Hsp27 Regulates Pro-Inflammatory Mediator Release in Keratinocytes by Modulating NF-κB Signaling

Runa Sur[1], Peter A. Lyte[1] and Michael D. Southall[1]

Heat-shock protein 27 (Hsp27) is a member of the small Hsp family that functions as molecular chaperones and protects cells against environmental stress. Hsp27 is expressed in the upper epidermal layers of normal human skin and has been reported to play a role in keratinocyte differentiation and apoptosis. In this investigation, we show an additional role of Hsp27 in the regulation of inflammatory pathways in keratinocytes. Downregulation of Hsp27 using Hsp27-specific small interfering RNA increased prostaglandin E_2 (PGE_2) production in both unstimulated and tumor necrosis factor-α (TNF-α)-stimulated keratinocytes. Moreover, downregulation of Hsp27 increased the release of the pro-inflammatory cytokine IL-8 from TNF-α-stimulated and UV-irradiated keratinocytes, and this increase was inhibited by pretreatment with the NF-κB inhibitor BAY11-7082. Further studies showed that downregulation of Hsp27 resulted in induction of NF-κB reporter activity in keratinocytes. This correlated with enhanced degradation of IκB-α protein and accumulation of phosphorylated IκB-α in Hsp27 knockdown cells. Moreover, Hsp27 associated with the IκB kinase (IKK) complex. As synthesis of the pro-inflammatory cytokine IL-8 and the prostanoid PGE_2 are regulated by NF-κB, this could be a probable mechanism by which Hsp27 modulates the production of these inflammatory cytokines. Thus, Hsp27 plays a protective role in regulating inflammatory responses in skin.

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INTRODUCTION

Environmental stress results in the preferential synthesis and accumulation of a conserved family of proteins in cells referred to as heat-shock proteins (Hsp) (Hightower, 1991). These proteins function as molecular chaperones maintaining protein solubility, cytoskeletal function and cellular homeostasis, and protect cells against oxidative stress and pathophysiological injuries (Hartl, 1996; Welsh and Gaestel, 1998; Beere, 2001).

Hsp27 is a member of the small Hsp family expressed in different adult cell types such as breast, uterus, cervix, placenta, skin, and platelets (Ciocca *et al.*, 1993). In addition to its protective function in heat shock and other conditions of stress, Hsp27 has been linked to different signaling pathways regulating critical cellular functions such as development (Michaud *et al.*, 1997; Jantschitsch *et al.*, 1998), apoptosis (Mehlen *et al.*, 1996; Gabai and Sherman, 2002), differentiation, and cell growth (Shakoori *et al.*, 1992; Kindas-Mugge and Trautinger, 1994; Spector *et al.*, 1995; Kindas-Mugge *et al.*, 1996). Furthermore, Hsp27 has been identified as an actin-associated protein that functions as a regulator of actin polymerization (Welsh and Gaestel, 1998). In skin, Hsp27 is expressed in normal human keratinocytes and has been shown to be related to keratinocyte differentiation (Trautinger *et al.*, 1993, 1995; Kindas-Mugge and Trautinger, 1994).

Hsp27 expression has also been shown to be induced during inflammation, although the role in inflammation is unclear. Inflammation is triggered by diverse factors that include physical agents (burns, UV radiation, trauma), chemical agents (toxins, heavy metals, and reactive oxygen species), infectious agents (bacteria, viruses, and parasites), and some immunological agents (antigens), those particularly involved in allergy and autoimmunity. Reactive oxygen species production, which is associated with inflammatory phagocytosis, has been shown to result in heat-shock factor (HSF) activation and subsequent induction of Hsp expression (Jacquier-Sarlin and Polla, 1996). p38 MAP kinase activates Hsp27 via MAPKAP kinase-2, and this pathway has been shown to be important for CXCL 12 and complement factor 5a (C5a) triggered cell migration that is critical for many processes including inflammation (Rousseau *et al.*, 2006). Heat-shock treatment and subsequent induction of Hsp27 expression and phosphorylation has been shown to protect against angiotensin II-induced hypertension and heart inflammation (Chen *et al.*, 2004a) by suppressing angiotensin II-induced activation of the transcription factor NF-κB (Chen *et al.*, 2004b). In contrast, Hsp27 has been shown to be required for IL-1-induced expression of the pro-inflammatory mediators, cyclooxygenase-2 (COX-2), IL-6, and IL-8 in HeLa

[1]*Preclinical Pharmacology, Johnson and Johnson Skin Research Center, CPPW, a Unit of Johnson & Johnson Consumer Companies Inc., Skillman, New Jersey, USA*

Correspondence: Dr Michael D. Southall, Preclinical Pharmacology, Johnson & Johnson, 199 Grandview Road, Skillman, New Jersey 08558, USA. E-mail: msoutha@cpcus.jnj.com

