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Tumor promoter TPA stimulates MMP-9 secretion from human keratinocytes by activation of superoxide-producing NADPH oxidase

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Abstract
Matrix metalloproteinase-9 (MMP-9) is involved in physiological tissue remodelling processes as well as in tumor invasion and metastasis. The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) increases MMP-9 secretion from normal human epidermal keratinocytes (NHEK) *in vivo* and *in vitro*. Here we show that the flavoprotein inhibitor diphenyleneiodinium (DPI) and the NADPH oxidase inhibitor apocynin block TPA-induced MMP-9 secretion of NHEK *in vitro*. Furthermore, N-acetyl-L-cysteine and L-cysteine lowered TPA-induced MMP-9 secretion, suggesting an involvement of reactive oxygen species(ROS). TPA exerts its effect on MMP-9 gene expression and secretion via the superoxide-producing enzyme NADPH oxidase: TPA rapidly stimulates generation of superoxide anion as well as gene expression of two cytosolic NADPH oxidase subunits (p47-phox and p67-phox) after 2 h, which is followed by induction of MMP-9 gene expression after 4 h. Taken together, the novel finding herein is the TPA-induced MMP-9 secretion from normal human epidermal keratinocytes through a NADPH oxidase dependent pathway.

Keywords: *Keratinocyte, gelatinase, MMP-9, superoxide, NADPH oxidase, TPA*

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-containing endopeptidases involved in remodelling and degradation of the extracellular matrix (ECM). MMPs are divided into five subgroups according to their substrate specificity and structural similarities: Collagenases, stromelysins, gelatinases, matrilysins and membrane-type MMPs [1]. Under normal physiological conditions, their gene expression and activity is tightly controlled. While the proteolytic activity of MMPs is regulated by cleavage of their prodomains as well as the activity of tissue inhibitors of MMPs (TIMPs), primarily regulation of MMP synthesis occurs at the level of transcription [1–3]. Induction of MMP expression has been described in many cell types after treatment with growth factors [4] or cytokines [5] and exposure to UV irradiation [6,7] or tumor promoters like 12-O-tetradecanoylphorbol-13-acetate (TPA) [8,9]. TPA-induced stimulation of gene expression for collagenase-1 (MMP-1), stromelysin-1 (MMP-3) and gelatinase-2 (MMP-9) is triggered by transcription factors such as AP-1 and Ets-1 via binding to adjacent AP-1 and PEA3 binding sites [2,10]. In the MMP-9 promoter, additional NFκB and Sp1 binding sites are needed for TPA-induced stimulation of MMP-9 [11].

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Enhanced expression and secretion of MMPs is associated with tumor progression and metastasis. These enzymes degrade major components of the extracellular matrix, and therefore promote migration of tumor cells to get access to the blood system [12,13]. The gelatinase MMP-9 contributes to keratinocyte hyperproliferation and progression to invasive cancer in a mouse model of oncogene-derived skin carcinogenesis [14]. Its expression and activity is enhanced in highly aggressive and invasive spindle cell carcinoma induced by treatment of mice skin with the chemical carcinogen anthracene and the tumor promoter TPA [15].

Transcriptional upregulation of MMPs can be mediated by increased production of reactive oxygen species (ROS). UVA-induced singlet oxygen and paraquat-induced ROS increase MMP-1 mRNA expression in human dermal fibroblasts [16,17]. Under normal physiological conditions, low levels of ROS are constitutively produced and involved as second messengers in different signal transduction pathways. Imbalance of ROS and antioxidative systems towards increased ROS amounts results in oxidative stress [18,19] and activation of stress genes contributing to proliferation and tumorigenic transformation of cells [20].

ROS are produced by cellular sources including enzyme systems of the mitochondrial electron transport chain, cytosolic xanthine oxidase and membrane-bound oxidoreductases [21]. NADPH oxidase catalyzes the one-electron reduction of molecular oxygen to superoxide, it is a multi-component enzyme consisting of 2 membrane-bound (p22-phox, gp91-phox) and 4 cytosolic (rac, p40-phox, p47-phox, p67-phox) subunits. In phagocytes, it produces high amount of superoxide as defense against microorganisms [22], but its subunits are also expressed in many non-phagocytic cells such as chondrocytes, fibroblasts, epithelial and endothelial cells [23]. However, the amount of NADPH oxidase-derived superoxide is, in general, much lower in non-phagocytic cells than in phagocytes. Among the agents described to stimulate the activity of NADPH oxidase in various cells, are the cytokines interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) [24,25], the regulator of blood pressure angiotensin II [26] and the tumor promoter TPA [27,28].

