The role of H_2O_2 as a mediator of UVB-induced apoptosis in keratinocytes

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1 Introduction

Apoptosis is an important and well-controlled form of cell death that occurs under a variety of physiological and pathological conditions. This process has been recognized to be of major importance for tissue homeostasis, neurodegeneration, carcinogenesis, and autoimmune diseases.

The skin, which represents one of the largest organ systems of the human body, is situated at the interface between the body and its environment and acts as a barrier to harmful effects of ultraviolet radiation (UVR) and exogenous chemicals. UV light is one of the most important environmental factors. UV light, in particular the middle wave length range (290-320 nm), UVB, can be a hazard to human health by inducing cancer, premature skin aging, immunosuppression, inflammation, and cell death (Young, 1987; Gilchrest, 1990; Fisher *et al*, 1996; Kraemer, 1997). A hallmark event of UV exposure is the occurance of sunburn cells with the epidermis (Danno and Horio, 1987). By using morphological criteria, these cells have been recognized as keratinocytes undergoing apoptosis. By applying more advanced techniques, it was latter confirmed that UV light induces apoptosis in keratinocytes and epithelial cell lines (Casciola-Rosen *et al*, 1994; Schwarz *et al*, 1995; Benassi *et al*, 1997; Gniadecki *et al*, 1997; Leverkus *et al*, 1997). The functional role of sunburn cells was regarded as a marker for severity of sun damage. Moreover, sunburn cell formation may also be important for preventing skin cancer (Ziegler *et al*, 1994). The cells of the skin contain a broad range of protective mechanisms to prevent DNA damage, including growth arrest followed by DNA repair and cell death by apoptosis. Both of these mechanisms prevent the transmission of mutations to daughter cells that can lead to transformation and carcinogenesis. Failure of these pathways can result in abnormal cell proliferation and skin carcinogenesis. UV-damaged keratinocytes that failed to repair the damage will die as apoptosis, thus escaping the risk of becoming malignant. Therefore, the formation of apoptotic cells can be regarded as a scavenging phenomenon protecting the individual from developing UV-induced skin cancer.

1.1 Differences between apoptosis and necrosis

Cell death can occur by either of two distinct mechanisms, apoptosis or necrosis (Schwartzman and Cidlowski, 1993; Vermes and Haanen, 1994). There are many observable morphological and biochemical differences between apoptosis and necrosis (Vermes and Haanen, 1994). Necrosis occurs when cells are exposed to extreme variance from physiological conditions which may result in damage to the plasma membrane. Necrosis begins with an impairment of the cells' ability to maintain homeostasis, leading to an influx of water and extracellular ions which causes the intracellular organelles, most notably the mitochondria, and the entire cells to swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response. Apoptosis, by contrast, is an active form of cell death that initiated by a variety of stimuli, such as elevation of intracellular Ca^{2+}, ionising and non-ionising irradiation, growth factor deprivation, and oxidative stress (Martin and Cotter, 1991; Nagata, 1997; Raff, 1998; Murphy *et al*, 2001). Cells undergoing apoptosis show characteristic morphological and biochemical features (Cohen, 1993), including detachment of the cells from neighbouring cells, chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasma and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. An important biochemical marker of apoptosis is the fragmentation of DNA at the linker regions between nucleosomes by endonucleases (Arends *et al*, 1990). *In vivo*, apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells (Savill *et al*, 1989). Due to this efficient mechanism for the removal of apoptotic cells *in vivo* no inflammatory response is elicited. *In vitro*, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of *in vitro* cell death has been termed "secondary necrosis".

1.2 Methods for the detection of apoptosis

Apoptosis plays an important role in a variety of cellular events, and consequently the analysis of apoptosis has been key of interest in many investigations. A number of assay methods have been developed for the detection of apoptosis. These methods include measurement of one of the following apoptotic parameters:

1.2.1 Morphological changes

The apoptotic process involves a sequence of cell shrinkage, increased cytoplasmic density, chromatin condensation and segregation into sharply circumscribed masses that are located around the nuclear membrane and can form blister-like protrusions ("budding"). The latter then separate to produce membrane-bound apoptotic bodies. Initially, the mitochondria and the Golgi apparatus show no signs of swelling and the nuclear membrane remains intact for an extended period of time. These morphological changes can be detected by electron microscopy (Kerr *et al*, 1972). Budding and formation of apoptotic bodies can also be observed by simple phase contrast light microscopy. On the other hand, the DNA binding dye Hoechst 33342 penetrates the plasma membrane and stains DNA in cells, without permeabilization. In contrast to normal cells, the nuclei of apoptotic cells include highly condensed chromatin that is uniformly stained by Hoeschst 33342. This can take the form of crescents around the periphery of the nucleus, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscopy. Apoptotic nuclear changes are also visible in permeabilized apoptotic cells stained with other DNA binding dyes such as DAPI. The characteristic condensed nuclei of apoptotic cells are clearly visible by DAPI staining.

1.2.2 Fragmentation of DNA

The degradation of nuclear DNA into nucleosomal units is one of the best characterized biochemical features of apoptotic cell death (Earnshaw, 1995). DNA fragmentation has been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+}-dependent nuclear endonuclease (Wyllie, 1980). This enzyme selectively cleaves DNA at sites located

between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments. These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit. DNA fragmentation can also be used to study apoptosis in individual cells. The methods used to assess DNA strand breaks are based on labeling/staining the cellular DNA. In general, two different labeling methods may be used to identify DNA in apoptotic cells. 1) Enzymatic labeling: Cellular DNA is labeled with modified nucleotides (e.g. biotin-dUTP, DIG-dUTP, fluorescein-dUTP) using exogenous enzymes (e.g. terminal transferase, DNA polymerases). The TUNEL (TdT-mediated X-dUTP nick end labeling) assay is the most commonly used method (Gavrieli *et al*, 1992). Terminal deoxynucleotidyl transferase (TdT) is able to label blunt ends of double-stranded DNA independent of a template. The labeled DNA is subsequently analyzed by flow cytometry, fluorescence microcopy or light microscopy. 2) Staining with fluorochromes: Cellular DNA is stained with fluorescent DNA-binding dyes (DNA fluorochromoes) capable of intercalating with DNA. Upon binding to DNA, these dyes become highly fluorescent. If the cells are permeabilized, the low molecular weight (LMW) DNA inside the cytoplasm of apoptotic cells leaks out during the subsequent rinse and staining procedure. The lower DNA content of these cells means they contain less DNA stained by fluorochrome. Thus, cells with lower DNA staining than that of G_1 cells (the so-called "sub-G_1" peaks) are considered to be apoptotic cells. The reduction in staining/DNA content of these cells is measured by flow cytometry.

