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Sodium Salicylate Activates p38MAPK Though a Specific-Sensing Mechanism, Distinct from Pathways Used by Oxidative Stress, Heat Shock, and Hyperosmotic Stress

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Sodium salicylate, a plant stress hormone that plays an important role(s) in defenses against pathogenic microbial and herbivore attack, has been shown to induce a variety of cell responses such as anti-inflammation, cell cycle arrest and apoptosis in animal cells. p38MAPK plays a critical role(s) in the cell regulation by sodium salicylate. However, the signal pathway for sodium salicylate-induced p38MAPK activation is yet unclear. In this study, we show that although sodium salicylate enhances reactive oxygen species (ROS) production, N-acetyl-L-cysteine, a general ROS scavenger, did not prevent sodium salicylate-induced p38MAPK, indicating ROS-independent activation of p38MAPK by sodium salicylate. Sodium salicylate-activated p38MAPK appeared to be very rapidly down-regulated 2 min after removal of sodium salicylate. Interestingly, sodium salicylate-pretreated cells remained fully responsive to re-induction of p38MAPK activity by a second sodium salicylate stimulation or by other stresses, H_2O_2 and methyl jasmonate (MeJA), thereby indicating that sodium salicylate does not exhibit both homologous and heterologous desensitization. In contrast, pre-exposure to MeJA, H_2O_2, heat shock, or hyperosmotic stress reduced the responsiveness to subsequent homologous stimulation. Sodium salicylate was able to activate p38MAPK in cells desensitized by other heterologous p38MAPK activators. These results indicate that there is a sensing mechanism highly specific to sodium salicylate for activation of p38MAPK, distinct from pathways used by other stressors such as MeJA, H_2O_2, heat shock, and hyperosmotic stress.

Key Words: Sodium salicylate, p38MAPK, Reactive oxygen species, Desensitization

INTRODUCTION

Sodium salicylate, a plant stress hormone that plays (an) important role(s) in defenses against pathogenic microbial and herbivore attack[1,2], has been well demonstrated to exert an anti-inflammatory effect[3]. In addition, sodium salicylate induces a variety of other cellular responses[4]. At concentrations compatible with amounts in plasma during chronic anti-inflammatory therapy, sodium salicylate protects nerve cells against toxicity elicited by the excitatory amino acid glutamate and ischemic stress[5]. Recently, it was also demonstrated that sodium salicylate has potent chemopreventive activity and can be used for treatment and prevention of a number of human cancers including those of colon, lung, and breast, and leukemia[6~9]. The chemopreventive activity of sodium salicylate has been ascribed to its ability to inhibit cell proliferation, and to induce differentiation and apoptosis[4,9,10].

Sodium salicylate is known to regulate a number of signal pathways including mitogen activated protein kinase (MAPK): ERK1/2, p38MAPK, and JNK/SAPK[4]. Although sodium salicylate exerts stimulatory or inhibitory effects on ERK1/2 and JNK/SAPK depending on the cellular context [11~13], it activates p38MAPK exceptionally in most cell types including normal human fibroblasts, human colorectal carcinoma cells, and COS cells[4,10,14]. Even though the mech-

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anisms by which sodium salicylate exerts its pharmacological actions in mammalian cells are unclear, p38MAPK has been demonstrated to play critical role in these actions of sodium salicylate. Recent evidence indicated that salicylate exerts an anti-inflammatory effect through preventing NF-κB activation via p38MAPK-dependent IKK inhibition [15~18]. Furthermore, p38MAPK has been reported to play a critical role(s) in sodium salicylate-induced apoptosis in a variety of cells including human fibroblasts and cancer cells [4,14,19], although p38MAPK-independent sodium salicylate-induced apoptosis has been also reported in human peripheral blood eosinophils[20]. Sodium salicylate-induced apoptosis can be blocked by the selective p38MAPK inhibitor SB203580[14,21]. In addition, salicylate-induced p38MAPK activation plays an important role in the sodium salicylate/recovery induced HSP72 expression (data not shown).

