Protein tyrosine phosphatase Shp2 positively regulates cold stress-induced tyrosine phosphorylation of SIRPα in neurons

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ABSTRACT

The membrane protein SIRPα is a cold stress-responsive signaling molecule in neurons. Cold stress directly induces tyrosine phosphorylation of SIRPα in its cytoplasmic region, and phosphorylated SIRPα is involved in regulating experience-dependent behavioral changes in mice. Here, we examined the mechanism of cold stress-induced SIRPα phosphorylation *in vitro* and *in vivo*. The levels of activated Src family protein tyrosine kinases (SFKs), which phosphorylate SIRPα, were not increased by lowering the temperature in cultured neurons. Although the SFK inhibitor dasatinib markedly reduced SIRPα phosphorylation, low temperature induced an increase in SIRPα phosphorylation even in the presence of dasatinib, suggesting that SFK activation is not required for low temperature-induced SIRPα phosphorylation. However, in the presence of pervanadate, a potent inhibitor of protein tyrosine phosphatases (PTPases), SIRPα phosphorylation was significantly reduced by lowering the temperature, suggesting that either the inactivation of PTPase(s) that dephosphorylate SIRPα or increased protection

of phosphorylated SIRPα from the PTPase activity is important for low temperature-induced SIRPα phosphorylation. Inactivation of PTPase Shp2 by the allosteric Shp2 inhibitor SHP099, but not by the competitive inhibitor NSC-87877, reduced SIRPα phosphorylation in cultured neurons. *Shp2* knockout also reduced SIRPα phosphorylation in the mouse brain. Our data suggest that Shp2, but not SFKs, positively regulates cold stress-induced SIRPα phosphorylation in a PTPase activity-independent manner.

Keywords: cold stress, neuron, protein tyrosine phosphatase, hypothermia

Abbreviations: SFKs, Src family protein tyrosine kinases; PTPases, protein tyrosine phosphatases; SIRPα, signal regulatory protein α

1. Introduction

Low body temperature is harmful to homeothermic mammals as it can lead to severe hypothermia, which is causes the deterioration of essential biological systems or cellular functions and can be fatal [1]. On the contrary, low temperature can protect the brain from injuries, such as ischemia, by suppressing inflammation, increasing growth factor expression, and allowing the repair of an injured brain [2]. The protective effect of low temperature on tissue damage has also been demonstrated in an animal model of neurodegenerative diseases [3]. Hypothermia has been reported to cause anemia in experimental animals and humans [4,5]. In addition, low temperature induces synaptic remodeling in experimental animals [3], suggesting that it has a significant effect on central nervous system functions. Understanding the neuronal response to low temperatures could reveal novel brain functions unidentified under normal physiological conditions and may provide novel application targets to treat brain injuries and diseases.

Signal regulatory protein α (SIRPα) , a membrane protein predominantly expressed in matured neurons, is a cold stress-responsive signaling molecule [6]. SIRPα, when tyrosine phosphorylated in its cytoplasmic region by the Src family protein tyrosine kinases (SFKs), binds to the cytoplasmic protein tyrosine phosphatase (PTPase), Shp2 [7-9]. SIRPα phosphorylation was markedly increased in the brain of mice in severe hypothermic conditions induced by forced swimming [6,9,10]. *In vitro* analyses also showed that cooling of cultured neurons induced SIRPα phosphorylation [6], suggesting that cold-stress directly induces SIRPα tyrosine phosphorylation. SIRPα knockout (KO) mice exhibited an experience-dependent increase in immobility during repeated forced swim, suggesting that SIRPα signaling is involved in higher brain functions, such as memory and behavior control [9]. The mechanism underlying the induction of SIRPα phosphorylation by hypothermia remain unclear. Our previous data showed that forced swim activated SFKs in the mouse brain, and might be the underlying mechanism for cooling-induced SIRPα phosphorylation [9]. However,

hypothermia failed to activate SFKs in the brains of anesthetized mice [6]. Thus, it is not clear whether low temperatures indeed activate SFKs.

Here, we show that the activation of SFKs is not the primary cause of cold stress-induced SIRPα phosphorylation in neurons. Our data suggest that Shp2 positively regulates cold stress-induced SIRPα phosphorylation *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Primary antibodies and reagents

Rabbit polyclonal antibodies (pAbs) specific to tyrosine-phosphorylated SIRPα (anti-pSIRPα) have been described previously [9]. Rabbit pAbs specific to SIRPα were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit monoclonal antibody (mAb) specific to SIRPα (D6I3M) and phospho-Src family (Tyr^{416}) (D49G4) were from Cell Signaling Technology (Danvers, MA). Rabbit pAbs specific to Shp2 were obtained from Santa Cruz Biotechnology (Dallas, TX). Dasatinib and NSC-87877 were from TOCRIS Bioscience (Bristol, UK). SHP099 was from Cayman Chemical (Ann Arbor, MI). Catalase and collagenase were from FUJIFILM Wako Pure Chemical (Osaka, Japan). Poly-L-lysine was from Sigma-Aldrich (St. Louis, MO).

