RESEARCH ARTICLE

SOCS1/2 controls NF-κB activity induced by HSP70 by degrading MyD88-adaptor-like protein (Mal) in porcine macrophages

Yanhong Yong¹,² Lianyun Wu¹,³ Minglong Ma² Biao Fang² Tianyue Yu² Junyu Li² Canying Hu³ Rumin Jia³⁷ Xianghong Ju¹,²

Abstract: Heat stress induces suppressor of cytokine signaling (SOCS) 1 and SOCS2 expression in the intestinal gut and disrupts inflammatory cytokine production in pigs. These changes may be important to the development of inflammatory bowel disease in heat-stressed pigs. However, the underlying mechanisms have not yet been completely elucidated. In the present study, we examined the roles of SOCS1 and SOCS2 in regulating the nuclear factor (NF)-κB pathway in CRL-2845 porcine macrophages. Ectopic expression of HSP70 significantly modulated NF-κB activity (p ≤ 0.05). Moreover, co-expression of SOCS1 or SOCS2 with HSP70 reduced NF-κB activity, which was abolished by SOCS1 or SOCS2 knockdown with small interfering RNA. Additionally, myeloid differentiation factor 88 (MyD88)-adaptor-like (Mal) protein was down-regulated in cells expressing SOCS1 and SOCS2. SOCS1 and SOCS2 were found to negatively regulate the activity of NF-κB induced by HSP70 overexpression by degrading Mal. These findings may facilitate the development of novel SOCS1-based and SOCS2-based therapeutic strategies for controlling heat stress-related disorders in pigs.

Keywords: heat stress, porcine macrophages, suppressor of cytokine signaling 1/2, nuclear factor-κB, MyD88-adaptor-like protein

1 Introduction

As global warming advances and highly intensive breeding processes are implemented, immunosuppression and cytokine disorders induced by heat stress have seriously endangered the safety of the pig industry[^1,2]. Tissieres et al. found that polyacrylamide gel electrophoresis can produce a new protein when the temperature is continuously increased, and subsequently demonstrated that the synthesis of this protein was associated with heat shock, this protein was thus named as heat shock protein[^3]. Heat shock proteins are categorized into families based on their molecular weights[^4] and primarily function as molecular chaperones for immature cells, abnormally folded proteins, mutated protein, or in cellular protection. As a member of the heat shock protein family, HSP70 responds to stress and shows the highest level of conservation in genetic evolution, is the most abundant HSP, is involved in the largest number of biological functions, and shows the greatest sensitivity to heat stress[^5]. Because of its specific structure, HSP70 is preferentially and highly expressed under heat stress. Although many studies have shown that HSP70 can act as an immunomodulatory agent by regulating cytokine production, experiments have suggested additional roles for HSP70[^6]. Therefore, further studies of the molecular pathways activated by HSP70 are needed.

The suppressor of cytokine signaling (SOCS) family contains signaling proteins that can negatively regulate inflammatory cytokines and includes eight members, SOCS1–7 and CIS. Structurally, members of the SOCS family have a central SH2 domain, SOCS box, and N-terminal domain[^7]. In addition to the common regions, SOCS1 and SOCS3 contain a kinase inhibition region in the N-terminal domain, which is involved in the precise regulation of these proteins and negative feedback adjustments[^8]. In terms of homology, CIS shows the highest similarity to SOCS2 and relatively low similarity to the other members. Among species, humans and rodents share the most similar homologues (over 99%). The structures of SOCS family members are important

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*Correspondence to: Xianghong Ju, Department of Veterinary Medicine, Guangdong Ocean University, Zhanjiang 524088, China. Email: zjoujrm@163.com; Rumin Jia, Department of Veterinary Medicine, Guangdong Ocean University, Zhanjiang 524088, China. Email: zjunajia@163.com

¹Shenzhen Institute of Guangdong Ocean University, Shenzhen 518018, China
²Department of Veterinary Medicine, Guangdong Ocean University, Zhanjiang 524088, China
³Department of Animal Science, Guangdong Ocean University, Zhanjiang 524088, China

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for their functions; the amino acids sequences at the N-terminus show different lengths, which impact SOCS function.

