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The phenylic hydroxyl group is essential for the induction of stress response by sodium salicylate

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Abstract

We have shown that sodium salicylate (SA) activates the heat shock promoter and induces the expression of heat shock proteins (Hsps) with a concomitant increase in the thermotolerance of cells. To identify the functional groups of SA necessary for the induction of Hsps, we evaluated the effect of various derivatives of SA using a mammalian cell line containing a reporter gene downstream of an *hsp105* promoter. Among the derivatives, the compounds in which the carboxyl group of SA was substituted activated the *hsp105* promoter at 37 °C as SA did, but the compounds in which the hydroxyl group was substituted did not. Thus, the phenylic hydroxyl group but not the carboxyl group of SA seemed to be necessary for a stress-induced response. In addition, the orientation of two functional groups on the benzene ring of SA derivatives was also important for the induction of a response. Among these compounds, salicylalcohol which strongly induced the expression of Hsps suppressed the protein aggregation and apoptosis caused by an expanded polyglutamine tract in a cellular model of polyglutamine disease. These findings may aid in the development of novel effective Hsp-inducers.

Keywords: Heat shock protein; Sodium salicylate; Hsp-inducer; Polyglutamine disease

Heat shock proteins (Hsps) are a set of highly conserved proteins produced in response to various forms of physiological and environmental stress, that serve to protect cells from the cytotoxic effects of a variety of stressors [1,2]. These proteins are also expressed under physiological conditions and have basic and indispensable functions in the life cycle of proteins as molecular chaperones [3–5]. Many studies indicate that Hsps have beneficial effects at both the cellular and tissue levels. For example, the expression of Hsps in animals following whole-body hyperthermia or gene transfer suppresses the tissue damage induced by ischemia-reperfusion [6–8]. In addition, overexpression of some Hsps can suppress the aggregation of mutant proteins that cause neurodegenerative disorders such as Huntington's disease [9], spinocerebellar ataxia I [10], and spinal and bulbar muscular atrophy (SBMA) [11,12]. Also, modulation of the cellular level of Hsps has gained attention as a potential therapeutic modality for cancer, trauma, transplantation surgery, and diabetes [13–16].

Several chemical compounds are known to induce the expression of Hsps [17]. Geldanamycin, a benzoquinone ansamycin, was initially identified through screening for anti-cancer drugs and subsequently developed for its potential in cancer therapy [18]. This compound binds to the ATP-binding domain of Hsp90 and depresses HSF1 activity leading to the induction of Hsps expression by the down-regulation of Hsp90 activity [19,20]. Geranylgeranylacetone, an acrylic isoprenoid, induces the expression of Hsps by way of the activation of HSF1 and pretreatment with geranylgeranylacetone markedly suppresses ischemia-reperfusion injury of the liver, small intestine, and heart [21]. Bimoclomol, a hydroxylamine derivative, is shown to have multilateral protective activities against various forms of stress or injury at the level of the cell, tissue, or organism [14,22]. This compound is effective only under conditions of stress and also active in various pathological

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animal models subjected to acute or chronic stress [23]. Thus, the small molecules that induce or enhance the expression of Hsps are useful for the treatment of various diseases.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as sodium salicylate (SA) and indomethacin are widely used as analgesic or antipyretic agents for the clinical treatment of inflammatory diseases. Most NSAIDs exhibit an inhibitory effect on cyclo-oxygenases, which catalyze the biosynthesis of prostaglandins and thromboxane from arachidonic acid. In addition to these anti-inflammatory effects, we have shown that SA and indomethacin activate the heat shock promoter and induce the expression of Hsps with a concomitant increase in the thermotolerance of cells [24,25]. Here, to clarify structure-activity relationships, we evaluated the effect of various derivatives of SA on the response to stress, and found that the phenylic hydroxyl group but not the carboxyl group of SA derivatives seemed to be necessary for the induction of the response to stress. In addition, the orientation of two functional groups on the benzene ring seemed to be important for a stress-induced response.

