Identification of copper/zinc superoxide dismutase as a nitric oxide-regulated gene in human (HaCaT) keratinocytes: implications for keratinocyte proliferation

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Recent studies have demonstrated an induction of expression of inducible nitric oxide synthase that is associated with several inflammatory diseases of the skin. To define the mechanisms of action of nitric oxide (NO) in the skin, we attempted to identify genes that are regulated by NO in keratinocytes. Using the human keratinocyte cell line HaCaT as a model system, we identified a Cu/Zn superoxide dismutase (SOD) that was strongly induced by high concentrations (500 μ M) of NO-donating agents {S-nitrosoglutathione, sodium nitroprusside and (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA-NO)}, but not by serum or by single recombinant growth factors and inflammatory cytokines or by treatment with superoxide anions. Furthermore, endogenously produced NO increased the expression of Cu/Zn SOD mRNA in keratinocytes. Moreover, treatment of HaCaT cells with NO was associated

with a biphasic effect on cell proliferation, because low doses (100 μ M) of different NO donors (S-nitrosoglutathione and DETA-NO) mediated a proliferative signal to the cells, whereas high concentrations (500 μ M) were cytostatic. To determine a possible correlation between the close regulation of Cu/Zn SOD expression and proliferation by NO in keratinocytes, we established a cell line (psp1CZ1N) carrying a human Cu/Zn SOD cDNA under the control of a ponasterone-inducible promoter construct. Ponasterone-induced overexpression of Cu/Zn SOD caused a cytostatic effect in proliferating psp1CZ1N cells. We therefore suggest that the up-regulation of Cu/Zn SOD expression by NO establishes an inhibitory mechanism on keratinocyte proliferation.

Key words: inflammation, psoriasis, skin.

INTRODUCTION

Within the past few years, the role of nitric oxide (NO) as an important mediator of physiological and pathophysiological processes has become evident. A prominent role for NO has been demonstrated in the regulation of blood vessel relaxation, the modulation of synaptic transmission, and bactericidal or tumoricidal activities of macrophages. NO is synthesized by three types of nitric oxide synthase (NOS). These NOS isoenzymes consist of two constitutive enzymes in neuronal and endothelial tissue, and an inducible NOS (iNOS) that releases large amounts of NO into the surrounding tissue during cytokine-triggered inflammatory processes [1–6]. All three NOS isoforms are homodimeric proteins dependent on NADPH, reduced flavins, haem-bound iron, and 6(R)-5,6,7,8-tetrahydrobiopterin as essential cofactors [7,8]. Among the different roles mediated by NO, it has become clear that NO affects the regulation of an increasing number of genes [9–13]. NO activates soluble guanylate cyclase [14,15] leading to an increase in intracellular levels of cGMP, which is considered to be the major second messenger for the physiological actions of NO.

The biological activities of NO in human skin include the regulation of vasodilation and melanogenesis and protection

against invading pathogens [16–18]. Evidence is emerging for an important role of iNOS in the pathogenesis of several skin diseases. Therefore the NO-producing activity of this enzyme might be important during inflammatory processes in the skin, providing novel clues to the role of NO in skin inflammation. In addition to its presence in inflammatory dermatoses [19], iNOS has been shown to be overexpressed in psoriatic skin lesions [20–23], indicating a function for NO in the progression of psoriasis, a chronic inflammatory skin disease characterized by the hyperproliferation and incomplete differentiation of epidermal keratinocytes in the involved lesions. We demonstrated very recently that a functionally active iNOS is a crucial prerequisite for proliferation of wound keratinocytes and therefore for normal wound re-epithelialization *in vivo* [24]. Moreover, studies *in vitro* clearly indicated a biphasic effect of NO on keratinocytes, because low concentrations of NO trigger cell proliferation, whereas high concentrations are cytostatic and characterized by a subsequent differentiation of the cells [25]. Because molecular mechanisms that underlie the action of NO in normal and diseased skin have not yet been elucidated, we were especially interested in genes that are regulated by this short-lived molecule in the skin. We have identified a Cu/Zn superoxide dismutase (SOD) that underlies a stringent NO-mediated ex-

Abbreviations used: BrdU, 5-bromo-2'-deoxyuridine; DETA-NO, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GSNO, S-nitrosoglutathione; IFN- γ , interferon γ ; IL, interleukin; iNOS, inducible nitric oxide synthase; L-NMMA, N^G-monomethyl-L-arginine; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; NO, nitric oxide; NOS, nitric oxide synthase; PDGF, platelet-derived growth factor; SIN-1, 3-morpholino-sydnnonimine; SOD, superoxide dismutase; SNP, sodium nitroprusside; TGF, transforming growth factor; TNF, tumour necrosis factor.

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pressional control in keratinocytes. To our knowledge, Cu/Zn SOD is the first gene identified that is stringently and exclusively regulated by NO in the skin.

SODs are enzymes regulating intracellular superoxide anion ($O_2^{\cdot-}$) levels in catalysing the dismutation of $O_2^{\cdot-}$ to H_2O_2 . Eukaryotic cells express three SOD isoenzymes that are present at high concentrations throughout the cell: Cu/Zn SOD in the cytosol, Mn SOD within the mitochondrial matrix, and an extracellular form similar to the Cu/Zn SOD isoform [26]. Immunohistochemical studies revealed an expression of Cu/Zn SOD protein in the keratinocytes forming the basal layer of the epidermis in normal and psoriatic skin [27,28]. The balanced intracellular concentration of $O_2^{\cdot-}$ is likely to be important in the proliferative behaviour of epidermal cells. The generation of $O_2^{\cdot-}$ was associated with the proliferation of cultured keratinocytes, because cells decreased their release of $O_2^{\cdot-}$ when they reached confluence [29]. In addition, the irradiation-mediated proliferation of keratinocytes could be inhibited by scavenging $O_2^{\cdot-}$ by SOD [30]. Moreover, the inhibition of keratinocyte growth by 3-morpholino-sydnonimine (SIN-1), a donor of both NO and $O_2^{\cdot-}$, could be reversed by scavenging the NO release from SIN-1, clearly indicating a close correlation between high levels of $O_2^{\cdot-}$ and proliferation [31]. Furthermore, our results suggest that the NO-mediated increase of Cu/Zn SOD levels in keratinocytes might be a mechanism for counteracting keratinocyte proliferation.

