

# Peroxynitrite Production, DNA Breakage, and Poly(ADP-ribose) Polymerase Activation in a Mouse Model of Oxazolone-Induced Contact Hypersensitivity

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Peroxynitrite-induced poly(ADP-ribose) polymerase activation has been implicated in the pathogenesis of various inflammatory conditions. Here we have investigated whether peroxynitrite and poly(ADP-ribose) polymerase may play a role in the pathophysiology of the elicitation phase of contact hypersensitivity. We have detected nitrotyrosine, DNA breakage, and poly(ADP-ribose) polymerase activation in the epidermis of mice in an oxazolone-induced contact hypersensitivity model. As tyrosine nitration is mostly mediated by peroxynitrite, a nitric-oxide-derived cytotoxic oxidant capable of causing DNA breakage, we have applied peroxynitrite directly on mouse skin and showed poly(ADP-ribose) polymerase activation in keratinocytes and in some scattered dermal cells. We have also investigated the cellular effects of peroxynitrite in HaCaT cells, a human keratinocyte cell line. We found that peroxynitrite inhibited cell proliferation and at higher concentrations also caused cytotoxicity. Peroxynitrite

activates poly(ADP-ribose) polymerase in HaCaT cells and poly(ADP-ribose) polymerase activation contributes to peroxynitrite-induced cytotoxicity, as indicated by the cytoprotective effect of the poly(ADP-ribose) polymerase inhibitor 3-aminobenzamide. The cytoprotective effect of 3-aminobenzamide cannot be attributed to inhibition of apoptosis, as apoptotic parameters (caspase activation and DNA fragmentation) were not reduced in the presence of 3-aminobenzamide in peroxynitrite-treated cells. Moreover, poly(ADP-ribose) polymerase inhibition by 3-aminobenzamide dose-dependently reduced interferon-induced intercellular adhesion molecule 1 expression as well as interleukin-1 $\beta$ -induced interleukin-8 expression. Our results indicate that peroxynitrite and poly(ADP-ribose) polymerase regulate keratinocyte function and death in contact hypersensitivity. **Key words:** apoptosis/inflammation/interleukin-8/keratinocyte/necrosis. *J Invest Dermatol* 117:74–80, 2001

**C**ontact hypersensitivity (CHS) is a form of delayed type hypersensitivity caused by various lipophilic compounds (Grabbe and Schwarz, 1998). The sensitization phase of CHS has been the focus of interest of dermato-immunologists for decades and it is therefore well characterized. The elicitation phase of the response is not well understood, however. Nitric oxide, a free radical synthesized from L-arginine by nitric oxide synthase enzymes (NOS), has been proposed to be a major mediator of inflammation in the elicitation phase of CHS (Morita *et al*, 1996; Ormerod *et al*, 1997; Rowe *et al*, 1997; Ross *et al*, 1998). Enhanced expression of the mRNA of inducible NOS (iNOS) has been detected in Langerhans cells and keratinocytes in the elicitation phase of 2,4-dinitrofluorobenzene-induced CHS (Ross *et al*, 1998). In the same model, as well as in picryl-chloride-induced CHS, pharmacologic

NOS inhibition suppressed the hypersensitivity reaction (Morita *et al*, 1996; Ross *et al*, 1998). The question arises, however, whether NO directly or indirectly through the formation of a more reactive intermediate such as peroxynitrite contributes to tissue injury in CHS.

Peroxynitrite, a cytotoxic oxidant formed in the near diffusion-limited reaction of nitric oxide and superoxide (Beckman and Koppenol, 1996; Groves, 1999), is an important mediator of tissue injury in various forms of inflammation, shock, and ischemia-reperfusion injury (Szabó, 1996a; 1996b; Ma *et al*, 1997). The cytotoxic effect of peroxynitrite is attributed to inhibition of the mitochondrial respiratory chain, inactivation of ion channels, initiation of lipid peroxidation, protein oxidation, DNA damage, and interference with protein tyrosine phosphorylation (Gow *et al*, 1996; Kong *et al*, 1996; Groves, 1999). In addition, peroxynitrite triggers an indirect suicidal pathway mediated by poly(ADP-ribose) polymerase (PARP). PARP, a nuclear nick sensor enzyme, cleaves NAD<sup>+</sup> to nicotinamide and ADP-ribose and polymerizes the latter on nuclear acceptor proteins such as histones, transcription factors, and PARP itself (de Murcia and Menissier de Murcia, 1994). Excessive PARP activation depletes cellular NAD<sup>+</sup> and ATP pools and results in necrotic cell death (Cochrane, 1991). Pharmacologic inhibition of PARP or the PARP<sup>-/-</sup> phenotype provided remark-

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Abbreviations: 3-AB, 3-aminobenzamide; iNOS, inducible nitric oxide synthase; PARP, poly(ADP-ribose) polymerase.