Materials and methods

Cell culture and treatment with chemical compounds. Mouse fibroblast C3H10T1/2 and African green monkey kidney COS-7 cells were obtained from Riken Bioresource Center Cell Bank (Tsukuba, Japan). C3H10T1/2 cells stably transfected with a plasmid containing the mouse *hsp105* promoter upstream of a luciferase reporter gene were designated pGL105/C3H cells [24]. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 in air at 37 °C.

The SA derivatives were dissolved in dimethyl sulfoxide (DMSO) or phosphate-buffered saline (PBS) at 1 M just before use. Cells were incubated in the medium with or without chemical compounds at 37 °C for 1 h, washed with PBS, and further incubated in fresh medium without the compounds at 37 °C for 6 h.

Measurement of hsp105 promoter activity. pGL105/C3H cells (2×10^5 cells/35-mm dish) were washed with PBS 3 times and lysed in 50 μl of cell lysis reagent (Promega). The cell lysates were centrifuged at 20,000×*g* for 10 min, and the supernatants recovered as cell extracts. Aliquots (5 μl) of the extracts were added to 50 μl of luciferase assay reagent (Promega), and luciferase activity was measured using a luminometer (Turner Designs, model TD-20/20).

Neutral red uptake assay. pGL105/C3H cells (5×10^4 cells/well) in 24-well plates were incubated for 3 h in the presence of 50 μg/ml of neutral red, then fixed with 1% formaldehyde containing 1% $CaCl_2$ for 1 min. The dye incorporated into viable cells was extracted with 50% ethanol containing 1% acetic acid and absorbance was measured at 540 nm.

Western blot analysis. C3H10T1/2 or COS-7 cells (5×10^5 and 6×10^5 cells/60-mm dish, respectively) were lysed in 200 μl of 0.1% SDS. Cellular proteins (15 μg) were separated by SDS–PAGE and blotted onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (20 mM Tris–HCl, pH 7.6, and 137 mM NaCl) containing 0.1% Tween 20 (TTBS), and incubated with rabbit anti-mouse Hsp105 [26], monoclonal anti-bovine Hsp70 (clone BRM-22: both Hsp70 and Hsc70 are detectable, Sigma Chemical) or monoclonal anti-human Hsp70 (clone C92F3A-5: Hsp70 specific, Stressgen) antibody for 16 h at 4 °C. After being washed with TTBS, the membrane was further incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Santa Cruz Biotechnology) at room temperature for 1 h. Hsp105α and Hsp70/Hsc70 were detected using ECL reagent (Santa Cruz Biotechnology).

Cellular model of polyglutamine (polyQ) disease. COS-7 cells grown on coverslips to 70–80% confluence were treated with or without drugs at 37 °C for 1 h and incubated at 37 °C for 6 h without chemical compounds. The cells were then transfected with the expression plasmid pcDNA-tAR97 which expressed a truncated androgen receptor containing 97 glutamine repeats fused to green fluorescence protein (tAR97) [11] with DMRIE-C reagent (Invitrogen). After incubation at 37 °C for 72 h, cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature, and stained with 10 μM Hoechst 33342 for 15 min at room temperature. The cells were observed using a fluorescence microscope (Nicon). The number of transfected cells with visible aggregates, and the number of transfected cells without aggregates were counted independently in randomly chosen microscopic fields in different areas of a coverslip. Approximately 300–600 transfected cells were analyzed in each experiment. Apoptotic cells were identified by their nuclear morphology stained with Hoechst 33342.

Results and discussion

We have shown that SA activates the heat shock promoter and induces the expression of Hsps with a concomitant increase in the thermotolerance of cells [24]. To clarify structure-activity relationships, we evaluated the effect of various derivatives of SA on the response to stress using pGL105/C3H cells containing a *hsp105* promoter-regulated luciferase reporter gene. When pGL105/C3H cells were treated with various concentrations of SA at 37 °C for 1 h and further incubated for 6 h without the SA, the luciferase activity was up-regulated in a dose-dependent manner (Fig. 1A). Approximately 25- and 40-fold increases were observed in cells pretreated with 60 and 80 mM SA, respectively, compared to control cells. In addition, compounds in which the carboxyl group of SA was substituted with $–CH_3$, $–CONH_2$, $–NHCOCH_3$, $–CH_2OH$, and $–NO_2$ such as *o*-methylphenol, salicylamide, *o*-acetamidophenol, salicylalcohol, and *o*-nitrophenol, respectively, also up-regulated the luciferase activity dose-dependently (Figs. 1B–F). These compounds increased the cellular levels of Hsp105 and Hsp70 in mouse C3H10T1/2 cells (Fig. 1), and also in monkey COS-7 and human HeLa cells (data not shown).