EXPERIMENTAL

Cell culture and treatment of cells

The human keratinocyte cell line HaCaT [32] was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal calf serum (FCS). Under these conditions, cells remain proliferative and undifferentiated. For the Cu/Zn SOD induction experiments, cells were grown to confluence without changing the medium and were rendered quiescent by a 24 h incubation in serum-free DMEM. Cells were then incubated for various periods in fresh DMEM containing 10 ng/ml epidermal growth factor, 10 ng/ml transforming growth factor (TGF)- β 2, 10 ng/ml platelet-derived growth factor (PDGF) BB, 10 ng/ml keratinocyte growth factor, 2 nM interleukin (IL)-1 β , 2 nM tumour necrosis factor α (TNF- α), 100 i.u./ml interferon γ (IFN- γ), 25 ng/ml IL-8, 100 μ M S-nitrosoglutathione (GSNO), 250 μ M GSNO, 500 μ M GSNO, 100 μ M sodium nitroprusside (SNP), 500 μ M SNP, 100 μ M (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA-NO), 500 μ M DETA-NO, 2 mM N^G-monomethyl-L-arginine (L-NMMA), 100 μ M xanthine and 20 or 100 m-units of xanthine oxidase respectively. For the experiment with proliferating cells, keratinocytes were treated with 500 μ M GSNO during the exponential growth phase. Aliquots of cells were harvested before and at different time points after treatment with these reagents and used for RNA isolation. Each experiment was done in triplicate. FCS and DMEM were purchased from Gibco Life Technologies (Eggenstein, Germany), growth factors and cytokines were from Boehringer Mannheim Biochemicals (Mannheim, Germany), SNP, xanthine and xanthine oxidase were from Sigma Biochemicals (Deisenhofen, Germany), and DETA-NO and L-NMMA were supplied by Alexis Corporation (Grünberg, Germany).

RNA isolation and RNase protection analysis

RNA isolation was performed as described [33]. Total RNA (20 μ g) from the different experimental time points of the cell-

culture experiments and 10 μ g of total RNA from control skin and psoriatic lesional skin biopsies respectively were used for RNase protection analysis. RNase protection assays were performed as described [34]. In brief, DNA probes were cloned into the transcription vector pBluescript II KS (+) (Stratagene, Heidelberg, Germany) and linearized. An anti-sense transcript was synthesized *in vitro* with T3 or T7 RNA polymerase and [α -³²P]UTP (800 Ci/mmol). RNA samples were hybridized at 42 °C overnight with 100 000 c.p.m. of the labelled anti-sense transcript. Hybrids were digested with RNases A and T1 for 1 h at 30 °C. Under these conditions, every single mismatch was recognized by the RNases. Protected fragments were separated on 5% (w/v) polyacrylamide/8 M urea gels and analysed with a Phosphor-Imager (Fuji, Straubenhardt, Germany). All protection assays were performed with at least three different sets of RNA from independent cell-culture experiments and skin biopsies from different patients.

Probe DNA species

The human Cu/Zn SOD cDNA probe was cloned by PCR with 5'-GAC TGA AGG CCT GCA TGG ATT CC-3' as a 5' primer and 5'-CAC AAG CCA AAC GAC TTC CAG CG-3' as a 3' primer. The amplified cDNA fragment corresponded to nt 181–506 of the published sequence [35].

Preparation of tissue lysates from psoriatic patients

Skin biopsies (5 mm in diameter) of normal control skin and psoriatic lesional skin of patients were taken and frozen immediately in liquid nitrogen. The study protocol and consent documents were approved by the Ethik Kommission of the Klinikum der Johann Wolfgang Goethe-Universität (Frankfurt am Main, Germany). Total skin samples were homogenized in 2 $\times$ homogenizing buffer [1 $\times$ homogenizing buffer being 20 mM Tris/HCl (pH 8.0)/137 mM NaCl/10% (v/v) glycerol/5 mM EDTA/1 mM PMSF/15 μ g/ml leupeptin]. The tissue extract was cleared by centrifugation and the supernatant was diluted 1:1 with water. The cytoplasmic and particulate fractions of total skin lysates were prepared by a single centrifugation step at 100 000 g for 60 min with an Optima TLX Ultracentrifuge (Beckman, München, Germany). The amount of protein in the cytoplasmic fractions of the lysates was determined by the Bio-Rad protein assay (Bradford method).

Preparation of cell lysates

Keratinocytes (HaCaT cell line) were grown to confluence in DMEM with 10% (v/v) FCS without a change of medium. Under these culturing conditions, cells remained undifferentiated. Cells were rendered quiescent by a 24 h incubation in serum-free DMEM. Subsequently, cells were treated for various durations with 500 μ M GSNO. Aliquots of cells were harvested before and at different time points after treatment. Cells were washed twice with PBS (3 mM KCl/1.5 mM KH_2PO_4 /0.15 mM NaCl/6 mM Na_2HPO_4) and subsequently scraped into 1 $\times$ homogenizing buffer. The cytoplasmic fraction of cell lysates was prepared as described above.

