HaCaT Keratinocytes Overexpressing the S100 Proteins S100A8 and S100A9 Show Increased NADPH Oxidase and NF-κB Activities

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The calcium- and arachidonic acid (AA)-binding proteins S100A8 and S100A9 are involved in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation in phagocytes. They are specifically expressed in myeloid cells, and are also found in epithelial cells in various (patho)physiological conditions. We have investigated the consequences of S100A8/A9 overexpression in epithelial cell lines on reactive oxygen species (ROS) generation and downstream signaling. Epithelial carcinoma HeLa cells, which exclusively express Nox2, showed dramatically increased activation of NADPH oxidase by phorbol 12-myristate 13-acetate after S100A8/A9 gene transfection. HaCaT keratinocytes overexpressing S100A8/A9 showed enhanced, transient ROS generation in response to the calcium ionophore A23187 compared to mock-transfected cells. Polymerase chain reaction analysis revealed mRNA transcripts for Nox1, Nox2, and Nox5 in HaCaT keratinocytes. Detailed transfection studies confirmed that NADPH oxidase activities in Nox1- and Nox5-transfected HeLa cells were enhanced after S100A8/A9 gene complementation. Furthermore, mutational analysis revealed that AA binding and Thr^{113} phosphorylation are important for S100A8/A9-enhanced activation of NADPH oxidase. Nuclear factor-κB (NF-κB) activation and interleukin-8 mRNA levels were increased in S100A8/A9-HaCaT keratinocytes, consistent with the view that NF-κB is a redox-sensitive transcription factor. Because they are expressed in epithelia under specific conditions, S100A8 and S100A9 might be involved in skin pathogenesis by modulating aspects of downstream signaling.

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INTRODUCTION

Members of the multigenic S100 protein family are non-ubiquitous cytoplasmic Ca^{2+}-binding proteins of the EF-hand type, differentially expressed in a wide variety of cell types. Two members of this family, S100A8 and S100A9, are predominantly expressed in myeloid cells. Their expression appears to be restricted to a specific stage of myeloid differentiation (Kerkhoff *et al.*, 1998; Nacken *et al.*, 2003), and is probably driven by a recently characterized regulatory element (Kerkhoff *et al.*, 2002). Although the exact functions of the S100 proteins remain unknown, S100A8 and S100A9 are normally associated as heteromeric complexes that can bind polyunsaturated fatty acids such as arachidonic acid (AA) in a calcium-dependent manner (Kerkhoff *et al.*, 1999a, b; Sopalla *et al.*, 2002). S100A8/A9 protein complexes account for the entire AA-binding capacity of neutrophil cytosol (Kerkhoff *et al.*, 1999a) and participate in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation in phagocytes by transferring AA to membrane-bound $gp91^{phox}$ during interactions with two cytosolic oxidase activation factors, $p67^{phox}$ and Rac-2 (Kerkhoff *et al.*, 2005). AA is an essential factor in the formation of active NADPH oxidase because its binding to $gp91^{phox}$ induces structural changes in cytochrome b_{558} (Doussiere *et al.*, 1996, 1999).

However, several studies also suggest potential roles for the two S100 proteins in epidermal wound repair, differentiation, and response to stress. They are expressed at only minimal levels in normal epidermis but are massively expressed in psoriasis (Madsen *et al.*, 1992). Both proteins are found in the granular layer and in the basal and spinous layers (Broome *et al.*, 2003; and references therein). Interestingly, the PSORS4 psoriasis susceptibility region has been mapped to chromosome 1q21 (Semprini *et al.*, 2002), where the two S100 genes are located within the epidermal

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Abbreviations: AA, arachidonic acid; DPI, diphenyleneiodonium; EGF, epidermal growth factor; IL-8, interleukin-8; NBT, nitroblue tetrazolium; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; wt, wild type

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differentiation complex together with other members of the S100 family, as well as epidermal differentiation markers such as several cytokeratins, profilaggrin, and involucrin (Hardas *et al.*, 1996; Mischke *et al.*, 1996). In addition, S100A8 is aberrantly overexpressed in various experimental models of cultured keratinocytes that exhibit an abnormal psoriasis-like phenotype (Nagpal *et al.*, 1996).

A role in wound healing has been attributed to S100A8 and S100A9, because they are significantly upregulated in differentiating suprabasal wound keratinocytes (Thorey *et al.*, 2001), especially in the first 12–24 hours after injury, with a gradual return to baseline expression over a 2-week period (Soo *et al.*, 2002). In addition, both proteins are strongly induced in human keratinocytes in response to UVB irradiation (Dazard *et al.*, 2003). In view of these findings, the two S100 proteins have been characterized as stress-induced proteins (Eckert *et al.*, 2004).

Originally, NADPH oxidase was thought to be restricted to phagocytes and used solely in host defense. However, it has become apparent over the past several years that similar NADPH oxidase systems are present in many nonphagocytic cells.

There are several homologs of Nox2 ($gp91^{phox}$) and of cytosolic *phox* proteins with specific expression patterns (Bokoch and Knaus, 2003; Lambeth, 2004). The NOX family includes Nox1 (initially called Mox1 or NOH-1) (Suh *et al.*, 1999; Banfi *et al.*, 2000), predominantly expressed in colon, Nox3 (also called gp91–3) cloned from fetal kidney (Kikuchi *et al.*, 2000), Nox4 (also termed Renox) found in kidney cortex (Geiszt *et al.*, 2000; Shiose *et al.*, 2001) and osteoclasts (Yang *et al.*, 2001), and Nox5, predominantly expressed in pachytene spermatocytes in the testis and in B- and T-lymphocyte-rich areas of the spleen and lymph nodes (Banfi *et al.*, 2001). NOXO1 and NOXA1 have also been identified as functional homologs of $p47^{phox}$ and $p67^{phox}$, respectively (Banfi *et al.*, 2003).

These novel $gp91^{phox}$ homologs have been proposed to regulate cell growth (Nox1 and Nox5), participate in host defense, (Nox1) or mediate oxygen sensing (Nox4) (Krause, 2004). However, their physiological functions are currently under intensive investigations.