The aim of this study was to find a link between two effects of the tumor promoter TPA in keratinocytes: The stimulation of ROS production and the induction of matrix metalloproteinase secretion. We demonstrate here that normal human epidermal keratinocytes constitutively express membrane-bound (p22-phox, gp91-phox) and cytosolic (p47-phox, p67-phox) components of the NADPH oxidase at the mRNA level and constitutively produce low levels of superoxide. TPA rapidly stimulates superoxide production of NHEK *in vitro* and increases gene expression of the regulatory cytosolic NADPH oxidase subunits. NADPH oxidase-derived superoxide is highlighted to be the mediator responsible for TPA-induced MMP-9 gene expression of and secretion from NHEK.

Material and methods

Reagents

Reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Roth (Karlsruhe, Germany). If not stated otherwise, all other reagents were obtained from Sigma (Taufkirchen, Germany). The NADPH oxidase inhibitor apocynin was from Merck Biosciences (Bad Soden, Germany). Primers for RT-PCR were synthesized by Invitrogen (Karlsruhe, Germany).

Cell culture and cell viability assay

Normal human epidermal keratinocytes were prepared from foreskin biopsies as described [29]. The cells were grown in keratinocyte-SFM medium (Invitrogen) containing supplements (human epidermal growth factor, bovine pituitary extract; Invitrogen) and gentamycin (5 μg/ml; Sigma) and were used for experiments between passages 2 and 5.

The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded in dishes of 6 cm diameter for the experiments and were growth-arrested at approximately 80% confluence by overnight incubation in supplement-free keratinocyte SFM prior to TPA stimulation.

Cell viability was determined by measurement of the ability of the cells to metabolise MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) to a purple formazan dye as described [30].

Stimulation with TPA

The phorbol ester TPA was used for MMP-9 stimulation as described [31]. Briefly, NHEK were treated with 1.5 μM TPA for 16 h. Thereafter, supernatants were collected and total RNA was isolated. Experiments were performed with NHEK from 3 different donors, prepared from foreskin biopsies of healthy Caucasian children with an average age of 7 years. Inhibitors were added to the cell medium 2 h prior to exposure to TPA and were present during TPA treatment. For time-course experiments, cells were harvested and RNA was prepared at the indicated time points after TPA treatment.

Zymography

Activity of gelatinases MMP-2 and MMP-9 in supernatants of NHEK was determined by gelatin zymography as described [32]. Briefly, zymography was performed by running aliquots of the supernatants under

denaturing, but non-reducing conditions in 10% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were incubated for 1 h in 2.5% Triton X-100, followed by an overnight incubation at 37°C in reaction buffer (50 mM Tris-HCl pH 7.3, 200 mM NaCl, 5 mM $CaCl_2$, 0,02% Brij 35). Gels were stained with Coomassie brilliant blue solution for 10 min and destained with 10% acetic acid for 1 h to visualize the bands of proteolytic activity. Molecular sizes of the bands were calculated by comparison with a prestained protein marker (Biomol, Hamburg, Germany). For quantification of the bands, the gels were scanned by an image-analysis system and analysed with AIDA image software (Raytest, Straubenhardt, Germany).

Western blotting

Aliquots of the supernatants were run on 10% SDS-polyacrylamide gels and the proteins were electroblotted onto nitrocellulose membranes (Hybond-C Extra, Amersham, Freiburg, Germany). Immunodetection was carried out using a mouse-MMP-9 antibody (Merck Biosciences) and a HRP-conjugated goat-anti mouse IgG antibody (Pierce, Bonn, Germany) as described [32]. MMP-9 was detected by a chemiluminescence system (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce) on Biomax light film (Kodak, Rochester, NY). Molecular sizes of the bands were calculated by comparison with a prestained protein marker (Biomol). For quantification of the bands, the developed films were scanned by an image-analysis system and analysed with the AIDA image software (Raytest).