1.2.3 Activation of apoptotic caspases

Regardless of the origin of the apoptotic stimulus, the commitment to apoptosis occurs through activation of caspases, a family of cysteine proteases present in growing cells as inactive precursors (Thornberry and Lazebnik, 1998). At least 14 members of the caspase family have been identified and several caspases are thought to mediate very early stages of apoptosis (Fraser and Evan, 1996). All these proteases are synthesized as pro-enzymes, activation involves cleavage at aspartate residues that could themselves be sites for the caspase family. Upon activation, these proteases cleave numerous substrates at the carboxy site of an aspartate residue. As caspases are probably the most important effector molecules for triggering the biochemical events which lead to apoptotic cell death, assays

for determination of caspase activation can detect apoptosis earlier than many other commonly used methods. The most elucidatory assay for these caspases involves western blot detection of proteolytic cleavage products of caspase substrates found in apoptotic cells, for example, employing anti-PARP antibody. This antibody can detect intact and cleaved forms of poly(ADP-ribose) polymerase, a target for some caspases (for example caspase 3 and 7). Another quantitative and specific measurement of caspase activity, e.g. caspase 3 activity, has also been developed. This assay is based on the detection of cleaved caspase substrates. However, most of the caspase substrates are not exclusively cleaved by a specific caspase but only preferentially, while other members of the caspases family act on these substrates to a lower extent.

1.2.4 Alterations in membrane asymmetry

In normal cells, the distribution of phospholipids is asymmetric, with the inner membrane containing anionic phospholipids (such as phosphatidylserine, PS) and the outer membrane having mostly neutral phospholipids. In apoptotic cells, however, the phosphatidylserine translocates from the cytoplasmic to the extracellular side of the cell membrane, which increases the amount of phosphatidylserine on the outer surface of the membrane and exposing PS to the surrounding liquid (Vermes *et al*, 1995). Annexin V, a calcium-dependent phospholipid-binding protein, has a high affinity for PS. Although it will not bind to normal living cells, Annexin V will bind to the PS exposed on the surface of apoptotic cells. Thus, Annexin V has proved suitable for detecting apoptotic cells.

1.2.5 Alterations in mitochondrial membrane potential

During apoptosis, the mitochondrial inner transmembrane potential $\Delta\Psi_m$ collapse, and the mitochondrial permeability transition (MPT), a large reductance channel is activated (Zoeteweij *et al*, 1992). This permeability transition leads to the release of cytochrome *c* and the apoptosis inducing factor (AIF), causing caspase activation and, finally, cell death. $\Delta\Psi_m$ changes can be assessed by mitochondrial staining with cationic, lipophilic fluorochromes such as 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], or rodamine 123 and flow cytometric analysis. Cells undergoing apoptosis show a reduction in the incorporation of $\Delta\Psi_m$-sensitive dyes (Vayssiere *et al*, 1994; Zamzami *et al*, 1995).

1.2.6 Nuclear poly(ADP-ribosylation)

Recently, several reports have suggested that poly(ADP-ribosylation) is an immediate cellular response to DNA damage and is thought to be involved in DNA repair, genetic recombination, apoptosis and other processes during which DNA strand breaks are formed. Poly(ADP-ribosylation) is a post-translational modification of nuclear proteins. The synthesis of protein-bound ADP-ribose polymers in eukaryotic chromatin is catalyzed by poly(ADP-ribose) polymerase (PARP), a homodimeric enzyme of 1014 amino acids (113 kDa)/subunit that utilizes NAD^+ as the ADP-ribose donor and DNA-binding proteins (heterodimeric), including itself, as ADP-ribose polymer covalent acceptors. The ADP-ribose polymerizing activity of this enzyme is strongly stimulated upon binding to DNA single or double strand breaks. As a result, PARP covalently modifies a number of nucleic acid-binding proteins with a very strong polyanion (Huletsky *et al*, 1985; Boulikas, 1990) (Fig 1). Thus, poly(ADP-ribosylation) plays a relevant role in DNA damage and its repair.

