Psoriasis Is Associated with Low Serum Levels of Hydrogen Sulfide, a Potential Anti-inflammatory Molecule

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Psoriasis, characterized by circumscribed, red, thickened plaques with an overlying silver-white scale, is a common T-cell-mediated chronic inflammatory skin disease. Although hydrogen sulfide (H2S) has been shown to be a signaling molecule with both pro- or anti-inflammatory effects, its relationship with psoriasis has not been elucidated. In the present study, 15 patients with chronic progressive psoriasis and 15 healthy volunteers were investigated. Serum H2S levels in psoriasis patients were significantly lower than those of healthy controls (16.69 ± 5.47 μM vs. 34.5 ± 6.39 μM). In contrast, serum levels of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) were significantly higher in psoriasis patients than healthy controls (22.88 ± 6.24 pg/ml vs. 12.07 ± 3.68 pg/ml; 61.47 ± 8.21 pg/ml vs. 31.54 ± 13.73 pg/ml; and 39.43 ± 8.56 pg/ml vs. 20.55 ± 6.45 pg/ml, respectively). The serum H2S levels negatively correlated with clinical disease severity. Furthermore, treatment of HaCaT human keratinocytes with TNF-α increased the levels of nitric oxide (NO), IL-6 and IL-8 (32.21 ± 5.71 μM vs. 3.22 ± 0.98 μM; 203.96 ± 13.16 pg/ml vs. 13.57 ± 3.75 pg/ml; and 301.24 ± 30.17 pg/ml vs. 29.06 ± 10.91 pg/ml, respectively) in the culture media. Exogenous H2S inhibited the TNF-α-mediated upregulation of NO, IL-6 and IL-8 in a dose-dependent manner. In addition, H2S inhibited TNF-α-mediated activation of p38, extracellular-signal-regulated kinase and nuclear factor kappa B. In conclusion, H2S may play a protective role in the pathogenesis of psoriasis. H2S-releasing agents may be promising therapeutics for psoriasis.

Keywords: hydrogen sulfide; inflammatory cytokines; mitogen-activated protein kinases; nuclear factor kappa B; psoriasis

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Psoriasis is a common chronic inflammatory skin disease characterized by circumscribed, red, thickened plaques with an overlying silver-white scale. Recent genetic and immunological advances have greatly increased our understanding of the pathogenesis of psoriasis (Cai et al. 2012; Sigurdardottir et al. 2012). Now, it is widely accepted that genetic predisposition and environmental factors have a profound effect on the immune system and play a crucial role in triggering psoriatic lesion development (Krueger and Bowcock 2005; Traub and Marshall 2007). Keratinocytes, the major cell type of the epidermis, constitute more than 90% of epidermal cells (Nestle et al. 2009). With the understanding of the immune mechanisms of psoriasis, the role of the keratinocytes in psoriasis has changed from a passive bystander to an active participant, which is supported by the ability of these cells to synthesize numerous proinflammatory cytokines, chemokines, and growth factors, such as tumor necrosis alpha (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), as well as prostaglandins (PGs) upon stimuli (Pei et al. 1998; Yu et al. 2002; Wolfram et al. 2009).

Hydrogen sulfide (H2S), which is known as a toxic gas, is endogenously generated from cysteine and homocysteine in many, if not all, tissues by two cytosolic enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Mizumura et al. 2010; You et al. 2011). Furthermore, it has also been shown that mitochondrial, 3-mercaptopypyruvate sulfurtransferase (3-MPST) plays an important role in the synthesis of sulfide (Wang 2002; Moore et al. 2003; Olson 2011). H2S, together with nitric oxide (NO) and carbon monoxide (CO), are included in the family of gaseous transmitters. Now the variety of H2S effects starts to be understood at both the cellular and molecular levels (Li et al. 2009; Li et al. 2011; Wallace et al. 2012). H2S is able to induce the upregulation of anti-inflammatory genes, including vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) receptor and several genes associated with the transforming growth factor-β (TGF-β) receptor pathway (Mancardi et al. 2009). However, H2S is also able to exert anti-inflammatory effect (Li et al. 2009).
In the brain and heart, it has been shown that H2S can be an antagonist of oxidative damage (Whiteman and Winyard 2011). However, the role of H2S in psoriasis has not been clearly elucidated.