The compound in which the hydroxyl group of SA was missing or substituted with a methyl group such as sodium benzoate and *o*-toluic acid, respectively, did not up-regulate the luciferase activity at concentrations of up to 80 or 120 mM, respectively (Fig. 2). Cellular levels of Hsp105 and Hsc70 were not increased by these compounds at up to 120 mM, whereas Hsp70 was slightly affected by 120 mM sodium benzoate or *o*-toluic acid (Fig. 2). Among the compounds used in Figs. 1 and 2, cell viability was only significantly reduced by the treatment with 25 mM salicylamide and 8–10 mM *o*-nitrophenol. Thus, the expression of Hsps induced by these compounds seemed not to be due to lethal stress, and the phenylic hydroxyl group not the carboxyl group of SA seemed to be necessary for the induction of a response to stress.

We next examined whether the orientation of two functional groups on the benzene ring of SA derivatives

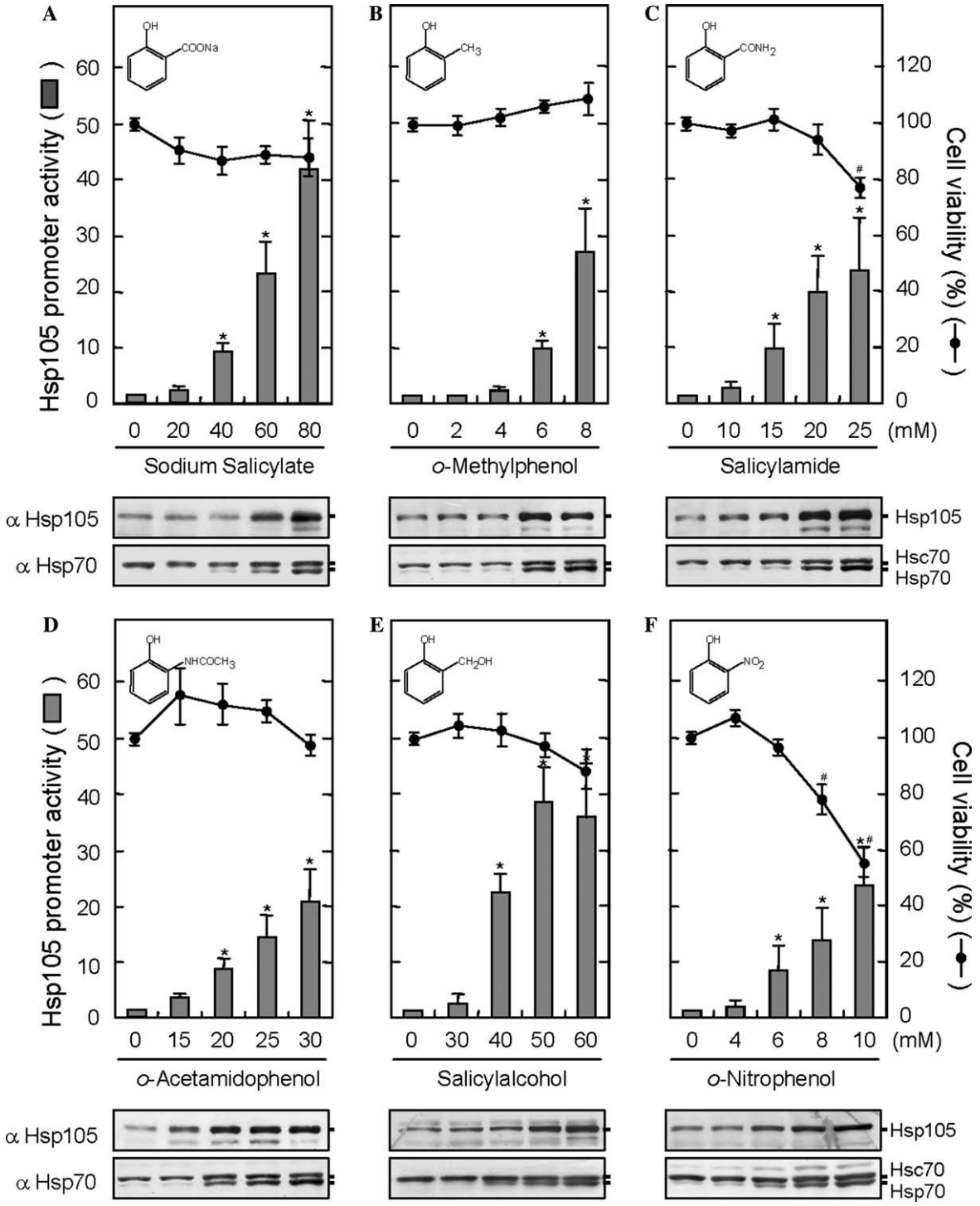

Fig. 1. The compounds in which the carboxyl group of SA is substituted induce the expression of Hsps at normal temperatures. pGL105/C3H cells were incubated with various concentrations of SA derivatives at 37 °C for 1 h and further incubated at 37 °C for 6 h without the compounds. Then, luciferase activity was assayed and relative activity is shown as a ratio to that of untreated control cells. Viability of cells was assessed by the neutral red uptake assay. Each value represents the means ± SD of four independent experiments. Statistical significance was determined with Student's *t*-test: * $p < 0.01$ *versus* respective control cells for luciferase activity; # $p < 0.01$ *versus* respective control cells for cell viability. Hsp105 and Hsp70/Hsc70 in C3H10T1/2 cells were detected by Western blotting using anti-Hsp105 or anti-bovine Hsp70 (both Hsp70 and Hsc70 are detectable) antibody, respectively.

is important for a stress-induced response. To this end, we compared *m*- and *p*-hydroxybenzoic acid with SA (*o*-hydroxybenzoic acid) (Figs. 3A and B). On the treatment with *m*- and *p*-hydroxybenzoic acid, the luciferase activity increased approximately 1.2–1.5-fold at 80 mM, although the activity was increased up to approximately 40-fold by 80 mM SA (Fig. 1A). The expression of Hsp105 in C3H10T1/2 cells was not increased by