2.2. Culture and treatment of primary cortical neurons

Primary cortical neurons were prepared as previously described, with some modifications [11]. Briefly, the cerebral cortex was dissected from the mouse embryos

at gestational days 18-19, and cortical neurons were isolated by treatment with trypsin (1.5 mg/mL) and collagenase (1 mg/mL) for 30 min at 30°C followed by trituration with a pipette. Dissociated cells were plated on 35 mm culture dishes, which were precoated with poly-L-lysine (0.1 mg/mL) overnight at 8°C and washed three times with sterilized water, at a density of 1.5×10^6 cells/dish in 1.5 mL Neurobasal A plus medium (Thermo Fisher Scientific, Waltham, MA) containing 1/50 volume of B27 plus supplement (Thermo Fisher Scientific), 0.5 mM GlutaMax (Thermo Fisher Scientific), and penicillin (100 unit/mL)/streptomycin (100 μg/mL) (FUJIFILM Wako Pure Chemical). Neurons were maintained for approximately 14-21 days before experiments by adding a fresh culture medium (0.5 mL) to each dish every week. For the treatment with inhibitors (dasatinib, SHP099, and NSC-87877), the volume of medium was adjusted at 1.5 mL/dish with the preconditioned medium, and inhibitors were added to the medium. Then cells were precultured for 2-3 h in a 5% CO_2 incubator at 37°C. After preculture, neurons were kept at 37°C in the same incubator or transferred to another

5% CO_2 incubator maintained at 23°C and cultured for an additional 24 h. The stock solutions of all inhibitors were prepared in dimethyl sulfoxide (DMSO). The final concentration of the inhibitor was achieved by diluting the stock solution (0.1% volume) with the culture medium. A similar concentration of DMSO was used for the control experiments. The final concentrations of dasatinib, SHP099, and NSC-87877 were 1, 10, and 10 μM, respectively. Pervanadate was prepared by mixing equal volumes of 100 mM Na_3VO_4 and 200 mM H_2O_2. After 15 min at room temperature, the mixture was diluted 2.5 times with the incubation medium containing 140 mM NaCl, 4.7 mM KCl, 11 mM glucose, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 15 mM HEPES (pH 7.4). Catalase was then added to the mixture to a final concentration of 420 U/mL to remove the remaining H_2O_2. The mixture was incubated for 15 min at room temperature, and the resulting 20 mM pervanadate stock solution was used within 1 h. For the pervanadate treatment, neurons cultured for 16 days were transferred from a CO_2 incubator to a water bath and were preincubated for 10 min at

37°C or 23°C in an incubation medium. The medium was then replaced with a fresh incubation medium with or without 100 μM pervanadate and then incubated for an additional 10 min at 37°C or 23°C in a water bath. After the treatment with inhibitors or pervanadate, media were removed, and neurons were washed once quickly and gently with PBS preincubated at 37°C or 23°C. Washed cells were frozen by sinking the culture dishes in liquid nitrogen and stored at -80°C until preparation of the cell lysate.

2.3. Animals

Forebrain neuron-specific Shp2 conditional KO (cKO) mice were generated by crossing the $Shp2^{flox/flox}$ [12] mice and CaMKIIα-Cre transgenic (CRE-159) [13] mice, as previously described [14]. Mice were bred and maintained at the Institute of Experimental Animal Research of Gunma University under specific pathogen-free conditions. They were housed in an air-conditioned room with a 12-h light/dark cycles. Hypothermia was induced in mice by forced swimming at 23°C as previously described

[6,9]. All animal experiments were approved by the Animal Care and Experimentation Committee of Gunma University (approval no. 13-015).

2.4. Immunoblot analysis

Cell lysates and brain homogenates were prepared from frozen cells and tissues, respectively, and were subjected to immunoblot analyses as previously described [10].

2.5. Statistical analysis

Quantitative data are presented as means ± SE and were analyzed by Tukey-Kramer test using the R statistical package (version 4.0.2) [15]. Statistical significance was set at $p < 0.05$.

3. Results

3.1. SFK activation was not required for low temperature-induced SIRPα phosphorylation in cultured neurons

In our previous study, we examined the tyrosine phosphorylation of SIRPα in cultured neurons after 10 min of cooling [6]. Therapeutic applications usually require 12-24 hours of cooling to obtain a protective effect on the patients [2]. Hence, in the present study, we attempted 24 h cooling to induce SIRPα phosphorylation in cultured neurons. After 24 h at 23°C, no obvious cell damage was observed under microscopic examination (**Fig. 1A**). Similar to that in the previous results, with short cooling, the phosphorylation of SIRPα markedly increased after 24 h of cooling (**Fig. 1B**), suggesting that the low temperature-induced increase in SIRPα phosphorylation persists for a long time *in vitro* as previously shown in hibernating animals [10]. To examine the contribution of SFKs to the low temperature-induced phosphorylation of SIRPα, we analyzed the effect of the SFK inhibitor dasatinib [16] on the phosphorylation of SIRPα