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able protection in disease models of hemorrhagic shock, stroke, myocardial ischemia, streptozotocin-induced diabetes, diabetes-associated endothelial dysfunction, arthritis, and traumatic brain injury, indicating that PARP activation may be involved in the pathomechanism of these diseases (Szabó and Dawson, 1998; Liaudet *et al.*, 2000; Soriano *et al.*, 2001). Very limited information is available in the literature regarding the possible role of peroxynitrite and PARP activation in skin pathology. Nitric oxide, a parent molecule of peroxynitrite, has been shown to be produced by iNOS in keratinocytes, Langerhans cells, and dermal microvascular endothelial cells (Arany *et al.*, 1996; Qureshi *et al.*, 1996; Hoffmann *et al.*, 1999). As superoxide is continuously "leaking" from the mitochondrial respiratory chain and is also produced by NADPH oxidase and xanthine oxidase enzymes under inflammatory conditions, the possibility exists that peroxynitrite is formed in the inflamed skin and PARP activation may also occur. Indeed, peroxynitrite has been shown to be produced by ultraviolet-B-irradiated keratinocytes and endothelial cells and has been implicated in ultraviolet-B-induced skin inflammation (Deliconstantinos *et al.*, 1996a; 1996b; 1996c). Furthermore, nitrotyrosine, as an indicator of *in vivo* peroxynitrite production, has been detected in skin homogenates from burns, chronic ultraviolet-B exposure, ischemia reperfusion, murine leishmaniasis, and systemic sclerosis (Giorgio *et al.*, 1996; Hattori *et al.*, 1996; Cotton *et al.*, 1999; Um *et al.*, 1999; Rawlingson *et al.*, 2000).

The aim of this study was to investigate (i) whether peroxynitrite is produced in the skin during CHS, (ii) whether DNA breakage and PARP activation occur in CHS, (iii) whether peroxynitrite can activate PARP in the skin, (iv) the cytostatic and cytotoxic effect of peroxynitrite on the human keratinocyte cell line HaCaT, (v) the role of PARP in the peroxynitrite-induced cytostasis and cytotoxicity in HaCaT cells, and (vi) whether PARP is involved in the cytokine-induced activation of HaCaT cells.

## MATERIALS AND METHODS

**Materials** Unless specified otherwise, all chemicals and materials listed were purchased from Sigma-Aldrich (St. Louis, MO). 3-Aminophenanthridinone was from the rare chemical library of Sigma-Aldrich. Peroxynitrite was a kind gift from Dr. Harry Ischiropoulos (University of Pennsylvania Medical Center, Philadelphia, PA).

**Animals** Animal experiments conform with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health and the treatment protocol was approved by the Institutional Animal Care and Use Committee. Female CD1 mice were used in the experiments and were allowed free access to food and water.

**CHS model and *in vivo* peroxynitrite treatment** A total of 12 mice were randomized into two groups (control group and CHS group). Sensitization was carried out on the shaved abdominal wall by the administration of 100  $\mu$ l 2% oxazolone solubilized in acetone:olive oil 4:1 (CHS group,  $n = 6$ ) or 100  $\mu$ l vehicle (control group,  $n = 6$ ). After 7 d, all animals were challenged by applying 20  $\mu$ l 0.5% oxazolone on each ear. After 24 h, mice were sacrificed by CO<sub>2</sub> and ears were immediately removed and placed in formalin or frozen in cryoembedding medium.

For *in vivo* peroxynitrite treatment, peroxynitrite was diluted in phosphate-buffered saline (PBS, pH 11.0) and 400 nmol was smeared on the shaved abdominal wall in a volume of 200  $\mu$ l. Control mice were treated with the vehicle (PBS, pH 11.0). After 30 min, skin was excised and frozen in cryoembedding medium.

**Immunohistochemistry** The immunohistochemical procedure was carried out basically as described previously (Virág *et al.*, 1998b) with slight modifications, as follows. Paraffin sections (5  $\mu$ m) were treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and then rinsed briefly in PBS. Non-specific binding was blocked by incubating the slides for 1 h in 2% goat serum (in PBS). To detect nitrotyrosine, rabbit polyclonal antinitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) was applied for 2 h in a dilution of 1:1000 at room temperature. (Control sections were incubated with either normal rabbit serum or with the primary antibody in the presence of 10 mM nitrotyrosine.) Following extensive washing (five  $\times$  5 min) with PBS, immunoreactivity was detected with a

biotinylated goat antirabbit secondary antibody and the avidin-biotin-peroxidase complex (ABC), both supplied in the Vector Elite kit (Vector Laboratories, Burlingame, CA). Color was developed using nickel-DAB substrate. Sections were counterstained with nuclear fast red for 2 min, dehydrated, and mounted in Permount medium.