Epithelial cells produce superoxide anions and hydrogen peroxide (H_2O_2) in response to a variety of extracellular stimuli including platelet-derived growth factor (Sundaresan *et al.*, 1995), epidermal growth factor (EGF) (Bae *et al.*, 1997), and other growth factors (Bokoch and Knaus, 2003). Several studies have shown that at the wound sites, reactive oxygen species (ROS) promote angiogenesis by inducing vascular endothelial growth factor (VEGF) expression in wound-related cells such as keratinocytes and macrophages (Khanna *et al.*, 2001; Sen *et al.*, 2002a, b). Furthermore, re-epithelialization of wounds involves the formation of peripheral cytoplasmic actin filaments, making the cells motile. ROS induce smooth muscle cell proliferation and migration (Nishio and Watanabe, 1997). Specifically, H_2O_2 has been shown to induce pro-matrix metalloproteinase (MMP)-2 activation and cell motility (Yoon *et al.*, 2002). It has been suggested that ROS act as autocrine second messengers modulating the redox status of individual components of signaling pathways such as growth factor receptors, Ras proteins, protein kinases, protein phosphatases, and transcription factors, for optimal activity.

To elucidate the role of the two S100 proteins in epithelial cells, we have investigated the consequences of S100A8/A9 overexpression in epithelial cell lines on ROS generation and downstream signaling. After S100A8/A9 gene complementation, phorbol 12-myristate 13-acetate (PMA)-elicited NADPH oxidase activation was enhanced in HeLa epithelial carcinoma cells, whereas ROS generation in response to the calcium ionophore A23187 was increased in HaCaT keratinocytes. Detailed transfection studies indicated that S100A8/A9 accelerated Nox1 and Nox5 activities. Further analyses demonstrated that AA-binding motif and Thr^{113} phosphorylation were important in the enhancement of epithelial NADPH oxidase activation. The enhancement of nuclear factor-κB (NF-κB) activation and interleukin-8 (IL-8) production after tumor necrosis factor-α (TNF-α) stimulation in HaCaT keratinocytes that overexpress S100A8/A9 point to an important role of S100A8/A9 in skin pathogenesis.

RESULTS

NADPH oxidase activity in epithelial cells after S100A8/A9 complementation

Recently, we established the functional role of S100A8/A9 in the activation of NADPH oxidase in phagocytes (Kerkhoff *et al.*, 2005). These proteins are specifically expressed in myeloid cells, and also in epithelium and involved epidermis in conditions such as psoriasis and malignant disorders (Brandtzaeg *et al.*, 1987; Wilkinson *et al.*, 1988; Madsen *et al.*, 1992). Here, we investigate the effect of S100A8/A9 complementation in the epithelial carcinoma HeLa cell line and keratinocyte-like HaCaT cells.

Both HeLa and HaCaT cells were transfected with the expression plasmid pVIVO-S100A8/A9, which ensures strong and sustained coexpression of S100A8 and S100A9. After 24 hours, aliquots of the cells were analyzed by Western blotting using specific polyclonal antibodies against S100A9 and S100A8, and NADPH oxidase activity was measured. ROS generation was monitored with nitroblue tetrazolium (NBT), a water-soluble dye, that is converted into an insoluble blue formazan by superoxide anions inside cells (Green and Pratt, 1990).

Western blotting revealed that HeLa cells were negative for S100A8 and S100A9, whereas HaCaT cells showed little endogenous expression of these proteins. After transfection, however, both S100 proteins were clearly detected in both cell lines (Figure 1a and b). The loading control β-actin was used to confirm that similar amounts of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). As reported previously (Teigelkamp *et al.*, 1991; Goebeler *et al.*, 1994; Rammes *et al.*, 1997), the two S100-specific antibodies crossreact as well as exhibiting different affinities for their antigens. Therefore, the different staining intensities did not reflect different amounts of protein.

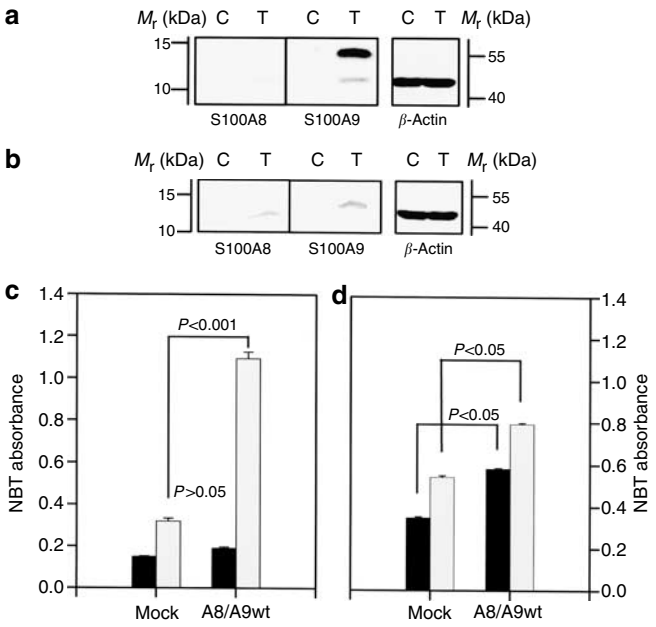

Figure 1. NADPH oxidase activity in mock- and S100A8/A9-overexpressing epithelial cell lines. (**a**, **b**) Western-blot analysis and (**c**, **d**) NADPH oxidase activity (**a**, **c**) in HeLa cells (**b**, **d**) and HaCaT keratinocytes after transfection with pVIVO-S100A8/A9. Cells were transfected with empty vector (mock) or pVIVO-S100A8/A9 expression vector for wild-type S100A8/A9 by Lipofectin as described in the Materials and Methods section, and incubated for 24 hours. For Western-blot analysis (**a**, **b**), SDS sample buffer was added to aliquots of the cells, the mixture was heated to 95°C for 5 minutes, and the proteins were subjected to 16.5% SDS–PAGE. Western blotting was performed using S100A8- and S100A9-specific antibodies. C, mock-transfected cells; T, S100A8/A9-overexpressing cells. The NADPH oxidase activity (**c**, **d**) induced by 100 nM PMA was determined by the formation of the formazan product, as described in the Materials and Methods section. Data are expressed as absorbance units/10^6 cells. The number of experiments was $n=3$. Control, ■; PMA, □.