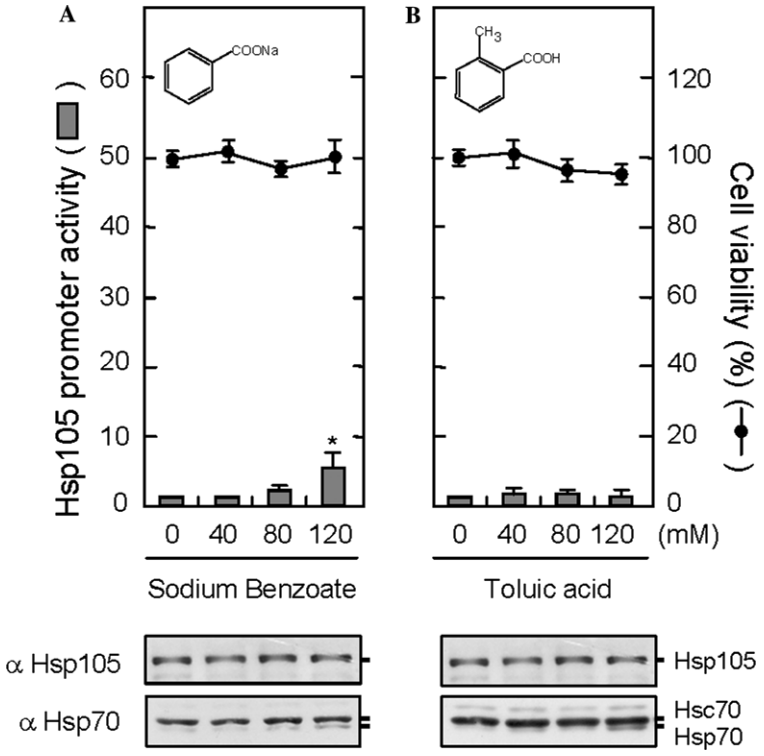

Fig. 2. The carboxyl group of SA is not necessary for a stress-induced response. pGL105/C3H cells were incubated with various concentrations of sodium benzoate or *o*-toluic acid at 37 °C for 1 h, and further incubated at 37 °C for 6 h without the compounds. Then, luciferase activity and cell viability were examined and Western blotting was conducted as described in Fig. 1. Each value represents the mean ± SD of four independent experiments. Statistical significance was determined with Student's *t*-test: * $p < 0.05$ *versus* respective control cells for luciferase activity.

m- and *p*-hydroxybenzoic acid at concentrations up to 160 mM, while Hsp70 was induced to express by 160 mM *m*-hydroxybenzoic acid and 120–160 mM *p*-hydroxybenzoic acid as observed by 60 mM SA.

Furthermore, we examined the effect of the orientation of another SA derivative, acetamidophenol (Figs. 3C and D). The luciferase activity was up-regulated in a dose-dependent manner, and an approximately 12-fold increase was observed with 30 mM *m*-acetamidophenol and 40 mM *p*-acetamidophenol, whereas an approximately 20-fold increase was detected at 30 mM *o*-acetamidophenol (Fig. 1D). Cellular levels of Hsp105 were also increased by 30–40 mM *m*-acetamidophenol and *p*-acetamidophenol. The expression of Hsp70 was also induced by 30–40 mM *m*-acetamidophenol and *p*-acetamidophenol, while it was markedly induced by 20 mM *o*-acetamidophenol. Cell viability was not significantly altered by the compounds used in these experiments (Fig. 3). Thus, as the *o*-isoform of hydroxybenzoic acid and acetamidophenol markedly induced a response to stress at lower concentrations than the *m*- or *p*-isoform, the orientation on the benzene ring of SA derivatives may be important for the induction of a response to stress.

Hsps such as Hsp105α, Hsp70, and Hsp40 have been identified as potent modulators of aggregation and/or cell death caused by the expression of proteins with an expanded polyQ tract in cellular models of neurodegenerative disease [9–12]. We next examined whether some of these SA derivatives, like salicylalcohol which markedly induced the expression of Hsps, suppress protein aggregation and apoptosis in a cellular model of SBMA (Fig. 4). When COS-7 cells were transfected with an expression plasmid for tAR97, approximately 25% of the cells expressing GFP fluorescence of tAR97 were found to contain protein aggregates and 15 % of GFP-positive cells were apoptotic. The aggregation of tAR97 and apoptosis were significantly suppressed by pretreatment of the cells with 40 and 50 mM salicylalcohol as they were with 45 mM SA. On the other hand, 45 and 60 mM *p*-hydroxybenzoic acid, which did not induce the expression of Hsp70, did not suppress the protein aggregation and apoptosis caused by an expanded polyQ tract. As several epidemiological studies have shown that prolonged use of NSAIDs reduces the risk of developing some neurodegenerative diseases including Alzheimer's and Parkinson's diseases [27–30], SA and its derivatives that induce the expression of Hsps may be used for the protection of cells against deleterious stressors and neurodegenerative diseases.

In this paper, we showed that the phenylic hydroxyl group, but not the carboxyl group, and the orientation