Western blot analysis

Protein (50 μ g) from cytoplasmic fractions of control and GSNO-treated cells, or control and psoriatic lesional skin, was separated by SDS/PAGE. After transfer to a PVDF membrane, Cu/Zn SOD and iNOS proteins were detected with the use of polyclonal antisera directed against human Cu/Zn SOD (Upstate Bio-

technology, Lake Placid, NY, U.S.A.) and human iNOS (Santa Cruz Biotechnologies, Heidelberg, Germany). A secondary antibody coupled to horseradish peroxidase and the enhanced chemiluminescence detection system were used to reveal Cu/Zn SOD and iNOS proteins. PMSF and leupeptin were supplied by Sigma Biochemicals (Deisenhofen, Germany), the antibody coupled to horseradish peroxidase was from Biomol (Hamburg, Germany), and the enhanced chemiluminescence detection system was obtained from Amersham (Braunschweig, Germany).

Cu/Zn SOD-overexpressing cell line

The human Cu/Zn SOD open reading frame was cloned from HaCaT keratinocytes with 5'-CTC GGA ACC AGG ACC TCG GCG-3' as a 5' primer and 5'-TTA TTG GGC GAT CCC AAT TAC-3' as a 3' primer. The amplified cDNA fragment corresponded to nt 30–529 of the published sequence [35]. The amplified cDNA included the Kozak sequence and ended with a TAA stop codon. The 499 bp Cu/Zn SOD cDNA was cloned into the *EcoRV* restriction site of the pBluescript II KS (+) vector. To verify the correct sequence, both strands were sequenced. Subsequently, the Cu/Zn SOD cDNA was subcloned into the pIND(SP1) ponasterone-inducible expression vector (Invitrogen, Groningen, The Netherlands) by using the *EcoRI* and *XhoI* restriction sites of the pBluescript II KS(+) and pIND(SP1) vectors (shown in Figure 5A). The correct orientation of the Cu/Zn SOD cDNA ligated into pIND(SP1) was determined by sequencing. The generation of cell lines was done in accordance with the instructions of the manufacturer of the ponasterone-inducible expression system. In brief, HaCaT keratinocytes (2×10^5 cells per plate) were co-transfected with the receptor plasmid (pVgRXR, zeocin resistance) and the pIND(SP1)-Cu/Zn SOD vector (G418 resistance) with Lipofectamine transfection reagent (Gibco). Cells were selected for the simultaneous integration of both plasmids by using both G418 (Gibco) and zeocin (Invitrogen) as selective antibiotics. After 6–8 weeks of selection, surviving clones were tested for ponasterone-inducible Cu/Zn SOD expression by an RNase protection assay with a specific probe permitting the detection of endogenous and recombinant Cu/Zn SOD mRNA species (see also Figure 5).

Proliferation assays

Cells (HaCaT, psp1CZ1N) were seeded into 96-well plates (Greiner, Frickenhausen, Germany). Each well contained 2000 cells in a total volume of 100 μ l of DMEM/10% (v/v) FCS. Under these conditions, cells remained proliferative and undifferentiated. Cells were then grown for 24 h. After 24 h, the proliferation rate of incubated cells was determined by using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Mannheim, Germany) in accordance with the instructions of the manufacturer. In brief, proliferation was quantified by measuring the amount of soluble formazan produced by the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS).

In addition, cell proliferation was assessed by determining the incorporation of 5-bromo-2'-deoxyuridine (BrdU) by using a BrdU cell proliferation ELISA (Roche Biochemicals, Mannheim, Germany) in accordance with the instructions of the manufacturer. In brief, BrdU was incorporated in place of thymidine into the DNA of proliferating cells. Incorporated BrdU was subsequently detected in fixed cells by using a peroxidase-coupled anti-BrdU antibody. Immune complexes were detected by a reaction with tetramethylbenzidine as a substrate. The reaction product was quantified by measuring A_{450} .

For the determination of total cell numbers, 5×10^5 cells were plated and subsequently grown for 24 h. After 24 h, cells were trypsin-treated ($1 \times$ trypsin/EDTA; Gibco), washed in PBS, diluted and counted in a Neubauer chamber.

Trypan Blue exclusion test for cell viability

psp1CZ1N keratinocytes from the 24 h experimental time point were trypsin-treated from the cell-culture plates ($1 \times$ trypsin/EDTA) and diluted in fresh DMEM. One part of the keratinocyte cell suspension was mixed with one part of 0.4% Trypan Blue (Gibco) and incubated for 3 min at room temperature. Subsequently, unstained (viable) and stained (non-viable) cells were counted in a Neubauer chamber.

Determination of SOD activity

Cells were grown as described above. Cells were washed twice with PBS, scraped into 1 ml of PBS and subsequently sonicated. SOD activities of GSNO-treated HaCaT cell lysates or ponasterone-treated psp1CZ1N cell lysates were determined as described by Elstner and Heupel [36]. The assay was based on the inhibition of nitrite formation from hydroxylamine in the presence of generators of superoxide ($O_2^{\cdot-}$). In brief, phosphate-buffered (pH 7.8) hydroxylammonium chloride (1 μ mol), xanthine (1.5 μ mol), xanthine oxidase (2.5 m-units) and 60 μ g of total protein from cellular lysates of keratinocytes were incubated at 25 °C for 20 min. The inhibition of the formation of nitrite from hydroxylamine driven by $O_2^{\cdot-}$ (released by the xanthine/xanthine-oxidase system) in cellular lysates was used a direct readout for SOD enzymic activity. Nitrite production was determined with the Griess reagent.

Statistical analysis

Results are shown as means $\pm$ S.D. and are presented as percentages of the unstimulated control (100%). Data were analysed by an unpaired Student's *t* test on raw data with SigmaPlot (Jandel Scientific, Erkrath, Germany).