RT-PCR

Total RNA was prepared from NHEK by using RNeasy Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. From each sample, 1 μg of RNA was transcribed into cDNA with SuperScript II reverse transcriptase (Invitrogen) and $p(dT)_{15}$ primers (Roche, Mannheim, Germany). PCR was performed with 100 ng cDNA per sample for the housekeeping gene hypoxanthin-phosphoribosyl-transferase (HPRT) in order to normalize the samples as well as for MMP-9 and for subunits of NADPH oxidase (gp91-phox, p22-phox, p47-phox, p67-phox) with Taq DNA polymerase (Qiagen) in a PCR thermocycler (Biometra, Goettingen, Germany). The sequences for sense and antisense primers are shown in Table I. The PCR for HPRT was performed for 3 min at 95°C followed by 31 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C as well as a final elongation step for 10 min at 72°C. For the other genes tested, the primer annealing temperatures (MMP-9: 65°C, gp91-phox: 58°C, p22-phox: 55°C, p47-phox: 63°C, p67-phox: 55°C) and the number of cycles (MMP-9: 31 cycles, gp91-phox: 40 cycles, p22-phox: 28 cycles, p47-phox: 32 cycles, p67-phox: 28 cycles) were changed according to previous optimisation experiments. The amplification products were run on a 1% agarose gel and visualized by ethidium bromide staining. For quantification, the gels were scanned by an image-analysis system and analysed with the AIDA image software (Raytest).

Determination of reactive oxygen species

To quantify the presence of ROS in NHEK, the cells were cultivated in 24-well dishes, starved at 80% confluency by overnight incubation in supplement-free keratinocyte medium and loaded with 50 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 1 h. H_2DCFDA is de-esterified intracellularly and metabolized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation [35]. Subsequently, the cells were washed 3 times with phosphate-buffered saline and mock-treated or stimulated with 1.5 μM TPA in triplicates for 15 min at 37°C in 5% CO_2. DCF fluorescence was measured with a Victor 1420 multilabel counter (Wallac, Freiburg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Determination of superoxide generation

The production of superoxide by NHEK was measured with the cytochrome c reduction assay as described [36]. Ferricytochrome c is reduced by superoxide radicals to ferrocytochrome c, creating an absorption peak at 550 nm. NHEK were cultured in 6 cm diameter dishes until 80% confluency. The cells were washed in Krebs-Ringer buffer (154 mM NaCl, 5.6 mM KCl, 25 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose), cytochrome c from horse heart in Krebs-Ringer buffer was

Table I. Primer sequences for RT-PCR.

	Sense-Primer	Antisense-Primer
HPRT	ATTCTTTGCTGACCTGCTGGATT	CTTAGGCTTTGTATTTTGCTTTTC
MMP-9	CACTGTCCACCCCTCAGAGC	GCCACTTGTCGGCGATAAGG
gp91-phox	CCTGTACCTGGCTGTGACCCTGTT	ACCCCAATCCCTGCTCCCACTAA
p22-phox	GTTTGTGTGCCTGCTGGAGT	TGGGCGGCTGCTTGATGGT
p47-phox	CGAGAACCGCCAGGGCACACTTAC	CCAACCGCTCTCGCTCTTCTCCAC
p67-phox	CGAGGGAACCAGCTGATAGA	CATGGGAACACTGAGCTTCA

Specific primers for HPRT, gp91- and p47-phox were designed, while primers for MMP-9 as well as for p22- and p67-phox were used as described [33,34].

added to a final concentration of 40 μM and cells were mock-treated or stimulated with 1.5 μM TPA. After 30 min incubation of the cells at 37°C in 5% CO_2 the samples were removed and the spectrum of each sample was measured between 500 and 600 nm with a Lambda 25 spectrophotometer (Perkin Elmer, Norwalk, CT). Superoxide production was determined using the extinction coefficient of cytochrome c at 550 nm ($\varepsilon = 21\,mM^{-1}\,cm^{-1}$).

Statistical analysis

All means were calculated from three independent experiments and the error bars represent standard error of the mean (S.E.M.). Analysis of statistical significance was done by Student's *t*-test with $^{\star}p < 0.05$ and $^{\star\star}p < 0.01$ as levels of significance.