Abbreviations: Hsp27, heat-shock protein 27; IκB-α, inhibitory κB-α; IKK, inhibitory κB-α kinase; PGE_2, prostaglandin E_2; siRNA, small interfering RNA; TNFα, tumor necrosis factor-α

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cells; thus, there is conflicting evidence on the role of Hsp27 in inflammation (Alford *et al.*, 2007). In keratinocytes, Hsp27 is induced upon UV irradiation and has been linked to the prevention of apoptosis, a contributing factor to the well-documented thickening of UVB-irradiated epidermis responsible for protecting stem cells in the stratum basale (Becker *et al.*, 2001). The inflammatory response often triggers a number of protective mechanisms associated with resolving and controlling inflammation, and recent evidence suggest that the production of Hsp may be involved with the regulation of inflammatory pathways (Polla *et al.*, 1998).

As Hsp27 is induced in keratinocytes during environmental stress, we therefore sought to determine the role of Hsp27 in keratinocyte inflammatory responses. In our studies, we show, for the first time, that Hsp27 regulates tumor necrosis factor-α (TNF-α)-induced pro-inflammatory mediators prostaglandin E_2 (PGE_2) and IL-8 production in keratinocytes. Hsp27 was also found to regulate the UV-induced release of the pro-inflammatory cytokines IL-8 and IL-1α from keratinocytes. Downregulation of the upstream p38 MAP kinase did not increase pro-inflammatory mediator production, suggesting that p38 MAP kinase-mediated activation of Hsp27 is not required for this effect. The synthesis of multiple inflammatory cytokines including PGE_2, IL-8, and IL-1α are regulated by the transcription factor NF-κB. We found that pretreatment of keratinocyte cells with the NF-κB inhibitor BAY11-7082 suppressed the potentiation of both TNF-α-independent and -dependent IL-8 production mediated by downregulation of Hsp27, suggesting the involvement of NF-κB signaling in these responses. Small interfering RNA (siRNA)-mediated downregulation of Hsp27 resulted in an induction of NF-κB reporter activity in keratinocytes, which was correlated with enhanced degradation of IκB-α protein and accumulation of phosphorylated IκB-α in Hsp27 downregulated cells. Moreover, Hsp27 associated with IκB kinase (IKK) complex without affecting phosphorylation, but interfering with the phosphorylation and subsequent degradation of the downstream IκB-α, thus decreasing NF-κB signaling and subsequent pro-inflammatory cytokine transcription in these cells. Taken together, these results demonstrate that Hsp27 plays a protective role in keratinocytes by regulating the production of NF-κB-dependent pro-inflammatory mediators.

RESULTS

Hsp27 regulates pro-inflammatory mediator production in keratinocytes

To elucidate the functional role of Hsp27 in keratinocyte inflammation, we investigated the role of Hsp27 on TNF-α-induced PGE_2 production. To this end, we knocked down Hsp27 in HaCaT keratinocytes by transfecting with Hsp27-specific siRNA for 72 hours. The level of total Hsp27 was determined by western blotting of whole cell extracts. In Hsp27 siRNA-transfected cells, Hsp27 expression was downregulated by about 80% compared to control siRNA (siCON)-transfected cells (Figure 1a). The same blot was reblotted with extracellular signal-regulated kinase-2 antibody to show equal protein loading. Hsp27 depletion had no effect on cell