in neurons. Although dasatinib markedly attenuated SIRPα phosphorylation in neurons at both control (37°C) and low temperature (23°C), SIRPα phosphorylation was significantly higher at 23°C compared to 37°C (**Fig. 1B**). We also examined the activation states of SFKs by immunoblot analysis using the antibodies for the active form (Tyr^{416}-phosphorylated) of SFKs [17]. The active SFK levels did not increase in neurons at 23°C (**Fig. 1C**). In the presence of dasatinib, active SFK levels significantly reduced in neurons, consistent with the decreased phosphorylation of SIRPα (**Fig. 1C**). These results suggest that the low temperature-induced increase in SIRPα phosphorylation was not due to the activation of SFKs, although SFK activity is important for the phosphorylation of SIRPα in neurons at both 37°C and 23°C.

3.2. SIRPα phosphorylation was reduced in the presence of pervanadate at low temperature in cultured neurons

Our data suggest that the low temperature-induced increase in SIRPα phosphorylation is not likely due to the activation of SFKs. Hence, activation of protein

tyrosine kinases (PTKs) other than SFKs might be involved in the low temperature-induced phosphorylation of SIRPα. To examine this possibility, neurons were incubated at low temperature in the presence of pervanadate (PV), a broad and potent inhibitor of PTPases [18]. In the presence of PV, the PTPase activity was inhibited, and the PTK activity directly determined the phosphorylation levels of their substrates. SIPRα phosphorylation was markedly increased in PV-treated neurons at both temperatures, consistent with the inhibition of PTPase activity by PV (**Fig. 2A**). In contrast to the low temperature-induced increase in SIRPα phosphorylation in control untreated neurons without PV, SIRPα phosphorylation was significantly decreased at 23°C in the PV-treated neurons compared with at 37°C in the presence of PV (**Fig. 2A**). Similarly, PV treatment markedly increased the autophosphorylation of SFK Tyr^{416} at both temperatures; however, it was significantly reduced at 23°C in PV-treated neurons, suggesting decreased SFK activity at 23°C (**Fig. 2B**). Thus, our results suggest that low

temperature does not increases, but rather reduces, the activity of PTKs catalyzing SIRPα phosphorylation.

3.3. An Shp2 inhibitor suppressed the SIRPα phosphorylation in cultured neurons

Low temperature-induced SIRPα phosphorylation was not likely due to the activation of PTKs. Another possible mechanism is the notable inactivation of PTPase(s) that dephosphorylate SIRPα. PTPase Shp2 is reported to dephosphorylate SIRPα in CHO cells [19]. Thus, if Shp2 is also involved in the dephosphorylation of SIRPα in neurons, inhibiting Shp2 activity may result in increased phosphorylation of SIRPα. We examined the effect of SHP099, a selective allosteric inhibitor of Shp2 [20], on the phosphorylation of SIRPα. SHP099 binds to Shp2 at the interface of the N-terminal SH2, C-terminal SH2, and protein tyrosine phosphatase domains, thus stabilizing the closed, inactive form of Shp2. Surprisingly, SHP099 strongly suppressed SIRPα phosphorylation at both 37°C and 23°C, suggesting that Shp2 does not catalyze the dephosphorylation of SIRPα in neurons (**Fig. 3A**). In the presence of SHP099,

SIRPα phosphorylation levels were comparable between neurons cultured at 37°C and 23°C, suggesting that the low temperature-induced increase in SIRPα phosphorylation was completely suppressed by SHP099 (**Fig. 3A**). We further examined the effect of NSC-87877 [21], another Shp2 inhibitor, on SIRPα phosphorylation. NSC-87877 is a competitive inhibitor that binds to the catalytic site and inhibits the PTPase activity of Shp2. However, NSC-87877 did not affect both control (37°C) and low temperature-induced (23°C) SIRPα phosphorylation. Autophosphorylation levels of SFKs tended to be reduced and increased by SHP099 and NSC-87877, respectively, but their effects were not significant (**Fig. 3B**).

3.4. Neuron-specific Shp2 knockout suppressed SIRPα phosphorylation in the mouse brain

To further investigate the functional significance of Shp2 in the regulation of SIRPα phosphorylation, we examined the cold stress-induced SIRPα phosphorylation in the brains of adult forebrain neuron-specific Shp2 conditional knockout (cKO) mice

[14]. Shp2 cKO mice were generated by crossing the Shp2-floxed mice with CaMKII-Cre transgenic mice to achieve forebrain neuron-specific ablation of Shp2. In the hippocampus, where Cre recombinase was most highly expressed in the forebrain [14], expression of Shp2 was reduced to approximately 45 % of the control mice lacking the CaMKII-Cre transgene (**Fig. 4A**). SIRPα phosphorylation significantly increased in the hippocampus after the forced swim at room temperature that induced severe hypothermia in control mice (**Fig. 4B**). The phosphorylation of SIRPα after the forced swim was significantly lower in Shp2 cKO mice than in control mice, although the forced swim induced an increase in SIRPα phosphorylation even in Shp2 cKO mice (**Fig. 4B**). As we previously reported, the forced swim induced an increase in the level of active form of SFKs in the hippocampus, but no significant differences were observed in the activation level of SFKs between control and Shp2 cKO mice (**Fig. 4C**).