Results

TPA-induced MMP-9 activity and secretion

Gelatinolytic activity in NHEK supernatants was determined by zymographic analysis and, consistent with previous studies[31,32], MMP-9 and MMP-2 activity was found for untreated cells (basal controls). Stimulation of NHEK with 1.5 μM TPA resulted in a 3-fold increase in MMP-9 activity after 16 h compared with untreated controls, whereas MMP-2 activity was not affected (Figure 1A and B). Both MMP-9 and MMP-2 were secreted as latent (uncleaved) pro-enzymes, depending on growth of NHEK on plastic surfaces [31,32,37]. In contrast, studies with fibroblasts, HaCaT keratinocytes and melanoma cells showed, that MMPs are cleaved into the active enzyme, if the cells are grown in a collagen matrix [38,39]. As the tumor promoter TPA increases ROS production in murine epidermal keratinocytes *in vivo* and *in vitro* [40], we addressed the question of whether ROS affect this TPA-stimulated MMP-9 activity of NHEK. Preincubation of NHEK with a non-toxic concentration (5 mM) of the antioxidants N-acetyl-L-cysteine or L-cysteine significantly lowered the TPA-induced increase in MMP-9 activity (Figure 1A).

In order to investigate the nature of the TPA-induced ROS and to find out by which enzymatic systems they are produced, NHEK were preincubated with non-toxic concentrations of the flavoprotein

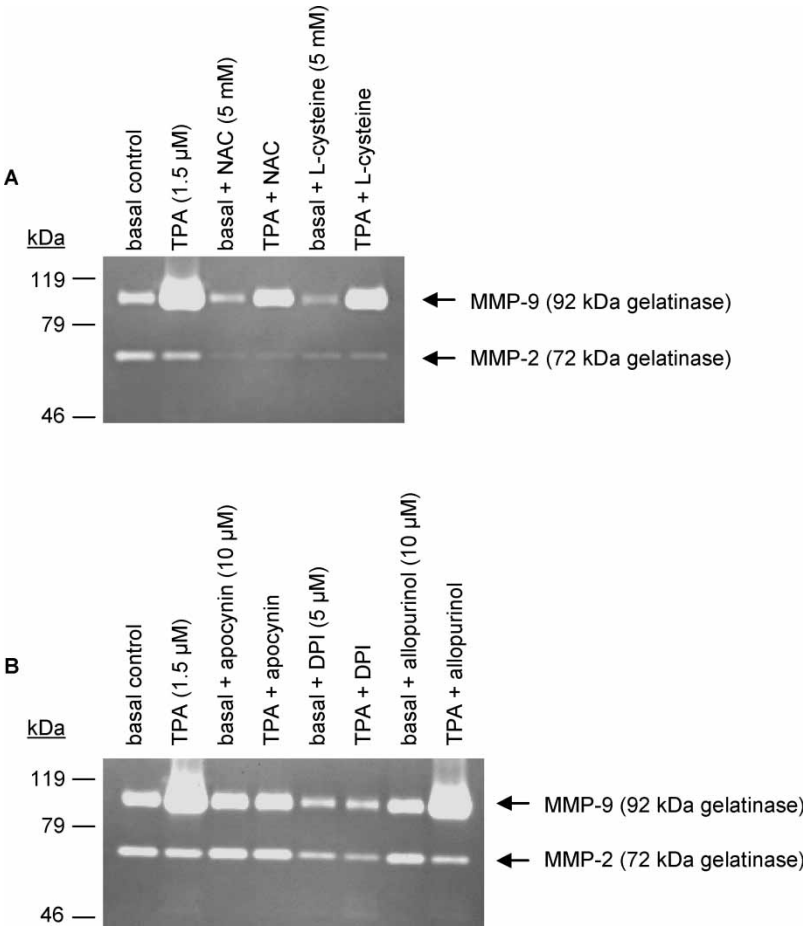

Figure 1. Effect of antioxidants (A) and inhibitors (B) on TPA-induced gelatinase activity in supernatants from normal human epidermal keratinocytes (NHEK). NHEK were treated with antioxidants or inhibitors at the indicated concentrations 2 h prior to TPA stimulation and were present during TPA treatment. Supernatants of the cells were collected 16 h upon treatment and gelatinolytic activity was assessed by zymography. The data represent 1 out of 3 independent experiments with similar results.