RESULTS

Induction of Cu/Zn SOD mRNA expression by GSNO

Several studies have demonstrated the presence of all three NOS isoforms in normal human skin [23,37,38] suggesting a potential role for NO in the homeostasis of normal skin function. Furthermore, an up-regulation of NOS isoenzymes, especially the iNOS isoform, has been shown for several pathological states of the skin including sunburn erythema, inflammatory dermatoses, psoriasis and even wound healing, suggesting that NO is an important mediator for these processes [19,39–41]. We are therefore interested in NO-mediated gene expression in keratinocytes to determine potential intermediates that might be involved in pathological states of human skin. With the human keratinocyte cell line HaCaT as a system *in vitro*, we found expression of the Cu/Zn SOD isoenzyme to be under the stringent control of exogenously added NO. As shown in Figure 1(A), basal levels of Cu/Zn SOD mRNA were detectable in quiescent keratinocytes. After the addition of increasing amounts of the NO donor GSNO, an induction of Cu/Zn SOD mRNA was observed. Maximal expression levels were detected at highest GSNO concentrations (500 μ M) after 24 h of treatment (Figure 1A, and Figure 1C, open bars). Note that a low concentration of GSNO (100 μ M) did not stimulate Cu/Zn SOD expression at all (Figure 1A, top panel). In addition, we investigated whether Cu/Zn SOD expression could be induced by exogenous NO in

Figure 1 Induction of Cu/Zn SOD mRNA expression by NO in cultured human keratinocytes (HaCaT)

(A) RNase protection assay demonstrating the induction of Cu/Zn SOD mRNA expression by the NO-donating agents GSNO, SNP or DETA-NO as indicated. Cells were rendered quiescent by serum starvation and stimulated with 100, 250 or 500 μ M GSNO, 500 μ M SNP or 500 μ M DETA-NO, for the indicated durations. Samples of 20 μ g of total cellular RNA were analysed for Cu/Zn SOD mRNA expression. The hybridization probe (1000 c.p.m.) was used as a size marker. (B) Proliferating keratinocytes were treated with 500 μ M GSNO for the indicated durations. Samples of 20 μ g of total cellular RNA were analysed for Cu/Zn SOD mRNA expression. The hybridization probe (1000 c.p.m.) was used as a size marker. (C) The increase in Cu/Zn SOD mRNA levels induced by 500 μ M GSNO in quiescent cells (open bars) or proliferating cells (filled bars) was assessed by PhosphorImager (Fuji) analysis of the radiolabelled gels. Results are expressed as percentages of unstimulated control (100%) and are means $\pm$ S.D. ($n = 3$ independent experiments). * $P < 0.05$; ** $P < 0.01$ compared with control. (D) Serum-starved keratinocytes were incubated with 100 μ M xanthine in the presence of 20 or 100 m-units of xanthine oxidase (XO). Total cellular RNA (20 μ g) was analysed for Cu/Zn SOD mRNA expression. The hybridization probe (1000 c.p.m.) was used as a size marker. For the RNase protection assays, one representative experiment was shown. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control in (A) and (B).

proliferating cells too. This was indeed so: exponentially growing keratinocytes responded to exogenously added GSNO (500 μ M) with an induction of Cu/Zn SOD expression (Figure 1B, and Figure 1C, filled bars). Stimulation of Cu/Zn SOD expression (approx. 3-fold) was also obtained with SNP or DETA-NO as additional NO donors (Figure 1A, bottom panels). The effects of SNP or DETA-NO on Cu/Zn SOD expression were transient, with increased Cu/Zn SOD mRNA levels at 5 h after stimulation.

Induction of Cu/Zn SOD protein expression by GSNO

Cu/Zn SOD is a homodimer consisting of subunits with a molecular mass of 15.7 kDa. To determine whether the observed induction of Cu/Zn SOD mRNA expression was correlated with the induction of immunoreactive protein, we performed a Western blot analysis of GSNO-treated cells (Figure 2A). In line with the kinetics of Cu/Zn SOD mRNA induction cells were harvested 8, 16, 24 and 32 h after treatment with GSNO. Total cytoplasmic protein (50 μ g) was subsequently analysed for the presence of Cu/Zn SOD protein by immunoblotting with an antiserum raised against human Cu/Zn SOD (see the Experimental section). As shown in Figure 2(A), the antiserum detected only one band of Cu/Zn SOD protein with an estimated molecular mass of 15 kDa. In comparison with basal Cu/Zn SOD protein expression, Cu/Zn SOD protein levels increased markedly (4-fold) after the addition of 500 μ M GSNO. As a next step we determined

whether the observed increase in Cu/Zn SOD protein was followed by an increase in $O_2^{\cdot-}$ -dismutating activities in the cells. For this reason we performed a SOD activity assay. A decrease in nitrite formation within the assay system was used to determine SOD enzymic activities (see the Experimental section). As shown in Figure 2(B), in comparison with heat-denatured cellular extracts (ctrl, inactivated), native extracts from untreated cells potentially decreased nitrite formation in the assay system, clearly indicating $O_2^{\cdot-}$ -dismutating activities of the cellular extracts. Moreover, treatment of the cells with GSNO markedly increased SOD activities in comparison with unstimulated control cells (Figure 2B). An increase in SOD activity after treatment with GSNO most probably reflected an increase in Cu/Zn SOD activity, because expression levels of manganese-type SOD (Mn SOD) were not altered by GSNO (results not shown). Taken together, these results demonstrate that the induction of Cu/Zn SOD mRNA by NO was also correlated with the induction of Cu/Zn SOD protein and activity.