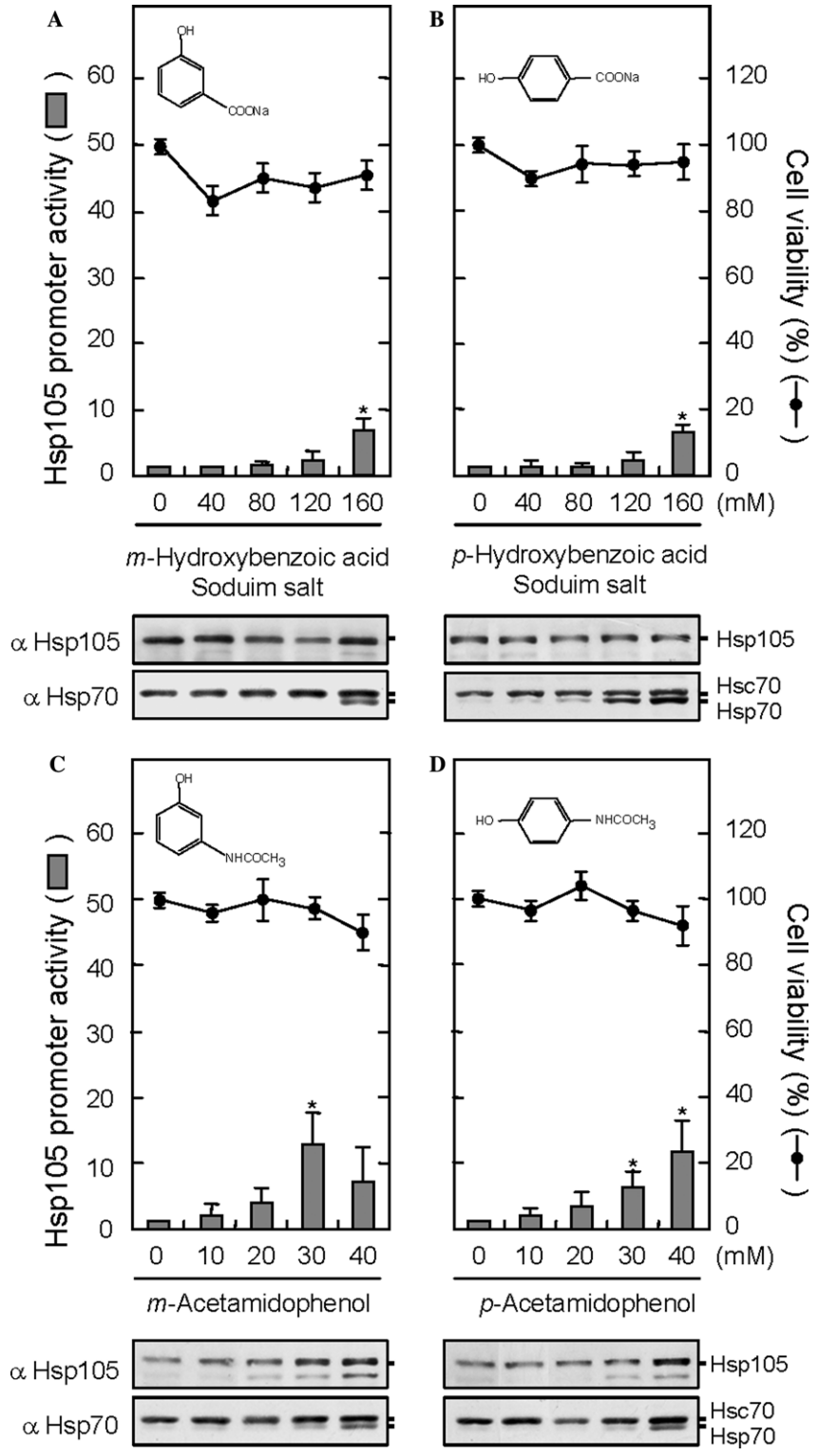

Fig. 3. Effect of the orientation of two functional groups on the benzene ring of SA derivatives on the induction of a response to stress. pGL105/C3H cells were incubated with various concentrations of *m*- or *p*-hydroxybenzoic acid and *m*- or *p*-acetamidophenol at 37 °C for 1 h, and further incubated at 37 °C for 6 h without the compounds. Then, luciferase activity and cell viability were examined and Western blotting was conducted as described in Fig. 1. Each value represents the mean ± SD of four independent experiments. Statistical significance was determined with Student's *t*-test: * $p < 0.01$ *versus* respective control cells for luciferase activity.

of two functional groups on the benzene ring of SA derivatives are important for a stress-induced response. These findings may help to develop novel effective Hsp-inducers in cells, although it remains to consider the availability of SA derivatives following extracellular administration.