4. Discussion

Our present data suggest that an increase in the active form of SFKs is not required for the low temperature-induced increase in the phosphorylation of SIRPα in cultured neurons. The levels of autophosphorylated (active) SFKs did not increase in neurons at low temperatures, while SIRPα phosphorylation increased under the same conditions. Low temperature also induced a significant increase in SIRPα phosphorylation, even in the presence of dasatinib, which strongly suppresses the active SFKs. In contrast, low temperature induced a decrease in SIRPα phosphorylation and SFK autophosphorylation in the presence of PV, a broad PTPase inhibitor. These data support the idea that low temperatures do not activate SFKs, but rather inactivate SFKs or other PTKs that catalyze SIRPα phosphorylation.

Strong inhibition of the dephosphorylation reaction is one possible mechanism for the low temperature-induced increase in SIRPα phosphorylation if its impact is much stronger than that of PTK inactivation at low temperatures. A similar mechanism has

been proposed for the low temperature-induced phosphorylation of tau protein [22]. A PTPase that catalyzes SIRPα dephosphorylation has still not been identified. Previous studies have shown that overexpression of a dominant-negative mutant of Shp2 induced over-phosphorylation of SIRPα in immortalized cell lines [19], suggesting that Shp2 is a possible candidate for the PTPase that dephosphorylates SIRPα. If Shp2 dephosphorylates SIRPα in neurons, inhibition of Shp2 activity would result in increased phosphorylation of SIRPα even at 37°C. We examined the effect of two Shp2 inhibitors, SHP099 and NSC-87877, on SIRPα phosphorylation. It was observed that SHP099 strongly suppressed SIRPα phosphorylation; however, NSC-87877 did not affect SIRPα phosphorylation in neurons. Suppression of SIRPα phosphorylation by SHP099 suggests that Shp2 does not dephosphorylate SIRPα but rather positively regulates SIRPα phosphorylation, at least in neurons. It has been proposed that Shp2 activates SFKs by dephosphorylating PAG, which regulates C-terminal Src kinase (Csk) [23]. A non-enzymatic (PTPase-independent) mechanism has also been reported for

Shp2-mediated SFK activation [24]. Inhibition of Shp2 may have resulted in the inactivation of SFKs and suppression of SIRPα phosphorylation. However, both the Shp2 inhibitors did not significantly affect the autophosphorylation of SFKs in neurons. The level of active form of SFKs tended to be slightly low and high in SHP099-treated and NSC-87877-treated neurons, respectively, but the differences were not significant. Although Shp2 inhibitors may affect the active state of SFKs, this effect seems to be limited in neurons. Thus, the inactivation of SFKs does not seem to be a major cause for the suppression of SIRPα phosphorylation by SHP099.

SHP099 is an allosteric inhibitor that binds to Shp2 at the interface of the N-terminal SH2, C-terminal SH2, and protein tyrosine phosphatase domains, thus stabilizing the closed (inactive) form of Shp2 [20], while NSC-87877 is a competitive inhibitor that binds to the catalytic center of Shp2, thus inhibiting its PTPase activity [21]. Open (active) form of Shp2 seems to be important for the positive regulation of SIRPα phosphorylation by Shp2, while its PTPase activity was not necessary for the

same because SHP099 reduced the phosphorylation levels of SIRPα, while NSC-87877 did not. The binding of SH2 domains to its target phosphorylation sites could protect their binding sites, phosphorylated tyrosine residues, from dephosphorylation by PTPase(s). Consistent with this, the phosphoproteome analyses in EGF-treated breast cancer cells reported that SHP099 decreased the phospho-abundance of proteins that can directly bind to the paired SH2 domains of Shp2 [25]. Thus, it is likely that Shp2 protects SIRPα from unknown PTPase(s) by binding its SH2 domains to the phosphorylated SIRPα, thereby sustaining SIRPα phosphorylation in neurons.

It should be noted that the low temperature-induced increase in the amount of phosphorylated SIRPα almost completely disappeared in SHP099-treated neurons. This result supports the idea that the low temperature-induced increase in SIRPα phosphorylation mainly depends on the open form of Shp2. If so, low temperature may induce the open form Shp2 or stabilize the interaction between the open-form Shp2 and phospho-tyrosine residues in SIRPα. Of the two hypotheses, the latter seems more likely

because the thermal vibration of atoms is reduced at low temperatures in general and hydrogen bonds important for stabilizing the closed-form Shp2 and the SH2 domain-phospho-tyrosine interaction are strengthened. However, the molecular mechanism underlying the Shp2-dependent increase in low temperature-induced SIRPα phosphorylation remains to be elucidated in further studies.