Regulation of Cu/Zn SOD mRNA levels by growth factors and inflammatory stimuli

As a next step we tested the potency of purified serum growth factors and inflammatory cytokines to stimulate Cu/Zn SOD expression, because no results were available on the regulation of Cu/Zn SOD expression in human keratinocytes. We tested

Figure 2 GSNO-stimulated Cu/Zn SOD protein expression and activity in cultured human keratinocytes (HaCaT)

(A) Serum-starved cells were harvested before and at different time points after treatment with GSNO, as indicated. Cytoplasmic fractions of these cells were analysed by immunoblotting for the presence of Cu/Zn SOD protein. Cu/Zn SOD protein of 15 kDa is indicated by an arrowhead. The positions of molecular-mass markers are indicated at the left. (B) Decrease in $O_2^{\cdot-}$ -triggered nitrite formation from hydroxylamine was used as a measure of total SOD activities of keratinocyte lysates. The SOD activity assay was performed with heat-denatured cell lysates [ctrl (inactivated); open bar], native lysates from quiescent cells (ctrl; filled bar) and GSNO-treated cells (500 μ M GSNO; hatched bar), as indicated. Results are expressed as percentages of inactivated control (100%) and are means $\pm$ S.D. ($n = 3$ independent experiments). * $P < 0.05$ compared with the conditions indicated by the bracket.

various growth factors (epidermal growth factor, PDGF BB, TGF- β 2 and keratinocyte growth factor) and cytokines (IL-1 β , TNF- α , IFN- γ and IL-8) for their potency in influencing Cu/Zn SOD mRNA levels in these cells. In summary, a single treatment of the cells by recombinant growth factors and inflammatory cytokines did not result in any modulation of Cu/Zn SOD expression (results not shown). Besides growth factors and cytokines, we further examined the potency of $O_2^{\cdot-}$ to induce Cu/Zn SOD expression in keratinocytes, because $O_2^{\cdot-}$ is likely to occur in inflammatory conditions. As shown in Figure 1(D), $O_2^{\cdot-}$ generated by the xanthine/xanthine oxidase system did not influence Cu/Zn SOD expression in keratinocytes. Note that the decrease in Cu/Zn SOD expression levels after 24 h was due to cell death after 24 h of treatment with $O_2^{\cdot-}$. These results further support our finding that Cu/Zn SOD expression is under stringent and exclusive control by NO (Figure 1).

Finally, we assessed whether a combination of cytokines was able to influence Cu/Zn SOD expression in the cells, because inflammatory conditions were characterized by the presence of a variety of inflammatory mediators. We therefore stimulated cells simultaneously with a combination of the inflammatory cytokines

Figure 3 Cu/Zn SOD mRNA expression is induced by endogenously produced NO

Keratinocytes were rendered quiescent by serum starvation and subsequently treated with a combination of inflammatory cytokines (2 nM IL-1 β , 2 nM TNF- α and 100 i.u./ml IFN- γ , identified in the figure as 'cytmix') in the presence (●) or absence (○) of 2 mM L-NMMA for different durations as indicated. (A) Nitrite accumulation in the cell-culture supernatants during the incubation period was used as a measure of iNOS enzymic activity. (B) Total cellular RNA was harvested before and at different time points after cytmix treatment in the presence or absence of 2 mM L-NMMA, and analysed simultaneously (20 μ g) for the presence of iNOS and Cu/Zn SOD mRNA by RNase protection assay. Both iNOS and Cu/Zn SOD anti-sense riboprobes hybridized simultaneously with the same batches of RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control for both representative experiments.

IL-1 β (2 nM), TNF- α (2 nM) and IFN- γ (100 i.u./ml) to determine a possible effect of this 'cytmix' on the expression of Cu/Zn SOD. We demonstrated recently that the cytmix also led to a large induction of iNOS expression in keratinocytes and subsequently to an accumulation of nitrite in the cell culture supernatants [42]. Thus we hypothesized that NO released from cytmix-induced iNOS might influence Cu/Zn SOD expression. To test this hypothesis we stimulated keratinocytes with cytmix in the presence or absence of the specific NOS inhibitor L-NMMA. As shown in Figure 3(A), we detected a 3-fold increase in nitrite concentration in the supernatants of cytmix-stimulated cells in comparison with unstimulated control cells, indicating the induction of a functionally active iNOS. Nitrite concentrations in the supernatants of cytmix/L-NMMA-treated cells were markedly decreased, thus clearly demonstrating the inhibitory effect of 2 mM L-NMMA on NOS enzymic activity. As a next step, we analysed total RNA species of the same experiment

Figure 4 Biphasic effect of NO on keratinocyte (HaCaT) proliferation

Keratinocytes were seeded into 96-well plates by using 2000 cells in a total volume of 100 μ l of DMEM/10% (v/v) FCS per well. After 24 h, fresh medium supplemented with different concentrations of NO-donating agent (GSNO or DETA-NO as indicated) was added (filled bars, 100 μ M; hatched bars, 500 μ M). After an additional 24 h, 20 μ l of MTS reagent was added to each well. The generation of soluble formazan was used as a direct measure of cell proliferation. Results are expressed as percentages of unstimulated control (100%) and are means $\pm$ S.D. ($n = 54$ counted wells from six independent experiments). * $P < 0.05$; ** $P < 0.01$ compared with control.

for the expression of Cu/Zn SOD and iNOS mRNA in simultaneously hybridizing both probes in the same set of RNA. Figure 3(B) clearly reveals a strong induction of iNOS mRNA levels after treatment of the cells with cytomix. Cu/Zn SOD mRNA levels remained unaffected after stimulation with cytomix in those experiments in which the release of endogenous NO was suppressed by inhibition of iNOS enzymic activity (Figure 3B, lower panel). In contrast, we observed an increase in Cu/Zn SOD mRNA levels at late time-points when iNOS induction resulted in maximal nitrite release in the absence of L-NMMA (Figure 3B, upper panel). Note that higher levels of iNOS mRNA were due not to unequal loading but to a positive feedback loop of NO on iNOS expression, as demonstrated by Mühl and Pfeilschifter [43]. Taken together, our results suggest that Cu/Zn SOD expression could also be induced by endogenously released NO.