To clarify the role of endogenous H2S in the pathogenesis of psoriasis, we examined the serum H2S level in patients with psoriasis and its relation to serum TNF-α, IL-6, IL-8 levels and disease severity, as evaluated by the psoriasis area and severity index (PASI). We also studied the effects of exogenous H2S on the inflammatory responses induced by TNF-α in keratinocytes and explored the potential mechanisms.

Material and Methods

Patients and controls

Fifteen patients with chronic progressive psoriasis were recruited from clinics at Sun Yat-sen Memorial Hospital, Sun Yat-sen University, China. None of the patients with progressive psoriasis had been treated with systemic immunosuppressants in 3 months before the study began. On the day of sampling, they began to receive treatment of cyclosporin A plus alfalcacidol (ALFA-D3, Alfarol Chugai Tokyo, Japan) based on the degree of severity. Treatments were often combined, or switched around every 12 to 24 months to reduce resistance and adverse reactions. The study was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital.

The control group comprised fifteen age- and gender-matched healthy volunteers. Venous blood samples (5 ml) were taken into vacutainer tubes under sterile conditions from patients and controls between 08:30-10:30 am. Blood samples were rapidly centrifuged at 560 × g for 20 min and then serum was collected and stored at −70°C until analysis. On the days of blood collection, clinical severity of the disease was evaluated by PASI score.

Measurement of serum H2S

Aliquots (75 μl) of serum were added to 300 μl of Zn acetate (2%) dissolved in 50 mM potassium phosphate buffer (pH 8.0). After incubation at room temperature for 90 min, 400 μl of 10% trichloroacetic acid was added into the reaction mixture. The mixture was centrifuged at 14,000 × g for 10 min at 4°C, and 400 μl of the clear supernatant was collected and mixed with N,N-dimethyl-p-phenylenediamine sulfate (20 μM; 40 μl) in 7.2 M HCl and FeCl3 (30 μM; 40 μl) in 1.2 M HCl. After 15 min of incubation, the absorbance of the resulting solution was measured at 670 nm with a microplate reader. All samples were assayed in triplicate, and H2S was calculated against a calibration curve. The calibration curve of absorbance versus H2S concentration was obtained using NaHS solution of varying concentrations. When NaHS is dissolved in water, H2S is released and forms H2S with H+.

Cell culture

HaCaT, an immortalized cell line of human epidermal keratinocytes, was obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Grand Island, NY, USA) with 0.45% glucose, 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G and 100 U/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2 and 95% air.

MTT assay

Cell viability was assessed by MTT assay. HaCaT cells were plated into 96-well culture plates at a density of 105 cells/well and incubated with medium or NaHS (20-400 μM) for 24 hours. Then, MTT assay was performed as previously described (Du et al. 2011). NaHS was purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

Cell treatment

Cells (2×104 cells/ml) were seeded in 100-mm dishes and incubated overnight. After washing with phosphate buffered solution (PBS), fresh medium was added before treatment. Cells were pretreated for 30 min with different concentrations of NaHS or pyrrolidine dithiocarbamate (PDTC), an inhibitor for NF-κB activation, and then were stimulated with TNF-α for indicated times. PDTC was purchased from Sigma-Aldrich.

Determination of cytokine concentrations

The levels of serum TNF-α, IL-6, IL-8 and culture supernatant IL-6, IL-8 were assayed by enzyme-linked immunosorbent assay (ELISA) using commercial kits from Shanghai ExCell Biology Inc. (Shanghai, China) according to the manufacturer’s instructions.

RNA preparation and Real time reverse transcription polymerase chain reaction

Cells were lysed directly in TRZol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted according to the supplier’s instruction. Total RNA (5 μg) was reverse-transcribed into cDNA using TIANScript kit (Beijing TIANGEN, Beijing, China). Primers specific for human IL-6, IL-8 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which sequences were shown in Table 1, were designed using the Primer Express 3.0 software. The amplification of the cDNA was accomplished in triplicate using an ABI7000 real-time polymerase chain reaction (PCR) instrument in the presence of the commercially available SYBR Green PCR Master Mix (Beijing TIANGEN). The cDNA was amplified under the following conditions: 95°C for 5 min for denaturation and subjected to 40 cycles of 95°C for 45 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed extension at 72°C for 10 min. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product, which was displayed as a single peak (data not shown). The relative expression ratio (R) of a target gene was expressed for the sample versus the control in comparison to GAPDH. R was calculated based on the following equation: $R = 2^{-\Delta\Delta Ct}$, where the cycle number at the threshold level of log-based fluorescence is defined as Ct value. $\Delta\Delta Ct$ was $(Ct_{\text{target}} - Ct_{\text{GAPDH}})$ treatment $-$ $(Ct_{\text{target}} - Ct_{\text{GAPDH}})$ control.