p38MAPK, also referred to as the stress-activated protein kinase 2 (SAPK2), is activated by various environmental stresses such as heat shock, oxidants, hyperosmolarity, as well as by growth factors and cytokines including tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1)[22~25]. This implies the existence of multiple proximal signaling pathways converging to the p38MAPK activation cascade. Two MAP kinase kinases, MKK3/6, have been shown to phosphorylate at Thr180 and Tyr182 and activate p38MAPK selectively, and several different MAP kinase kinase kinases including MLK-2/3, MEKK1, ASK1, and TAK1 can potentially activate MKK3/6[22,23]. The MAP kinase kinase kinases are themselves activated either by kinases of the ste-20-like family of protein kinases or more directly by interacting with adaptors of specific receptors[22,23]. In addition, ligation of Fas to its ligand activates p38MAPK through the adaptor Daxx-ASK1-MKK6 activation cascade. H_2O_2 and TNF-α also use ASK1 to activate p38MAPK, but in this case the signal is generated through the oxidative stress sensor thioredoxin, which acts as a regulator of ASK1[22,23,26]. G-proteins are also involved in p38MAPK activation by agonists of serpentine receptors, although the molecules coupling G-proteins to the p38MAPK pathway are still unknown[27]. However, the proximal signaling events activated by sodium salicylate and the signaling mechanism that leads to p38MAPK activation is yet unknown.

In this study, to investigate the signaling pathway for sodium salicylate-induced p38MAPK activation, we performed desensitization experiments. It has been demonstrated that the activity of kinase in the presence of continuing agonist stimulation exhibits desensitization if it uses the same activation pathway[28]. Thus, desensitization study is one of the most suitable and useful tools for examining an unknown signaling pathway and comparing it with other known signaling pathways. Several mechanisms have been demonstrated for homologous desensitization of receptor-mediated responses to agonists including direct down-regulation of the receptor expression and feedback inhibition of essential signaling components by phosphorylation or other mechanisms. Herein, we show that sodium salicylate-pretreated cells remained fully responsive to re-induction of p38MAPK activity by a second sodium salicylate stimulation or by other stresses, H_2O_2 and methyl jasmonate (MeJA), thereby indicating that sodium salicylate does not exhibit both homologous and heterologous desensitization. In contrast, pre-exposure to MeJA, H_2O_2, heat shock, or hyperosmotic stress reduced the responsiveness to subsequent homologous stimulation. These findings demonstrate that sodium salicylate activates p38MAPK through a unique-sensing mechanism, distinct from pathways used by other stressors such as MeJA, H_2O_2, heat shock, and hyperosmotic stress.

MATERIALS AND METHODS

1. Materials

Sodium salicylate, hydrogen peroxide, sorbitol, N-acetylcysteine (NAC) and sodium orthovarnadate were purchased from Sigma. Methyl jasmonate was from Serva. Phospho-p38MAPK antibody and p38MAPK antibody were from Cell Signaling Technology (New England Biolabs, INC.). Okadic acid and cyclosporine A was from Tocris Cookson Ltd. Calyculin A was from Calbiochem. Chemicals for electrophoresis were obtained from Sigma.

2. Cell culture and treatments

C6 glioma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% penicillin-streptomycin in a 37℃ humidified incubator with 5% CO_2. Exponentially growing cells (10^6 cells/60 mm culture dish the day before the experiment) were used for all experiments. For heat shock treatment, the dishes were sealed with parafilm and immersed into a circulating water bath at 45℃ for 20 min.

3. Measurement of intracellular reactive oxygen species

C6 cells were grown on cover slips for 24 h and exposed to sodium salicylate for the indicated times. To measure intracellular ROS, prewarmed (37℃) growth medium containing the 2',7'-dichlorofluorescin diacetate (DCFH-DA; 5 /ml; Molecular Probe) was added to culture dishes. The cells were incubated for 45 min under growth condition and then replaced with fresh prewarmed medium and observed under a laser-scanning confocal microscope (LSM-510, Carl Zeiss). DCF fluorescence was excited at 488 nm using an argon laser, and evoked emission was filtered with a 515 nm long-pass filter.