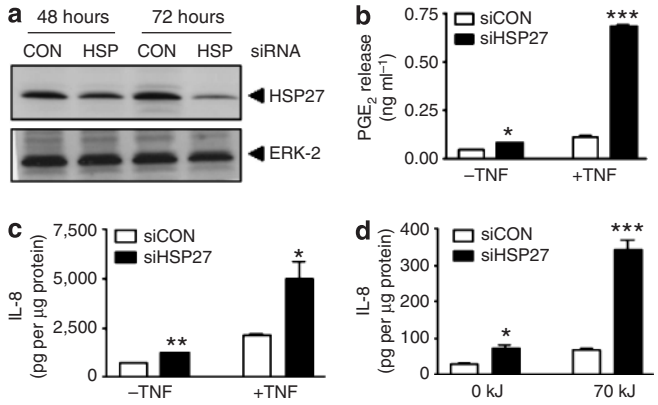

Figure 1. Hsp27 regulates pro-inflammatory mediator production in keratinocytes. (**a**) HaCaT keratinocytes were transfected with siCON (100 nM) or Hsp27 siRNA (100 nM) for 72 hours. Whole cell extracts (20 µg protein) were subjected to western blotting and probed with Hsp27 antibody to visualize protein knockdown. The same blot was re-probed with extracellular signal-regulated kinase-2 antibody to confirm equal protein loading and siRNA specificity. (**b**) HaCaT keratinocytes were transfected with siCON or Hsp27 siRNA for 48 hours, after which the cells were either left untreated or treated with 100 ng ml^{-1} TNF-α for 24 hours. Supernatants were assessed for PGE_2 levels by ELISA. *$P<0.05$ compared with untreated siCON-transfected keratinocytes. ***$P<0.001$ compared with siCON-transfected keratinocytes treated with TNF-α. (**c**) Human primary epidermal keratinocyte cells were either transfected with siCON (100 nM) or Hsp27 siRNA (100 nM) for 48 hours after which the cells were either left untreated or treated with 100 ng ml^{-1} TNF-α. After 24 hours, supernatants were assessed for IL-8 levels by ELISA. **$P<0.01$ compared with untreated siCON transfected keratinocytes. *$P<0.05$ compared with siCON transfected keratinocytes treated with TNF-α. (**d**) Human primary epidermal keratinocyte cells were either transfected with siCON (100 nM) or Hsp27 siRNA (100 nM) for 48 hours, after which the cells were either left unexposed (0 kJ) or exposed to 70 kJ UV radiation. After 24 hours, supernatants were assessed for IL-8 levels by ELISA. *$P<0.05$ compared with unexposed siCON-transfected keratinocytes. ***$P<0.001$ compared with siCON-transfected keratinocytes treated with 70 kJ UV radiation. Results represent mean ± SD from at least three different experiments.

viability as measured by lactose dehydrogenase release from cells and on cell proliferation, as measured by alamar blue uptake by proliferating cells (data not shown). Next, we assessed TNF-α-induced PGE_2 release from either siCON or Hsp27 siRNA-transfected cells. In siCON-transfected cells TNF-α increased PGE_2 release by approximately seven-fold from 0.015 ± 0.001 to 0.116 ± 0.004 ng ml^{-1} and in Hsp27 knockdown cells, TNF-α-induced PGE_2 levels were found to be potentiated by approximately 34-fold from 0.026 ± 0.003 to 0.692 ± 0.007 ng ml^{-1}, indicating that Hsp27 regulates TNF-α-induced PGE_2 production in keratinocytes (Figure 1b). Interestingly, the approximately two-fold greater basal cellular levels of PGE_2 in Hsp 27 knockdown keratinocytes were significantly ($P<0.05$) higher than siCON-transfected cells.

Similarly, in TNF-α-stimulated primary keratinocytes, Hsp27 knockdown showed a 2.35-fold potentiation in IL-8 production, increasing IL-8 release from 2,125.19 ± 115.02 to 5,008.07 ± 1,473.40 pg ml^{-1} (Figure 1c). In unstimulated cells, Hsp27 knockdown also increased basal IL-8 production by 1.7-fold, which was significant (**$P<0.01$) compared with unstimulated

siCON-transfected keratinocytes. Next, to determine whether the Hsp27 effect was stimuli specific, we assessed whether Hsp27 regulated UV-induced release of the cytokines IL-8 and IL-1α from keratinocytes. Primary human keratinocyte cells were transfected with siCON or Hsp27 siRNA followed by UV irradiation. Results showed that UV irradiation induced an increased production of both IL-8 (Figure 1d) and IL-1α (data not shown) from siCON-transfected cells between 0 kJ/m^2(kJ) control and 70 kJ/m^2(kJ) UV radiation from a solar simulator. Consistent with the TNF-α-induced responses, there was a potentiation of UV-induced IL-8 and IL-1α release in Hsp27 downregulated keratinocytes compared to siCON-transfected cells. In the presence of 70 kJ UV irradiation, IL-8 increased five-fold from 68.02 ± 4.76 pg ml^{-1} in siCON-transfected cells to 342.79 ± 49.29 pg ml^{-1} in Hsp27-transfected cells (Figure 1d), and similarly IL-1α increased 1.7-fold with knockdown of Hsp27 (data not shown). Even under untreated conditions (0 kJ), Hsp27 downregulation showed a three-fold higher IL-8 level in cells (Figure 1d).

Regulation of mediator production by Hsp27 is independent of Hsp27 activation

The p38 MAP kinase phosphorylates Hsp27 upon activation via MAPKAP kinase-2, thereby regulating Hsp27 activity (Wong *et al.*, 2000; Garmyn *et al.*, 2001). To investigate whether the TNF-α-induced PGE_2 production in keratinocytes required the p38-dependent phosphorylation of Hsp27, we looked at this effect in the absence of the upstream p38 MAP kinase by knocking down p38 MAP kinase with p38-specific siRNA for 72 hours. The level of total p38 MAP kinase was determined by western blotting of whole-cell extracts. In p38 siRNA-transfected cells, p38 expression was downregulated by about 80% compared to siCON-transfected cells (Figure 2a). The same blot was reblotted with extracellular signal-regulated kinase-2 and Hsp27 antibodies to show equal protein loading. Next, we assessed TNF-α-induced PGE_2 release in the supernatants of siCON and p38 siRNA-transfected cells. TNF-α treatment of siCON-transfected cells increased PGE_2 release by four-fold from 0.087 ± 0.012 to 0.397 ± 0.128 ng ml^{-1}. Interestingly, we found that in p38 siRNA-transfected cells there was a four-fold decrease of TNF-α-induced PGE_2 production from 0.397 ± 0.128 to 0.095 ± 0.016 ng ml^{-1}, which was opposite to the effects of Hsp27 knockdown (Figure 2b), suggesting that activation of Hsp27 was not required for the anti-inflammatory effect.