Assay of nitrite

The concentration of nitrite in the culture medium related to the amount of NO secreted by HaCaT cells was measured. Briefly, an aliquot of the culture supernatants (100 μl) was placed in triplicate in a 96-well plate and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride, 2.5% H3PO4) at room temperature for 10 min. The absorbance at 540 nm was determined using a microplate reader. Nitrite was determined using sodium nitrite as a standard.
Protein extraction and western blot analysis

For western blot analysis, the whole cell extracts and nuclear protein were extracted and the concentrations of all the samples were measured by bicinchoninic acid (BCA) method. Protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then was transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with Tween-20 (TBST) for 1 hour at room temperature and then incubated overnight at 4°C with specific primary antibodies in blocking solution. iNOS antibody, phospho-I-κBα antibody (Ser32/36), NF-κB p65 antibody, GAPDH antibody and histone H2.X antibody were purchased from Santa Cruz Biotechnology, inc (Santa Cruz, CA, USA). Phospho p38 mitogen-activated protein kinase (p-p38 MAPK) (Thr180/Tyr182) antibody, p38 MAPK antibody, Phospho extracellular-signal-regulated kinase (pERK) (Thr202/Tyr204) antibody, ERK antibody, phosphor-c-Jun N-terminal kinases (pJNK) (Thr183/Tyr185) antibody and JNK antibody were purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). After being washed three times in TBST, the membrane was incubated with appropriate horseradish peroxidase conjugated secondary antibody for 1 hour and the antibody-specific protein was visualized by enhanced chemiluminescence detection system.

Statistical analysis

Results were expressed as means ± standard error of the mean (SEM). Statistical analyses were performed by the Student’s t-test to express the difference between two groups. Regression analysis was performed by use of Spearman rank correlation coefficients; p < 0.05 was considered significant. SPSS 13.0 computer program was used for statistical analysis.

Results

Serum levels of H₂S, TNF-α, IL-6, and IL-8 in patients with psoriasis and their correlation with disease severity

Fifteen patients with chronic progressive psoriasis (6 males, 9 females, mean age 35.5 ± 18.4, range: 9-68 years) and 15 healthy controls (8 males, 7 females, mean age 33.4 ± 20.8, range: 8-69 years) were enrolled in this study. The cell counts in whole blood and C-reactive protein of all participants were in normal ranges. The average PASI score in patients with chronic progressive psoriasis was 11.9 ± 4.8 (range: 5.3-22.6).

Serum H₂S levels were lower in patients with psoriasis compared with those of healthy controls (16.69 ± 5.47 µM vs. 34.5 ± 6.39 µM, Fig. 1). In contrast, serum levels of TNF-α, IL-6 and IL-8 were significantly higher in patients with psoriasis than those of healthy controls (22.88 ± 6.24 pg/ml vs. 12.07 ± 3.68 pg/ml; 61.47 ± 8.21 pg/ml vs. 31.54 ± 13.73 pg/ml; and 39.43 ± 8.56 pg/ml vs. 20.55 ± 6.45 pg/ml, respectively, Fig. 2). As shown in Table 2, serum levels of H₂S in patients with psoriasis were correlated negatively with serum levels of IL-6, IL-8, and TNF-α and with PASI scores.

Effect of H₂S on cell viability in HaCaT cells

H₂S exerts various effects on a number of biological targets resulting in responses that range from cytotoxic effects to cytoprotective actions (Whiteman and Winyard 2011). In HaCaT cells used here, no obvious cytotoxicity was observed when the cells were exposed to NaHS (up to 400 µM) for 24 hours (Fig. 3).
Effects of H$_2$S on the secretion of IL-6 and IL-8 in HaCaT cells