**Detection of DNA strand breaks** DNA breakage was detected with a commercially available kit (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions. Dewaxed and rehydrated sections were treated with 20  $\mu$ g per ml proteinase K (in 10 mM Tris/HCl pH 7.8) for 30 min at 37°C. DNA breaks were labeled with terminal deoxyribonucleotide transferase (TdT) and a deoxyribonucleotide mix containing fluorescein isothiocyanate (FITC) labeled dUTP for 60 min at 37°C. After washing, sections were incubated with anti-FITC peroxidase conjugate (30 min at room temperature), and peroxidase was detected as described above for immunohistochemistry.

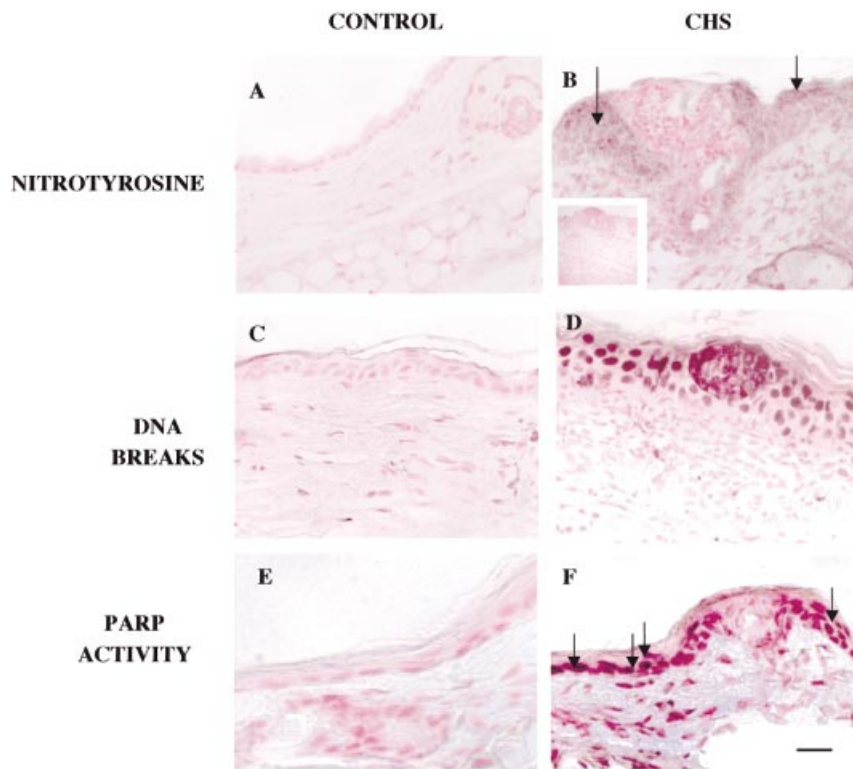
***In situ* PARP activity assay** PARP activity in tissues was detected by an *in situ* histochemical reaction utilizing biotinylated NAD<sup>+</sup> as substrate of PARP (Zhang, 1997). Cryosections were fixed in 95% ethanol at -20°C and then rinsed in PBS. Reaction mixture (10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 70  $\mu$ M biotinylated NAD<sup>+</sup>, in 100 mM Tris, pH 8.0) was applied to the sections for 30 min at room temperature. A reaction mix containing 5 mM 3-aminobenzamide (3-AB) or biotinyl-NAD<sup>+</sup>-free reaction mix was used as control. After three washes in PBS, incorporated biotin was detected by peroxidase-conjugated streptavidine (Trevigen, Gaithersburg, MD). Color development, counterstaining, and mounting were carried out exactly as described for the immunohistochemistry.

**Cell culture and peroxynitrite treatment** HaCaT cells (kindly provided by Dr. Ulrich Rodeck, Thomas Jefferson University, Philadelphia, PA, with the permission of Professor Norbert E. Fusenig, German Cancer Research Center, Heidelberg, Germany) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mM glutamine, 10 mM HEPES, 100 U per ml penicillin, and 100  $\mu$ g per ml streptomycin. The concentration of authentic peroxynitrite was determined spectrophotometrically by measuring absorbance at 302 nm using an extinction coefficient of 1670 M<sup>-1</sup> cm<sup>-1</sup>. Peroxynitrite was diluted in PBS (pH 11.0) and was added to the cells in 1/10 of the volume of the cell suspension. Under these conditions addition of peroxynitrite does not shift the pH of the medium. Control samples were treated with PBS (pH 11.0) only. The effect of decomposed peroxynitrite (kept in PBS pH 7.2 at 37°C for 30 min) has also been tested in all of the assays and was found to have no effect on any parameters measured.

