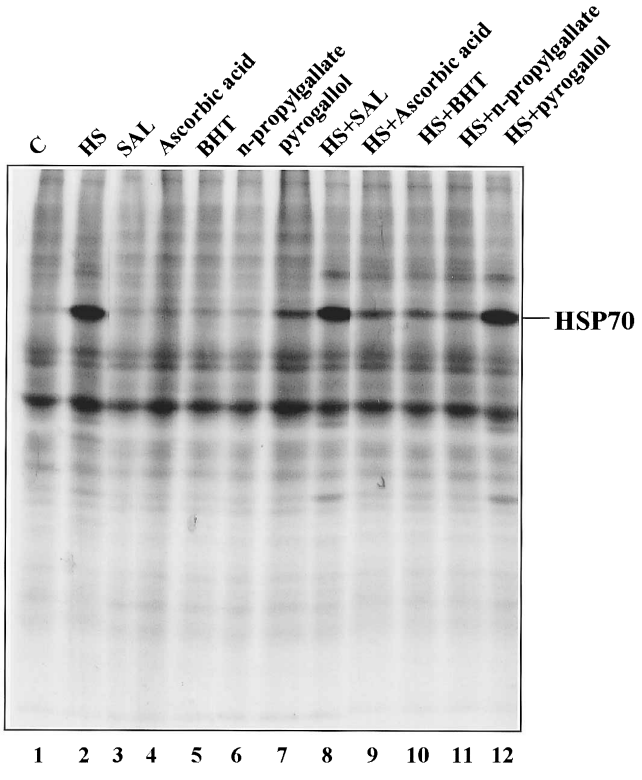

Fig. 3. Effects of anti-oxidants and oxidants on the HSP70 synthesis. CHSE-214 cells were exposed to 24°C heat shock and/or 50 mM sodium salicylate (SAL), 5 μM ascorbic acid, 5 μM butylated-hyroxy-toluene (BHT), 5 μM n-propylgallate and 5 μM pyrogallol for 3 hr. The cells were labeled with [^{35}S]-methionine and the labeled polypeptides were then analyzed by SDS-PAGE and fluorography.

Sodium salicylate potentiates the synthesis of heat shock proteins in mild heat shock conditions, which can not induce the synthesis of the heat shock proteins.

To investigate if sodium salicylate could exert synergistic effects on the HSP70 expression at lower temperature that could not induce the HSPs, CHSE-214 cells were exposed to 20, 22 and 24°C in the presence or absence of sodium salicylate. The induction of HSP70 by 24°C heat shock was enhanced and prolonged by sodium salicylate. While mild heat shock alone (20 and 22°C) could not induce the expression of HSP70, combined treatment of mild heat shock and sodium salicylate highly induced and prolonged the HSP70 expression (Fig. 4). The increased HSP70 level was observed at 3 hr and maintained until 9 hr of heat shock treatment. Similar results were obtained with HeLa cells. While mild heat shock alone (40°C and 41°C) slightly induced the expression of HSP70, co-treatment of heat shock and sodium salicylate highly potentiated and prolonged the HSP70 expression (Fig. 5).

Sodium salicylate may potentiate the synthesis of the heat shock proteins via activation of heat shock transcription factor

To examine whether the effects of sodium salicylate occur at the transcriptional level, cells were pretreated with actinomycin D prior to exposure to heat shock and sodium salicylate. Pretreatment of actinomycin D completely blocked the sodium salicylate-enhanced HSP70 synthesis (Fig. 6). Therefore, sodium salicylate is likely to modulate the heat shock response at the transcriptional level.