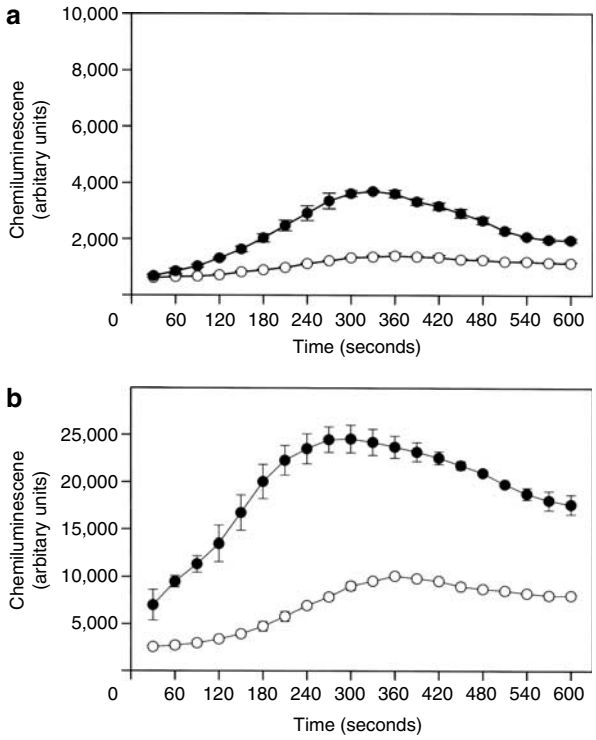

Figure 2. NADPH oxidase activity in mock- and S100A8/A9-overexpressing HaCaT keratinocytes in response to A23187. (**a**) NADPH oxidase activity in HaCaT cells and (**b**) S100A8/A9-overexpressing HaCaT cells after elevation of intracellular Ca^{2+} level. HaCaT cells were transfected with the pVIVO-S100A8/A9wt expression plasmid by Lipofectin as indicated, and incubated for 24 hours. The NADPH oxidase activity induced by 1 μM A23187 was determined by isoluminol-amplified chemiluminescence (Liu *et al.*, 1996) as described in the Materials and Methods section. The number of experiments was $n=3$. Control, ○; A23187, ●.

Complementation of HeLa cells with S100A8/A9 had no effect on unstimulated NADPH oxidase activity. However, after PMA stimulation, HeLa cells overexpressing S100A8/A9 showed a marked increase in ROS generation (Figure 1c). Complementation of HaCaT cells with S100A8/A9 had a slight but significant effect on unstimulated NADPH oxidase activity (Figure 1d). After PMA stimulation, HaCaT cells overexpressing S100A8/A9 showed a small significant increase in ROS generation compared to mock-transfected cells. ROS generation in both cell lines was attenuated by cotreatment with diphenyleneiodonium (DPI), an inhibitor of NADPH oxidases. The inhibitory effect of DPI was concentration-dependent (data not shown), indicating that flavoprotein-dependent oxidase are involved in ROS generation.

The NOX family of NADPH oxidases, comprising five homologs including the phagocyte NADPH oxidase, has been characterized in recent years. These homologs are expressed in a variety of different tissues and differ in their cofactor dependence, the stimuli inducing enzyme activity, and, most importantly, their putative cellular functions (Krause, 2004). Therefore, we determined NOX gene expression in both HeLa cells and HaCaT keratinocytes. Semiquantitative polymerase chain reaction (PCR) analysis revealed that HeLa cells expressed only $gp91^{phox}$ (Nox2), whereas HaCaT cells expressed mRNA transcripts for Nox1, Nox2, and Nox5 (Table S1).

A calcium-inducible NADPH oxidase activity in HaCaT cells

Nox5, a homolog of the $gp91^{phox}$ subunit of phagocyte NADPH oxidase, is distinguishable from other NADPH oxidases by its unique N terminus, which contains three canonical EF-hands representing Ca^{2+}-binding domains (Banfi *et al.*, 2001, 2004), and by its induction when intracellular calcium levels are elevated in response to the calcium ionophore A23187.

HaCaT cells were stimulated with the calcium ionophore A23187, and ROS generation was followed by isoluminol-amplified chemiluminescence. Under basal culture conditions, these cells exhibit little or no isoluminol-amplified chemiluminescence (Figure 2a). The elevation of intracellular calcium levels induced a small but significant increase in NADPH oxidase activity. The response was maximal in <5 minutes and returned to the original baseline within 10 minutes. A23187-induced isoluminol-amplified chemilu-

minescence was attenuated by cotreatment with the NADPH oxidase inhibitor DPI (4 μM) (data not shown), strongly indicating that HaCaT keratinocytes express a calcium-dependent NADPH oxidase activity.

After HaCaT cells were complemented with S100A8/A9, elevation of intracellular calcium resulted in a marked increase of NADPH oxidase activity (Figure 2b). The response was maximal in <4 minutes and had not returned to the original baseline within 10 minutes. In comparison to nontransfected cells, S100A8/A9 overexpressing HaCaT cells showed significantly higher levels of ROS generation, indicating that S100A8/A9 enhances Nox5 activation.

To confirm this finding, HeLa cells were transfected with either Nox5 expression plasmid alone or a set of Nox5 and S100A8/A9 expression plasmids. After Nox5 complementation, HeLa cells exhibited A23187-inducible NADPH oxidase activity, and concomitant expression of S100A8/A9 and Nox5 resulted in enhanced ROS generation after A23187 stimulation (data not shown).

From these data it is likely that HaCaT keratinocytes express a calcium-inducible oxidase activity that is promoted by S100A8/A9.

Expression studies of phox homologs in the two epithelial cell lines

In addition to several homologs of the NOX family of NADPH oxidases, functional homologs of $p47^{phox}$ and $p67^{phox}$ have also been identified in recent years (Banfi *et al.*, 2003). Stimulus-independent NADPH oxidase activation has been ascribed to these functional homologs. Therefore, we used PCR to investigate the expression of cytosolic *phox* proteins and their analogs, as well as the GTPases Rac1 and Rac2 in the two epithelial cell lines. These analyses revealed mRNA transcripts of $p22^{phox}$, $p67^{phox}$, NOXA1, NOXO1, and Rac-1 in HeLa cells, whereas mRNA transcripts of $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, NOXA1, NOXO1, Rac-1, and Rac-2 were detected in HaCaT cells (Table S1). Thus, the PCR analysis revealed that the two cell lines differed in the expression of several NOX and *phox* homologs.

Effect of S100A8/A9 mutants in HeLa cells

Complementation of HaCaT keratinocytes with S100A8/A9 significantly increased the unstimulated NADPH oxidase (Figure 1d). As HeLa cells express only Nox2, they appear to be a suitable cellular model to evaluating the effect of S100A8/A9 on Nox1 by means of a detailed transfection study. To confirm this assumption, we investigated the properties of S100A8/A9-enhanced NADPH oxidase activity in HeLa cells.