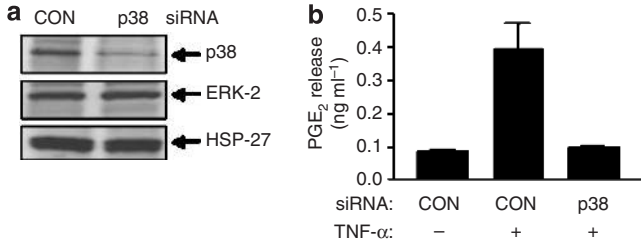

Figure 2. Hsp27 acts independently of p38 MAP kinase in regulation of PGE_2 production in keratinocytes. (**a**) HaCaT keratinocytes were transfected with siCON (100 nM) or p38 MAPK siRNA (20 nM) for 72 hours. Whole-cell extracts (20 μg protein) were subjected to western blotting and probed with p38 antibody to visualize p38 knockdown. The same blot was re-probed with extracellular signal-regulated kinase-2 and Hsp27 antibodies to confirm equal protein loading and siRNA specificity. (**b**) HaCaT keratinocytes were transfected with siCON or p38 MAPK siRNA for 48 hours, after which the cells were either left untreated or treated with 100 ng ml^{-1} TNF-α for another 24 hours. Supernatants were assessed for PGE_2 levels by ELISA. Results represent mean ± SD from at least three different experiments.

Hsp27 regulates NF-κB signaling in keratinocytes

Since the transcription factor NF-κB regulates the release of multiple pro-inflammatory mediators including IL-8, IL-1α, and PGE_2 and as Hsp27 has been reported to associate with the IKK complex and downregulate NF-κB signaling in cells (Park *et al.*, 2003; Kammanadiminti and Chadee, 2006), we hypothesized that Hsp27 was regulating cytokine production in keratinocytes by modulating NF-κB signaling. To this end, we looked at basal, TNF-α-dependent and UV-dependent IL-8 production in Hsp27 knockdown keratinocytes in the presence of the NF-κB inhibitor BAY11-7082. Interestingly, pretreatment of keratinocyte cells with BAY11-7082 suppressed the increase of basal and both TNF-α- and UV-dependent IL-8 production mediated by downregulation of Hsp27, suggesting the involvement of NF-κB signaling in these responses (Figure 3a and b).

To assess the role of Hsp27 on NF-κB signaling, we first examined the TNF-α-independent and -dependent NF-κB transcriptional activity in the presence and absence of Hsp27. Unstimulated NF-κB luciferase activity increased from 18.81 ± 1.97% in control cells to 29.89 ± 1.08% in Hsp27

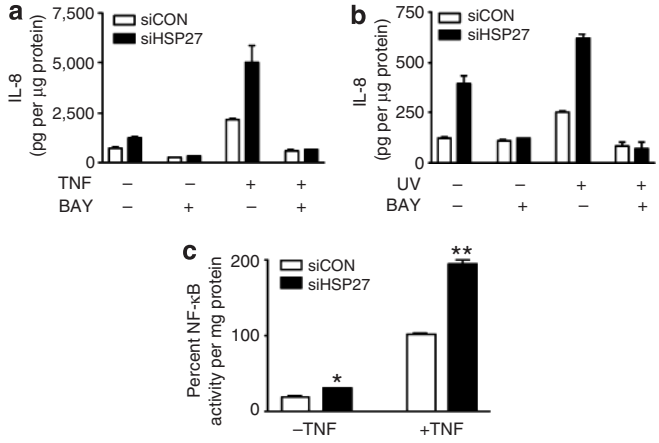

Figure 3. Hsp27 regulates NF-κB activity in keratinocytes. Human primary epidermal keratinocyte cells were either transfected with siCON (100 nM) or Hsp27 siRNA (100 nM) for 48 hours, after which the cells were either left untreated or treated with 100 ng ml^{-1} TNF-α (**a**) or 70 kJ UV radiation (**b**) following 30 minutes pretreatment with the NF-κB inhibitor BAY11-7082. After 24 hours, supernatants were assessed for IL-8 levels by ELISA. (**c**) Human primary adult epidermal keratinocytes were co-transfected with pNF-κB-Luc reporter plasmid and Hsp27 siRNA or siCON. Cells were either left untreated or treated with 100 ng ml^{-1} TNF-α for 24 hours. Luciferase assay was performed as described in the Materials and Methods section and the values were normalized to total protein in each well. Results represent mean ± SD from three different experiments. *P<0.05 compared with untreated siCON-transfected keratinocytes. **P<0.01 compared with siCON-transfected keratinocytes treated with TNF-α.

downregulated cells, a 1.6-fold increase (Figure 3c). Similarly, TNF-α-stimulated NF-κB luciferase activity increased from 101.98 ± 2.80% in control cells to 195.65 ± 5.80% in Hsp27 downregulated cells, a two-fold increase (Figure 3c), suggesting that Hsp27 regulates both TNF-α-independent and -dependent NF-κB transcriptional activity in keratinocytes. As a control, cells were transfected with a negative control plasmid (pCIS-CK) with siCON and Hsp27 siRNA and these showed very low luciferase activity (data not shown).