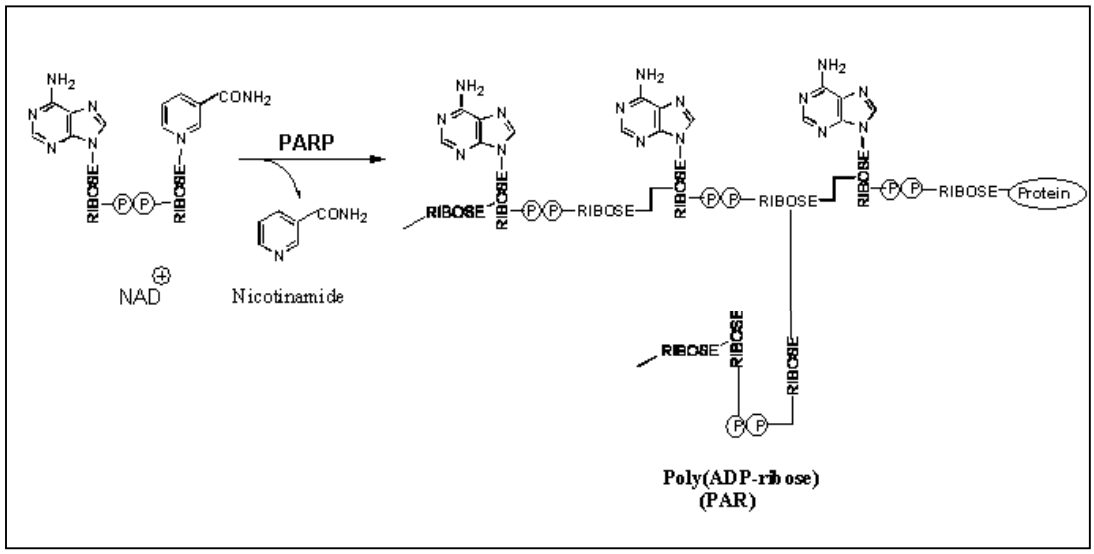

Figure 1. Scheme of poly(ADP-ribose) synthesis. *PAR is synthesized after activation of poly(ADP-ribose) polymerase (PARP). PARP is activated by DNA stand breaks and utilizes NAD^+ as a substrate to catalyze the addition of long branched chains of PAR to a variety of nuclear proteins.*

Three distinct structural domains of PARP have been identified: (i) a 46 kDa DNA-binding amino-terminal domain; (ii) a 22 kDa centrally located automodification domain; and (iii) a 54 kDa carboxy-terminal catalytic domain. The DNA binding domain contains a duplicated sequence in which 35 amino acids are highly conserved. These peptides show the typical "zinc-finger" structure that is found in several DNA-binding proteins. Zinc fingers 1 and 11 allow the enzyme to specifically bind double (Ikejima *et al*, 1990) and single strand-breaks on DNA (Gradwohl *et al*, 1990), respectively. The automodification domain of PARP contains protein-protein binding motifs, e.g. peptide sequences involved in both homo- and heterodimerization (Buki *et al*, 1995) recognition. In the catalytic dimerization process, protein-protein interactions appear to stabilize homodimeric and heterodimeric (PARP)-DNA complexes (Mendoza-Alvarez and Alvarez-Gonzalez, 1993) which, in turn, facilitate protein-poly(ADP-ribosyl)ation. This automodification domain also contains 15 highly conserved Glu residues (Uchida *et al*, 1993) which include some of the auto-poly(ADP-ribosyl)ation sites. The carboxy-terminal region of PARP, the 54 kDa NAD-binding domain, contains a highly conserved amino acid residue that is directly involved in the catalysis of the ADP-ribose transfer reaction carried out by prokaryotic and eukaryotic enzymes. The enzymatic automodification reaction of PARP involves three chemically distinct reactions. First, PARP is responsible for the Glu-specific binding of one ADP-ribose unit from NAD^+ to a protein acceptor amino acid side chain, which usually involves mono-ester bond formation (initiation reaction) (Kawaichi *et al*, 1980). Secondly, PARP proceeds to the characteristic (2'-1") ribose-ribose glycosidic bond formation of the ADP-ribose chain elongation reaction (Ueda *et al*, 1979; Alvarez-Gonzalez, 1988). Finally, the (2"-1'") ribose-ribose bonding between ADP-ribose units (branching reaction) (Rolli *et al*, 1997) takes place at the ratio of one every forty ADP-ribose units polymerized. The ADP-ribose polymerization (elongation and branching reactions) take place via a highly processive mechanism.

Recently, several reports suggest a modulation of this nuclear poly(ADP-ribosylation) process during cell death by apoptosis (Negri C, 1997, Scovassi A.I., 1998, 1999) and identified poly(ADP-ribose) synthesis as a useful marker for identifying apoptotic cells. It was demonstrated that endogenous poly(ADP-ribose) production was indeed stimulated in HeLa cells undergoing apoptosis, and that the detection of PAR formation, combined with morphological and other biochemical parameters, facilitated the detection of apoptosis (Donzelli *et al*, 1997; Negri *et al*, 1997).

1.3 Oxidative stress and apoptosis

A disturbance in the pro-oxidant/anti-oxidant balance in favour of the former leading to oxidative modification of macromolecules is defined as "oxidative stress" (Sies, 1985). Oxidative stress occurs when redox homeostasis within the cells is altered, either as a result of increased exposure to oxidants or from decreased protection against oxidants. The first level of cellular response to oxidative stress is to use its antioxidant defence. The balance between pro- and anti-oxidants determines whether cells undergo death or proliferation.

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and superoxide anion radicals (O_2^-) are known mediators of intracellular signaling cascades. Studies have demonstrated that ROS and the resulting oxidative stress play a pivotal role in apoptosis (Kannan and Jain, 2000). ROS can react with a wide range of biological macromolecules, i.e. lipids (Thiele *et al*, 1998), proteins (Thiele *et al*, 1999), nucleic acids and carbohydrates (Stadtman, 1992). The initial reaction generates a second radical, which reacts with a second macromolecule and so on in a continuing chain reaction (Fig 2). Therefore, excessive production of ROS may lead to oxidative damage, loss of cell function, and ultimately apoptosis or necrosis (Cadenas, 1989; Davies, 1995; Fridovich, 1995).