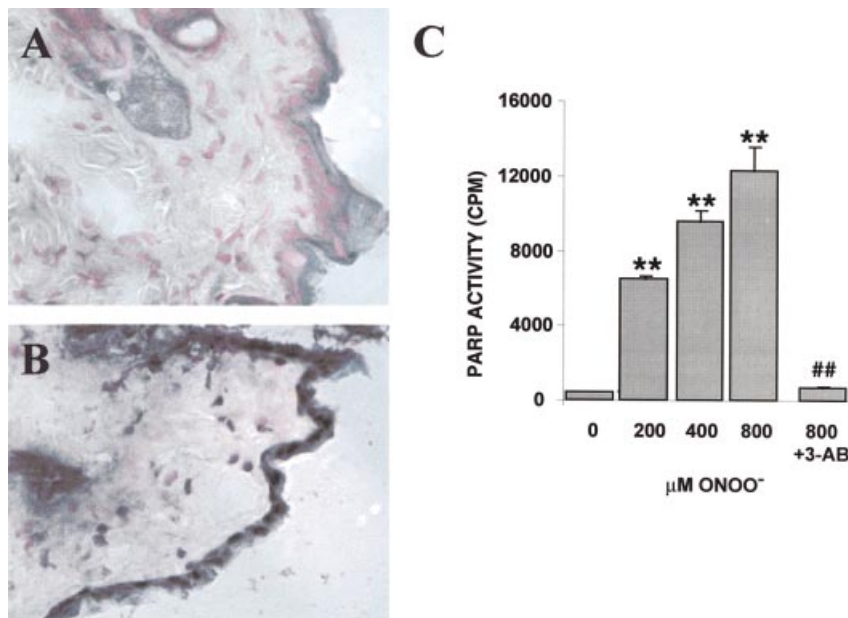
**Cell proliferation and cytotoxicity assays** Cell proliferation was determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Amersham Pharmacia Biotechnology, Piscataway, NJ) based on the measurement of BrDU incorporation. The assay was carried out in 96-well tissue culture plates following the manufacturer's instructions. Cytotoxicity was quantitated with the colorimetric MTT test, as described previously (Virág *et al.*, 1995).

**Cellular PARP activity assay** PARP activity was measured as previously described (Virág *et al.*, 1998a). Medium was removed from the cells 20 min after peroxynitrite treatment and cells were incubated at 37°C in 0.5 ml assay buffer [56 mM Hepes pH 7.5, 28 mM KCl, 28 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.01% digitonin, and 0.125  $\mu$ M <sup>3</sup>H-NAD (0.5  $\mu$ Ci per ml)]. Cells were then scraped and transferred into Eppendorf tubes. Next, 200  $\mu$ l ice-cold 50% trichloroacetic acid (TCA) was added to the samples and tubes were incubated for 4 h at 4°C. Samples were then spun (10,000g, 10 min) and the pellets were washed twice in ice-cold 5% TCA and solubilized overnight in 250  $\mu$ l 2% sodium dodecyl sulfate/0.1 N NaOH at 37°C. The contents of the tubes were added to 7 ml ScintiSafe Plus scintillation liquid (Fisher Scientific) and radioactivity was determined in a liquid scintillation counter (Wallac, Gaithersburg, MD).

**Measurement of caspase activation** Caspase activity was measured by the cleavage of the fluorogenic tetrapeptide-amino-4-methylcoumarine conjugate (DEVD-AMC), as described previously (Virág and Szabó, 1998). Cells were harvested 6 h after peroxynitrite treatment, washed in 1  $\times$  PBS, and then lysed in a lysis buffer (10 mM HEPES, 0.1% CHAPS, 5 mM dithiothreitol, 2 mM ethylenediamine tetraacetic acid, 10  $\mu$ g per ml aprotinin, 20  $\mu$ g per ml leupeptin, 10  $\mu$ g per ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride, pH 7.25). Cell lysates and substrates (50  $\mu$ M) were combined in triplicate in the caspase reaction buffer (100 mM HEPES, 10% sucrose, 5 mM



**Figure 1. Tyrosine nitration, DNA breakage, and PARP activation in CHS.** Ears were obtained from sensitized mice 24 h after the second challenge with oxazolone. Paraffin-embedded sections were used for nitrotyrosine staining (A, B) and for the detection of DNA breaks (C, D). PARP activity was visualized by *in situ* PARP assay in frozen sections (E, F). For all three reactions control ears from nonsensitized mice (A, C, E) were stained in parallel with CHS ears (B, D, F). Keratinocytes from CHS stain positive for nitrotyrosine (B), display massive DNA breakage (D), and show PARP activation (F). Infiltrating cells of microabscessi are negative for nitrotyrosine but many of them contain DNA breaks. Insert shows negative staining of a CHS section stained with normal rabbit serum instead of rabbit polyclonal antinitrotyrosine antibody. (Scale bar, 6  $\mu$ m.)



**Figure 2. Peroxynitrite-induced PARP activation in the skin and in HaCaT cells.** Peroxynitrite (400 nmol in 200  $\mu$ l) was applied to the skin of mice. After 30 min, skin samples were excised and frozen in cryoembedding medium. Cryosections were stained by *in situ* PARP enzyme histochemistry. Intense PARP activation was found in the peroxynitrite-treated skin (B) but not in the control (vehicle-treated) skin (A) (scale bar, 6  $\mu$ m). HaCaT cells were treated with the indicated concentrations of peroxynitrite for 30 min and PARP activity was then determined by <sup>3</sup>H-NAD incorporation (C). Peroxynitrite induced a significant (\*\**p* < 0.01) PARP activation. Pretreatment of cells with 3 mM PARP inhibitor, 3-AB, abolished PARP activation (##*p* < 0.01).

dithiothreitol, 0.1% CHAPS, pH 7.25). AMC liberation was monitored over time with a Shimadzu fluorimeter using 380 nm excitation and 460 nm emission wavelength.