In our previous study, we reported that binding of AA to S100A8/A9 is essential for the enhancement of NADPH oxidase in human phagocytes. This follows from the finding that mutant S100A8/A9 protein complexes, which are unable to bind AA, failed to enhance NADPH oxidase activity in a cell-free activation system (Kerkhoff *et al.*, 2005). Therefore, HeLa cells were transfected with either pVIVO-S100A8/A9wt or pVIVO-S100A8/A9(H103,104,105K106A) expression plasmids and NADPH oxidase activity was determined. Western blotting confirmed that the S100 proteins had similar expression levels in the mutants (Figure 3a).

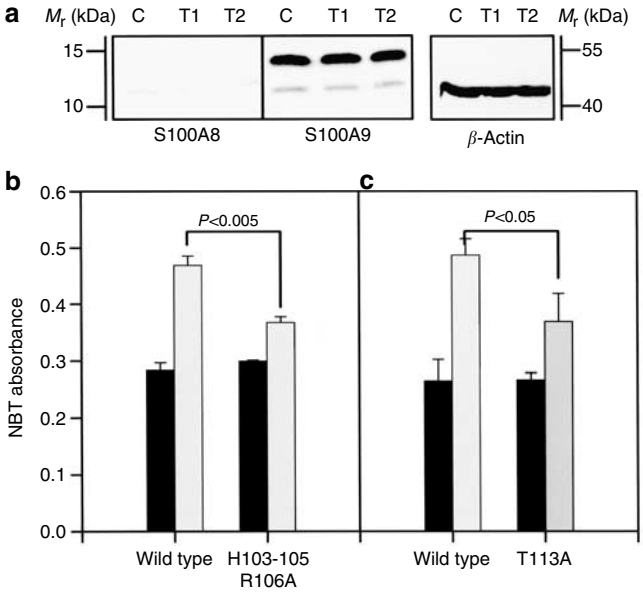

Figure 3. NADPH oxidase activity in HeLa cells transfected with 8100A8/A9wt and S100A8/A9 mutants. (**a**) Western-blot analysis and (**b**) NADPH oxidase activity in HeLa cells transfected with pVIVO-S100A8/A9(H103,104,105K106A) (**c**) and pVIVO-S100A8/A9(T113A). HeLa cells were transfected with pVIVO-S100A8/A9wt, pVIVO-S100A8/A9(H103,104,105K106A), or pVIVOS100A8/A9(T113A) expression plasmids by Lipofectin as described in the Materials and Methods section, and incubated for 24 hours. (**a**) Westernblot analysis was performed using S100A8- and S100A9-specific antibodies. C, S100A8/A9wt-transfected cells; T1, S100A8/A9(H103,104,105K106A)-transfected cells; T2, S100A8/A9(T113A)-transfected cells. (**b**, **c**) The NADPH oxidase activity induced by 100 nM PMA was determined by the formation of the formazan product, as described in the Materials and Methods section. Data are expressed as absorbance units/10^6 cells. The number of experiments was $n=3$. Probability values of $P<0.05$ were considered to represent significant differences. Control, ■; PMA, □.

As shown in Figure 3b, S100A8/A9(H103,104,105K106A), which cannot bind AA, had significantly less ability to enhance NADPH oxidase activation than S100A8/A9wt ($P<0.005$; $n=3$). These data are consistent with our earlier finding and further increases our knowledge about the cellular function of AA binding to S100A8/A9, as S100A8/A9(H103,104,105K106A) failed to promote NADPH oxidase activity in intact cells.

S100A9 can be phosphorylated at its Thr113 residue (Edgeworth *et al.*, 1991), and it has been suggested that this phosphorylation is important for its cellular function (Nacken *et al.*, 2003). Therefore, we constructed a mutant of S100A9 (S100A9-T113A) in which Thr113 was substituted by alanine. HeLa cells were transfected with either pVIVO-S100A8/A9wt or pVIVO-S100A8/A9(T113A) expression plasmids and NADPH oxidase activity was determined. S100A8/A9(T113A) had a significantly lower stimulatory effect on NADPH oxidase activity than S100A8/A9wt ($P<0.05$; $n=5$) (Figure 3c). This is the first evidence that phosphorylation of S100A9 represents a regulatory mechanism by which the enhancing effect of S100A8/A9 on NADPH oxidase activation is modulated.

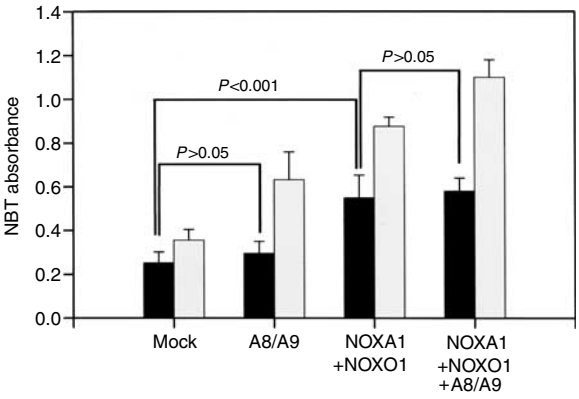

Figure 4. Transfection studies with S100A8/A9, NOXO1, or NOXA1. HeLa cells were transfected with different combinations of either pVIVO-S100A8/A9wt, pcDNA3.1-hNOXO1, or pCMV-SPORT6-hNOXA1 expression plasmids by Lipofectin as indicated, and incubated for 24 hours. The NADPH oxidase activity induced by 100 nM PMA was determined by the formation of the formazan product, as described in the Materials and Methods section. Data are expressed as absorbance units/10^6 cells. The number of experiments was $n=3$. Probability values of $P<0.05$ were considered to represent significant differences. Control, ■; PMA, □.

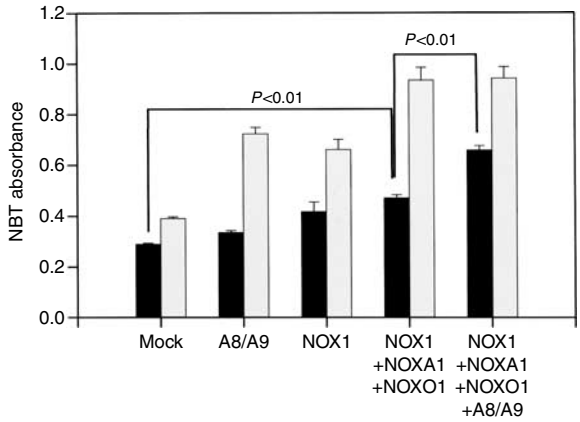

Figure 5. Transfection studies with S100A8/A9, NOXO1, NOXA1, or Nox1. HeLa cells were transfected with different combinations of pVIVO-S100A8/A9wt, pcDNA3.1-hNOXO1, pCMV-SPORT6-hNOXA1, or pcDNA3.0-hNox1 expression plasmids by Lipofectin as indicated, and were incubated for 24 hours. The NADPH oxidase activity induced by 100 nM PMA was determined by the formation of the formazan product, as described in the Materials and Methods section. Data are expressed as absorbance units/10^6 cells. The number of experiments was $n=3$. Probability values of $P<0.05$ were considered to represent significant differences. Control, ■; PMA, □.