To mechanistically determine how Hsp27 modulates NF-κB transcriptional activity, we followed IκB-α degradation in siCON and Hsp27 siRNA (siHSP27) transfected keratinocytes. In control cells, there was 10 and 30% greater IκB-α at 15 and 30 minutes post-TNF-α stimulation, respectively, compared to Hsp27 downregulated cells (Figure 4a). This result coincided well with decreased phosphorylated IκB-α levels in control cells compared to Hsp27 downregulated cells 15 and 30 minutes post-TNF-α stimulation (Figure 4b). The blots were probed with tubulin antibody to account for equal protein loading (Figure 4a and b). This suggests that Hsp27 regulates degradation of IκB-α in keratinocytes and thus there is greater NF-κB activity in the absence of Hsp27 correlating with greater NF-κB luciferase activity in Hsp27 siRNA-transfected cells (Figure 3b). Next, we looked at whether Hsp27 regulates the phosphorylation of IKK that activates IκB-α. There was no difference in TNF-α-induced IKK phosphorylation observed between control cells and Hsp27 downregulated cells (Figure 5a). The same blot was blotted with the IKK antibody to account for equal protein in each well (Figure 5a). However, immunoprecipitation studies using Hsp27 antibodies showed that Hsp27 associates with IKK in keratinocytes (Figure 5b). Lysates were incubated with rabbit serum as control, which confirmed that the co-immunoprecipitation of IKK was not nonspecific. These results suggest that in keratinocyte cells, Hsp27 associates with IKK without affecting its activation but functions to prevent the phosphorylation and subsequent degradation of the downstream IκB-α, thus modulating NF-κB signaling.

DISCUSSION

Recent studies have suggested that Hsp27 is produced in skin during periods of inflammation and may protect cells against oxidative stress; however, the functional role of Hsp27 in inflammation remains unknown. Hsp27 has been reported to function in IL-1-induced cell signaling and pro-inflammatory gene expression in human dermal fibroblasts and HeLa cells (Alford *et al.*, 2007). In particular, downregulation of Hsp27 using RNAi in these cells augmented the IL-1-induced expression of the pro-inflammatory cytokines COX-2, IL-6, and IL-8 (Alford *et al.*, 2007). In this investigation, we have shown, for the first time, that Hsp27 regulates unstimulated and TNF-α-stimulated PGE_2 and IL-8 production in keratinocytes. Downregulation of Hsp27, using gene-specific siRNA, potentiated PGE_2 and IL-8 production in these cells (Figure 1b and c). This effect is opposite of what was observed in human dermal fibroblasts where deletion of Hsp27 decreased the release of pro-inflammatory cytokines, suggesting that Hsp27 exerts different effects in different cell types.

TNF-α-induced production of PGE_2 is regulated by COX-2 (Robertson, 1998), and COX-2 expression is stabilized by p38 MAP kinase signaling (Grewe *et al.*, 1993) in keratinocytes. Additionally, p38 MAP kinase lies upstream to Hsp27 and activates it via phosphorylation (Wong *et al.*, 2000; Garmyn *et al.*, 2001). Thus, we addressed whether TNF-α-induced PGE_2 production in keratinocytes required the p38 MAP kinase-dependent activation of Hsp27. Downregulation of the upstream p38 MAP kinase by siRNA was found to reduce

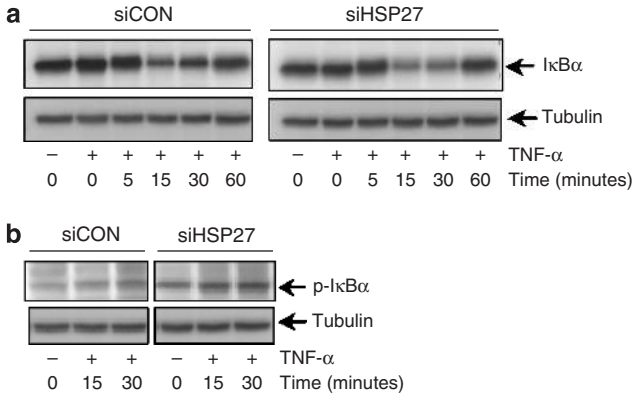

Figure 4. Hsp27 regulates NF-κB signaling in keratinocytes. (**a**) Primary keratinocytes were grown in six-well dishes and transfected with siCON or Hsp27 siRNA (siHSP27). Cells were treated with 20 ng ml^{-1} TNF-α for the indicated times. Cells were lyzed in radioimmunoprecipitation buffer and whole-cell extracts (20 μg protein) were subjected to SDS-PAGE followed by blotting with IκB-α antibody and tubulin antibody. (**b**) Whole-cell extracts (20 μg protein) of keratinocytes transfected with siCON or siHSP27 were subjected to SDS-PAGE followed by blotting with phospho-IκB-α antibody and tubulin antibody.