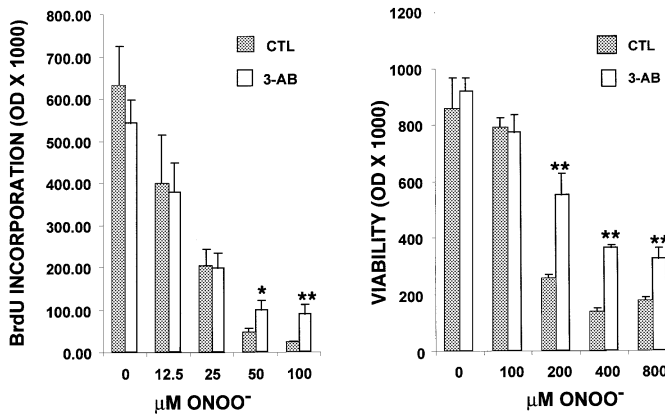
**Measurement of DNA fragmentation** DNA fragmentation was measured with a commercially available ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions, as previously described (Virág *et al*, 1998a). The assay is based on the detection of histone-associated DNA fragments from the cytoplasmic fraction of cell lysates. HaCaT cells ( $2 \times 10^5$ ) were seeded into 12-well plates in 1000  $\mu$ l culture medium and treated with different concentrations of peroxynitrite. After 6 h incubation (37°C, 5% CO<sub>2</sub>) cells were washed with cold PBS, scraped into 400  $\mu$ l incubation buffer (supplied with the kit), and incubated at 4°C for 60 min. Cell lysates

were then transferred to Eppendorf tubes and centrifuged (10,000g, 10 min). Supernatants were diluted 10 $\times$  and measured in an ELISA (antihistone capturing antibody, peroxidase-conjugated anti-DNA secondary antibody, ABTS substrate). Absorbance was then measured (405 nm) *versus* substrate solution as a blank using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA).

**Determination of intercellular adhesion molecule 1 (ICAM-1) expression** HaCaT cells were stimulated by 30 ng per ml (300 U per ml) recombinant human interferon- $\gamma$  (R&D Systems, Minneapolis, MN) for 24 h. Cells were then scraped and washed twice in ice-cold PBS. Staining was performed on ice with FITC-conjugated monoclonal antihuman ICAM-1 antibody (Pharmingen, San Diego, CA) and isotype-matched monoclonal antibody (Pharmingen) as a control for 1 h.

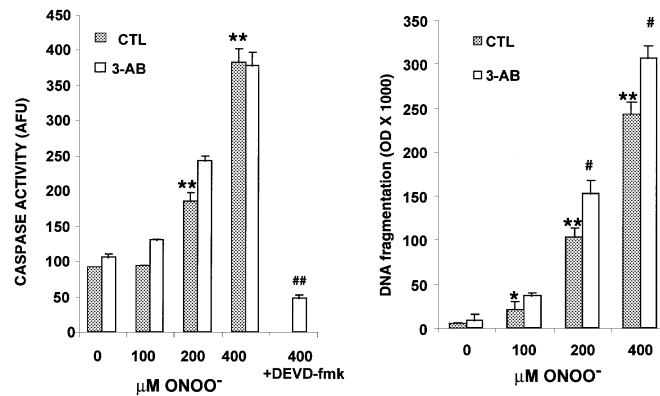
Cells were then washed twice in ice-cold PBS and fixed in 1% paraformaldehyde. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA) and ICAM-1 expression was determined as an increase in mean fluorescence intensity.

**Measurement of interleukin-8 (IL-8) production** HaCaT cells were stimulated for 24 h with 20 ng per ml human recombinant IL-1 $\beta$  (R&D Systems) in the presence or absence of PARP inhibitors. Supernatants were then collected and assayed for IL-8 in a commercially available sandwich ELISA system (R&D Systems).