4. SDS-PAGE and Western blot analysis

SDS-polyacrylmide gel electrophoresis (SDS-PAGE) and Western blot analysis were carried out as described previously[29,30]. Briefly, cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in TNE buffer (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 2 mM EDTA, 1% NP-40) containing 1 mM PMSF, 50 mM NaF, 10 mM Na_3VO_4, 1 mM β-glycophosphate and 1% protease inhibitor cocktail (Sigma) at 4℃ for 10 min. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad). Equal amount of proteins was separated by SDS-PAGE. The resulting gels were either stained with Coomassie Blue or transferred to nitrocellulose membrane (Bio-Rad). For Western blotting, the membrane was blocked with 5% skim milk in PBS-T (0.1% tween-20 in PBS) for 1 h at room temperature on a shaker. After washing the membrane with PBS-T, it was incubated with 1:1000 diluted anti-phospho-p38MAPK anti-p38MAPK (Cell signaling) for overnight at 4℃. After washing the membrane with PBS-T, it was incubated with 1:1000 diluted HRP-conjugated secondary antibody and the antibody-specific proteins were visualized by the enhanced chemo-luminescence detection system according to the recommended procedure (Amersham Corp.).

RESULTS AND DISCUSSION

1. Sodium salicylate activates p38MAPK independently of reactive oxygen species production

Sodium salicylate has been demonstrated to regulate a number of protein kinase signal pathways including ERK1/2, JNK/SAPK, and p38MAPK[4]. With the exception of reported salicylate-induced activation of p38MAPK, observed effects on ERK1/2 and JNK/SAPK are either stimulatory or inhibitory depending on the used cell types. For instance, sodium salicylate was shown to inhibit the activity of ERK1/2 in mouse epidermal cells[11,12] and in C6 cells (data not shown), where it activated ERK1/2 in HCT116 cells[31] and A549 cells (data not shown). Moreover, sodium salicylate inhibited the TNF-α-induced activation of JNK in human fibroblasts, but activated JNK in colon cancer cell and COS cells[4,10,14]. However, sodium salicylate has been reported to activate p38MAPK in most cell types such as normal human fibroblasts, human colorectal carcinoma cells (HT29, HCT116), human lung cancer cells (A549) and COS cells[4,13]. Since p38MAPK plays a crucial role(s) in its anti-inflammatory and anti-neoplastic effects, we investigated the signal pathway responsible for p38MAPK activation by sodium salicylate.

First, we examined activation of p38MAPK in response to sodium salicylate in C6 glioma cells. Treatment of C6

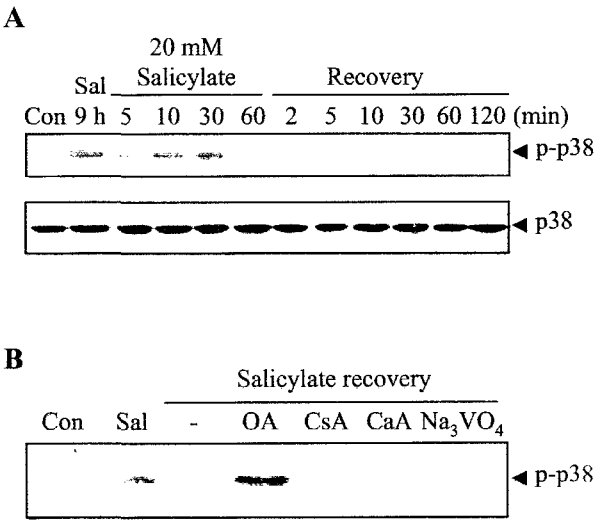

Fig. 1. Activation of p38MAPK by sodium salicylate. **(A)** C6 glioma cells were exposed to 20 mM sodium salicylate (Sal) for various times (5~60 min and 9 h). After treatment of 20 mM Sal for 60 min, the cells were recovered with normal growth media for 2~120 min. At varying times, the cells were harvested and the cell lysates were analyzed by SDS-PAGE and Western blotting using anti-phospho p38MAPK and p38MAPK antibodies. **(B)** C6 glioma cells were pretreated with okadaic acid (OA, 500 nM), cyclosporine A (CsA, 1 μM), calyculin A (CaA, 3 nM), or sodium vanadate (Na_3VO_4, 100 μM) for 1 h and exposed to 50 mM sodium salicylate for 20 min and recovered for 10 min in the presence of the inhibitors and the cellular proteins were analyzed by Western blotting with antibody to phospho-p38MAPK.