Keratinocytes have been shown to synthesize both IL-6 and IL-8 in response to various stimuli, including phorbol esters and TNF-α (De Vry et al. 2005; Ikuta et al. 2008; Sun et al. 2012). As shown in Fig. 4A, resting HaCaT cells secreted low levels of IL-6 ($13.57 \pm 3.75$ pg/ml) and IL-8 ($29.06 \pm 10.91$ pg/ml). However, in the presence of 10 ng/ml TNF-α, HaCaT cells secreted larger amounts of IL-6 ($203.96 \pm 13.16$ pg/ml) and IL-8 ($301.24 \pm 30.17$ pg/ml). The upregulation of IL-6 and IL-8 was significantly inhibited by the treatment with PDTC ($30.98 \pm 8.57$ pg/ml and $72.26 \pm 16.00$ pg/ml, respectively). Dose-response experiments showed that NaHS was able to prevent the TNF-α-induced IL-6 and IL-8 protein secretion in HaCaT cells in a dose-dependent manner. Likewise, TNF-α remarkably increased the expression levels of IL-6 and IL-8 mRNAs, but induction was prevented with NaHS (Fig. 4B).

Effects of H$_2$S on the secretion of IL-6 and IL-8 in HaCaT cells

To investigate whether H$_2$S could inhibit the TNF-α-induced NO production (Yamaoka et al. 2000), nitrite in the culture medium was increased to $32.21 \pm 5.71$ μM from $3.22 \pm 0.98$ μM, and the expression of iNOS protein was increased (Fig. 4C and D). Importantly, NaHS inhibited the release of NO and the expression of iNOS in a dose-dependent manner.

Effects of H$_2$S on TNF-α-mediated activation of MAPKs and NF-κB pathways in HaCaT cells

Accumulating data show that the TNF-α-mediated upregulation of iNOS, IL-6 and IL-8 depends on MAPKs and NF-κB pathways (Cho et al. 2007; Amigo et al. 2008). However, whether the effect of H$_2$S is exerted through inhibiting MAPKs and NF-κB is unknown yet. As shown in Fig. 5A, there was no obvious change in total protein levels of each MAPK kinase, but the p-p38 MAPK, pERK and pJNK were dramatically increased in the TNF-α-treated cells. NaHS treatment inhibited the phosphorylation of p38 MAPK and ERK, but not JNK. In non-activated cells, NF-κB is bound to IkappaB (I-κB) and is retained in the cytoplasm. Activation of NF-κB is contingent upon phosphorylation, polyubiquitination, and proteolysis of IκBs with subsequent NF-κB translocation to the nucleus. As shown in Fig. 5B, nuclear p65 and cytoplasmic phosphorylated I-κBα were significantly increased in TNF-α-treated HaCaT cells compared with control cells. The TNF-α-induced NF-κB activation was inhibited with PTDC or...
NaHS in a dose-dependent manner.

**Discussion**

In the present study, we investigated the role of H\(_2\)S in psoriasis and the potential anti-inflammatory effect in vitro. H\(_2\)S has been implicated to play an important role in several physiological and pathophysiological processes such as long term synaptic potentiation, vasorelaxation, pro- and anti-inflammatory conditions, cardiac inotropism regulation, cardioprotection, and several other physiological mechanisms (Gobbi et al. 2009). In the present study, we found that the serum level of H\(_2\)S in psoriasis patients was down-regulated and the levels of several inflammatory cytokines were increased significantly. In vitro study showed that H\(_2\)S could inhibit TNF-\(\alpha\)-induced inflammatory responses in keratinocytes. Further studies indicate that p38 MAPK, ERK and NF-\(\kappa\)B pathways are involved in the anti-inflammatory effect of H\(_2\)S.

It has been reported that serum levels of several cytokines are increased in psoriatic patients, at both local and systemic levels, including interferon gamma (IFN-\(\gamma\)), TNF-\(\alpha\), IL-6, IL-8, IL-2, IL-12 and IL-18, all of which are regarded as hallmark cytokines in psoriatic cytokine network (Kemeny et al. 1994; Arican et al. 2005; Zalewska et al. 2006). In this study, we also observed high serum levels of TNF-\(\alpha\), IL-6 and IL-8 in psoriasis patients, whereas the serum H\(_2\)S levels were very low in patients with psoriasis. In addition, the serum levels of H\(_2\)S correlated negatively with serum levels of IL-6, IL-8, and TNF-\(\alpha\) and PSAI score, the latter of which is often quoted in clinical trials to measure the severity and extent of psoriasis based on area coverage and plaque appearance. These results indicate that psoriasis is associated with low serum levels of H\(_2\)S, which may play a protective role in the pathogenesis of psoriasis.