Biphasic effect of NO on keratinocyte (HaCaT) proliferation

A biphasic effect of exogenous NO on proliferation has recently been described for primary keratinocytes [25]. We therefore investigated the effects of low (100 μ M; did not induce Cu/Zn SOD expression) and high (500 μ M; mediated a strong induction of Cu/Zn SOD expression) concentrations of NO-donating agents on the proliferation of the stable keratinocyte cell line HaCaT [32]. As shown in Figure 4, we observed a markedly

Figure 5 Ponasterone-inducible overexpression of Cu/Zn SOD in the keratinocyte cell line psp1CZ1N

(A) Map of human Cu/Zn SOD cDNA ligated into pIND(SP1). Note that the probe (arrow) was the anti-sense RNA transcript for the RNase protection assay. Use of this anti-sense probe in RNase protection assay resulted in the simultaneous protection of the recombinant transcript (425 nt protected fragment, identified as 1) and the endogenous Cu/Zn SOD (345 nt protected fragment, identified as 2). (B) Simultaneous detection of recombinant Cu/Zn SOD and endogenous Cu/Zn SOD transcripts in ponasterone (PoA, 5 μ M)-induced keratinocytes of the psp1CZ1 inducible cell line.

Figure 6 Overexpression of Cu/Zn SOD mediates a cytostatic effect on keratinocytes

psp1CZ1N keratinocytes were seeded into 96-well plates by using 2000 cells in a total volume of 100 μ l of DMEM/10% (v/v) FCS per well. After 24 h, fresh medium supplemented with different concentrations of ponasterone (PoA), GSNO and DMSO was added as indicated. Because ponasterone was dissolved in DMSO, DMSO alone was used as a control in all experiments. After an additional 24 h we performed proliferation assays. **(A)** MTS assay: 20 μ l of MTS reagent was added to each well; the generation of soluble formazan was used as a direct measure of cell proliferation. The effect of ponasterone on HaCaT keratinocytes is shown in the right panel. **(B)** BrdU assay: the incorporation of BrdU into proliferating cells was measured by a peroxidase-coupled anti-BrdU antibody reaction with tetramethylbenzidine as a substrate. Open bar, control (DMSO); filled bar, ponasterone. In **(A)** and **(B)**, results are expressed as percentages of unstimulated control (100%) and are means $\pm$ S.D. ($n = 27$ counted wells from three independent experiments). $*P < 0.05$; $**P < 0.01$ compared with conditions indicated by brackets. **(C)** Cells (5×10^5) were plated and grown in DMEM/10% (v/v) FCS for 24 h. Total cell numbers were determined after 24 h. Viable cells (open bars) were determined with the Trypan Blue assay; filled bars indicate non-viable cells. Results are expressed as total cell numbers and are means $\pm$ S.D. ($n = 3$ independent experiments). $**P < 0.01$ compared with conditions indicated by the bracket. **(D)** The decrease in O_2^{2-} -triggered nitrite formation from hydroxylamine was used as a measure of total SOD activities in keratinocyte lysates. The SOD activity assay was performed with heat-denatured cell lysates [ctrl (inactivated); open bar], native lysates from quiescent cells [ctrl (DMSO); filled bar], and ponasterone-treated cells (PoA; hatched bar) as indicated. Results are expressed as percentages of inactivated control (100%) and are means $\pm$ S.D. ($n = 3$ independent experiments). $*P < 0.05$ compared with the conditions as indicated by the bracket.

increased proliferation rate of cells incubated at low concentrations (100 μ M) of GSNO or DETA-NO that was independent of the NO donor used. In contrast, high concentrations of NO decreased keratinocyte proliferation.

Overexpression of Cu/Zn SOD mediates a cytostatic effect on HaCaT keratinocytes

Recent studies have demonstrated a possible correlation between the presence of high levels of O_2^{2-} and keratinocyte proliferation [29–31]. We therefore speculated that NO-triggered Cu/Zn SOD expression might contribute to the observed anti-proliferative effects of high doses of NO in keratinocytes. To this end, we stably integrated a recombinant human Cu/Zn SOD cDNA into HaCaT keratinocytes (Figure 5A). In this cell line, expression of the surplus recombinant Cu/Zn SOD is under the stringent control of a promoter that is inducible by the ecdysone derivative ponasterone. To obtain exclusive inducibility by ponasterone, these cell lines constitutively express an altered steroid receptor that does not interact with mammalian steroid hormones. After the addition of ponasterone, the activated steroid receptor

became bound to the glucocorticoid-responsive element of the inducible promoter and triggered Cu/Zn SOD expression. As shown in Figure 5, we detected recombinant and endogenous Cu/Zn SOD mRNA simultaneously within the same set of total RNA isolated from the cell lines by using an RNase protection assay. Screening of different clones identified one cell line (psp1CZ1N) that demonstrated a strong inducibility of recombinant Cu/Zn SOD expression after treatment with ponasterone (Figure 5B). With this ponasterone-inducible cell line (psp1CZ1N) we were able to mimic the action of NO on Cu/Zn SOD expression and therefore to exclude the toxic effects of high concentrations of NO, which might have contributed to the cytostatic effects seen in these cells.