cells with sodium salicylate rapidly activated p38MAPK through its phosphorylation at Thr 180/Tyr 182, without affecting its quantity (Fig. 1A). During recovery, p38MAPK was inactivated again and it takes about 2 minutes for sodium salicylate induced p38MAPK activity to be fully recovered to basal level (Fig. 1A). p38MAPK inactivation by sodium salicylate recovery was very rapid compared to that of other p38MAPK activators that require 1~6 hours to fully inactivate p38MAPK when sodium salicylate was removed[28]. Pretreatment of okadaic acid and calyculin A, PP2A and PP1 inhibitors, but not of cyclosporin A and sodium vanadate, PP2B and tyrosine phosphatase inhibitors, respectively, blocked dephosphorylation of p38MAPK by recovery from sodium salicylate, suggesting that disappearance of p38MAPK activity by removal of p38MAPK is mediated by PP2A and PP1 (Fig. 1B).

Recently, sodium salicylate has been shown to enhance cellular ROS production in animal cells as well as in plant cells[1,9]. Moreover, MAPK family protein kinases including p38MAPK have been demonstrated to be regulated in a ROS-dependent manner[32~36]. As shown in Fig. 2, H_2O_2 activated p38MAPK in C6 cells. We examined whether ROS is involved in salicylate-induced p38MAP activation. First, we examined if sodium salicylate increases ROS level in C6 glioma cells. As shown in Fig. 2A, fluorescent analysis using 2',7'-dichlorodihydrofluorescin diacetate revealed that sodium salicylate enhanced ROS (H_2O_2) production in C6 cells in dose- and time-dependent manners and the ROS production declined to the control level during salicylate recovery. Significant ROS production was observed in C6 cells treated with sodium salicylate at concentration of above 35 mM. The ROS production was prevented by N-acetyl-L-cysteine (NAC), a general ROS scavenger (Fig. 2A).

Next, we examined whether p38MAPK activation by sodium salicylate is linked to ROS production. Pretreatment of C6 cells with NAC did not exert significant inhibitory effects on p38MAPK activation by sodium salicylate (Fig. 2B), indicating ROS-independent p38MAPK activation by sodium salicylate. In parallel, it was also examined whether methyl jasmonate, another plant stress hormone that is known to induce ROS in plant cells, induces ROS production in C6 cells. As shown in Fig. 2, cellular ROS level was prominently increased in response to 5 mM methyl jasmonate in C6 cells, that can be suppressed by NAC pretreatment. Interestingly, methyl jasmonate activated p38MAPK (Fig. 2) and this induction was prevented by NAC pretreatment, demonstrating that methyl jasmonate induces p38MAPK activation dependently of ROS production.