The HSF is a key regulator for the heat shock response (Morimoto, 1993; Sorger, 1991). In mammals, sodium salicylate is known to activate DNA binding of the HSF (Jurivich *et al.*, 1995). To find out whether the HSF is activated by sodium salicylate and the stimulatory effect of sodium salicylate on the heat shock-induced HSP70 expression is mediated by activation of the HSF, EMSA was carried out using oligonucleotides containing the HSE consensus sequence (5'-GATCCTCGAATGTTCGCGAAAAG-3'). As shown in Fig. 7 (A and C), sodium salicylate alone triggered the HSF-HSE binding in CHSE-214 as well as in HeLa cells, although this drug alone was not sufficient to induce the synthesis of HSP70. Co-treatment of sodium salicylate and heat shock prevented the attenuation of HSF-DNA binding activity, resulting in prolonged HSF activation. Aspirin exerted similar effects to sodium salicylate, in activating the HSF-HSE binding activity

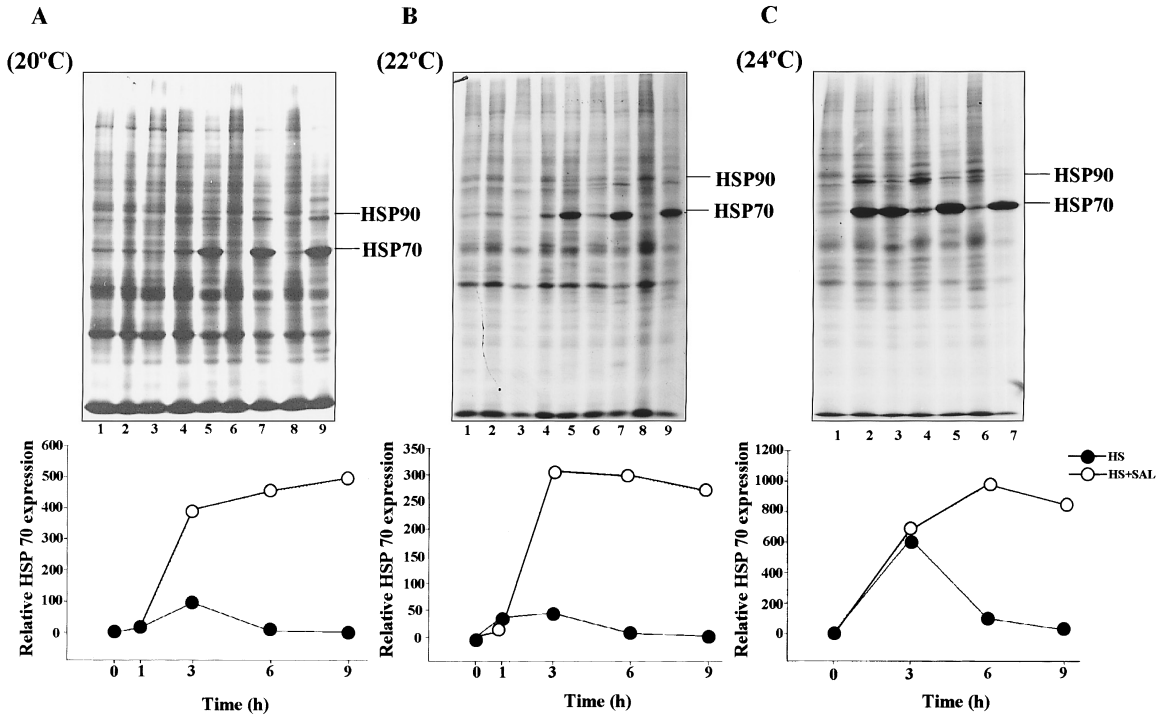

Fig. 4. Effects of sodium salicylate on the HSP70 synthesis at various heat shock temperatures in CHSE-214 cells. CHSE-214 cells were exposed to 20°C (panel A), 22°C (panel B), or 24°C (panel C) in the presence of 50 mM sodium salicylate for 1, 3, 6, and 9 hr. Lane 1, control; lanes 2, 4, 6 and 8, exposed to heat shock alone for 1, 3, 6 and 9 hr, respectively; lanes 3, 5, 7 and 9, exposed to heat shock in the presence of sodium salicylate for 1, 3, 6 and 9 hr, respectively. The cells were labeled with [^{35}S]-methionine and the labeled polypeptides were then analyzed by SDS-PAGE and fluorography. The relative synthesis of HSP70 was determined by densitometric scanning (lower panels).