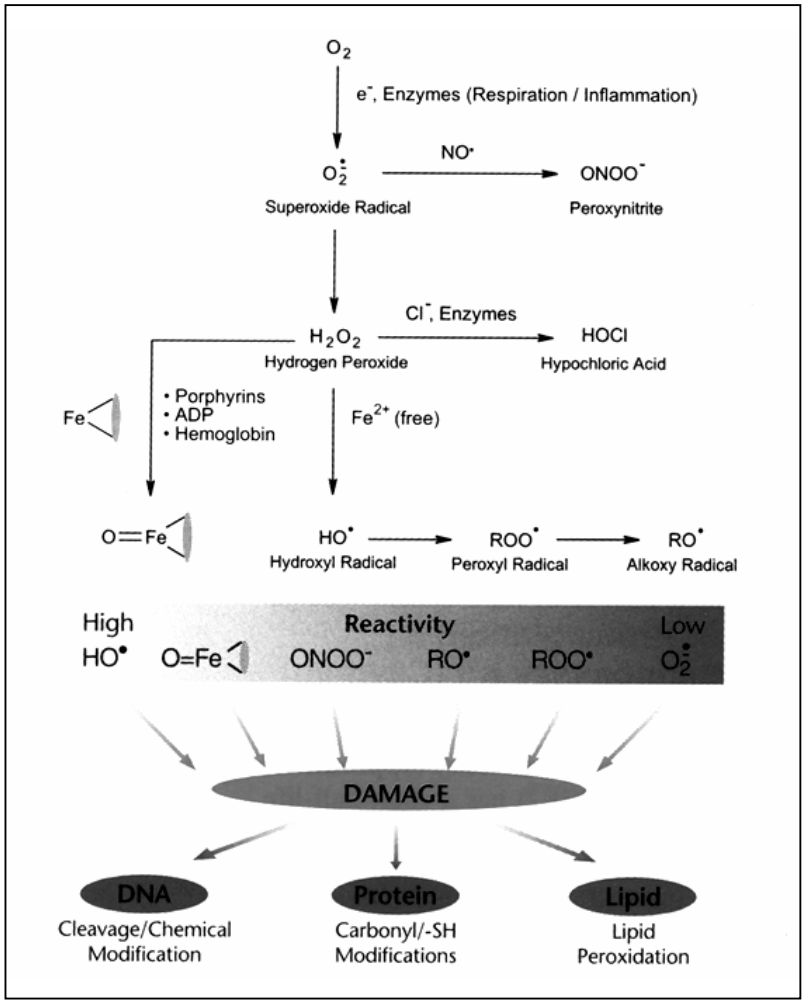

Figure 2. Reactive oxygen species and oxiditive damage. *ROS can react with DNA, protein, and lipid, and lead to oxiditive damage.*

The mechanisms of oxidative stress induced apoptosis involve loss of mitochondria transmembrane potential (Zamzami *et al*, 1997), release of cytochrome *c* (cyt *c*) to the cytoplasm (Reed, 1997), loss of bcl-2 (Kane *et al*, 1993), down-regulation and degradation of mitochondrially encoded mRNA, rRNA, and DNA (Crawford *et al*, 1997a; Crawford *et al*, 1997b; Crawford *et al*, 1998), and diminished transcription of the mitochondrial genome. Thus, mitochondria play a central role in ROS-induced apoptosis.

1.3.1 Mitochondria damage during apoptosis

Mitochondria are well known to play a key role in energy metabolism (Mitchell and Moyle, 1965), ion homeostasis (Azzone and Massari, 1973) and redox regulation (Lee, 1995). The damage to mitochondria has long been linked to apoptosis. Mitochondrial oxidative phosphorylation is one of the major sites of production of ROS (Halliwell and Gutteridge, 1989), and mitochondrial structures are very susceptible to oxidative stress as well. Several studies have shown that the mitochondrial dysfunction under oxidative stress could contribute to apoptosis (Zamzami *et al*, 1996; Green and Reed, 1998; Morel and Barouki, 1999). Damage by ROS to mitochondrial components includes lipid peroxidation, protein oxidation and mitochondrial DNA mutations (Lenaz, 1998). One major effect of oxidative stress on the mitochondrial function is the activation of mitochondrial permeability transition (MPT) that is associated with apoptosis in many systems (Kroemer *et al*, 1997). MPT involves a sudden increase of the inner mitochondrial membrane permeability to solutes greater than 1500 kDa. MPT functions as a voltage sensor, a thiol sensor, a sensor of oxidation-reduction equilibrium of adenine nucleotide pool, and as a sensor of divalent cations. As a consequence, defective MPT opening to larger molecules causes uncoupling of the respiratory chain resulting in the hypergeneration of ROS, cessation of ATP synthesis, matrix Ca^{2+} outflow and depletion of reduced glutathione and other reductants. Following the inner membrane permeability and the release of matrix solutes, a colloidal osmotic pressure arises in the mitochondrial matrix due to the high concentrations of proteins, which are slow to equilibrate (Igbavboa *et al*, 1989; Richter *et al*, 1995). In order to correct the osmotic balance, the diffusion of H_2O results in a massive swelling of the mitochondria (Gunter and Pfeiffer, 1990), and eventually causes outer membrane rupture, releasing caspase-activating molecules, such as cytochrome *c* and apoptosis-inducing factor (AIF). The release of cytochrome *c* from mitochondria is necessary for activation of caspases (such as caspase-3/CPP32/apopain/yama) and DNA fragmentation (Susin *et al*, 1998; Yang and Cortopassi, 1998).

1.3.2 Other intracellular targets for oxidation during apoptosis

Following their production, intracellular oxidants have the potential to trigger apoptosis in other pathways. Oxidation of intracellular proteins may modify either their function or their ability to be recognized by other proteins. For example, oxidative damage can increase the susceptibility of some proteins to degradation by non-lysosomal proteinases complex (Davies and Goldberg, 1987). Proteinase inhibitors are known to block thymocytes apoptosis (Weaver *et al*, 1993), and it is possible that they are protecting the same intracellular targets as antioxidants from degradation. Protein oxidation may also be involved in changing nuclear gene transcription such that the apoptotic pathway is activated. Several transcription factors have critical cysteine residues involved in DNA binding (for example fos and jun (Staal *et al*, 1990)). Thiol oxidation causes a large decrease in the efficiency of DNA binding. By contrast, DNA binding activity of the nuclear factor kappa B (NFκB) is indirectly activated by oxidative events (via enhanced proteolysis of its inhibiting factor, IκB), thereby initiating transcription of NFκB responsive genes (Staal *et al*, 1990; Brown *et al*, 1993; Meyer *et al*, 1993). Oxidation therefore has the potential to alter the phenotype of a cell via changes in gene transcription, and at least in some situations this may provide an entrance into apoptosis (Slater *et al*, 1995). Moreover, it is likely that direct oxidative damage to DNA can also initiate an apoptotic response (Yamada and Ohyama, 1988; Zhivotovsky *et al*, 1993).