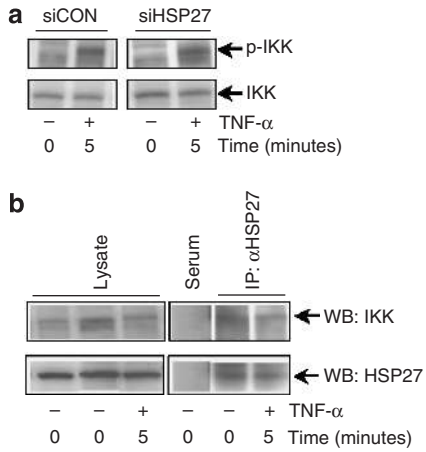

Figure 5. Hsp27 associates with IKK in keratinocytes. (**a**) Primary keratinocytes were grown in six-well dishes and transfected with siCON or Hsp27 siRNA (siHSP27). Cells were treated with 20 ng ml^{-1} TNF-α for 5 minutes. Cells were lyzed in radioimmunoprecipitation buffer and whole-cell extracts (20 μg protein) were subjected to SDS-PAGE followed by blotting with phospho-IKK antibody and IKK antibody. (**b**) Primary keratinocytes were either left untreated or treated with TNF-α. The lysates were then immunoprecipitated by incubation with 10 μl of anti-Hsp27 antibody or rabbit serum as control, as described under the Materials and methods section. Immunoprecipitated samples were subjected to SDS-PAGE and immunoblotted with anti-IKK and anti-Hsp27 antibodies.

TNF-α-induced PGE_2 production, an opposite effect to that of Hsp27 downregulation (Figure 2b), suggesting that p38 MAP kinase-mediated activation of Hsp27 is not required for Hsp27 regulation of PGE_2 production in keratinocytes.

Exposure of skin to UV irradiation has been known to induce the release of various cytokines such as TNF-α, IL-1α, IL-6, and IL-8, which drive cutaneous inflammatory responses (Kondo, 1999; Aubin, 2003). UV radiation has also been shown to activate p38 MAP kinase and downstream Hsp27 in murine skin and, moreover, UVB-induced pro-inflammatory cytokine production from murine skin has been blocked by the p38 inhibitor (Kim *et al.*, 2005). Our results show that downregulation of Hsp27 potentiates the release of UV-induced pro-inflammatory cytokines IL-1α (data not shown) and IL-8 from keratinocytes (Figure 1d). Thus, it is probable that Hsp27 regulates the production of TNF-α- and UV-induced cytokines independent of p38-mediated activation. These results also suggest that Hsp27 plays an anti-inflammatory role in keratinocytes independent of the inflammatory stimuli.