inhibitor DPI, the specific NADPH oxidase inhibitor apocynin or the xanthine oxidase inhibitor allopurinol prior to TPA treatment. Preincubation with 5 μM DPI or 10 μM apocynin completely abolished the TPA-induced increase in MMP-9 activity. In contrast, preincubation with 10 μM of the xanthine oxidase inhibitor allopurinol had no effect (Figure 1B). The metabolic activity of the NHEK after treatment with TPA and/or the inhibitors were tested in an MTT assay to ensure that the concentrations of the reagents were not cytotoxic (data not shown). The concentrations of the inhibitors were chosen so that they did not influence basal MMP-9 activity of untreated NHEK.

In order to confirm the predominant 92 kDa band in the zymograms as MMP-9, Western blotting of NHEK supernatants with a monoclonal MMP-9 antibody was performed. Figure 2A shows that TPA-induced MMP-9 secretion of NHEK is indeed lowered by NAC and L-cysteine, while inhibitors of NADPH oxidase (DPI and apocynin) completely blocked TPA-induced MMP-9 secretion (Figure 2B). In summary, these experiments provide evidence that TPA-stimulated enhanced MMP-9 secretion and activity in supernatants of NHEK is mediated by NADPH oxidase-generated superoxide. The response to TPA and the different inhibitors was similar in the NHEK of 3 different donors (data not shown).

TPA induces a rapid increase of ROS production in NHEK

Human keratinocyte cell lines H357 and HaCaT produce low amounts of superoxide by constitutive enzymatic activity of NADPH oxidase [36,41]. Therefore, ROS production and superoxide generation of NHEK were determined by measurement of DCF fluorescence as well as cytochrome c reduction assay. NHEK constitutively produced intracellular ROS and released superoxide into their medium. Intracellular ROS level of NHEK was significantly increased upon treatment with TPA, which resulted in increased release of superoxide into the medium: 1.5 μM TPA increased intracellular ROS level in NHEK 1.5 fold after 15 min and resulted in a 2.2 fold increase in superoxide release after 30 min compared with untreated controls (Table II). The TPA-induced increase in superoxide generation was inhibited by 2 h pretreatment of the NHEK with 5 μM of the flavoprotein inhibitor DPI (data not shown).

TPA stimulates mRNA expression of regulatory NADPH oxidase subunits prior to MMP-9 expression

Some components of the phagocytic NADPH oxidase were detected in three different human keratinocyte cell lines on the mRNA and on the protein level [36,41]. Therefore, steady-state mRNA levels of 2 membrane-bound (gp91-phox, p22-phox)