1.4 UV irradiation and DNA damage

Exposure to UVA and UVB causes cellular DNA damage within skin cells. This damage is elicited directly through absorption of energy (UVB), and indirectly through intermediates (UVA). DNA damage is detected as strand breaks, base deletion, or base modification. UVB induces direct DNA damage by formation of cyclobutane pyrimidine dimers, whereas UVA exerts its genotoxic effects indirectly, most likely through sensitizer molecules that generate reactive oxygen species (ROS), and induces 8-hydroxydeoxyguanosine (8-OHdG) formation.

1.4.1 Mechanisms of UV-induced damage

1.4.1.1 Direct effects - UVB

DNA is the most prominent cellular chromophore for the absorption of UVB (Setlow and Woodhead, 1994). The absorption of energy causes the excitation of a single electron to a higher, less stable energy level. This reactive intermediate can degrade or react with a variety of biomolecules. The predominant UVB-induced DNA photoproducts are formed between adjacent bipyrimidine bases on one strand of DNA. Two types of bipyrimidine photoproducts are created in high quantity, cyclobutane pyrimidine dimers, and (6-4) photoproducts (Fig 3.1). The relative yields of these two types of photoproducts vary with the irradiation wavelength, the ratio of AT to GC, the neighbouring nucleotides, and other factors, but the ratio is generally between 2 and 5 in favor of pyrimidine dimers (Brash *et al*, 1987; Tornaletti *et al*, 1993). A dimer is produced when one of the pyrimidines in an excited state forms covalent bonds with a neighbouring pyrimidine via the atoms in the carbon-carbon double bonds, thus forming a four-membered cyclobutane ring. The probability of forming a dimer is generally TT > TC > CC (Brash *et al*, 1987). (6-4) photoproducts, like dimers, are created when one pyrimidine base in an excited state reacts with an adjacent pyrimidine on the same strand. The formation of (6-4) photoproducts, the atoms in the carbon-carbon double bond of the 5'-pyrimidine to the atoms of the carbon-nitrogen (for cytosine) or carbon-oxygen (for thymine) bond at the 4-position of the adjacent pyrimidine. The rearrangement of the initial product yields the (6-4) photoproduct (Fig 3.1). The probability of formation of (6-4) photoproduct is influenced by the base sequence (TC > CC > TT), the adjacent sequences, and the irradiation wavelength (Brash and Haseltine, 1982). In contrast to cyclobutane pyrimidine dimers, (6-4) photoproducts are not stable when exposed to wavelengths greater than 290 nm and are converted to new bicyclic structures called Dewar photoproducts (Cadet *et al*, 1992) (Fig 3.2). Other DNA photoproducts are produced at much lower efficiencies than are dimers and (6-4) photoproducts upon UVB irradiation. Purine-purine covalently linked products, cytosine photohydrates, purine photoproducts, single-stand breaks, and DNA-protein crosslinks have been reported upon irradiation of isolated DNA or intact cells with high UVB doses (Bose *et al*, 1983; Peak *et al*, 1987; Gallagher and Duker, 1989; Mitchell *et al*, 1991). Furthermore, there is also evidence for the induction of 8-OHdG following UVB exposure (Stewart *et al*, 1996).

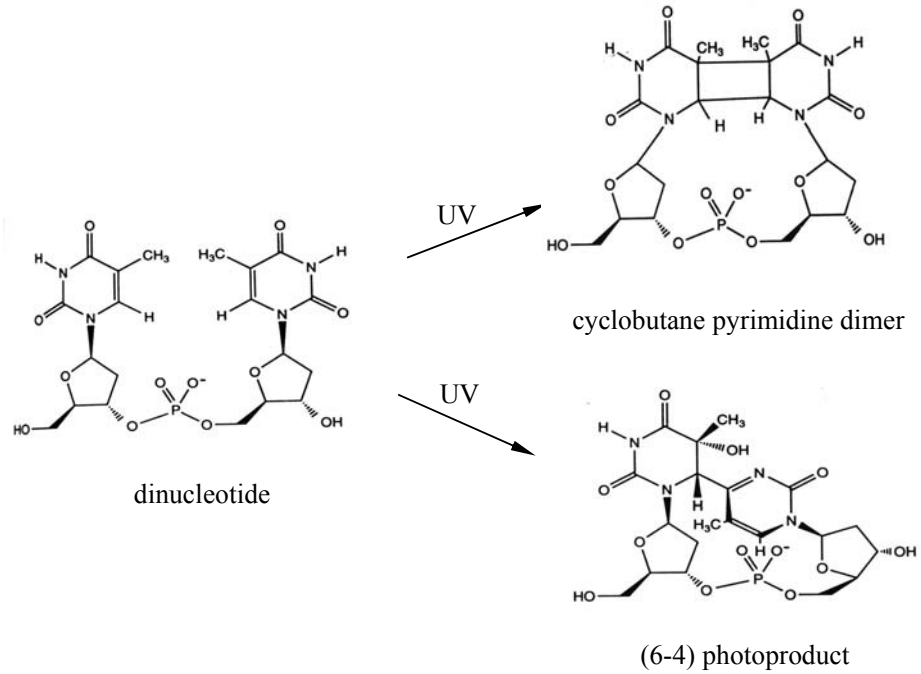

Figure 3.1. Formation of major types of dipyrimidine photoproducts in DNA*. The cyclobutane pyrimidine dimer results from photoaddition between the carbon-carbon double bonds in adjacent pyrimidines. The (6-4) photoproduct is formed by the photoaddition of the carbon-oxygen (or carbon-nitrogen) bond of the 3' pyrimidine to the carbon-carbon double bond of the 5' pyrimidine and subsequent rearrangement to a ring-opened structure.*