Positive regulation by Shp2 of SIRPα phosphorylation was also evident in the Shp2 cKO mouse brain. Hypothermia-induced SIRPα phosphorylation was significantly reduced in Shp2 cKO mice compared with that in control mice. The reduced amount of Shp2 molecules in the Shp2 cKO mouse brain is more closely related to the SHP099-treated neurons than to the NSC-87877-treated neurons because NSC-87877 may not affect the amount of open and closed forms of Shp2. Inconsistent with our *in vitro* data, a small but significant increase in SIRPα phosphorylation was observed even in the brain of Shp2 cKO mice after the forced swim, but this could be due to the substantial residual Shp2 (~45% of control mice) in the mutant mice. Unlike *in vitro* results, forced

swim-induced *in vivo* hypothermia increased the levels of autophosphorylated SFKs in the brain. However, we have previously reported that hypothermia in anesthetized mice failed to activate SFKs in the brain [6], which is consistent with our present *in vitro* results. Thus, activation of SFKs in the brain of awaked mice was probably due to the psychiatric or physiologically sensible stress during the forced swim.

Overall, our data suggest that Shp2 may positively regulate SIRPα phosphorylation both *in vitro* and *in vivo*, and the PTPase activity may not be required for the regulatory function of Shp2, as observed in cultured neurons. Although the detailed mechanism of this reaction remains to be elucidated, our findings provide a basis for understanding the cellular response of neurons to low temperatures.

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Figure legends

Fig. 1. Low temperature induced an increase in the phosphorylation of SIRPα, but not in the level of active SFKs in cultured neurons. Cultured mouse cortical neurons were preincubated in the medium at 37℃ or 23℃ for 2-3 h in the presence or absence of 1 μM dasatinib (**Dasat**) and then cultured at the indicated temperature for 24 h in the continuous presence or absence of dasatinib. After the treatment, cell images were acquired by phase-contrast microscopy (**A**). Cell lysates were prepared and subjected to immunoblot analyses with antibodies specific to phosphorylated SIRPα (**pSIRPα**), SIRPα (**B**), or active SFKs **(Src-pY^{416})** (**C**). The pSIRPα/SIRPα ratio of the band (**B**) or the intensity of Src-pY^{416} band (**C**) was expressed as fold increase relative to the value at 37℃ control condition without dasatinib (**DMSO**); Individual circles represent each data from three independent experiments. Summary data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ (Tukey-Kramer test)

Fig. 2. Low temperature induced a decrease in the phosphorylation of SIRPα and level of activated SFKs in pervanadate-treated neurons. Cultured neurons were preincubated in the medium at 37℃ for 10 min and then incubated at the indicated temperature for 10 min in the presence or absence of 100 μM pervanadate (**PV**). After the treatment, cell lysates were prepared and subjected to immunoblot analyses as described in **Fig. 1**. The pSIRPα/SIRPα ratio of the band (**A**) or the intensity of Src-pY^{416} band (**B**) was expressed as a fold increase relative to the value at 37℃ control condition without pervanadate (**Ctrl**); Individual circles represent each data from two independent experiments. Summary data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ (Tukey-Kramer test)

Fig. 3. Effect of Shp2 inhibitors on SIRPα phosphorylation in cultured neurons. Cultured neurons were treated with SHP099 (**SHP**) or NSC-87877 (**NSC**) and cultured at 37℃ or 23℃ for 24 h, and cell lysates were prepared and subjected to immunoblot

analyses as described in **Fig. 1**. The pSIRPα/SIRPα ratio of the band (**A**) or the intensity of Src-pY^{416} band (**B**) was expressed as in **Fig. 1**; Individual circles represent each data from five independent experiments. Summary data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005 (Tukey-Kramer test)

Fig. 4. Effect of genetic ablation of neuronal Shp2 for hypothermia-induced SIRPα phosphorylation *in vivo*. Control (**Ctrl**) and forebrain neuron-specific Shp2 knockout (**cKO**) mice were subjected to forced swim (**FS+**) to induce hypothermia or allowed to stand in shallow water (**FS-**) for control, after which homogenates of the hippocampus were immediately prepared and subjected to immunoblot analysis with antibodies specific to Shp2 (**A**), phosphorylated SIRPα (**pSIRPα**), SIRPα (**B**), or active SFKs **(Src-pY^{416})** (**C**). The intensities of Shp2 bands (**A**) or Src-pY^{416} bands (**C**) and the pSIRPα/SIRPα ratio (**B**) were expressed as fold change relative to the value of control mice without forced swim; Individual circles represent data from one animal. Summary

data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ (Tukey-Kramer test)