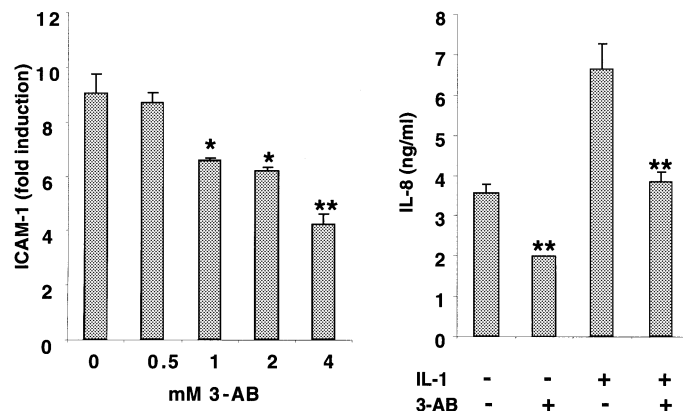


**Figure 3. Antiproliferative and cytotoxic effect of peroxynitrite in HaCaT cells.** Proliferation (*left panel*) and viability (*right panel*) of HaCaT cells have been determined after treatment of cells with the indicated concentrations of peroxynitrite. Treatment was carried out in the presence or absence of 3-AB (3 mM). Asterisks indicate significant (\* $p < 0.05$ , \*\* $p < 0.01$ ) protection provided by the PARP inhibitor.

**Figure 4. Peroxynitrite-induced caspase activation and DNA fragmentation in HaCaT cells.** HaCaT cells were treated with increasing concentrations of peroxynitrite in the presence or absence of 3 mM 3-AB. After 6 h, caspase activity (*left panel*) and DNA fragmentation were determined. Asterisks indicate significantly (\* $p < 0.05$ , \*\* $p < 0.01$ ) elevated caspase activity or DNA fragmentation. Hash marks indicate significant difference between 3-AB-treated and untreated cells.



**Figure 5. Effect of PARP inhibition on ICAM-1 expression and IL-8 production of immune-stimulated HaCaT cells.** (A) ICAM-1 expression; (B) IL-8 production. HaCaT cells were stimulated for 24 h with 30 ng per ml human recombinant interferon- $\gamma$  to induce ICAM-1 expression or with human recombinant IL-1 $\beta$  to induce IL-8 secretion. Induction was carried out in the presence or absence of 3-AB. 3-AB (3 mM) caused significant (\* $p < 0.05$ , \*\* $p < 0.01$ ) inhibition of both ICAM-1 expression and IL-8 secretion.



**Statistical analysis** Results are reported as mean  $\pm$  SD of quadruplicate samples. Experiments were repeated at least  $n = 3-4$  times. Student's  $t$  test was used to compare mean values. Statistical differences were declared significant for  $p \leq 0.05$ .

## RESULTS

**Tyrosine nitration in the inflamed skin during CHS** As peroxynitrite nitrates the tyrosine residues of various proteins, *in vivo* peroxynitrite production is indicated by the presence of nitrotyrosine-containing proteins in the tissues. Nitrotyrosine could be detected by immunohistochemistry in the sections of ears from CHS mice but not from control (nonsensitized) ones (**Fig 1**). Tyrosine nitration of keratinocytes was most intense in the vicinity of inflammatory foci (microabscessi). Somewhat surprisingly, only a small number of infiltrating granulocytes displayed immunoreactivity for nitrotyrosine. Nitrotyrosine was not detectable in control nonsensitized skin (**Fig 1**).

**Increased DNA breakage during CHS-induced skin inflammation** As the formation of nitrotyrosine is most probably caused by peroxynitrite, which is a potent inducer of DNA breakage, we performed TUNEL staining on skin samples from CHS and control nonsensitized animals (**Fig 1**). Only faint positivity could be detected in some scattered cells in control skin tissues. In contrast, CHS-induced dermatitis resulted in a massive increase of TUNEL-positive cells both in the infiltrating cells of microabscessi and in keratinocytes (**Fig 1**). It is worthwhile noting here that the TUNEL assay is widely used to detect apoptotic cells; however, what it detects are free 3'-OH ends in DNA, which can be caused by either single or double strand breaks. In fact, the TUNEL assay has been used to detect single strand breakage in several systems (Didier *et al*, 1996; Ahmadi and Ong, 1999; Jung *et al*, 2000; Mihm *et al*, 2000; Shen and Ong, 2000). Moreover, it has also been reported recently that proteinase K treatment (applied in

TUNEL kits) enhances the detection of single strand breaks in non-apoptotic cells (Gal *et al*, 2000). In our system, we believe that DNA single strand breaks are responsible for the majority of TUNEL positivity, as most TUNEL-positive nuclei do not show apoptotic morphology. Moreover, the vast majority of TUNEL-positive cells stain negative for p85 (a fragment of PARP generated by caspase-mediated cleavage of the 116 kDa enzyme during apoptosis) (not shown), indicating that no caspase activation occurs. Moreover, we found that almost 100% of keratinocytes surrounding the inflammatory foci are TUNEL positive, which would be an improbably high apoptotic percentage.

**PARP activation in CHS** We detected PARP activation in CHS by *in situ* PARP activity assay. PARP activity was not detectable in control sections (Fig 1E). In CHS, we found strong staining in keratinocytes and also in various dermal cells (Fig 1F). When the reaction was carried out in the presence of 5 mM 3-AB, no staining could be detected (not shown).