Expression of the pro-inflammatory cytokines IL-1α (Fenton, 1992; Mori and Prager, 1996), IL-8 (Yasumoto *et al.*, 1992), and COX-2 (Chun *et al.*, 2004) that catalyses the synthesis of PGE_2 are transcriptionally regulated by NF-κB. In majority of cells, the NF-κB proteins are sequestered in the cytoplasm bound to inhibitory proteins referred to as IκB. Exposure of cells to extracellular stimuli such as TNF-α leads to the activation of the IKK complex including two catalytic subunits, IKKα and IKKβ, resulting in the phosphorylation and ubiquitination of the IκB proteins and their proteosome-mediated degradation (Beg *et al.*, 1993; Alkalay *et al.*, 1995; Brown *et al.*, 1995; Maniatis, 1999). Hsp27 has been shown to differentially modulate NF-κB signaling based on the cell type. In U937 human leukemic cells, Hsp27 overexpression was found to enhance NF-κB activity (Parcellier *et al.*, 2003). However, in vascular smooth muscle cells, Hsp27 has been reported to suppress angiotensin II-induced NF-κB activity (Chen and Currie, 2006) and heart inflammation (Chen *et al.*, 2004a, b). Additionally, Hsp27 has been reported to suppress *Entamoeba histolytica*-induced NF-κB activation by associating with the IKK complex and inhibiting IKK activity and intestinal inflammation (Kammanadiminti and Chadee, 2006). Therefore, we hypothesized that Hsp27 regulated cytokine production in keratinocytes by modulating NF-κB signaling. Interestingly, we found that pretreatment of keratinocyte cells with the NF-κB inhibitor BAY11-7082 suppressed the increase in IL-8 production mediated by downregulation of Hsp27, suggesting the involvement of NF-κB signaling in these responses (Figure 3a and b). To further assess the role of Hsp27 on NF-κB signaling, we first looked at the role of Hsp27 in regulating NF-κB transcriptional activity in keratinocytes. Unstimulated NF-κB luciferase activity increased by 1.6-fold (Figure 3c) and TNF-α stimulated NF-κB luciferase activity increased by two-fold (Figure 3c) in Hsp27 downregulated cells, suggesting that Hsp27 regulates both TNF-α-independent and -dependent NF-κB transcriptional activity in keratinocytes. To determine a probable mechanism for how Hsp27-modulated NF-κB transcriptional activity, we followed IκB-α degradation in siCON and Hsp27 siRNA-transfected keratinocytes. In control cells, there was 10 and 30% greater IκB-α at 15 and 30 minutes, respectively, and less phospho-IκB-α accumulation at these time points post-TNF-α stimulation compared to Hsp27 downregulated cells (Figure 4a and b). This suggests that Hsp27 regulates degradation of IκB-α in keratinocytes and that there is greater NF-κB activity in the absence of Hsp27 correlating with greater NF-κB luciferase activity in Hsp27 siRNA-transfected cells (Figure 3c). Downregulation of Hsp27 did not affect phosphorylation of IKK that phosphorylates IκB-α (Figure 5a). However, immunoprecipitation studies using Hsp27 antibodies showed that Hsp27 associates with IKK in keratinocytes (Figure 5b). These results suggest that in keratinocyte cells, Hsp27 associates with IKK without affecting IKK activation but instead prevented the phosphorylation and subsequent degradation of the downstream IκB-α, possibly by preventing the binding of IKK to the active phosphorylation recognition sequence on IκB-α, thereby modulating NF-κB signaling. Thus, greater NF-κB activity in Hsp27 downregulated cells results in the potentiation of pro-inflammatory mediator release from keratinocytes.

Immunological studies of skin demonstrate that Hsp27 is expressed in epidermis and increases with differentiation, with the highest expression localized in the upper epidermal layers (Trautinger *et al.*, 1995), while decreased Hsp27 expression is found in the skin of patients with disorders of keratinization or incomplete keratinocyte differentiation (Jonak *et al.*, 2005). Hsp27 expression in skin of patients with psoriasis or with dermatitis was increased compared to uninvolved skin (Trautinger *et al.*, 1995). As psoriatic skin manifests with a hyperproliferative epidermis and subsequent decreased differentiation, the increased Hsp27 expression in the skin of patients with psoriasis conflicts with the positive association of Hsp27 and differentiation. Unrelated to differentiation, this increased expression of Hsp27 in inflammatory skin lesions may be mediated by the increased release of pro-inflammatory mediators to regulate inflammation. Indeed, expression of Hsps is upregulated in murine fibroblasts after exposure to pro-inflammatory mediators such as TNF-α (Watanabe *et al.*, 1997), and our studies demonstrate that Hsp27 plays a protective role in keratinocytes by regulating the production of NF-κB-dependent pro-inflammatory mediators, such as IL-8, IL-1α, and PGE_2. Hsp expression can be induced by non-steroidal, anti-inflammatory agents, such as sodium salicylate (Jurivich *et al.*, 1992) and indomethacin (Lee *et al.*, 1995), and increased Hsp expression protected cells against stress-induced cellular damage. Interestingly, the induction of Hsps may provide a mechanism of action to account for the therapeutic efficacy of non-steroidal anti-inflammatory drugs (NSAIDS), as the anti-inflammatory activity and potency of many NSAIDS cannot be explained by inhibitions of COX alone. Thus, Hsps may have a ubiquitous role in protecting cells against numerous types of environmental stress and inflammation. Taken together, our findings reveal an additional function in skin for the small Hsp27 distinct from the well-characterized role in keratinocyte differentiation and apoptosis, as a regulatory factor controlling the production of pro-inflammatory mediators.

MATERIALS AND METHODS

Materials

Lipofectamine 2000 transfection reagent was obtained from Invitrogen Corporation (Carlsbad, CA). Recombinant human TNF-α was obtained from Peprotech Inc. (Rocky Hill, NJ). IκB-α antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). p-IκB-α, IKKα/β, p-IKK, Hsp27, p38, extracellular signal-regulated kinase-2, and Tubulin antibodies were obtained from Cell Signaling Technology (Danvers, MA). Protein A/G Plus Agarose beads were from Santa Cruz Biotechnology. PathDetect NF-κB luciferase reporter construct (pNF-κB-Luc) and negative control plasmid (pCIS-CK) were purchased from Stratagene (La Jolla, CA). BAY11-7082 was from Calbiochem (San Diego, CA).