As shown in Figure 6(C), ponasterone-induced overexpression of Cu/Zn SOD markedly decreased the proliferation of psp1CZ1N keratinocytes in comparison with uninduced control cells. We observed an overall decrease in proliferation rates of psp1CZ1N cells (approx. 30%) after treatment of cells with ponasterone, and therefore an induction of additional Cu/Zn SOD expression. We confirmed decreased proliferation rates in the presence of ponasterone by (1) the use of an MTS assay

Figure 7 Increased Cu/Zn SOD mRNA and protein levels are correlated with the induction of iNOS in psoriatic lesional skin

The same batches of total cellular RNA and cytoplasmic protein fractions from normal unaffected skin and affected lesional skin of patients with psoriasis were analysed for co-expressed Cu/Zn SOD (left panels) and iNOS (right panels) mRNA and protein levels by RNase protection (A) and Western blot analysis (B). The expression levels are shown for two representative patients (Pat. A and Pat. C).

measuring mitochondrial reduction capacities (Figure 6A), (2) the incorporation of BrdU into proliferating cells (Figure 6B) and (3) counting total cell numbers (Figure 6C). Treatment of psp1CZ1N cells with ponasterone was directly associated with an increase in SOD activities of the cellular lysates (Figure 6D). The increase in SOD activities was due to Cu/Zn SOD activity, because ponasterone did not influence Mn SOD expression levels (results not shown). In the absence of ponasterone, 100 μ M GSNO stimulated the proliferation of psp1CZ1N cells (Figure 6A). To exclude an effect of ponasterone on HaCaT proliferation, we incubated the cells with ponasterone as a control (Figure 6A, right panel). Moreover, the observed decrease in cellular proliferation rates of psp1CZ1N cells after the addition of ponasterone was not due to a decrease in cell viability as assessed by the determination of non-viable cells with the Trypan Blue assay (Figure 6C).

Overexpression of iNOS and Cu/Zn SOD mRNA and protein in hyperproliferative skin lesions of psoriatic patients

Cu/Zn SOD is an important cytosolic scavenger of reactive oxygen species, which are produced continuously in the epidermis, particularly on exposure to UV or several xenobiotics. Immunohistochemical studies demonstrated the presence of Cu/Zn SOD protein expressed almost exclusively in the basal cell layer of the epidermis of normal skin and the affected lesional skin of psoriatic patients [27,28]. Furthermore, the expression of iNOS has been shown to be associated with psoriatic lesions [21–23]. To determine a correlation between iNOS and Cu/Zn SOD expression, we investigated the expression of both genes at the mRNA and protein level in control skin and lesional skin of psoriatic patients ($n = 4$ individuals) by using RNase protection and Western blot analysis. As shown in Figure 7, we detected a strong correlation between iNOS and Cu/Zn SOD expression in

the lesional skin of all four psoriatic patients examined. Cu/Zn SOD is present at low levels in unaffected skin, thus confirming previous results. For the first time we demonstrated a strong up-regulation of Cu/Zn SOD (3–10-fold) at the mRNA and protein levels in psoriatic lesions (Figure 7, left panels). As a next step, we determined iNOS mRNA and protein in the same patients. Psoriatic lesions display a large induction of iNOS mRNA and protein that are not present at detectable levels in unaffected skin of the patients (Figure 7, right panels). In summary, these results obtained from the skin of psoriatic patients, thus representing the situation *in vivo*, clearly support a direct correlation between the expression of iNOS and the presence of elevated Cu/Zn SOD levels.

DISCUSSION

It has been well established that all three NOS isoforms are expressed in skin tissue. Expression of the constitutive neuronal-type NOS isoform (nNOS) has been observed in keratinocytes and melanocytes [23,44–47]; expression of the endothelial-type NOS (eNOS) could be detected in keratinocytes of the basal epidermal cell layer and in dermal fibroblasts, endothelial capillaries and eccrine gland cells [38,48,49]. The presence of constitutively expressed nNOS and eNOS isoforms in the skin, that are activated by elevated intracellular Ca^{2+} levels and generate only small amounts of NO, suggests an involvement in maintaining skin homeostasis in regulating resting blood flow and responses to local warming and UV-B radiation [16]. These constitutive, Ca^{2+} /calmodulin-dependent NOS isoforms are activated after UV irradiation in keratinocytes; the NO produced subsequently is responsible for the stimulation of melanogenesis or the development of sunburn erythema [18,39,50]. In contrast, the iNOS isoenzyme is not expressed in resident cells but is strongly induced by inflammatory cytokines and endotoxins.

The induction of iNOS then results in the release of large amounts of NO from the cells [4,5]. In general, the presence of iNOS is associated with pathological conditions, which is also true of skin tissue. The expression of iNOS has been described for inflammatory diseases of the skin, including inflammatory dermatoses and psoriasis [19–22]. Therefore a potential role for NO as a novel mediator serving the communication between cells by triggering cellular gene expression, as described previously [9–13], became evident also for the skin. For these reasons our interest focused on the identification of novel NO-regulated genes in keratinocytes to gain new insights into the origin of these diseases. We identified Cu/Zn SOD as a novel NO-regulated gene in keratinocytes, whose expression is strongly induced by exogenously added and also endogenously produced NO *in vitro*. It is remarkable that the induction of Cu/Zn SOD is clearly restricted to NO, because none of the many growth factors and inflammatory cytokines tested were able to influence Cu/Zn SOD expression at all. The observed dependence on NO might reflect a general mechanism of Cu/Zn SOD regulation by NO, because we have also demonstrated NO-triggered Cu/Zn SOD expression in rat mesangial cells *in vitro* and during endotoxic shock in rats *in vivo* [9].