**Peroxynitrite induces PARP activation in the skin and in HaCaT cells** As peroxynitrite has been shown to cause DNA single strand breaks in human keratinocytes (Spencer *et al*, 1996), we investigated whether poly(ADP-ribose) polymerase, a nuclear nick sensor enzyme, becomes activated in peroxynitrite-treated keratinocytes. To prove that peroxynitrite can activate PARP in the skin, we applied 400 nmol peroxynitrite on the skin of mice for 30 min. Control animals were treated with vehicle (PBS, pH 11.0). We found intense PARP activation in the peroxynitrite-treated skin but not in control skin, as detected by *in situ* PARP activity assay (Fig 2A, B). PARP activation was most intense in keratinocytes; however, some dermal cells also stained positive. The presence of the PARP inhibitor 3-AB (5 mM) in the reaction mixture abolished the staining.

We also investigated the cellular effects of peroxynitrite in a human keratinocyte-derived cell line (HaCaT). Peroxynitrite induced a concentration-dependent PARP activation (Fig 2C), which was completely blocked by the PARP inhibitor 3-AB.

**Antiproliferative and cytotoxic effect of peroxynitrite in HaCaT cells** Peroxynitrite caused a dose-dependent inhibition of cell proliferation (Fig 3A) and also caused cytotoxicity (Fig 3B). PARP is not likely to be involved in proliferation inhibition caused by the lowest concentrations of peroxynitrite, as 3-AB had no effect on this parameter. At higher concentrations, the antiproliferative effect of peroxynitrite was in part mediated by PARP activation, as indicated by the significant cytoprotective effect of 3-AB. 3-AB also provided significant protection against peroxynitrite-induced cytotoxicity (Fig 3B). Similar results were obtained with another PARP inhibitor, 3-amino-phenanthridinone (5  $\mu$ M) (not shown).

**Peroxynitrite-induced caspase activation and DNA fragmentation in HaCaT cells** Peroxynitrite has been reported to cause apoptosis in various cell types including thymocytes, HL-60 cells, and PC12 cells (Estevez *et al*, 1995; Lin *et al*, 1995; Virág and Szabó, 1998; Virág *et al*, 1998a). Here we show that peroxynitrite also causes apoptosis in HaCaT cells, as indicated by increased caspase-3-like activity (Fig 4A) and DNA fragmentation (Fig 4B). With other cell types we have previously shown that pharmacologic PARP inhibition or the absence of PARP (in PARP<sup>-/-</sup> cells) shifts the oxidant-induced necrotic cell death toward apoptosis and toward the normal phenotype (Virág *et al*, 1998a; 1998c). The increase in DNA fragmentation is usually more pronounced than the increase in caspase activation, indicating that DNA fragmentation is more sensitive to the consequences (e.g., ATP depletion) of PARP activation. In peroxynitrite-treated HaCaT cells, in the presence of 3-AB we have detected a moderately increased DNA fragmentation but virtually unchanged caspase activity (Fig 4). 3-AB (2–16 mM) alone, however, did not cause caspase activation or DNA fragmentation (data not shown).

**Involvement of PARP in the regulation of ICAM-1 expression and IL-8 production** Human recombinant interferon- $\gamma$  (30 ng per ml) induced a ninefold increase in the cell surface expression of ICAM-1. Interferon- $\gamma$ -induced ICAM-1 expression was dose-dependently inhibited by 3-AB. The highest concentration (4 mM) of the PARP inhibitor caused a 50% reduction in the expression of ICAM-1. Similar results were obtained with another PARP inhibitor, 3-amino-phenanthridinone (5  $\mu$ M) (not shown).

In serum-containing media, HaCaT cells are known to spontaneously produce IL-8 (Stein *et al*, 1997). We found that IL-1 $\beta$  increased the production of IL-8 by HaCaT cells. Both the basal and the IL-1 $\beta$ -induced IL-8 production were inhibited by 3-AB (4 mM), indicating a possible involvement of PARP in the regulation of IL-8 expression.

## DISCUSSION

Here we provide evidence that intense tyrosine nitration occurs in the epidermis during CHS. Detection of nitrotyrosine in tissues is regarded as an indicator of *in vivo* peroxynitrite production (Beckman and Koppenol, 1996). Although microabscessi in the skin contained a large number of neutrophil granulocytes, the vast majority of these cells did not stain for nitrotyrosine. In contrast to infiltrating cells, keratinocytes showed the most intense staining for nitrotyrosine, thus representing the prime targets of peroxynitrite in the skin during CHS. The most likely scenario for increased tyrosine nitration is that inflammatory cytokines induce the upregulation of iNOS synthase and overproduction of superoxide. iNOS-derived NO and superoxide, in turn, react to form peroxynitrite, which nitrates tyrosine. Other mechanisms of *in vitro* tyrosine nitrations, most notably the one mediated by myeloperoxidase, have also been described (Sampson *et al*, 1998); however, the *in vivo* significance of these alternative tyrosine nitration pathways remains to be proven. Our finding that inflammatory cells in microabscessi were negative for nitrotyrosine makes it unlikely that myeloperoxidase-mediated tyrosine nitration occurs in our model.