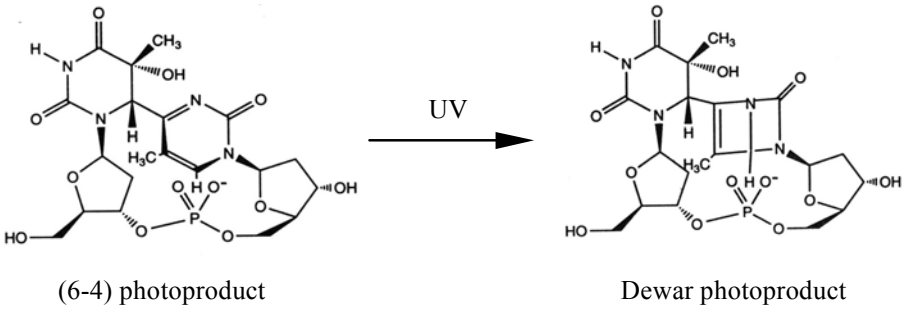

Figure 3.2. Formation of the Dewar photoproduct from the (6-4) photoproduct. *The pyrimidinone structure isomerizes to a bicyclic structure.*

1.4.1.2 Indirect damage – UVA

UVA radiation is by far the most prevalent component of UV radiation in sunlight (Seite *et al*, 2000), and historically UVA radiation has been considered harmless. However, the genotoxicity of UVA has been firmly established from *in vitro* experiments. Owing to the low absorptivity of DNA between 315 nm and 400 nm, the damage induced is largely due to the absorption of UVA photons by endogenous non-DNA chromophores. This energy is indirectly transferred to DNA via reactive oxygen intermediates or radicals generated on the absorbing chromophore. In principle, an excited endogenous photosensitizer molecule could react with DNA without further intermediates (type I reaction) or via ROS (type II reaction). Ample evidence supports a pivotal role for singlet oxygen in a type II reaction. Alternatively, hydroxyl radicals, generated via superoxide and a Fenton reaction, could be involved and possibly, unlike singlet oxygen, generate DNA strand breaks directly (Epe *et al*, 1993).

UVA excited photosensitizers promote the formation of three major base lesions, in addition to a much lower level of base loss (Cadet *et al*, 1992). These are 1) 8-hydroxy-deoxyguanosine (8-OHdG)- singlet oxygen appears to mediate the formation of 8-OHdG from guanosine (Devasagayam *et al*, 1991). 8-OHdG is reported to be induced by UVA at a 10-fold higher rate than stand breaks (SB) in mammalian cells (Pflaum *et al*, 1994); 2) hydroxyhydroperoxides- using thymine in solution, in the presence of menadione as a photosensitizer, UVA indirectly generates the radical cation of thymine that, in the presence of oxygen, gives rise to isomeric hydroxy-hydroperoxides (Fisher and Land, 1983); 3) Pyrimidine photoproducts, formation of pyrimidine photoproducts has been reported following absorption of UVA photones, but a sixfold greater energy is required at 365 nm compared with 254 nm to induce the same order of lesions (Tyrrell, 1973). However, the (6-4) photoproducts are not observed (Umlas *et al*, 1985; Mori *et al*, 1988).

1.4.2 Cellular responses to UV-induced DNA damage

The cells can respond to the DNA damage by repairing DNA to avoid harmful mutations, or if the damage is too great, by inducing apoptosis to remove potential cancer cells from the population (Nataraj *et al*, 1995). A primary response of cells to UV-induced DNA damage is initiating DNA repair mechanisms. In general, DNA repair mechanisms fall into one of three categories. The first is photoreactivation, or photoenzymatic repair, in

which an enzyme, photolyase, binds to specific photolesions, such as cyclobutane pyrimidine dimers, and, after absorption of visible light, reverses the damage in situ. The second, excision repair, is more complicated and involves a variety of proteins which recognize damaged nucleotides, incise surrounding DNA and remove the DNA fragment containing the photodamage. The third type of repair, postreplication or recombinational repair, utilizes newly-synthesized DNA daughter strands to fill in gaps generated during replication past unrepaired photolesions.

Furthermore, UV-irradiation also stimulates the cell to produce early response gene that activate a cascade of signaling molecules (e.g. protein kinases), activate antioxidant defence system and detoxification system.

However, failure of these DNA repair pathways can result in permanent mutations in the DNA sequence, and therefore abnormal cell proliferation. Consequently, cells which can not repair UV-induced extensive DNA damage sufficiently ultimately initiate apoptosis (Brash *et al*, 1996). It is generally accepted that the severity of DNA damage determines whether programmed cells death is initiated. Thus, apoptosis plays an important role in preventing photocarcinogenesis.