Transfection studies with NOX and *phox* homologs

To study the putative activating effect of S100A8/A9 on Nox1, different combinations of NOXA1, NOXO1, and S100A8/A9 were first overexpressed in HeLa cells and NADPH oxidase activity was determined (Figure 4). Expression of NOXA1 and NOXO1 mRNA transcripts was analyzed by Taqman analysis using a gene-specific forward primer and a reverse primer directed against the plasmid polyadenylation sequence as described in the Materials and Methods section. Overexpression of NOXO1 and NOXA1 resulted in a higher level of unstimulated NADPH oxidase activity and this was not affected by the simultaneous expression of S100A8/A9. After treatment with PMA, NADPH oxidase activity increased, and a further enhancement was observed in cells simultaneously overexpressing NOXA1, NOXO1, and S100A8/A9 (Figure 4).

NOXA1 and NOXO1 are physiologically relevant partners of Nox1 and mediate stimulus-independent oxidase activation (Banfi *et al.*, 2003). However, they also interact with other NOX homologs (Banfi *et al.*, 2003; Takeya *et al.*, 2003). Consequently, the overexpression of NOXA1 and NOXO1 in HeLa cells resulted in an enhanced unstimulated oxidase activity. NOXO1 lacks both the autoinhibitory domain and the protein kinase C (PKC) phosphorylation sites of $p47^{phox}$, whereas NOXA1 lacks the first Src homology 3 domain of $p67^{phox}$ and contains a hydrophobic stretch (Banfi *et al.*, 2003). These differences may cause permanent association with membrane-bound NOX homologs, resulting in constitutive NADPH oxidase activity. However, it is obvious that S100A8/A9 did not enhance the NOXO1/NOXA1-mediated constitutive activation of endogenous Nox2 activity.

Next, analogous studies were performed with different combinations of Nox1, NOXA1, NOXO1, and S100A8/A9 (Figure 5), and their expression was analyzed by Taqman analysis as described in the Materials and Methods section. Overexpression of Nox1 in HeLa cells resulted in higher levels of unstimulated NADPH oxidase activity. This activity was enhanced by PMA stimulation. Concomitant overexpression of NOXO1 and NOXA1 led to a further but small increase in unstimulated NADPH oxidase activity compared with HeLa cells overexpressing Nox1 alone. Concomitant expression of S100A8/A9 enhanced NADPH oxidase activity in comparison with Nox1- or Nox1-NOXA1-NOXO1-transfected HeLa cells (Figure 5). Similar, overexpression of Nox1 and S100A8/A9 also enhanced NADPH oxidase activity in comparison with Nox1-transfected HeLa cells (data not shown). These data indicate that S100A8/A9 promotes Nox1. This conclusion is consistent with other reports demonstrating that Nox1 is closely similar to the phagocyte NADPH oxidase (Geiszt and Leto, 2004).

Increased NF-κB activation in S100A8/A9-transfected HaCaT cells

It has been suggested that ROS modulate the redox status of individual components of various signaling pathways. For example, they regulate gene expression by modulating the redox status of various transcription factors, including NF-κB (DiDonato *et al.*, 1997; Ghosh and Karin, 2002). Interestingly, it has been reported that TNF-α signaling causes both NF-κB activation and oxidant production (for review see Janssen-Heininger *et al.* (2000); and references therein). Therefore, transient transfection assays were performed using a luciferase reporter gene driven by NF-κB binding sites.

Figure 6 shows that TNF-α-induced reporter expression was significantly increased in S100A8/A9wt-overexpressing HaCaT cells compared to mock-transfected and S100A8/A9 mutant-transfected cells. Luciferase activity was negligible in cells cotransfected with a combination of negative control

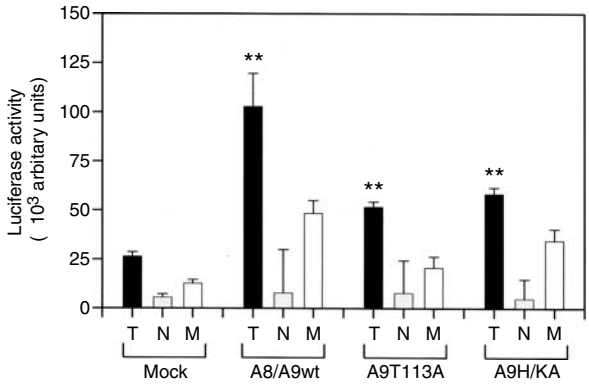

Figure 6. NF-κB activation in S100A8/A9 mutant- and S100A8/A9wt-overexpressing HaCaT cells. HaCaT cells were co-transfected with different combinations of NF-κB-regulated luciferase reporter construct and various S100A8/A9 expression vectors as indicated. Forty-eight hours after transfection, the cells were treated with 50 ng/ml TNF-α for 4 hours. The luciferase activity was determined for three independent transfection reactions. The luminescence of unstimulated cells was substracted from that of TNF-α-stimulated cells, and the data were normalized relative to β-galactosidase activities. The asterisks indicate statistically significant differences in the S100A8/A9wt- and mutant-transfected cells. ■, TNF-α; ▨, TNF-α + 10 μM NF-κB inhibitor III; □, TNFα + 5 μM Mn-Cpx.