SOCS is a classical negative regulatory protein involved in the JAK/STAT signaling pathway. The expression of SOCS changes as the levels of inflammatory factors are increased. Inflammatory factors activate the JAK/STAT pathway, which mediates the expression of SOCS1, SOCS2, SOCS3, and CIS, following which the SOCS family negatively regulates JAK. However, these negative regulatory mechanisms differ, and SOCS1 specifically binds to JAK kinase to inhibit its kinase activity. When SOCS1 is overexpressed, it can inhibit the activity of STAT mediated by interferon (IFN) and interleukins (ILs). SOCS1 blocks JAKs through its SH2 domain and 24 amino acid residues to inhibit their N-terminal activity. JAKs are also inhibited by the unique structure of SOCS1, which can directly degrade the active pathway of JAKs. This structure is the previously mentioned kinase inhibitory region, which ultimately inhibits the JAK-STAT signal transduction pathway. SOCS1 also contains a site that binds to STAT to inhibit its activation. Various pathways are regulated by SOCS1, including the nuclear factor (NF)-κB signaling pathway, which is mediated by numerous cellular inflammatory factors. Studies have widely shown that SOCS1 inhibits the NF-κB signaling pathway by degrading p65/RelA, and thus p65 is an important factor affecting NF-κB activity. In the NF-κB pathway, researchers identified an important protein as the primary response gene for myeloid differentiation known as TIRAP. By binding to TIRAP, SOCS1 can inhibit myeloid differentiation factor (MyD88)-adaptor-like (Mal) activation to block the up-regulation of NF-κB activity. Therefore, among the NF-κB signaling pathways, the p65 and Mal proteins have been identified as markers of functional changes.

SOCS2 not only regulates the expression of other members of the family, but also causes changes in inflammatory factors. It also directly participates in various signaling pathways and regulates the expression of inflammatory factors such as IFN-γ, IL-6, and IL-12. In addition, SOCS2 affects the Th1/Th2 balance and exerts an anti-inflammatory effect by suppressing tumor necrosis factor-α (TNF-α) expression. During parasitic infection, SOCS2 is associated with the inflammatory response and neutrophil aggregation. Gernot-Posselt et al. demonstrated that in the dendritic cells of mice in which SOCS2 had been knocked out showed increased expression of the inflammatory factors IL-1 and IL-10; further, SOCS2 was found to negatively regulate Toll-like receptor (TLR)-mediated activation of dendritic cells. SOCS2 is preferentially expressed in regulatory T cells (Tregs), and the expression of IFN, IL-13, and IL-4 is increased when SOCS2 is knocked out in T cells. The Treg balance can be restored by blocking IL-4 in T cells by knocking out SOCS2. This suggests that SOCS2 plays an important role in balancing Tregs.

Our previous study showed that heat stress can significantly up-regulate the expression of TLR2/4 in pigs and significantly alter the expression of inflammatory factors such as IL-2, IL-12, IL-8, and IFN-γ. In addition, porcine SOCS3 but not SOCS4 negatively regulates HSP-induced NF-κB by degrading Mal in porcine cells. However, the mechanisms mediating the interaction between SOCS1/2 and HSP70 remain unclear. In this study, we investigated the mechanisms of SOCS1/2 regulation of HSP70-mediated NF-κB activity in CRL-2845 porcine macrophages.

2 Materials and methods

CRL-2845 porcine macrophages were obtained from the Collection of Cell Lines in the College of Veterinary Medicine, Guangdong Ocean University, China.

2.1 Construction of vectors expressing porcine SOCS1, SOCS2 and HSP70

Porcine SOCS1, SOCS2, and HSP70 sequences containing the corresponding full-length open-reading frame were ligated separately into the mammalian expression vector pcDNA3.1/V5-His-TOPO TA (Invitrogen, Carlsbad, CA, USA). The sequences of small interfering RNAs were as follows:

**SOCS1**: (forward) 5'-ACACAACCAGGUUGGCAGCGACAAG-3',
(reverse) 5'-AUUGUCGGCUGCCACCUGGUUGU-3'.

**SOCS2**: (forward) 5'-AGUUCGCUCUUGCCACCUGGUUGU-3',
(reverse) 5'-UUAGUAGUGUAGUCUGAGUGCGA-3'.

**HSP70**: (forward) 5'-GGGCUUUUGGUGGUGCCAUUU-3',
(reverse) 5'-UUAGUAGUAGUAGUGUAGGCGA-3'.

Precipitation experiments to evaluate knockdown efficiency showed that both small interfering RNAs reduced expression by approximately 90%. Plasmid DNAs were prepared with an endotoxin-free Plasmid Maxi Kit (Qiagen, Hilden, Germany). CRL-2845 porcine macrophages were transfected with plasmid DNAs using Lipofectamine-3000 (Invitrogen).