Fig. 1
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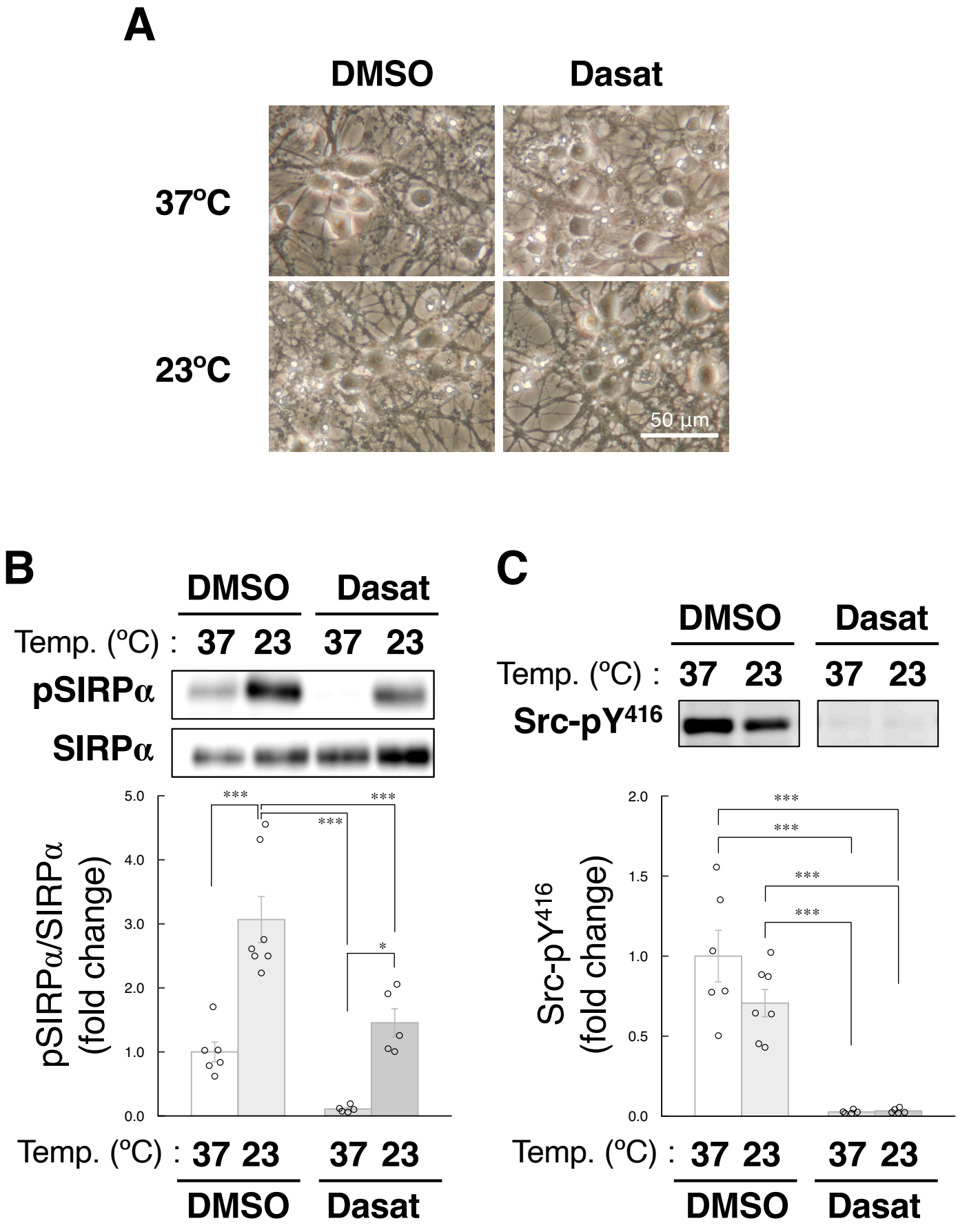