Cells and cell culture

Human HaCaT keratinocytes (a gift from Dr NE Fusenig, Heidelberg, Germany) were maintained in DMEM (Invitrogen Corporation) containing 10% fetal bovine serum, 4.5 mg ml^{-1} glucose, 2 mM L-glutamine, 1% penicillin, and streptomycin. Cells were maintained at <80% confluency at 37°C in 5% CO_2 (vol/vol). Normal human epidermal neonatal keratinocytes were obtained from Cascade Biologics (Portland, OR) and maintained in serum-free Epilife medium (Cascade Biologics) supplemented with human keratinocyte growth supplement containing 0.2% (v/v) bovine pituitary extract, 5 μg ml^{-1} bovine insulin, 0.18 μg ml^{-1} hydrocortisone, 5 μg ml^{-1} bovine transferrin, and 0.2 ng ml^{-1} human epidermal growth factor.

Measurement of PGE_2 release

Keratinocytes transfected with the respective siRNA were incubated without or with 100 ng ml^{-1} TNF-α for 24 hours. PGE_2 levels in the supernatant were measured by ELISA according to the manufacturer's instructions (Assay Designs Inc., Ann Arbor, MI).

Gene specific knockdown using siRNA

Primary keratinocytes were plated in 24-well plates at a density of 4×10^4 cells per well and incubated overnight at 37°C, 5% CO_2. Cells were transfected with signalsilence negative siCON or signalsilence Hsp27 siRNA (Cell Signaling Technology) at 100 nM concentration or signalsilence pool p38 MAPK siRNA (Cell Signaling Technology) at 20 nM concentration using lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were then grown for 72 hours at 37°C, 5% CO_2.

NF-κB luciferase reporter assay

Primary keratinocytes seeded in six-well tissue culture dishes were co-transfected with 1 μg pNF-kB-Luc reporter plasmid or pCIS-CK negative control plasmid and 50 pmol of Hsp27 siRNA or siCON using Lipofectamine 2000 reagent. At 48 hours post-transfection, cells were either left untreated or treated with 100 μg ml^{-1} TNF-α for 24 hours. The cells were then lysed and luciferase readings were obtained using the Promega assay kit (Promega Corporation, Madison, WI) according to the manufacturer's protocol.

Western blotting

Cells were washed with phosphate-buffered saline and lysed with radioimmunoprecipitation lysis buffer containing 65 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8), 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitor cocktail (Sigma, St Louis, MO). Protein (20 μg) was loaded on SDS-PAGE followed by immunoblotting with the specific antibodies.

Immunoprecipitation

Adult human primary keratinocytes grown in 10 cm^2 dishes were either left untreated or treated with TNF-α. Cells were then washed with phosphate-buffered saline buffer and lysed with radioimmunoprecipitation lysis buffer. The lysate was centrifuged at 13 306 *g* for 10 min, and then immunoprecipitated by incubation with 10 μl of anti-Hsp27 antibody or control rabbit serum. Immune complexes were captured with protein A/G plus agarose beads (20 μl) overnight at 4°C. After immunoprecipitation, the beads were washed four times with lysis buffer, eluted with SDS sample buffer, resolved by SDS-PAGE and visualized by immunoblotting with anti-IKK and anti-Hsp27 antibodies.

Measurement of UV-induced cytokine release

Primary human keratinocytes were seeded into 24-well plates at 30,000 cells per well and next day transfected with siCON or Hsp27-specific siRNA. After 24 hours of transfection, the media was replaced with supplement-free media and the plate was returned to the incubator. After 48 hours of transfection, just prior to UV irradiation, the supplement-free media was replaced with 250 μl phenol-free Hank's balanced salt solution and the cells were either left unexposed or exposed to UV irradiation. The UV light source was an Oriel 1,000 W solar UV simulator (Oriel Corporation, Stratford, CT), equipped with an atmospheric attenuation filter (Schott WG 320; 1 mm thick) and a visible-infrared filter (Schott UG 11; 1 mm thick). The irradiance of the source, uniformity of the beam, and the spectral power distribution was measured with a model 754 spectroradiometer (Optronic Labs, Orlando, FL) and determined to comprise 12% UVB (290–320 nm) and 88% UVA (320–400 nm). This filtered xenon light source provided a simulated solar UVR spectrum (290–400 nm) that was nearly devoid of visible and infrared radiation. Fresh supplement-free EpiLife media was added to the plates and incubated at 37°C and 5% CO_2 for an additional 24 h period, following which the media was analyzed for IL-1α and IL-8 release using commercially available immunoassay multiplex kits (Upstate Biotechnology, Charlottesville, VA) on a Luminex L100 (Luminex Corporation, Austin, TX), a validated assay with a sensitivity that is comparable to that of conventional ELISA (de Jager *et al.*, 2003).

Statistical analysis

Data are presented as mean ± SD. Student's *t*-test was used for comparisons between two groups. A value of $P<0.05$ was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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