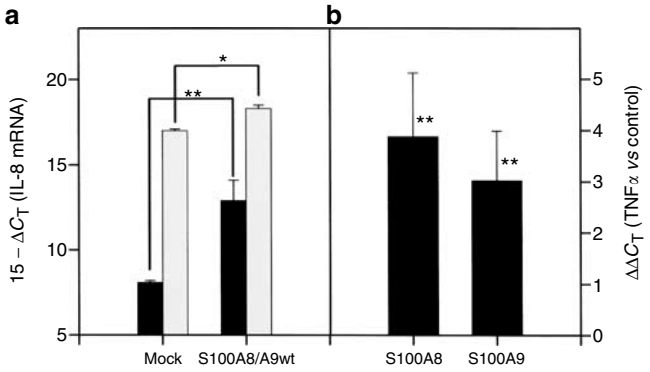

Figure 7. TNFα-modulated gene expression in mock- and S100A8/A9-overexpressing HaCaT keratinocytes. (**a**) TNF-α-induced enhancement of IL-8 and (**b**) S100 gene expression. (**a**) HaCaT cells were transfected with empty pVIVO or the pVIVO-S100A8/A9wt expression plasmids. Twenty-four hours after transfection, the cells were cultured in the presence and absence of 50 ng/ml TNF-α for an additional 18 hours. Total RNA was then extracted from the cells, and IL-8 gene expression analysis was performed as described in the Materials and Methods section. C_T values of IL-8 were normalized to GAPDH and final data are shown as $15-\Delta C_T$ values. The number of experiments was $n=6$. (**b**) HaCaT cells were cultured in the presence or absence of 50 ng/ml TNF-α for 18 hours. Total RNA was then extracted from the cells, and S100 gene expression was analyzed as described in the Materials and Methods section. S100 gene expression is given in relation to the corresponding S100 gene expression in unstimulated cells: $\Delta\Delta C_T$ S100 (TNF-α) = ΔC_T S100 (TNF-α) $-\Delta C_T$ S100 (nonstimulated). The asterisks indicate statistically significant differences in the S100A8/A9wt- and mutant-transfected cells. The number of experiments was $n=4$.

plasmid and either the S100A8/A9 mutant or the S100A8/A9wt expression plasmid (data not shown). The TNF-α-induced reporter expression was completely blocked by cotreatment with NF-κB inhibitor III. Interestingly, the cell-permeable superoxide dismutase mimetic Mn-Cpx3 partly reduced the TNF-α-induced expression of luciferase. The precise signaling mechanism by which NOX-generated ROS accelerate NF-κB activation still remains to be elucidated. However, these data indicate that S100A8/A9-enhanced NADPH oxidase affects downstream signaling.

Increased IL-8 production in S100A8/A9-transfected HaCaT cells

Normally, cells secrete very low amounts of IL-8, a member of the CXC chemokine family. However, its production is rapidly induced by proinflammatory cytokines, bacterial or viral products, or cellular stress via NF-κB activation (Hoffmann *et al.*, 2002). Therefore, we investigated IL-8 expression in mock-transfected and S100A8/A9wt-overexpressing cells by real-time PCR. As shown in Figure 7a, IL-8 gene expression is $n^{4.8}=27.9$ and $n^{1.3}=2.5$-fold increased in S100A8/A9-overexpressing HaCaT keratinocytes compared to unstimulated and TNF-α-treated mock-transfected cells, respectively. These data indicate enhanced NF-κB activation in S100A8/A9-overexpressing HaCaT keratinocytes.

Remarkably, TNF-α-stimulation also resulted in increased levels of S100 mRNA transcripts. S100A8 and S100A9 gene expression was increased $n^{3.9}=14.7$ and $n^{3.02}=8.1$-fold compared to unstimulated cells, respectively (Figure 7b). This is consistent with a recent analysis of NF-κB-dependent TNF-α-regulated genes in epidermal keratinocytes (Banno *et al.*, 2005). Conversely, as shown in our study, S100 gene overexpression enhances NF-κB-dependent gene expression, probably representing another loop by which the inflammatory response could be prolonged.

DISCUSSION

This study strongly indicates that in addition to phagocyte NADPH oxidase, the two S100 proteins also promote NADPH oxidase activation in nonphagocytic cells. This finding has important implications because the two S100 genes, S100A8 and S100A9, are expressed in epidermis in response to stress (for review see Eckert *et al.* (2004)).

In our initial experiment, overexpression of S100A8/A9 had different effects on NADPH oxidase activation in epithelial carcinoma HeLa and keratinocyte-like HaCaT cells after PMA stimulation. On the basis of PCR analyses, these differences were attributed to distinct patterns of expression of various NOX and *phox* homologs. Epithelial HeLa cells expressed only Nox2. Their complementation with S100A8 and S100A9 by gene transfection caused a significant increase in NADPH oxidase activity after PMA stimulation. This is consistent with another study (Berthier *et al.*, 2003).

S100A8 and S100A9 form heteromeric protein complexes that specifically bind AA (Kerkhoff *et al.*, 1999a). This AA-binding capacity plays an important role in the molecular mechanisms by which S100A8/A9 enhances NADPH oxidase (Kerkhoff *et al.*, 2005). Consequently, the mutant S100A8/A9(H103,104,105 K106A) protein complex, which is unable to bind AA, failed to promote PMA-induced NADPH oxidase

activity in intact HeLa cells. These data increase our knowledge about the cellular function of AA binding to S100A8/A9, as the failure of the S100A8/A9(H103,104,105K106A) mutant complex to promote phagocyte NADPH oxidase activity has been demonstrated by *in vitro* assays (Kerkhoff *et al.*, 2005). Transfer of AA via S100A8/A9 is clearly important for S100A8/A9-mediated NADPH oxidase activation.

A number of neutrophil responses, including the respiratory burst activity, depend on activation of the p38 mitogen-activated protein kinase (MAPK) pathway (Nick *et al.*, 1996; McLeish *et al.*, 1998; Ward *et al.*, 2000; Brown *et al.*, 2004), and S100A9 has been shown to be a p38 MAPK substrate (Vogl *et al.*, 2004; Lominadze *et al.*, 2005). Overexpression of the S100A8/A9(T113A) mutant in HeLa cells significantly reduced the stimulatory effect on NADPH oxidase activity in comparison with S100A8/A9wt, indicating that phosphorylation of S100A9 might have a regulatory role. S100A9 phosphorylation has been implicated in various pathways, such as translocation of S100A8/A9 to membranes and the cytoskeleton in monocytes (Guignard *et al.*, 1996; van den *et al.*, 1996). Furthermore, S100A8 and S100A9 are associated with low-density detergent-resistant membranes (DRMs) (Nacken *et al.*, 2004), and the NADPH oxidase complex is assembled in cholesterol-enriched membrane microdomains (lipid rafts) (Vilhardt and van Deurs, 2004). It is noteworthy that phosphorylation does not affect the substrate affinity of S100A8/A9 for AA (Kerkhoff *et al.*, 1999b).