Fig. 2
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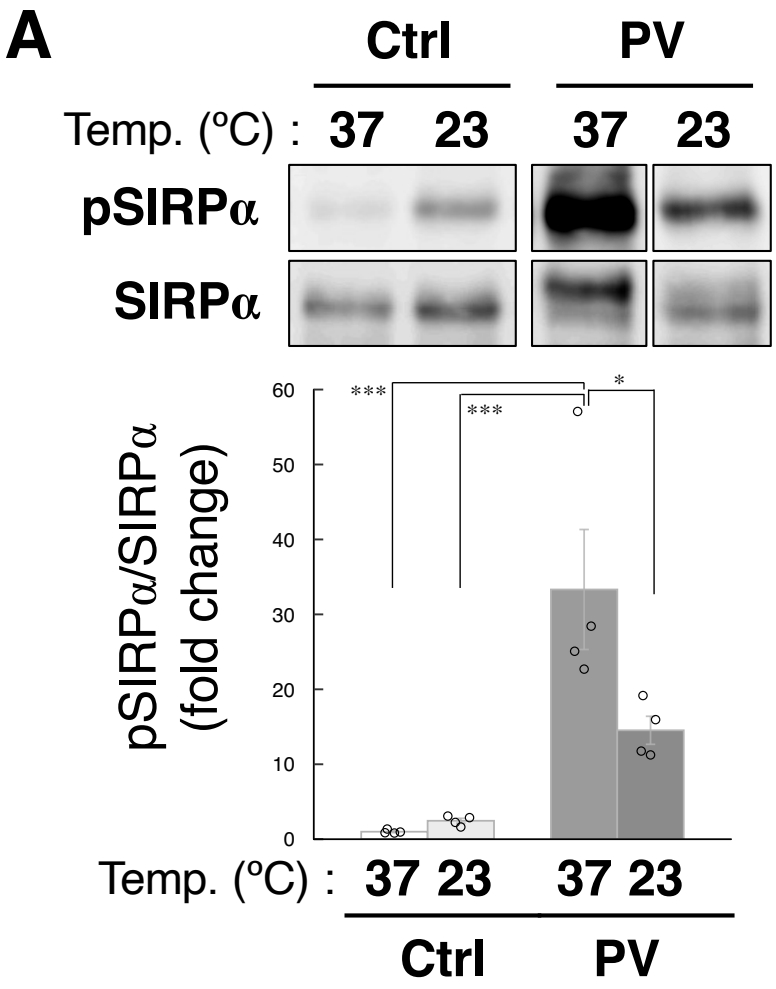