1.5 MAPK and apoptosis

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that play a number of important roles in transmitting signals from the membrane or cytoplasm to the nucleus (Seger and Krebs, 1995). MAPKs are involved in a wide range of cellular responses, including cell proliferation, differentiation, survival, and apoptosis (Su and Karin, 1996). As cytoplasmic protein kinases, MAPKs mediate the transcription of many early genes and play a major role in modulating and coordinating these gene responses (Karin, 1995). Three major MAPK signal transduction pathways have been identified. These include p42/p44 extracellular signal-releated kinase (ERK1 and 2) (Boulton *et al*, 1991), c-Jun N-terminal protein kinase (JNK)/stress-activated protein kinase (SAPK) (Kyriakis *et al*, 1996) and p38 MAP kinase (Han *et al*, 1994), respectively. They are structurally releated but biochemically and functionally distinct. ERKs are the best characterized and most distally situated enzymes in a three-kinase cascade. This cascade is initiated by growth factor binding, which stimulates receptor tyrosine kinases. The sequential activation of the GTP-binding protein Ras and the serine kinases Raf then

initiates (Howe *et al*, 1992; Avruch *et al*, 1994; Burgering and Bos, 1995). Raf then phosphorylates and activates MAPK kinase (MEK1 and MEK2), a threonine/tyrosine dual specificity kinase that directly activates ERK by dual phosphorylation on tyrosine and threonine residues within the protein kinase subdomain VIII (Crews *et al*, 1992; Marshall, 1994). ERK activation culminates in the phosphorylation of downstream cytosolic and nuclear factors that control a variety of cellular processes (Davis, 1993) (Fig 4).

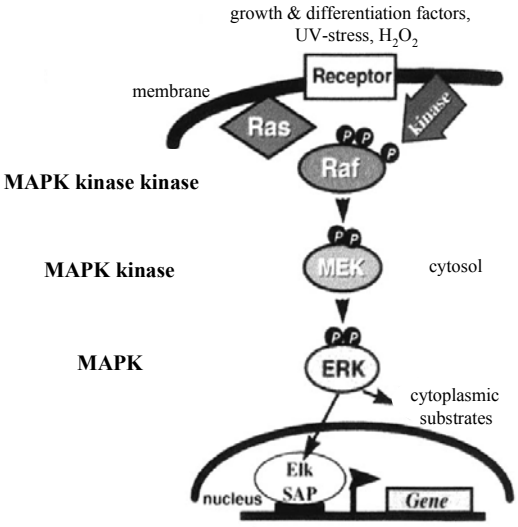

Figure 4. Schematic representation of the structure of MAPK pathways. *Growth factors stimulate Ras and Raf (MAPK kinase kinase) activation. Raf then phosphorylates MAPK kinase (MEK),which in turn activate ERK by phosphorylation (modified from Kolch (Kolch, 2000)).*

ERK is centered on multiple signal transduction pathways to accomplish a variety of functions. A number of studies have shown that ERK is activated by UV irradiation and ROS. (Guyton *et al*, 1996; Wang *et al*, 1998). Activation of ERK through different pathways leads to fundamentally different cellular responses, including proliferation, differentiation, survival and apoptosis (Bergmann *et al*, 1998; Kurada and White, 1998; Impey *et al*, 1999; Kolch, 2000). The role of ERK-1/2 activation in cell survival appears to depend on the cell type as well as the stimulus and the cellular environment. ERK-1/2

inhibition had no significant effect on the insulin- or brain-derived neurotrophic factor-induced survival of cerebellar granule cells (Gunn-Moore *et al*, 1997). In cardiac myocytes, inhibition of ERKs increased apoptotic cell death (Aikawa *et al*,) and cardiotrophin 1 promotes survival via activation of a signaling pathway requiring ERKs (Sheng *et al*, 1997). In hematopoietic cells, activation of ERK partially prevented apoptotic death (Kinoshita *et al*, 1997). However, Fas-mediated apoptosis in a neuroblastoma cell line was blocked by interference with the ERK pathway (Goillot *et al*, 1997).

Another member of the MAPK superfamily, p38 MAPK, is also activated in a time-dependent and transient fashion by UVB. P38 MAPK has sequence homology to an enzyme in yeast termed HOG-1 (high-osmolarity glycerol response) (Brewster *et al*, 1993) and is only poorly activated by epidermal growth factor (Derijard *et al*, 1995). The enzyme is activated by phosphorylation of tyrosine and threonine residues by a wide range of cellular stresses such as osmotic shock, heat shock, inflammatory cytokines and UV light (Rouse *et al*, 1994; Raingeaud *et al*, 1995; Wesselborg *et al*, 1997). Signals are transmitted via MEK kinase 3, 4 and 6 and small GTP-binding proteins (Lamarche *et al*, 1996). An expanding group of substrates for p38 have been identified including transcription factors such as ATF-2 (Raingeaud *et al*, 1995) and other protein kinases including MAPK-activated protein kinases 2 and 3 (Rouse *et al*, 1994; McLaughlin *et al*, 1996). Upon activation, MAPK-activated protein kinase 2 can induce expression of the transcription factor such as ATF-1. The role of p38 MAPK in apoptosis is controversial. p38 MAPK activation has shown to be protective against hypericin-induced apoptosis of HeLa cells (Assefa *et al*, 1999). By contrast, other investigators have observed p38 MAPK activation occured upstream of caspase and mediated apoptosis induced by various stresses (UVC, hyperosmolarity, sphingosine) in human neutrophils (Frasch *et al*, 1998). Assefa *et al* also reported UVB induced apoptosis of HaCaT cells, as well as p38 MAPK activation, inhibition of p38 MAPK counteracted apoptosis (Assefa *et al*, 2000).

The JNK or stress-activated protein kinase cascade participates in growth factor signaling in many cell types, but JNK are mainly activated in response to various stress events that also activate p38 MAPK and include UVC, heat shock, osmotic imbalance, and cytokines (Canman and Kastan, 1996). Like other members of the MAPK family, JNK requires threonine and tyrosine phosphorylation for its enzymatic activity (Raingeaud *et al*, 1995). Upon activation, JNK translocates to the nucleus and induces transcription of AP-1-

containing genes by specifically phosphorylating Ser 63 and Ser 73 of the transcription factor c-*jun* (Cui and Douglas, 1997). Studies have shown that physiological doses of UVB induce transient and rapid JNK activation. A number of studies have reported on the role of JNK in coupling cellular stress signals and specifically in the UV response leading to the apoptotic cell death (Chen *et al*, 1996; Zanke *et al*, 1996; Butterfield *et al*, 1997).