In contrast to HeLa cells, PMA did not significantly enhance NADPH oxidase activity in HaCaT keratinocytes after S100A8/A9 gene complementation. PCR analysis revealed the presence of mRNA transcripts for Nox1, Nox2, and Nox5. The presence of Nox5 in HaCaT keratinocytes is a novel finding, as Nox1 has been identified as the major ROS-generating enzyme in these cells by two recent studies (Chamulitrat *et al.*, 2003, 2004). However, the authors did not investigate whether Nox5 is also expressed. The relevance of Nox5 mRNA transcripts was demonstrated by the response of NADPH oxidase activity to elevated intracellular Ca^{2+}. Nox5 is distinguished from other NADPH oxidases by its unique Ca^{2+}-binding N-terminal EF-hands (Banfi *et al.*, 2001, 2004). This also accords with other reports showing transient ROS production in HaCaT cells after stimulation with EGF, thapsigargin, and A23187 (Goldman *et al.*, 1997, 1998). However, Nox5 needs to be confirmed by Western blotting in further studies.

In response to A23187, HaCaT cells overexpressing S100A8/A9 showed a drastic increase in NADPH oxidase activity compared to mock-transfected cells, strongly indicating that S100A8/A9 also enhances the calcium-inducible oxidase activity. Correspondingly, HeLa cells exhibited an A23187-inducible NADPH oxidase activity after transfection with the Nox5 expression plasmid. The concomitant expression of S100A8/A9 resulted in enhanced ROS generation after A23187 stimulation (data not shown).

Like Nox5, Nox1 was also promoted by S100A8/A9. Concomitant expression of Nox1, NOXO1, NOXA1, and S100A8/A9 increased the stimulus-independent NADPH oxidase activity in comparison to HeLa cells co-transfected with Nox1, NOXO1, and NOXA1. Low levels of NOXA1 and NOXO1 mRNAs were found in HeLa cells by PCR analysis. In accordance with this finding, overexpression of Nox1 and S100A8/A9 also enhanced NADPH oxidase activity in comparison with that in Nox1-transfected HeLa cells (data not shown).

NOXA1 and NOXO1 are physiologically relevant partners of Nox1 (Banfi *et al.*, 2003), but they also interact with other NOX homologs thereby mediating stimulus-independent oxidase activation (Banfi *et al.*, 2003; Takeya *et al.*, 2003). Consequently, overexpression of NOXA1 and NOXO1 resulted in enhanced unstimulated oxidase activity, which might resemble the constitutive activation of the endogenous Nox2. Interestingly, S100A8/A9 did not enhance this NOXA1/NOXO1-mediated stimulus independent oxidase activation.

In early work exploring the function of Nox1, heterologous overexpression of Nox1 in NIH-3T3 cells was associated with increased cell proliferation and resulted in tumor formation when these cells were injected into nude mice (Suh *et al.*, 1999). However, further studies revealed that these Nox1-transfected NIH-3T3 cell lines also carry a mutant form of Ras that could account for the enhanced proliferation and transformation (for review see reference Lambeth (2004)). Other observations suggest that Nox1 could serve as a host defense oxidase (Kawahara *et al.*, 2001, 2004). This is an interesting observation as S100 protein expression is induced under inflammatory conditions, so S100 proteins may enhance NADPH oxidase activity to perform host defense functions.

What consequences might S100A8/A9-enhanced ROS generation have in HaCaT keratinocytes? Reactive superoxides are known to modulate the redox status of individual components of signaling pathways, thereby affecting downstream signaling. For example, NF-κB activation, which in turn induces the expression of different gene encoding proteins involved in the inflammatory and immune responses such as TNF-α, IL1, IL-6, and IL8, is regulated by H_2O_2 (Zhang and Chen, 2004). In accordance with these findings, we demonstrated that S100A8/A9-overexpressing HaCaT keratinocytes showed increased NF-κB activity as well as elevated levels of IL-8 mRNA transcripts after TNF-α stimulation. Both responses have important implications for the (patho)physiological circumstances in which the expression of the S100 genes is induced in epithelial cells, such as the stress response.

NF-κB, or components of the system such as IKK-α, is involved in epidermal proliferation and differentiation. For example, mice overexpressing a constitutively nuclear form of the NF-κB subunit p50 in the skin showed growth inhibition of the epidermis (Seitz *et al.*, 1998). IKKα knockout has a severe cutaneous phenotype with incomplete epidermal differentiation (Hu *et al.*, 1999; Takeda *et al.*, 1999). Mice with an epidermis-specific deletion of IKK-β develop a severe inflammatory skin disease, which is caused by a TNF-α-mediated, $\alpha\beta$ T-cell-independent inflammatory response

that develops shortly after birth (Pasparakis *et al.*, 2002). Conversely, overexpression of inhibitory κB causes hyperplasia and inflammation and leads to squamous-cell carcinomas (van *et al.*, 1999; Lind *et al.*, 2004).

IL-8 is regulated by NF-κB, and exhibits a dual function in wound healing because it contributes to the regulation of re-epithelialization, tissue remodeling, and angiogenesis (Gillitzer and Goebeler, 2001), and is also involved in recruiting leukocytes to the site of inflammation (Baggiolini, 1998). Moreover, IL-8 primes the oxidative burst in neutrophils (Guichard *et al.*, 2005). Thus, alterations of NF-κB activation play a crucial role in the development of skin diseases.

Moreover, excessive ROS generation may propel the cellular redox balance to a more prooxidant state that favors oxidative damage and the apoptotic pathway. In fact, there is growing evidence that ROS are involved in the development of psoriatic lesions (Utas *et al.*, 2002; Briganti and Picardo, 2003; Yildirim *et al.*, 2003). Uncontrolled production of ROS leads to peroxidative damage to membranes in the skin, a tissue that is particularly vulnerable to the effects of these species (Trenam *et al.*, 1992). Both increased ROS and insufficient antioxidant activity have been found in psoriatic lesions (Trenam *et al.*, 1992; Yildirim *et al.*, 2003). Together with the accumulation of S100 proteins in psoriasis keratinocytes and their enhancement of both NADPH oxidase and NF-κB activation, as shown in this study, it is tempting to speculate that these events may lead to an exacerbated inflammatory response that fails to resolve, leading eventually to activation of lymphocytes that may propagate disease development through autoimmune mechanisms. Therefore, both enhancement of NF-κB activation and increased IL-8 levels indicate that S100A8/A9 has a prominent role in skin pathology.

MATERIALS AND METHODS

Plasmids

For the construction of pVIVO-S100A8/A9, both hS100A9 and hS100A8 cDNAs were used as templates to generate PCR fragments with suitable cloning ends. The PCR fragments were purified and cloned into pVIVO-mcs (InvivoGen, San Diego) according to standard protocols. Individual clones were analyzed by PCR and SDS–PAGE.

Both pVIVO-S100A8/A9(H103,104,105K106A) and pVIVO-S100A8/A9(T113A) were generated using a QuickChangeTM Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions.