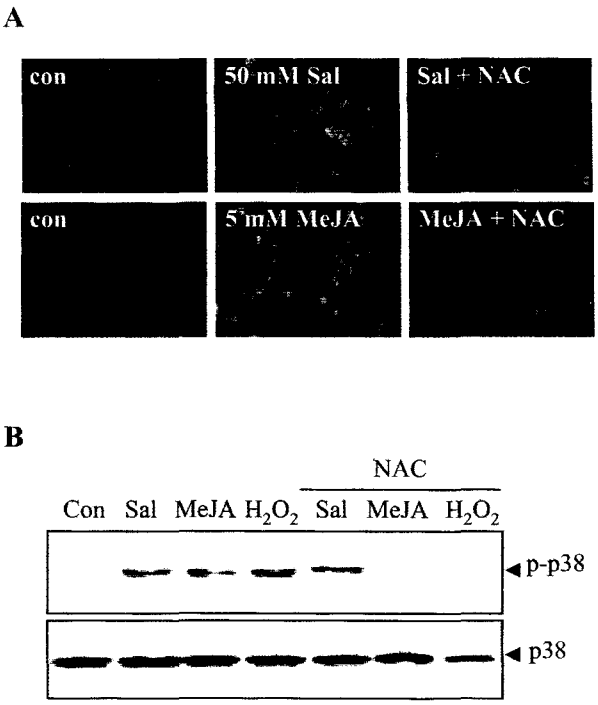

Fig. 2. Sodium salicylate-induced ROS production is not linked to p38MAPK activation. **(A)** C6 glioma cells were pretreated with NAC (20 mM) for 1 h and exposed to 50 mM sodium salicylate (Sal) or 5 mM methyl jasmonate (MeJA) for 20 min. The cells were treated with DCFH-DA (Molecular Probes) and the cellular ROS level determined using a confocal microscope (X400, Cal Zeiss LSM510). **(B)** C6 glioma cells were pre-treated with NAC (20 mM) for 1 h and exposed to 50 mM sodium salicylate (Sal), 5 mM methyl jasmonate (MeJA), or 1 mM H_2O_2 for 20 min in the presence of NAC and the cellular proteins were analyzed by SDS-PAGE and Western blotting with antibodies against phospho-p38.

2. Sodium salicylate-induced p38MAPK activation does not exhibit homologous and heterologous desensitization

To investigate the signal pathways for p38MAPK activation by sodium salicylate, we performed desensitization experiments. It has been demonstrated that the activity of kinase in the presence of continuing agonist stimulation exhibits desensitization if it uses the same activation pathway[28]. Thus, desensitization study is one of the most suitable and useful tools for examining an unknown signaling pathway and comparing it with other known signaling pathways. First, to investigate if the p38MAPK signal pathway

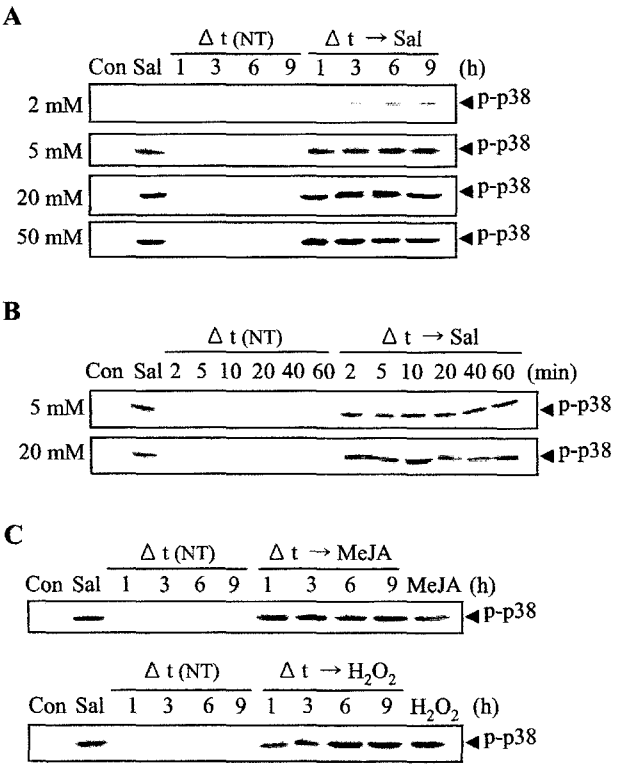

Fig. 3. Sodium salicylate-activated p38MAPK signaling pathway does not exhibit desensitization. **(A-B)** C6 cells were treated with 2~50 mM sodium salicylate (Sal) for 20 min. After varying time (Δt, recovery with normal growth media: panel A, 1~9 h; panel B, 2~60 min), the cells were treated with no drug (NT) or Sal for 20 min. **(C)** C6 cells were treated with 50 mM Sal for 20 min. At Δt thereafter the cells were exposed to no drug treatment (NT), 5 mM MeJA, or 1 mM H_2O_2 treatment for 20min. Immediately after the second treatment, the cells were harvested and the cell lysates were analyzed by SDS-PAGE and Western blotting using anti-phospho p38MAPK.