2.2 Detection of genes by qRT-PCR

Total RNAs were isolated from cells using the RNAiso Plus kit (TaKaRa, Shiga, Japan). RNAs were reverse-transcribed into cDNAs using QUANTITECT (Qiagen).
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Gene detection was performed using a SYBR green polymerase mix (TaKaRa). The primers used for quantitative transcription polymerase chain reaction (qRT-PCR) were designed with Primer3 software (http://www.broad.mit.edu/cgi-in/primer/primer3-www.cgi). Primer pairs (Table 1) were selected for their specificity based on dissociation curves. For assay validation, the purified products were cloned into pMD19-T (TaKaRa) and sequenced to verify the correct target amplification. PCR products were amplified using a LightCycler 480 (Roche, Basel, Switzerland) (95°C, 15 s; 61°C, 15 s; 72°C, 15 s, 30 cycles). The efficiency of PCR amplification was greater than 97% for each primer set using six serial dilutions of cloned products as templates. The data are presented as the means ± standard deviations. Data normalization was carried out based on expression of the β-actin gene, and quantification was carried out using the delta Ct method.

2.3 Luciferase reporter assay

Cells were seeded into 96-well plates at 4 × 10⁴ cells/well in complete Dulbecco’s modified Eagle’s medium (Hyclone, Logan, UT, USA). Cells were transfected with plasmid DNAs after 24 h using Lipofectamine-3000 (Invitrogen) according to the manufacturer’s instructions [24]. Briefly, cells in each well were transfected with the following plasmids: 60 ng pNF-κB (expressing firefly luciferase under control of NF-κB; Invitrogen) and 0.2 ng PRL-YK (expressing Renilla luciferase constitutively via a β-actin promoter and served to normalize transfection variables; Invitrogen). Twenty-four hours post-transfection, the cells were stimulated with ligands TNF-α (PeproTech, Rocky Hill, NJ, USA) for 6 h. The cells were washed with phosphate-buffered saline and lysed in passive lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase values were normalized by dividing the light output of firefly luciferase by that of Renilla luciferase. The experiments were repeated three times.

2.4 Protein extraction

Cells (2 × 10⁶) were rinsed twice with cold phosphate-buffered saline, and cytoplasmic proteins were extracted using M-TERTM mammalian protein extraction reagent containing a protease inhibitor mixture (Roche). Protein concentrations were measured using a BCA-200 protein assay kit (Pierce, Rockford, IL, USA).

2.5 Western immunoblotting

Immunoblotting was performed as described previously [25]. Briefly, proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose membranes. After blocking in 25 mM Tris-HCl (pH 7.5), 1.25 mM NaCl, and 0.1% Tween 20 with 5% fat-free milk, the blots were incubated with primary antibodies for 2 h and then with horseradish-peroxidase-conjugated secondary antibodies for 1 h. Rabbit anti-human IκBα polyclonal antibodies and rabbit anti-human phospho-IκBα polyclonal antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and rabbit anti-pig Mal monoclonal antibodies and rabbit-anti-β-actin IgG

| Gene       | Sequence                                      | Reference | Amplicon | Length (bp) | Tm (℃) |
|------------|-----------------------------------------------|-----------|----------|-------------|--------|
| SOCS1      | 5'-AGGATGGTAGCACACAAC-3'                     | NM_001204768.1 | 119      | 62          |
|            | 5'-GAGGAGGAGGATGAAGATG-3'                    |           |          |             |        |
| SOCS2      | 5'-AGATGTAAGCACACAAC-3'                      | NM_001097461.1 | 155      | 60          |
| HSP70      | 5'-GCCCTGAATCCCGAGAATT-3'                    | NM_001123127.1 | 152      | 58          |
|            | 5'-TCCCCACAGTAGAAAACG-3'                     |           |          |             |        |
| SOCS1-ORF  | 5'-ATGTTAACACACAACTTGGTGACCC-3'              | NM_001204768.1 | 119      | 62          |
|            | 5'-TCATATCTGGAAGGGAAAGGACGTCCAGA-3'         |           |          |             |        |
| SOCS2-ORF  | 5'-ATGGCCCTGCGGTGGCCCCTCC-3'                 | NM_001097461.1 | 597      | 53          |
| HSP70-ORF  | 5'-ATGGCCGCTGCAAGAGAAGTGGCCATAG-3'          | NM_001123127.1 | 1933     | 55          |
|            | 5'-GGCCCTGCCATATTGGAGGATGTTGATTA-3'         |           |          |             |        |
| β-actin    | 5'-CGGCGACCCAGCCTGAGTTG-3'                   | DQ845171.1 | 119      | 64.2        |