The pcDNA3.1-hNOXO1, pcDNA3.0-hNox1, and pCMV-SPORT6-hNOXA1 expression plasmids were kindly provided by Dr JD Lambeth (Emory University School of Medicine, Atlanta, GA, USA). The various cDNAs were amplified by PCR and cloned into the appropriate expression vectors using specific primers with specific restriction sites. All constructs were tested by sequencing.

Cell culture

HeLa (human cervix epithelial carcinoma cell line) and HaCaT (human keratinocyte cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biochrom AG, Berlin, Germany) containing 10% heat-inactivated fetal bovine serum (Biowest, Nuaille, France), 100 U of penicillin (Biochrom AG) and 2 mM L-glutamine (Biochrom AG) at 37°C in a humidified atmosphere with 5% CO_2. The Ethical Committee of the University Hospital (Muenster, Germany) approved all described studies. The study was conducted according to the Declaration of Helsinki Principles.

Expression studies of Nox and *phox* homologs

Total RNA was extracted from HeLa and HaCaT cells using an RNA Isolation Kit (Qiagen, Hilden, Germany), and first strand cDNA was synthesized according to common molecular biological techniques. The presence of mRNA transcripts for Nox1, Nox2, Nox3, Nox4, Nox5, $p22^{phox}$, $p67^{phox}$, $p47^{phox}$, NOXA1, NOXO1, Rac-1, and Rac-2 was determined by real-time PCR using specific primers (Table S2). The primers were obtained from MWG (Ebersberg, Germany).

Transient expression of S100A8, S100A9, and Nox homologs

For transient transfections HeLa and HaCaT cells were seeded in six-well plates 24 hours before transfection (2.5×10^5 cells/well). The Lipofectamine (Invitrogen, Karlsruhe, Germany) transfection reagent was used according to the manufacturer's instructions with some modifications. In brief, 1.5 μg of total plasmid DNA and 7 μl Lipofectamine were diluted to a final volume of 800 μl with pure DMEM and added to the cells. In co-transfection experiments, 1:1 ratios of the reporter and/or expression plasmids were used. The final concentration of transfected DNA was kept constant in all co-transfection assays. The medium was changed 5 hours after transfection. The cells were cultured for 24 and/or 48 hours, and used for the appropriate tests. Cell viability was determined 12 hours post-transfection by the trypan blue exclusion assay.

S100A8 and S100A9 expression was confirmed by Western blotting using antibodies directed against each protein. Expression of the other transfected genes was shown by Taqman analysis using a gene-specific forward primer and a reverse primer directed against either the bovine growth hormone polyadenylation sequence (for pcDNA3.1 and pcDNA3.0 expression plasmid) or the SV40 polyadenylation sequence (for pCMV-SPORT6 expression plasmid).

Measurement of NADPH oxidase activity

Superoxide production by the indicated cells was determined by a quantitative NBT assay or by an isoluminol-amplified chemiluminescence method as described previously (Liu *et al.*, 1996). Briefly, for the NBT test, the cells ($0.5–2 \times 10^6$) were incubated in phosphate-buffered saline (PBS) containing 2 mg/ml NBT (Roth, Karlsruhe, Germany) with or without 100 ng/ml PMA (Sigma-Aldrich, München, Germany) for 1 hour at 37°C. The expression levels of the S100 genes and the Nox isoforms were not altered during stimulation (data not shown). Finally, the cells were fixed and washed with methanol to remove the nonreduced NBT. The formazan product was obtained by centrifuging the sample at 700 *g* for 10 minutes. The supernatant was discarded and the reduced formazan was dissolved in 230 μl 2 M potassium hydroxide and in 280 μl DMSO. The absorbance of the solution was measured at 630 nm. The data are expressed as absorbance units/10^6 cells.

For chemiluminescence measurements, cells were suspended at 1×10^5 cells/ml in PBS (Biochrom AG) supplemented with 10 μM isoluminol (Sigma) and 4 U/ml horseradish peroxidase (Roche AB, Roche Applied Science, Mannheim, Germany) in the presence or absence of 100 ng/ml calcium ionophore A23187 (Sigma). Samples

were detected directly. Photon emission was followed at room temperature in a Centro LB 960 luminometer.

Transient transfection and luciferase activity assays

Transient transfection of HaCaT cells with different combinations of NF-κB luciferase reporter plasmid and eVIVO, pVIVO-S100A8/A9wt, pVIVOS100A8/A9(H103,104,105K106A), and pVIVO-S100A8/A9(T113A) expression plasmids was performed using Lipofectamine according to the manufacturer's recommended protocol. Luciferase assays were performed using the Promega luciferase assay system (Promega). Following treatments, cells were washed twice with cold PBS and lysed with 75 μl cell culture lysis reagent (Promega). About 5 μl of the resulting supernatant was mixed with 50 μl luciferase assay solution and the luminescence was determined using a Centro LB 960 luminometer. All experiments were performed in triplicate. The luminescence of unstimulated cells was substracted from that of TNF-α-stimulated cells, and the data were normalized relative to β-galactosidase activities. The data are shown as means $\pm$ SEM.

Quantitative PCR

HaCaT cells were transfected with either empty pVIVO (mock) or pVIVO-S100A8/A9 expression plasmids. After 24 hours, the cells were cultured in the presence or absence of 50 ng/ml TNF-α for an additional 18 hours. Total RNA was then extracted using an RNA Isolation Kit (Qiagen) and first strand cDNA was synthesized according to common molecular biological techniques. The IL-8, S100A8, and S100A9 mRNA levels were estimated by real-time PCR using specific primers (Table S2). The C_T values of target genes were normalized to GAPDH and are thus presented as ΔC_T (C_T of target minus C_T of GAPDH). Final data are shown as 15-ΔC_T values. This presentation was used to show that high ΔC_T values represent high expression of the target RNA. S100 gene expression is given in relation to the corresponding S100 gene expression in unstimulated cells: ΔC_T S100 (TNF-α) $-$ ΔC_T S100 (nonstimulated) $=$ $\Delta\Delta C_T$ S100 (TNF-α)

Data analysis

Data from measurements of ROS production, NF-κB activation, and IL-8 gene expression are given as means $\pm$ SEM. Statistical significance of differences between means was established by Student's *t*-test, and probability values of $P<0.05$ were considered to represent significant differences.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Expression of various Nox and *phox* homologues, and of GTPases Rac1 and Rac2 in HeLa cells and HaCaT keratinocytes as shown by PCR analysis.

Table S2. DNA sequences of RT-PCR primers.

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