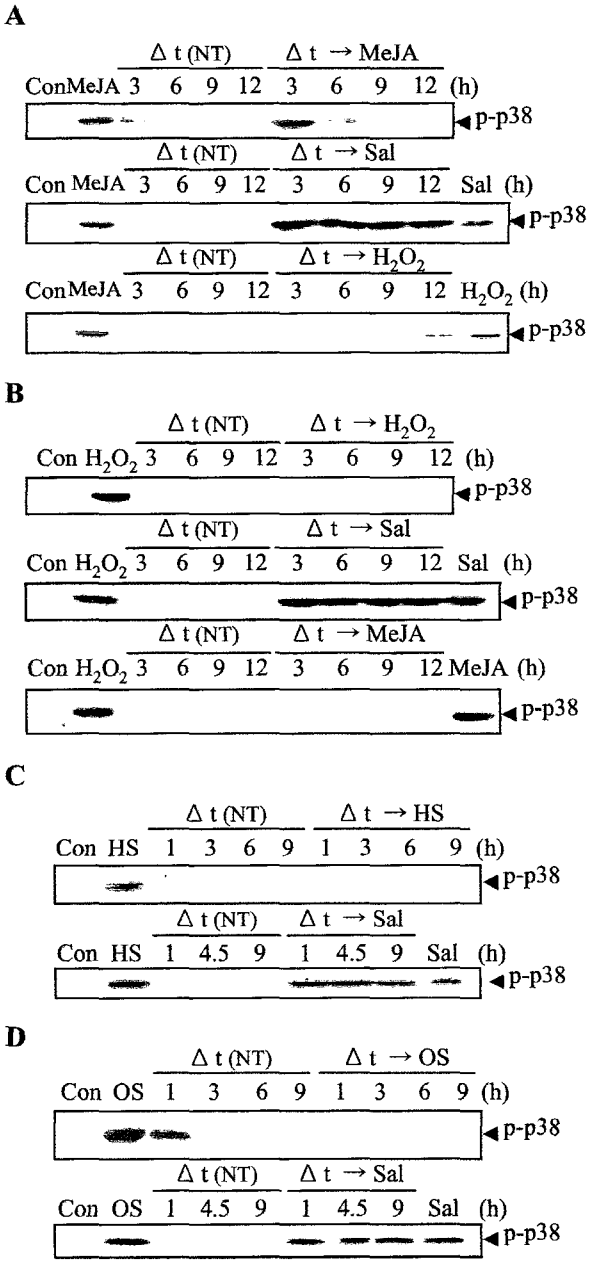

Fig. 4. Activation of p38MAPK by sodium salicylate in methyl jasmonate-, H_2O_2-, heat shock-, or osmotic shock-desensitized cells. C6 cells were exposed to either 5 mM methyl jasmonate (MeJA, panel **A**), 1 mM H_2O_2 (panel **B**), heat shock (HS) of 45℃ (penel **C**), or 0.5 M sorbitol (OS, panel **D**) for 20 min. At varying times (Δt), the cells were treated with no drug (NT), or various p38MAPK activators (MeJA, sodium salicylate (Sal), H_2O_2, heat shock, sorbitol) for 20 min. Immediately after the second treatment, the cells were harvested and the cell lysates were analyzed by Western blot using anti-phospho p38MAPK antibody.