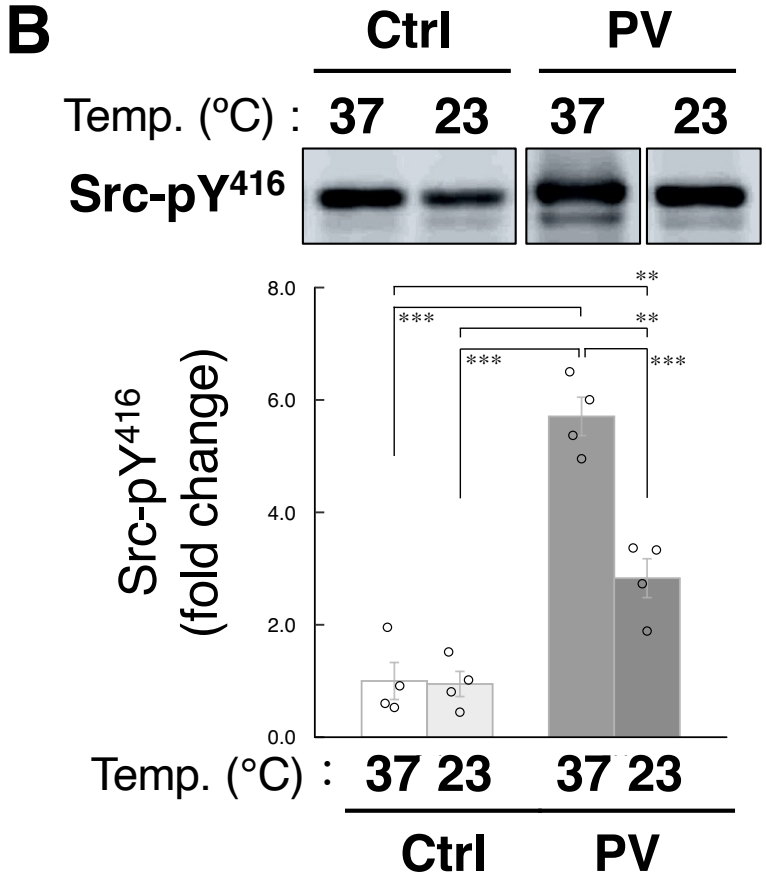

Fig. 3
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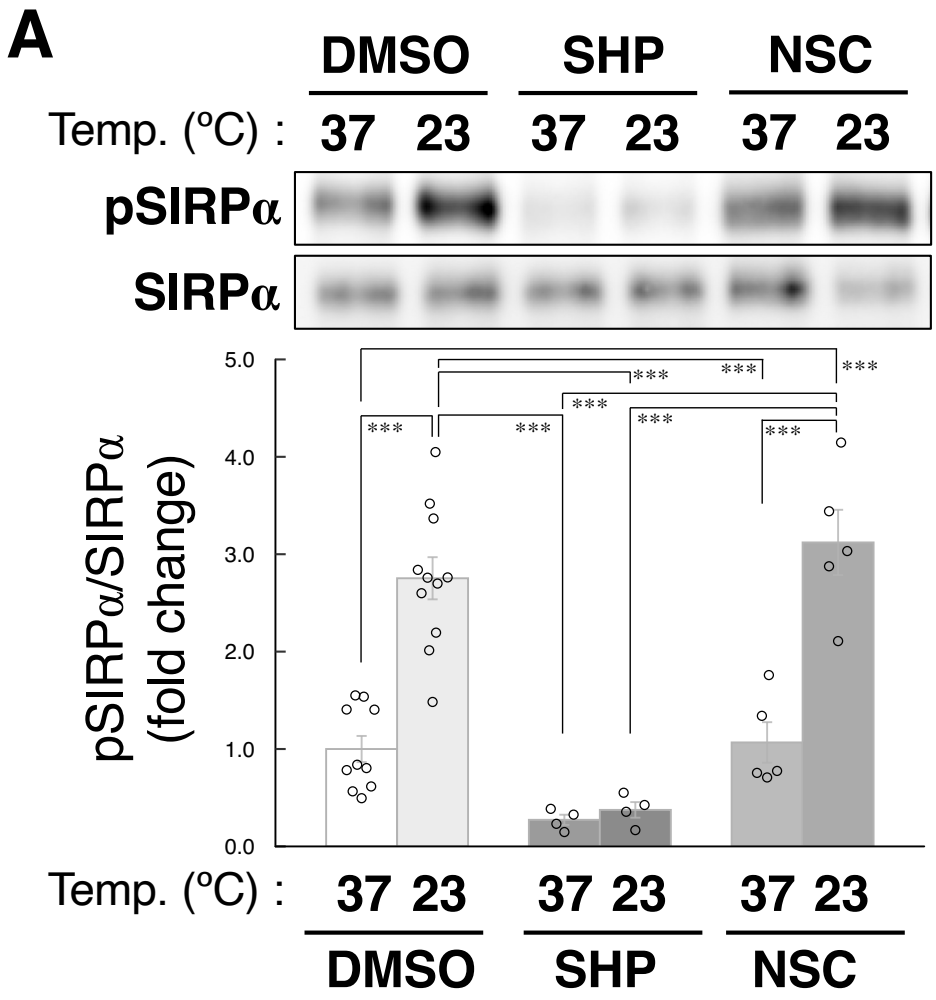

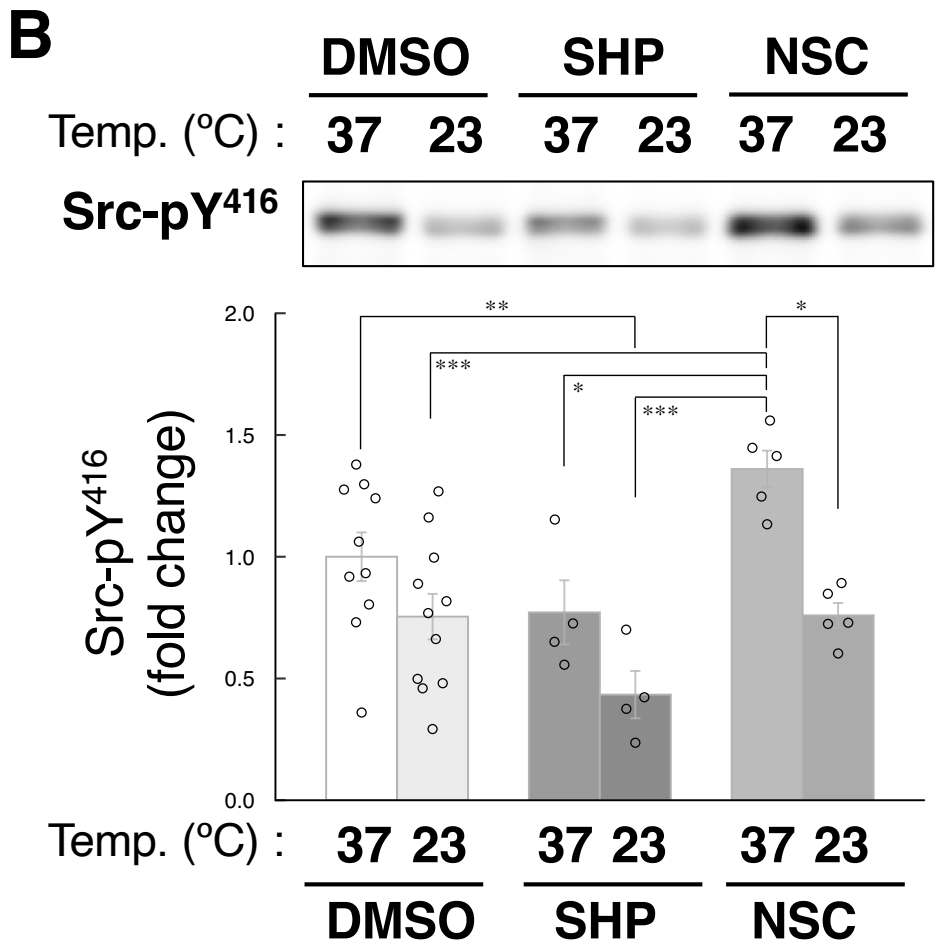

Fig. 4
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