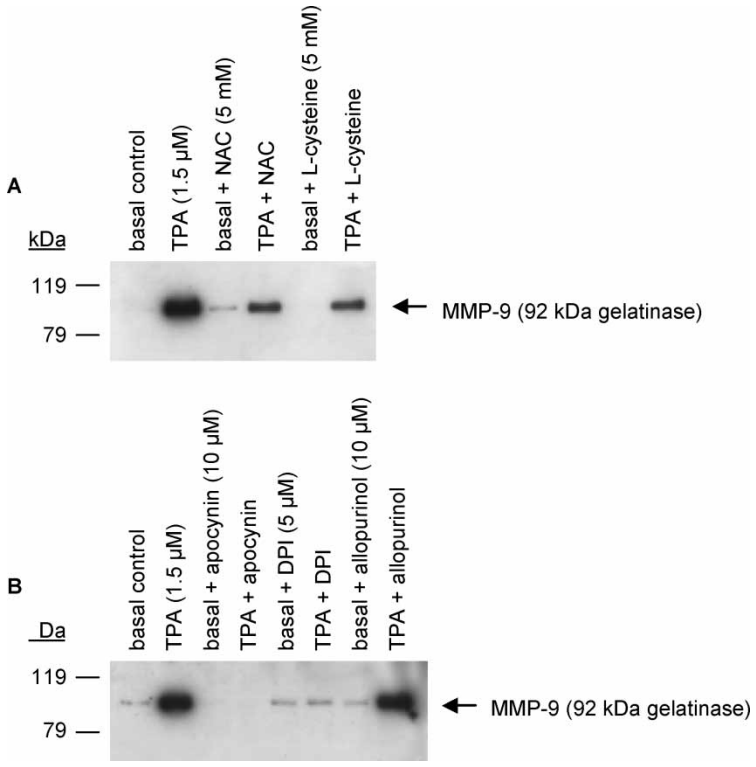

Figure 2. Effect of antioxidants (A) and inhibitors (B) on TPA-induced MMP-9 secretion of NHEK. Conditions were as described in Figure 1. MMP-9 protein level was determined by Western Blotting. The data represent 1 out of 3 independent experiments with similar results.

Table II. Effect of TPA treatment on ROS generation of NHEK.

Basal (untreated)	TPA-treated
A. ROS production	
100%	145.7 ± 2.2% (*)
B. superoxide production	
13.0 ± 0.1 nmol/h/10^6 cells	29.3 ± 0.3 nmol/h/10^6 cells (**)

NHEK were mock-treated or treated with 1.5 μM TPA. Intracellular ROS production was measured 15 min after treatment by DCF fluorescence (A) and superoxide production was determined 30 min after treatment by cytochrome c reduction assay (B).
Data represent means ± S.E.M. from 3 independent experiments; $*p < 0.05$, $**p < 0.01$ (Student's *t*-test).

and 2 cytosolic (p47-phox, p67-phox) subunits of NADPH oxidase were semi-quantitatively measured by RT-PCR upon TPA treatment of NHEK. Constitutive mRNA expression was found for all four subunits in untreated NHEK, although expression of gp91-phox was very low and only detectable if 40 cycles were used in the RT-PCR. Steady-state mRNA levels of the NADPH oxidase subunits were investigated at different time points between 2 h and 16 h upon stimulation of NHEK with 1.5 μM TPA. TPA treatment did not affect the steady-state mRNA levels of the two membrane-bound subunits gp91-phox and p22-phox, but strongly increased the mRNA amount of the two cytosolic subunits p47-phox and p67-phox. Transcriptional upregulation already started 2 h upon TPA treatment and peaked after 6 h (p47-phox) or 8 h (p67-phox). The steady-state mRNA levels of both cytosolic subunits were increased 3.7 fold by TPA compared with untreated controls (Figure 3).

The time course of TPA-induced MMP-9 stimulation was compared with the time course analysis of steady-state mRNA levels of the NADPH oxidase subunits. As shown in Figure 3, a slight up-regulation of MMP-9 steady-state mRNA level was observed 4 h upon TPA treatment, which gradually increased, reaching 3 fold stimulation after 16 h.

Discussion

Many cell types including epidermal keratinocytes increase MMP production in response to agonists such as the tumor promoter TPA [31]. MMPs are involved in early and late stages of carcinogenesis by stimulating the proteolytic release and activation of cytokines and growth factors as well as by catalyzing the degradation of extracellular matrix components. MMP-9 is one of the key enzymes associated with tumor progression and is a marker of malignant transformation in various human cancers [42,43].

The mechanisms responsible for TPA-induced MMP-9 expression include activation of protein kinase C[44] and of the MAPKs ERK1/2[45] and JNK[44] and binding of transcription factors AP-1, Ets-1 and NFκB [11], but the early events in TPA signaling are still not completely understood. As TPA stimulates ROS production in various cell types [27,46–48], we addressed in the present study the question of whether ROS might be involved in TPA-induced MMP-9 expression and secretion of normal human epidermal keratinocytes. In a study with initiated mouse keratinocytes, which develop tumors after TPA treatment and grafting into athymic nude mice, TPA-induced ROS did not cause direct oxidative DNA damage, leading to the hypothesis of an indirect role of those ROS as second messengers in signal transduction [46]. Indeed, we found that pre-treatment with NAC or L-cysteine lowered the TPA-induced secretion and gelatinolytic activity of MMP-9 from NHEK. As thiol compounds, NAC and L-cysteine are a source of intracellular sulfhydryl groups and may act on the detoxification of ROS such as hydrogen peroxide, hydroxyl radicals or superoxide [49].