Note: bp: base pairs; Tm: melting temperature

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were purchased from Proteintech (Rosemont, IL, USA). Peroxidase-conjugated AffiniPure Goat anti-Rabbit IgG antigen/antibody complexes on the membranes were detected using a SuperSignal Ultra chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein levels were quantified by densitometry analysis and are expressed in OD units of target protein relative to β-actin.

2.6 Statistical analysis

Data are presented as the means ± standard errors. Statistical significance was determined by analysis of variance, followed by Student’s t-tests. Differences with \( p \) values of less than 0.05 were considered as significant.

3 Results

3.1 Ectopic expression of porcine SOCS1, SOCS2 and HSP70

To construct expression vectors for SOCS1, SOCS2, and HSP70, we used a PCR technique to isolate cDNA fragments of these genes (Figure 1(a, b)) from the porcine spleen and inserted the cDNA fragments into the PMD18-T vector (TaKaRa). Porcine SOCS1 (pSOCS1) and SOCS2 (pSOCS2) cDNA gene fragments were then ligated into the pcDNA3.1/V5-His-TOPO vector with a His-tag at the C-terminus, yielding pcDNA3.1/V5-SOCS1-His and -SOC2-His.

To establish a co-transfection model system, DNA plasmids of pSOCS1, pSOCS2, and pHSP70 and the NF-κB reporter system (pNF-κB and pRL-YK) were co-transfected into IPEC-J2 cells. An NF-κB reporter luciferase assays showed that ectopic expression of SOCS1 or SOCS2 resulted in suppression of pHSP70-induced NF-κB gene expression in CRL-2845 porcine macrophages (\( p < 0.01 \), Figure 2(b, c)). Expression of SOCS1 or SOCS2 also reduced the levels of HSP70-induced phosphorylated IκBα (\( p < 0.01 \); Figure 2(e, f, h, i)). These results indicate that SOCS1 and SOCS2 negatively regulated the activity of NF-κB induced by HSP70 overexpression in CRL-2845 porcine macrophages.

3.2 HSP70 overexpression upregulated the activity of NF-κB in cultured cells

To determine the effects of HSP70 on NF-κB activity, plasmids encoding pHSP70 and the NF-κB reporter system (pNF-κB and pRL-YK) were transiently co-transfected into CRL-2845 porcine macrophages. Stimulation of cells with TNF-α (50 ng/mL) resulted in increased activity of NF-κB in treated cells but not in control cells (Figure 2(a, c)). Immunoblotting showed that the expression of IκBα (an inhibitor of NF-κB) was lower in pHSP70-transfected cells and that the levels of phosphorylated IκBα were higher than those in controls (Figure 2(d, f, g, i)). These results indicate that the activity of NF-κB was induced in HSP70-overexpressing cells.

3.3 Both SOCS1 and SOCS2 negatively regulated HSP70 induced NF-κB activity

To evaluate the interactions of SOCS1, HSP70, and NF-κB, the pSOCS1, pHSP70, and NF-κB reporter genes (pNF-κB and pRL-YK) were co-transfected into IPEC-J2 cells. An NF-κB reporter luciferase assays showed that ectopic expression of SOCS1 or SOCS2 resulted in suppression of pHSP70-induced NF-κB gene expression in CRL-2845 porcine macrophages (\( p < 0.01 \), Figure 2(b, c)). Expression of SOCS1 or SOCS2 also reduced the levels of HSP70-induced phosphorylated IκBα (\( p < 0.01 \); Figure 2(e, f, h, i)). These results indicate that SOCS1 and SOCS2 negatively regulated the activity of NF-κB induced by HSP70 overexpression in CRL-2845 porcine macrophages.