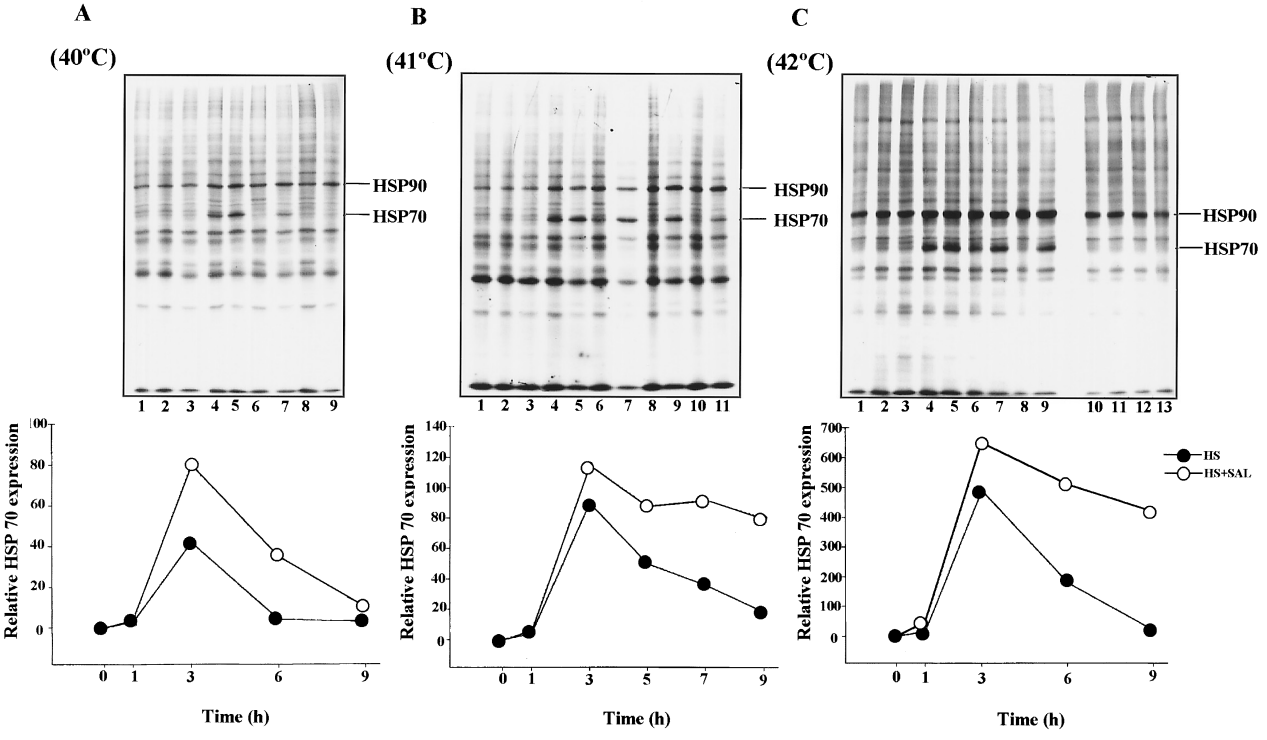

Fig. 5. Effects of sodium salicylate on the HSP70 synthesis at various temperatures in HeLa cells. HeLa cells were exposed to 40°C (panel A), 41°C (panel B), or 42°C (panel C) in the presence of 20 mM sodium salicylate for 1, 3, 5, 7, and 9 hr. Lane 1, control; lanes 2, 4, 6, 8, and 10, exposed to heat shock alone for 1, 3, 5, 7, and 9 hr, respectively; lanes 3, 5, 7, 9, and 11, exposed to heat shock in the presence of sodium salicylate for 1, 3, 5, 7, and 9 hr, respectively. The cells were labeled with [^{35}S]-methionine and the labeled polypeptides were then analyzed by SDS-PAGE and fluorography. The relative synthesis of HSP70 was determined by densitometric scanning (lower panels).