It is known from studies in macrophages, monocytes, fibroblasts and synoviocytes that TPA causes a respiratory burst of superoxide generation by activating the multi-component enzyme NADPH oxidase [27,28,48,50]. Herein, we show that two inhibitors of the NADPH oxidase, DPI and apocynin, prevented the TPA-induced MMP-9 secretion and gelatinolytic activity from NHEK. DPI, although widely used in studies, is a rather unspecific inhibitor of flavoproteins, blocking the catalytic center of flavocytochromes [51]. Apocynin, a catechol compound of the Himalayan herb *Picrorhiza kurroa*, inhibits the assembly of NADPH oxidase [52]. Apocynin was recently shown to prevent the TPA-induced and superoxide-mediated stimulation of cyclooxygenase-2 in monocytes, providing a mechanistic rationale for its possible therapeutic application in inflammatory disorders [48]. As we found that apocynin is not cytotoxic to NHEK even in much higher concentrations than used in this study (up to 1 mM apocynin were tested in MTT assay; data not shown), this compound might also have pharmacological potential in cancer treatment by preventing enhanced MMP-9 secretion.

Human keratinocyte cell lines H357 and HaCaT were reported to possess phagocyte-like NADPH oxidases, which constitutively generate low amounts of superoxide at a rate of 8.4 nmol/h/10^6 cells [36,41]. In our study, unstimulated NHEK produced superoxide at a rate of 13 nmol/h/10^6 cells, which was similar to the rate of keratinocyte cell lines. In contrast to the NHEK studied herein, both keratinocyte cell lines were reported not to stimulate superoxide production in response to TPA [36,41]. Recently, it was demonstrated that the HaCaT keratinocyte cell line does not activate ERK1/2 and JNK signal transduction pathways and therefore does not stimulate MMP-1 expression upon TPA treatment [53]. In contrast, TPA also stimulates MMP-1