is desensitized by sodium salicylate, C6 cells were exposed to sodium salicylate for 20 min and then recovered and reactivation of p38MAPK by sodium salicylate was examined at various times after a priming sodium salicylate treatment. As shown in Fig. 3, p38MAPK was reactivated immediately when the cells were retreated with sodium salicylate at any time after priming homologous sodium salicylate treatment. This data turns out that sodium salicylate-induced p38MAPK activation does not exhibit homologous desensitization. Next, we examined whether other p38-MAPK activator can activate p38MAPK in sodium salicylate-treated cells. Cells were first exposed to sodium salicylate and then restimulated with various stresses, H_2O_2, methyl jasmonate, and heat shock. Sodium salicylate pretreated cells remained fully responsive to restimulation with all other agonists, H_2O_2 and methyl jasmonate, tested at 1~9 h after sodium salicylate treatment, suggesting that sodium salicylate-induced p38MAPK activation pathway

does not exhibit homologous and heterologous desensitization.

3. Activation of p38MAPK by sodium salicylate in H_2O_2-, and methyl jasmonate-, heat shock-, or osmotic shock-desensitized cells

We further examined whether sodium salicylate activates p38MAPK in C6 cells that were desensitized by other chemicals that activated p38MAPK: H_2O_2, methyl jasmonate, osmotic shock, and heat shock. As shown in Fig. 4, after H_2O_2 treatment, cells became refractory to reinduction of the p38MAPK pathway by a second H_2O_2. Moreover, H_2O_2-treated cells showed desensitization of p38MAPK activation to stimulation with methyl jasmonate. Conversely, methyl jasmonate-primed cells did not respond to successive stimulation with methyl jasmonate or H_2O_2 until 12 h after methyl jasmonate-priming, further supporting that MeJA activates p38MAPK dependently of ROS production and indicating that heterologous desensitization of p38MAPK activation occur between H_2O_2 and MeJA. Desensitization starts at 2 h after the priming treatment, peaks at 5 h, at which time no reinduction of p38MAPK activation is observed, and vanishes by 15 h. However, H_2O_2-desensitized cells and methyl jasmonate-desensitized cells remained fully responsive to stimulation with sodium salicylate (Fig. 4). Similarly, the ability of sodium salicylate to activate p38MAPK was unaffected in heat-desensitized cells and osmotic shock-desensitized cells, while heat shock- or osmotic shock-desensitized cells became refractory to reinduction of the SAPK2/p38 pathway by a second heat shock or osmotic shock (Fig. 4). These results indicate that the cells were not desensitized to sodium salicylate by other treatments that activated p38MAPK.

A recent report demonstrated that H_2O_2-induced p38MAPK activation was related with direct inhibition of protein phosphatase PP2A, PP1, but not PP2B[37,38]. H_2O_2 also use ASK1 to activate p38MAPK, and in this case the signal is generated through the oxidative stress sensor thioredoxin, which acts as a regulator of ASK1[39]. In case of osmotic shock, it is known to activate p38MAPK through a small GTPase Rac1[40,41]. Heat shock also activates p38MAPK through activating its specific pathway that involves a short-lived protein whose activity depends on a tight regulation between synthesis and degradation[28,29,41]. We propose that there is a sensing mechanism highly specific to sodium salicylate for activation of p38MAPK, which is not shared by other p38MAPK activators including H_2O_2, methyl jasmonate, osmotic shock, and heat shock. No significant refractoriness to restimulation was specific to sodium salicylate. The proximal signaling events activated by sodium salicylate and the signaling mechanism that leads to p38MAPK activation cascade is unclear. One possibility is that sodium salicylate may induce autophosphorylation of p38MAPK. Recently, p38MAPK has been demonstrated to be activated by TAB1-dependent autophosphorylation independently of MAPKK[42] and the dual phosphorylation motif located in loop12 of p38MAPK influences auto-phosphorylation and substrate specificity, but not upstream kinase selection[43]. Sodium salicylate may interact with this motif. In fact, sodium salicylate has been shown to interact with several proteins such as BiP[44] or IKKβ[15,45] and modulate their activities. Otherwise, the target of sodium salicylate may be proximal upstream molecules of p38MAPK such as MKK3/6 or ASK1[23,46~49]. Further studies on the signal pathways by which sodium salicylate activates p38MAPK will help to understand the molecular action mechanism of this drug *in vivo*.

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