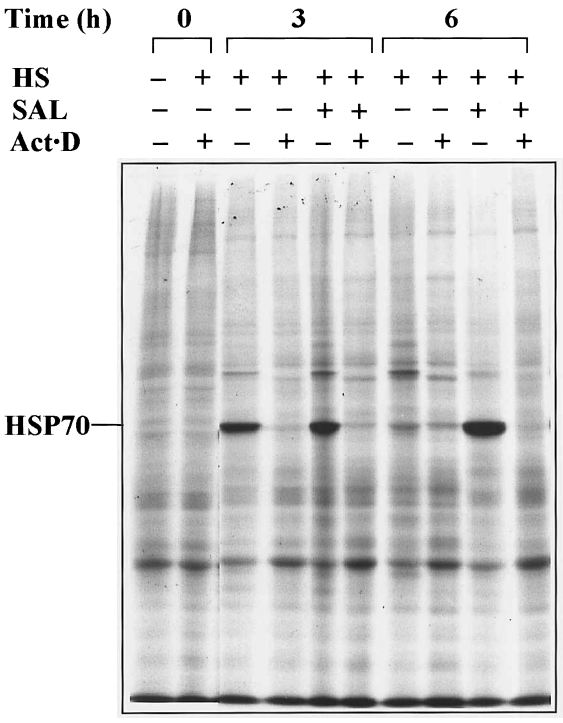

Fig. 6. Effects of actinomycin D on the HSP70 synthesis by sodium salicylate. CHSE-214 cells were incubated in the presence or absence of actinomycin D (20 μg/ml) for 30 min and then exposed to 24°C heat shock in the presence or absence of 50 mM sodium salicylate. The cells were labeled with [^{35}S]-methionine and the labeled polypeptides were then analyzed by SDS-PAGE and fluorography.

(data not shown). Thus, the effects of sodium salicylate and aspirin on heat shock response are probably mediated by activation of HSF-HSE binding. In contrast, indomethacin did not induce the HSF-HSE binding in CHSE214 cells (Fig. 7 B). Furthermore, exposure of CHSE214 cells to indomethacin did not prevent the attenuation of HSF-DNA binding activity, which could be observed after 3 hr of heat shock (Fig. 7 B).

DISCUSSION

It has been reported that fishes and fish cells induce the expression of the HSPs in response to a variety of stressful agents, e.g., heat shock, heavy metals, and hypoxia (Bols *et al.*, 1992; Gamperl *et al.*, 1998; Gedamu *et al.*, 1983; Krone *et al.*, 1997; Misra *et al.*, 1989; Schmidt *et al.*, 1998). The expression of the HSPs is also increased by inflammation and bacterial and virus infection (Bols *et al.*, 1992; Cho *et al.*, 1997). Although the role(s) of the heat shock response in aquatic organisms have not been clearly elucidated, the HSPs have been implicated in thermotolerance and may have protective roles against pollutant-induced cytotoxicity (Bols *et al.*, 1992; diIorio *et al.*, 1996).

HSP70, a major heat shock protein in fishes, increases by an elevated temperature and its synthesis declines despite continuous exposure to heat shock (Bols *et al.*, 1992; Kong *et al.*, 1996). Such attenuation in the HSP70 expression is also observed in other cell systems including yeast and human cells (Morimoto, 1993; Sorger, 1991). Heat shock-induced HSP expression is mediated through activation and HSE-binding of the HSF. As DNA binding activity of HSF attenuates, the rate of heat shock gene expression declines proportionally. Our data indicated that in fish CHSE-214 cells, DNA binding of the HSF was initially activated upon exposure to heat shock and then attenuated by prolonged heat shock treatment (Fig. 7).