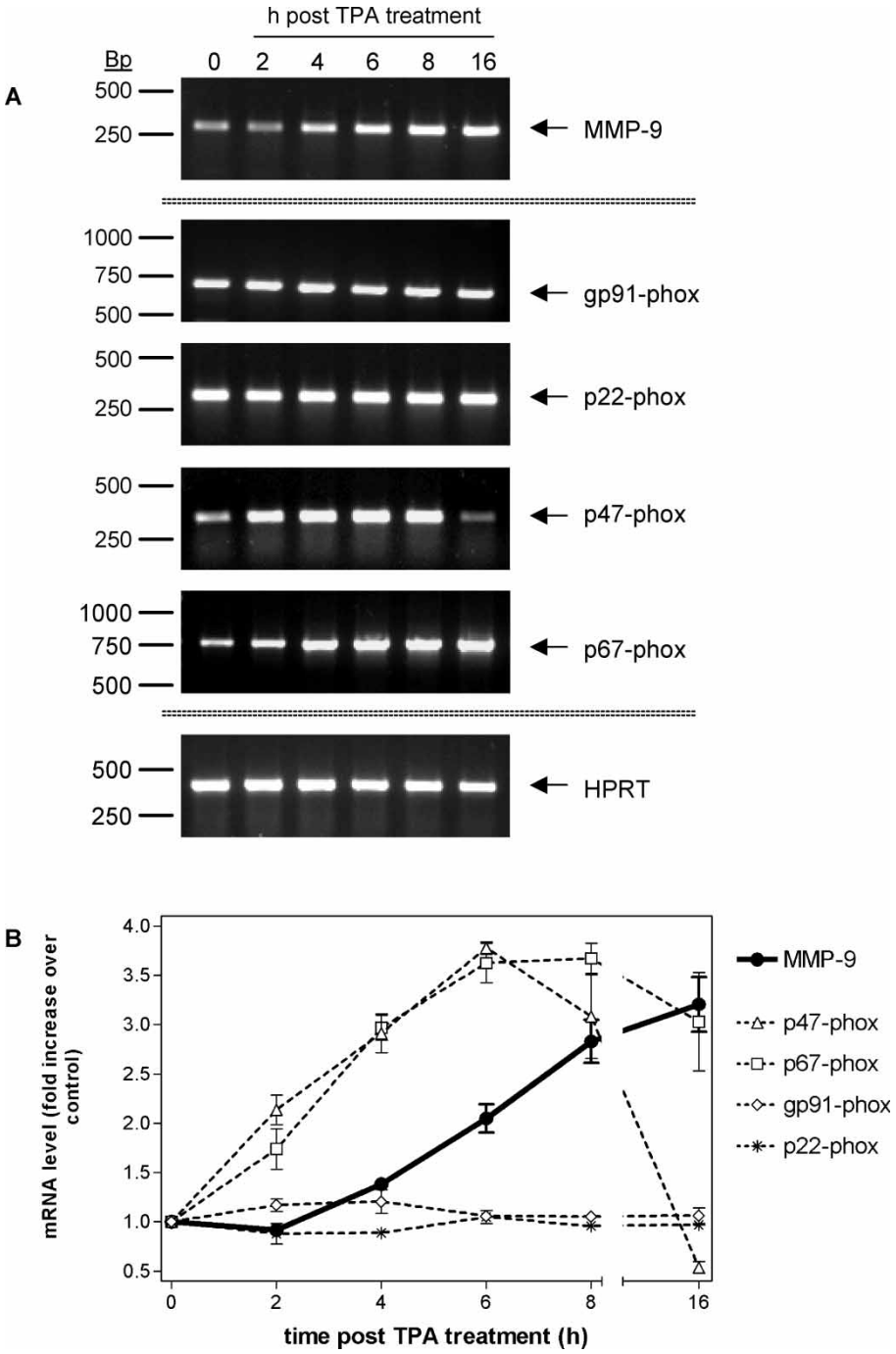

Figure 3. Time course of MMP-9 and NADPH oxidase subunit steady-state mRNA levels after TPA treatment of NHEK. NHEK were stimulated with 1.5 μM TPA, and total RNA was isolated at the indicated time points after treatment. Steady-state mRNA levels of MMP-9 as well as gp91-, p22-, p47- and p67-phox (NADPH oxidase subunits) were detected by RT-PCR. The housekeeping gene HPRT was used as an internal control to standardise the samples for mRNA content. Representative RT-PCRs of 3 independent experiments are shown (A). Densitometric values represent mRNA increases over control, which was set at 1.0. The data represent means ± S.E.M. from 3 independent experiments (B).

expression in NHEK [31]. These findings make clear that TPA effects can differ completely between NHEK and keratinocyte-derived cell lines. The inability of immortalized keratinocyte cell lines to generate enhanced superoxide levels upon TPA treatment might explain the defect in TPA-mediated signal transduction in the HaCaT cell line.

We found that NHEK constitutively express gp91-phox (nox2), p22-, p47- and p67-phox at the mRNA level. These findings are consistent with studies reporting the presence of p22-, p47- and p67-phox mRNA in H357 oral keratinocyte cell line [36] as well as rac1, p22-, p40- and p67-phox protein and gp91-phox (nox2) mRNA in HaCaT and GM16

keratinocyte cell lines [41]. In recent years, homologues of nox2 (termed nox1 and nox3-5) were cloned, which are expressed in different non-phagocytic cells instead of or additionally to nox2 [54]. Nox1 and nox4 were detected at very low mRNA levels in HaCaT and at significant mRNA levels in GM16 keratinocyte cell lines, in addition to the presence of nox2 mRNA [41]. Therefore, we can not exclude that NHEK also express other gp91-phox homologs.

In our study, TPA stimulates the mRNA expression of the cytosolic NADPH oxidase subunits p47- and p67-phox in NHEK. Similarly, other NADPH oxidase-activating agents were described to upregulate the expression of some components of this enzyme system: In rabbit aortic adventitial fibroblasts angiotensin II induces p67-phox expression [26] and in cultured human blood monocytes the cytokines TNF-α and IFN-γ upregulate gp91- and p22-phox expression [25].

In summary, our findings provide evidence for a role of superoxide as a second messenger in gene regulation of MMP-9 in normal human epidermal keratinocytes. We demonstrate the constitutive expression of membrane-bound and cytosolic subunits of the superoxide-generating enzyme NADPH oxidase in NHEK, which is activated by TPA. As enhanced levels of MMP-9 are associated with malignant transformation in carcinogenesis, our study might contribute to further understanding of the mechanism of TPA-induced skin tumor promotion.

Acknowledgements

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