3.4 Knockdown of SOCS1 or SOCS2 upregulated NF-κB in HSP70-expressing cells

We also used specific small interfering RNAs (siRNAs) to knock-down SOCS1 and SOCS2. After co-transfection, NF-κB activity was increased when SOCS1 or SOCS2 expression was reduced in CRL-2845 porcine macrophages (Figure 3(a, b)). The protein levels of IκBα were also reduced by knocking down SOCS1 (Figure 3(c, e)) or SOCS2 (Figure 3(d, f)); however, phospho-IκBα levels were increased significantly compared to in controls. These results further indicate that SOCS1 and SOCS2 negatively regulate NF-κB in HSP70-expressing cells.

3.5 Both SOCS1 and SOCS2 decreased the expression of Mal protein induced by HSP70

To further evaluate the mechanisms by which pSOCS1 and pSOCS2 regulate HSP70-induced NF-κB activity, we studied the Mal protein, an upstream modulator of the NF-κB pathway. Ectopic expression of HSP70 resulted in elevation of Mal protein in CRL-2845 porcine macrophages (Figure 4(a,b,c)). Co-expression of SOCS1 or SOCS2 with HSP70 blocked the activity of Mal (Figure 4(a,b,c)). Knockdown of SOCS1 and SOCS2 with siRNA restored the HSP70-induced elevation in Mal (Figure 4(d,e,f,g,h,i)). The results showed that both SOCS1 and SOCS2 controlled the activity of NF-κB induced by HSP70 by degrading Mal protein.
4 Discussion

NF-κB is a key nucleoprotein factor that regulates inflammatory factors and is commonly found in various tissues and cells. Activation of NF-κB induces the expression of various inflammatory factors such as IL-1β, IL-2, and IL-8 [26] which participate in the immune process and inflammatory response. As a molecular chaperone, the expression level of HSP70 increases with increasing ambient temperature [27]. In heat-stressed pigs, HSP70 can remain in the plasma for a long time and is extremely sensitive; thus, HSP70 levels reflect the degree of heat stress, suggesting that the HSP70 content can be used as a biomarker of the heat stress response [28]. Studies of the effects of HSP70 on the NF-κB pathway have shown inconsistent results. In some studies on the effect of HSP70 on the NF-κB pathway, HSP70 was found to activate NF-κB [29]. However, other studies suggested that HSP70-overexpression can inhibit NF-κB activity [30]. In the present study, co-transfection of both cells showed that high expression of HSP70 significantly up-regulated NF-κB activity, which was amplified by exposure to TNF-α (50 ng/mL). These results indicate that the effect of HSP70 on NF-κB activity may be specific for the transfected cells. However, the underlying specific mechanism requires further analysis.

SOCS protein is an important inhibitor of cytokine signaling pathways [31] and is involved in regulating various pathways such as JAK/STAT and NF-κB [32,33]. SOCS1 is a ubiquitin ligase of NF-κB subunit p65. When SOCS1 is expressed at high levels, the p65 protein level is significantly decreased and ubiquitination occurs, thereby negatively regulating NF-κB activation [34]. NF-κB is an important nuclear transcription factor that plays an important role in immune defense function [35–38]. It has been reported that heat stress can cause phosphorylation of IκB through a series of signal transductions [39], which in turn activates the NF-κB signaling pathway [40]. Additionally, Amaral et al. found that cooling of heat-stressed cows significantly reduced the expression of SOCS1 mRNA in lymphocytes, indicating that SOCS1 is closely related to temperature [41]. Our previous studies also confirmed that SOCS1 shows a significant heat stress response. In the present study, when SOCS1 inhibited HSP70-induced NF-κB activation, the expression level of IκBα protein was significantly up-regulated, while the expression level of p-IκBα protein was significantly down-regulated. This indicates that SOCS1 can inhibit the phosphorylation of IκBα during HSP70-induced NF-κB activation. Knockout of the SOCS1 gene eliminated these effects. This further demonstrates that...
SOCS1 negatively regulates HSP70-induced NF-κB activation, which is consistent with the results of previous studies.