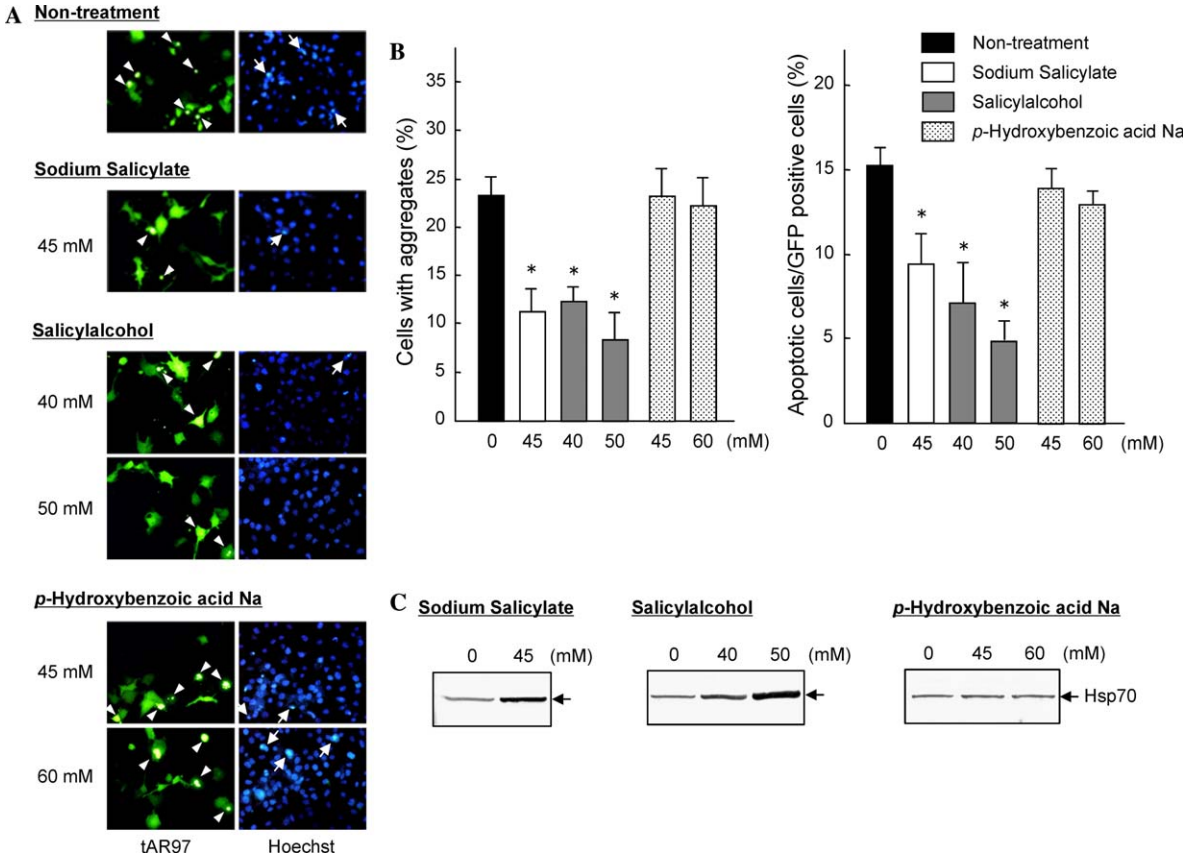

Fig. 4. Effects of salicylalcohol on protein aggregation and cytotoxicity caused by an expanded polyQ tract. (A) COS-7 cells were treated with SA, salicylalcohol, or *p*-hydroxybenzoic acid at 37 °C for 1 h, and further incubated at 37 °C for 6 h without the compounds. These cells were then transfected with the tAR97 expression plasmid. After 72 h, the cells were fixed, stained with Hoechst 33342, and observed using a fluorescence microscope. Arrowheads and arrows represent typical cells containing aggregated tAR97 and condensed chromatin, respectively. (B) Proportions of cells containing tAR97 aggregates or condensed chromatin among GFP-positive cells were calculated and represent the mean ± S.D. of four independent experiments. Statistical significance was determined with Student's *t*-test: * $p < 0.01$ *versus* respective controls. (C) The expression of Hsp70 in COS-7 cells treated with or without the compounds was detected by Western blotting using anti-human Hsp70 antibody (Hsp70 specific antibody).

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