As the major resident cell type in the epidermis, keratinocytes produce various cytokines in response to diverse stimuli and subsequently affect Langerhans cells, lymphocytes, vascular endothelial cells, and keratinocytes themselves to regulate immune responses and inflammatory reactions (Grossman et al. 1989; Sabat et al. 2007; Hattori et al. 2008; Hong et al. 2010). Among the proinflammatory cytokines, IL-6 and IL-8 have been implicated in epidermal function and cutaneous inflammation. IL-6 mediates T-cell activation and the acute phase responses at the beginning of acute inflammation and IL-8 exerts a critical role as a potent chemoattractant for neutrophils and T lymphocytes. Both IL-6 and IL-8 can stimulate the proliferation of keratinocytes. The abnormally high levels of these cytokines and the expression pattern of both receptors in psoriasis would be associated with the hyperproliferation of keratinocytes.
and accumulation of neutrophils, which are characteristic findings of psoriatic lesions. TNF-α could induce the upregulation of IL-6, IL-8, IL-12, and IL-18 and constitute an important link in the cytokine network in the pathogenesis of psoriasis (Schottelius et al. 2004; Jeong et al. 2010). In this study, we observed that the constitutive production of IL-6 and IL-8 by resting keratinocytes was low. However, the production of both cytokines increased significantly in the presence of TNF-α. Pretreatment with NaHS was able to prevent IL-6 and IL-8 protein secretion and gene transcription in HaCaT cells in a dose-dependent manner.

NO is a highly reactive molecule produced from a guanidino nitrogen of L-arginine in a reaction catalyzed by a family of NO synthase (NOS), of which iNOS is capable of producing high amounts of NO during disease, injury, and inflammation. iNOS is not expressed in normal skin (Ormerod et al. 1998), but it can be induced by many cytokines and bacteria-derived products in keratinocytes (Higuchi et al. 1990; Portugal-Cohen and Kohen 2009). Recent evidence showed that iNOS was significantly upregulated in psoriatic skin (Ormerod et al. 1998) and it is the key component of the angiogenic cascade, keratinocyte proliferation, differentiation and psoriasis inflammation. It has been reported that H₂S could inhibit the production of NO and activation of NF-κB in macrophages stimulated by lipopolysaccharide (Oh et al. 2006), but the effect of H₂S on NO system in skin inflammation has not been investigated. Our results showed that H₂S inhibited markedly the NO release and iNOS expression in HaCaT cells stimulated with TNF-α, indicating H₂S is a potential anti-inflammatory molecule.

Transcription of TNF-α, IL-6, IL-8 and iNOS has been documented to be regulated by MAPKs and NF-κB pathways (Cho et al. 2007; Wang et al. 2010). Phosphorylated MAPKs can phosphorylate their cascade proteins on serine or/and threonine residues and direct the execution of appropriate genetic programs, including protein synthesis, cell death and differentiation. Our results showed that TNF-α induced the activation of MAPKs. The activation of p38 MAPK and ERK was inhibited by H₂S, while the phosphorylation of JNK was not inhibited. NF-κB is sequestered in the cytoplasm by IκB which could be phosphorylated, and degraded by the proteasome with subsequent NF-κB translocation to the nucleus (Kurylowicz and Nauman 2008; Tsuruta 2009). Blocking NF-κB activity with a specific inhibitor PDTC abolished the increase of IL-6, IL-8 and NO production in keratinocytes induced by TNF-α, indicating NF-κB is also involved in TNF-α-induced inflammatory response in keratinocytes (Kwon et al. 2011; Lee et al. 2011). In this study, pretreatment with H₂S also inhibited the activation of NF-κB, suggesting that the NF-κB pathway may also participate in the anti-inflammatory effect of H₂S.

In conclusion, our results indicate that endogenous H₂S may play a protective role in the pathogenesis of psoriasis. H₂S may exert anti-inflammatory effect via suppressing the activation of p38 MAPK, ERK and NF-κB pathways. Therefore, H₂S-releasing agents may be promising therapeutics for psoriasis.

Conflict of Interest
The authors declare no conflict of interest.

References
Arican, O., Aral, M., Sasmaz, S. & Ciragil, P. (2005) Serum levels of TNF-alpha, IFN-gamma, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. Mediators Inflamm., 2005, 273-279.


Wang, R. (2002) Two’s company, three’s a crowd: can H2S be the...