In this study, we reveal that in fish cells, sodium salicylate activates the HSF without inducing the expression of HSP70, which is in agreement with the observations in *Drosophila* and mammal research (Jurivich *et al.*, 1995; Lee *et al.*, 1995; Winegarden *et al.*, 1996). In addition, co-treatment of sodium salicylate/aspirin and heat shock enhanced and prolonged the heat shock-induced HSP70 synthesis. The effects of NSAIDs appeared to be linked to inhibition of attenuation in the HSF DNA-binding activity. Interestingly, the heat shock-induced HSP90 expression was prevented by these NSAIDs. Therefore, factors other than HSF might be involved in the regulation of HSP70 and HSP90 expression.

We also show that treatment of sodium salicylate resulted in the induction of HSP70 in mild heat shock conditions, which could not induce the HSPs expression if sodium salicylate is not co-treated. Thus, sodium salicylate appears to lower the temperature threshold for induction of the HSPs expression. This result raises the possibility that sodium salicylate and aspirin could be used as drugs for potentiating thermotolerance in fishes.

In mammals, indomethacin, another cyclooxygenase inhibitor, is known to be able to induce the HSF activation (Lee *et al.*, 1995). Thus, it was thought that the effects of sodium salicylate and aspirin on the heat shock response are mediated by their ability to inhibit the cyclooxygenase activity. In addition, prostaglandin and arachidonic acid can also induce the heat shock response in mammalian cells (Amici *et al.*, 1992; Jurivich *et al.*, 1994), indicating that arachidonic acid metabolism is likely to be closely linked to the heat shock response. However, we failed to observe any stimulatory or inhibitory effects of indomethacin on the HSP70 and HSP90 expression in fish CHSE-214 cells (Fig. 1). We also found that indomethacin did not trigger the HSF-HSE binding activity (Fig. 7). Furthermore, arachidonic acid metabolism inhibitors (4-BPB and NDGA) have no effects on the heat shock response (Fig. 2). Thus, at least in fish cells, the effect of sodium salicylate and aspirin on heat shock response may not be due to their ability to inhibit the cyclooxygenase activity. Recently, sodium salicylate and aspirin were reported to possess other activities than cyclooxygenase inhibition. Sodium salicylate and aspirin inhibit NFkB activation and iNOS induction independent of cyclooxygenase (Amin *et al.*, 1995; Farivar and Brecher, 1996; Kopp and Ghosh, 1994; Lyons-Alcantara *et al.*, 1998; Pierce *et al.*, 1996). As sodium salicylate is known to possess an antioxidant activity, we tested the relationship between sodium salicylate and oxygen radicals. While treatment of antioxidants before heat shock could