The involvement of antioxidant enzyme systems in the regulation of keratinocyte proliferation has been reported recently [29–31]. Irradiation-stimulated keratinocyte proliferation was inhibited by the addition of the antioxidative enzymes SOD and catalase, thus demonstrating that the scavenging of $O_2^{\cdot-}$ and H_2O_2 suppressed the enhanced proliferation of these cells [30]. This proposal is supported by the observation that proliferating keratinocytes decreased their release of $O_2^{\cdot-}$ when cells reached confluence [29]. However, the situation turned out to be more complex. The application of SIN-1, a donor releasing both NO and $O_2^{\cdot-}$, resulted in the cytostasis of keratinocyte cultures. This cytostatic effect of NO/ $O_2^{\cdot-}$ action could be reversed by scavenging either the NO or the $O_2^{\cdot-}$ released from SIN-1, thus transforming SIN-1 to a $O_2^{\cdot-}$ or NO donor respectively [31]. Furthermore, it has been shown that exogenously applied NO stimulated keratinocyte proliferation in a biphasic manner. Low concentrations of NO mediated keratinocyte proliferation, whereas high doses of NO acted in an anti-proliferative manner and subsequently initiated differentiation [25]. Moreover, we have recently demonstrated the function of NO as a growth factor *in vivo* for keratinocytes too. The inhibition of a functional iNOS during the inflammatory phase of cutaneous wound repair resulted in markedly decreased keratinocyte proliferation, atrophied hyperproliferative epithelia at the wound edges and thus a severely impaired re-epithelialization [24]. Therefore the dependence of Cu/Zn SOD expression on NO (the present study) clearly suggests a functional link between the actions of NO and $O_2^{\cdot-}$. Remarkably, we observed a differential effect of NO on Cu/Zn SOD expressional regulation, because low doses (100 μ M) of NO donors did not alter Cu/Zn SOD levels, whereas high concentrations (500 μ M) strongly induced Cu/Zn SOD mRNA and protein levels. It is noteworthy that this expressional regulation was exclusively under the control of NO, because growth factors, cytokines and $O_2^{\cdot-}$ did not alter Cu/Zn SOD expression levels. Thus large amounts of NO are most likely to decrease intracellular $O_2^{\cdot-}$ levels by inducing the $O_2^{\cdot-}$ dismutating enzyme Cu/Zn SOD in keratinocytes. Because $O_2^{\cdot-}$ levels have been discussed as being involved in keratinocyte proliferation, we speculated that NO-elevated Cu/Zn SOD levels in keratinocytes might contribute to an anti-proliferative effect observed at high concentrations of NO. This would be in line with the observation that NO-triggered keratinocyte proliferation at low concentrations [25] (see Figure 4) was associated with unaltered

Cu/Zn SOD expression levels. Indeed our results suggest that the induction of Cu/Zn SOD levels was directly involved in the proliferative behaviour of keratinocytes, because the cell line psp1CZ1N revealed markedly decreased proliferation after the ponasterone-induced overexpression of Cu/Zn SOD (Figure 6). Moreover, we could mimic the anti-proliferative actions of high doses of NO by the NO-independent induction of Cu/Zn SOD within the cells. These results suggest that NO-regulated Cu/Zn SOD might contribute to the cytostatic effects observed at high concentrations of NO in keratinocytes.

To evaluate the possible relevance of our results *in vitro* to a situation *in vivo*, we chose to investigate iNOS and Cu/Zn SOD expression in the inflammatory skin disease psoriasis. Histological examination of psoriatic plaques reveals three major pathogenic factors: abnormal keratinocyte differentiation, hyperproliferation of keratinocytes and infiltration of inflammatory cells into the skin [51], indicating that iNOS-inducing cytokines are present in psoriatic skin. Several studies demonstrated the involvement of different inflammatory cytokines, chemokines and their receptors in psoriasis [52–57]. The presence of these proinflammatory cytokines (especially IL-1, IL-6, IL-8 and TNF- α) explains the infiltration of immune cells into psoriatic diseased skin [58]. These cytokines trigger T-cell activation in lesional psoriatic epidermis [59], further amplifying the inflammatory process. These results suggest that inflammatory cytokines that are potent inducers of iNOS might trigger a NO-dependent Cu/Zn SOD overexpression *in vivo*. Recent studies independently demonstrated an increased expression of iNOS in psoriatic skin lesions [20–22]. Additionally, Cu/Zn SOD is expressed in normal human skin and psoriatic plaques [27,28]. In line with our observations *in vitro*, we should have been able to detect, at least, increased Cu/Zn SOD levels that were directly correlated with overexpressed iNOS in psoriatic lesions in comparison with uninvolved skin. This was indeed true (Figure 7), because a strong induction of iNOS at the mRNA and protein levels occurred in all examined biopsies of lesional skin, whereas no iNOS expression was detected in control skin. We found a marked increase in Cu/Zn SOD mRNA and protein levels in the same psoriatic lesions that were strongly positive for iNOS expression. It is worth emphasizing the strong increase in Cu/Zn SOD levels in psoriatic plaques (Figure 7), although immunohistochemical studies indicated an expression of Cu/Zn SOD that was almost exclusively restricted to the basal cells of the lowest parts of the elongated rete ridges in psoriasis [27]. Interestingly, *in situ* hybridization against iNOS also revealed that keratinocytes of the highly proliferative basal epidermal layer at the tips of the rete ridges expressed the largest amounts of iNOS mRNA [21]. This situation suggests that a correlation between the presence of iNOS and Cu/Zn SOD expression in keratinocytes might be of importance in inflammatory skin diseases *in vivo*. Bruch-Gerharz et al. [60] discussed the role of NO as a cytostatic mechanism to counteract excessive mitogenesis in psoriasis. In line with this hypothesis, our results suggest an anti-psoriatic role of NO in decreasing the epidermal proliferation of the diseased skin: the overexpression of iNOS in psoriatic lesions might contribute, at least partly, to the observed high levels of Cu/Zn SOD. Furthermore, our results suggest that the up-regulation of Cu/Zn SOD in keratinocytes might be involved in mechanisms initiated to counteract the onset of keratinocyte hyperproliferation in psoriatic skin.

We thank Nicole Kolb for her excellent technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 553) and by grants from the Commission of the European Communities (Biomed 2, PL 90979) and the Adolf Messer-Stiftung.

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Received 28 June 1999/11 November 1999; accepted 4 January 2000