We have also shown that, in CHS, intense DNA breakage occurs in keratinocytes as well as in infiltrating cells. Based on the lack of apoptotic morphology, lack of caspase activation, and the unanimous staining of keratinocytes surrounding the inflammatory foci, we propose that single stranded DNA breakage occurs in the keratinocytes during CHS. In fact, peroxynitrite has been shown to cause DNA damage in various cell types including human keratinocytes (Spencer *et al*, 1996; Szabó, 1996b). In light of the proapoptotic effect of peroxynitrite, however, it cannot be excluded that a fraction of TUNEL-positive cells are apoptotic.

We have investigated the cellular effects of peroxynitrite in HaCaT cells with special regard to the role played by PARP. Nitric oxide, one of the parent molecules of peroxynitrite, has been shown to enhance the proliferation of HaCaT cells (Stallmeyer *et al*, 1999). Peroxynitrite at lower concentrations was antiproliferative, further emphasizing the differences in the cellular effects of NO and peroxynitrite. The differences in the chemistry, reactivity, and target molecule spectrum may account for the often divergent cellular effects of NO and peroxynitrite. At higher concentrations peroxynitrite induced cytotoxicity. Similarly to our previous observations (Szabó *et al*, 1998; Virág *et al*, 1998a; 1998c) we found cytoprotection by PARP inhibitors against peroxynitrite-induced cytotoxicity in HaCaT cells. The cytoprotection provided by 3-AB is not related to inhibition of apoptosis, as caspase activation and DNA fragmentation were not reduced. On the contrary, 3-AB caused a moderate increase in peroxynitrite-induced apoptotic DNA fragmentation. This is not due to the cytotoxic effect of 3-AB, as 3-AB alone (2, 4, 8, 16 mM) caused neither cytotoxicity nor caspase activation or DNA fragmentation (data not shown). The increased output of apoptotic parameters in 3-AB-treated and oxidatively stressed cells is in line with our previous findings as well as reports from other laboratories showing

a diversion by PARP inhibitors of oxidant-induced necrosis toward apoptosis (Virág *et al*, 1998a; Filipovic *et al*, 1999; Ha and Snyder, 1999). Switching the mode of cell death from necrosis to apoptosis may be anti-inflammatory *in vivo*, as it prevents the leakage of the proinflammatory cell content into the tissues. In oxidatively injured cells, the mechanism of the necrosis to apoptosis switch in the presence of PARP inhibitors is probably the preservation of NAD<sup>+</sup> and ATP pools that would be depleted by PARP activation. In HaCaT cells, however, the increase in DNA fragmentation in the presence of 3-AB is less than in other cell types. This is likely to be due to differences in the metabolism of different cell types.

The question arises, however, whether PARP inhibitors may have therapeutic potential in CHS. We believe that PARP inhibition may prevent oxidant-induced cell dysfunction and cytotoxicity, by preserving cellular energy stores. PARP inhibitors may also have beneficial effects in other human skin diseases such as Stevens–Johnson syndrome or toxic epidermal necrolysis where keratinocyte necrosis is accompanied by iNOS expression (Lerner *et al*, 2000); therefore the NO–peroxynitrite–PARP activation–necrosis pathway may be involved in the pathogenesis of these diseases.

Another important mechanism by which PARP may be involved in CHS and other inflammatory dermatoses is the regulation of keratinocyte activation. Upon cytokine stimulation, keratinocytes are known to produce numerous humoral factors, among them chemokines such as IL-8. IL-8 is responsible for the recruitment of neutrophil granulocytes into the sites of inflammation, so that inhibition of IL-8 production by PARP inhibitors may reduce inflammation. Upregulation of adhesion molecules is also important in the regulation of cellular trafficking during inflammation; thus the inhibitory effect of PARP inhibition on ICAM-1 expression might be anti-inflammatory (Zingarelli *et al*, 1998). The transcription factor NFκB is a key regulator of the expression of inflammatory proteins (Baeuerle and Baltimore, 1996), including chemokines and adhesion molecules. PARP has been shown to enhance the effect of NFκB (Oliver *et al*, 1999), indicating that inhibition of NFκB activation by PARP inhibitors may be responsible for the reduced IL-8 production and ICAM-1 expression in 3-AB-treated HaCaT cells.

## CONCLUSIONS

(i) CHS leads to peroxynitrite production, DNA breakage, and PARP activation in keratinocytes. (ii) Peroxynitrite activates PARP in the skin. (iii) Peroxynitrite causes apoptosis characterized by caspase activation and DNA fragmentation in HaCaT cells. (iv) At higher concentrations, peroxynitrite causes necrosis in HaCaT cells. (v) Peroxynitrite activates PARP in HaCaT cells. (vi) PARP plays a role in the cytokine-induced activation of keratinocytes.

Our findings provide rationale for experimental therapies of CHS and possibly other inflammatory dermatoses by targeting peroxynitrite or PARP.

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