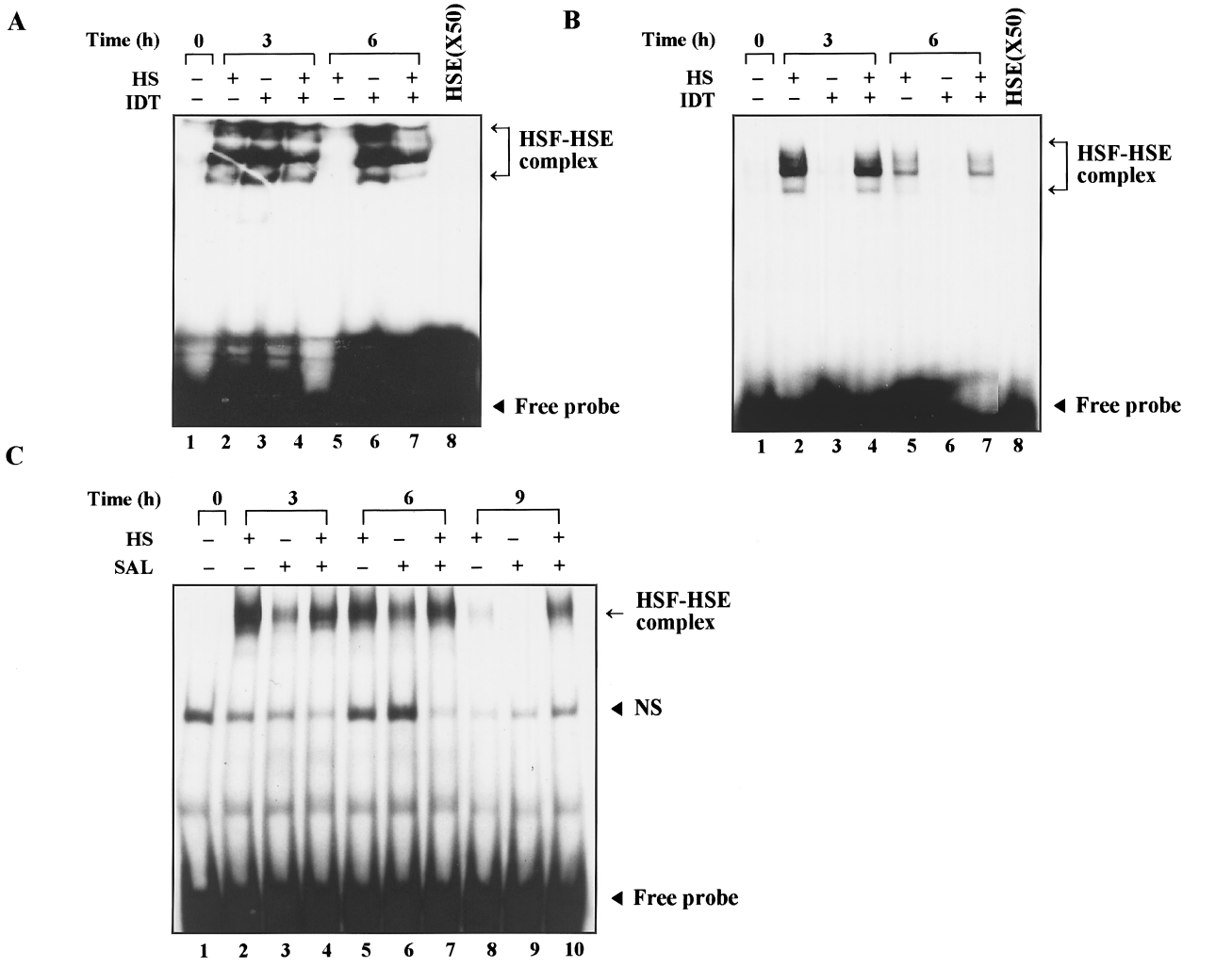

Fig. 7. Electromobility shift analysis for HSF DNA-binding activity. CHSE-214 (panels A and B) and HeLa cells (panel C) were exposed to heat shock (24°C in CHSE-214 cells; 42°C in HeLa cells) in the presence or absence of 50 mM sodium salicylate and 30 μM indomethacin for 3, 6 and 9 hr and then nuclear extracts from cells were prepared and electromobility shift analysis was performed as described in Materials and Methods. Binding reaction was also carried out with nuclear extracts, from CHSE-214 cells exposed to heat shock, in the presence of 50-fold excess of cold HSE as a competitor DNA (HSE X50). HSF-HSE complex (indicated by arrow) indicates specific HSF-DNA complexes and NS indicates nonspecific protein-DNA complexes.

inhibit the HSP70 expression, sodium salicylate had a stimulatory effect. These results suggest that the effects of sodium salicylate and aspirin may not be due to their antioxidant activity. Recently, it has been demonstrated that sodium salicylate activates the specific signal pathway involving p38 kinase (Schwenger *et al.*, 1997; Schwenger *et al.*, 1998). Thus, it will be interesting to examine the possible involvement of p38 in the modulation by sodium salicylate of the heat shock response.

In summary, we show that sodium salicylate and aspirin, which alone do not induce the HSF-DNA binding activity, could act synergistically with heat shock to induce the heat shock response. The ability of sodium salicylate to lower temperature threshold for the heat shock response may have important implications in modulating thermotolerance in fishes, probably increasing cellular protection. And synergistic effects of sodium salicylate and aspirin and elevated temperature on the HSPs expression could provide fishes with a protective mechanism against cytotoxic and pathogenic conditions.

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