As a major member of the SOCS family, SOCS2 can reduce the release of inflammatory factors such as IL-6 and induce protease-dependent degradation of TRAF6 to control innate immune responses and exert anti-inflammatory effects\(^{42,43}\). Reportedly, SOCS2 can regulate the activity of NF-κB\(^{44}\). In macrophages, SOCS2 negatively regulates the expression of many inflammatory factors, such as IL-1 and IL-6\(^{45}\). Because of the similar molecular structures and functions of SOCS1 and SOCS2, our previous studies showed that SOCS1 can significantly inhibit HSP70-induced upregulation of NF-κB activity in macrophages through a mechanism involving phosphorylation degradation of IκBα. In the present study, when SOCS2 inhibited the induction of NF-κB by HSP70, the expression level of IκBα protein was significantly up-regulated, while p-IκBα protein was significantly down-regulated. This indicates that SOCS2 can inhibit the phosphorylation of IκBα during activation of NF-κB by HSP70. These effects were eliminated by interference with SOCS2 gene expression. This further demonstrates that SOCS2 negatively regulates HSP70-induced NF-κB activation.

The TLR is an important pattern recognition receptor in the body. When combined with the pathogen-associated molecular pattern of a microorganism, TLR dimerizes, and the adaptor protein MyD88 or Mal is then recruited, which induces a phosphorylation cascade and activates NF-κB. After activation, NF-κB enters the nucleus to activate the target gene and induce the expression of inflammatory factors\(^{46}\). As a key molecule in the NF-κB signaling pathway, activation of Mal plays an important role in the transmission of TLRs signaling\(^{47}\). Its function is not only positively regulated by TLR activation, but also negatively inhibited by SOCS family molecules\(^{48,49}\). Mansell et al. used gene transfection technology to show that high expression of the SOCS1 gene in cells caused degradation of Mal protein through the ubiquitination pathway. The mechanism involved a tyrosine phosphorylation interaction between SOCS1 and Mal, which eventually led to the degradation of Mal\(^{13}\). In mouse macrophages in which Mal protein was knocked out, SOCS2 mRNA expression was lower than that in the control group\(^{50}\).

In the present study, when HSP70 activated NF-κB activity, the expression of Mal protein was significantly increased. When the activation of NF-κB by HSP70 was inhibited by SOCS1, the expression level of Mal protein was significantly down-regulated. Therefore, SOCS1 can inhibit the expression of Mal protein activated by HSP70. Additionally, in CRL-2845 porcine macrophages after silencing of the SOCS1 gene, the expression of Mal protein was significantly increased by excessive HSP70 expression. In the current study, when HSP70 activated NF-κB activity, the expression of Mal protein was significantly increased. When NF-κB activation by HSP70 was inhibited by SOCS2, the expression level of Mal protein was significantly down-regulated.
Figure 3. SOCSs knockdown abolished NF-κB activity in HSP70-expressing cells. Cells were transfected with the NF-κB reporter system (pNF-κB-Luc and pRL-YK) and si socs1 + socs1 + hsp70 (a) or si socs2 + socs2 + hsp70 (b), and the activation of NF-κB was measured using dual luciferase reporter assays. The levels of IκBα and phosphor-IκBα were detected by western blotting in CRL-2845 porcine macrophages transfected with siSOCS1 + SOCS1 + HSP70 (c, e), and siSOCS2 + SOCS2 + HSP70 (d, f). Data in (e, f) are from one representative experiment with three technical replicates (means ± standard deviations) and were statistically analyzed by one-way ANOVA. ** and *** p ≤ 0.01. All experiments were repeated three times.

Figure 4. SOCSs decreased the expression of Mal protein induced by HSP70. Change in the expression of Mal protein in HSP-overexpression cells and cells with SOCSs co-transfection (a-i). Data in (g-i) are from one representative experiment with three technical replicates (means ± standard deviations) and were statistically analyzed by one-way ANOVA. ** and *** p ≤ 0.01. All experiments were repeated three times.
This indicates that SOCS2 can inhibit the expression of Mal protein activated by HSP70. After silencing of the SOCS2 gene, Mal protein expression was significantly increased under the induction of excessive HSP70 expression. Thus, SOCS1 and SOCS2 negatively regulate HSP70-induced NF-κB activation by degrading Mal protein under heat stress conditions.

Competing interests

The authors declare that they do not have any competing or financial interests.

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