1.6 Aim of the study

The aim of the present study was to establish a sensitive method for the early detection of apoptosis induced by UVB and H_2O_2 in human keratinocytes, and to elucidate the role of H_2O_2 in UVB-induced HaCaT cells apoptosis.

UVB is known to generate intracellular H_2O_2 in keratinocytes dose-dependently, and the effects of UVB-irradiation may be partly mediated by H_2O_2 (Peus *et al*, 1999). As discussed earlier, poly(ADP-ribose) synthesis was suggested to be a useful marker for identifying apoptosis in HeLa cells. Based on these findings, a hypothesis was constructed in which PAR formation is an early and sensitive marker of UVB-induced apoptosis in human keratinocytes, and UVB-induced apoptosis is mediated by H_2O_2 generation. To assess this, human immortalized keratinocytes (HaCaT) were UVB irradiated or exposed to H_2O_2, and thereafter PAR formation was measured by indirect immunofluorescence using a monoclonal antibody against PAR. Cells were harvested at 4-24 hours after single exposures to UVB doses of 0-30 mJ/cm^2, or at 16 hours after 0-1 mM H_2O_2-treatment. Apoptotic cells were also identified by morphological changes, DNA laddering, and TUNEL assay. In order to identify if H_2O_2 is a mediator of UVB-induced apoptosis, experiments using antioxidants catalase and NAC were performed thereafter.

There is contradictory data on the effect of ERK phosphorylation on apoptosis, and apparently, the role of ERK activation is cell type- and stimulus-dependent. This work was also set up to investigate the direct influence of ERK activation on UVB- and H_2O_2-induced HaCaT cells apoptosis.

2. Materials and Methods

2.1 Materials

2.1.1 Medium and substances for cell culture

1 PBS: 170 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4.
1 0.25% Trypsin: 1 mM EDTA (1:1) for cell dissociation, Gibco BRL, Karlsruhe, Germany.
2 Cell culture medium: Dulbecco's modified Eagles medium (DMEM), Biochrom KG Seromed, Berlin, Germany.
3 Fetal calf serum: Biochrom KG Seromed.
4 Antibiotics: 100 U/ml penicillin/ 0.1 mg/ml streptomycin. Sigma, Munich, Germany.
5 H_2O_2: Sigma, Munich, Germany.
6 Catalase: 2,000-5,000 U/mg, Sigma, Munich, Germany.
7 NAC: Sigma, Munich, Germany.

2.1.2 Reagents for immunofluorescence

1 10% trichloroacetic acid for fixation, Merck, Darmstadt, Germany.
2 4% paraformaldehyde for fixation, Merck, Darmstadt, Germany.
3 10% goat serum in PBS, Gibco BRL, Karlsruhe, Germany.
4 0.5% bovine serum albumin in PBS, Gibco BRL, Karlsruhe, Germany.
5 Vectashield® Mounting Medium: Vector laboratories Inc., Burlingame, CA, USA.

2.1.3. Antibodies for immunofluorescence

1 Monoclonal antibody against poly(ADP-ribose): 10H, Alexis, Grünberg, Germany.
2 Monoclonal antibody against cyclobutane thymine dimer: clone KTM53, Kamiya Biomedical company, Seattle, WA, USA.
3 Secondary antibody: Cy3™-conjugated goat anti-mouse secondary antibody, Dianova, Hamburg, Germany.

2.1.4 Reagents for western blot

1 Lysis buffer: 50mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, 20 µg/ml Aprotinin, 20 µg/ml Leupeptin, 10 mM Benzamidin.
2 BCA protein assay reagent: used for protein concentration measurements, Pierce, St-Augustin, Germany.
3 5 x Loading buffer: 50% glycerol, 500 mM Tris, 5 mM EDTA, 10% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue, pH 6.8.
4 SDS-PAGE electrophoresis buffer: 25 mM Tris, 250 mM Glycine, 0.1% SDS.
5 Transfer buffer: 39 mM Glycine, 48 mM Tris, 0.037% SDS, 20% methanol.
6 TBS-Tween buffer: 137 mM NaCl, 2.68 mM KCl, 25 mM Tris, 0.1% Tween-20, pH 7.4.
7 Stripping buffer: 63 mM Tris, 2% SDS, 0.1 M ß-mercaptoethanol.
8 ECL Western Blot detection reagents: Amersham Pharmacia Biotech, Freiburg, Germany.

2.1.5 Antibodies for western blot

1 Monoclonal antibody against ERK1+2 (panERK): BD Transduction Laboratories, Heidelberg, Germany.
2 Monoclonal antibody against phospho-ERK: Cell Signaling Technology, Frankfurt, Germany.
3 Secondary antibody: HRP-conjugated goat anti-mouse IgG, Santa Cruz Biotechnology, Inc., Heidelberg, Germany.

2.1.6 Reagents for DNA electrophoresis

1 6 x loading buffer: 0.25% bromophenol blue (Sigma), 0.25% Xylene cyanol FF (Sigma), 30% glycerol.
2 1 x TBE electrophoresis buffer: 0.09 mM Tris-borate, 0.002 mM EDTA.
3 Ethidium bromide stock solution, 10 mg/ml, Serva Electrophoresis GmbH, Heidelberg, Germany.

4 200 bp DNA marker for DNA electrophoresis, Eurogentec, Köln, Germany.

2.1.7 Reagents for TUNEL assay

1 Fixation solution: 4% paraformaldehyde in PBS, pH 7.4.
2 Permeabilisation solution: 0.1% Triton X-100 in 0.1% sodium citrate.

2.1.8 Reagents for fluorescence measurement of intracellular peroxides

1 2',7'-dichlorofluorescein-diacetate (DCF-DA) stock solution: 1 mM DCF-DA (Mo Bi Tec, Göttingen, Germany) in ethanol, freeze at –20°C, avoid light.
2 DCF-DA working solution: 1 ml DCF-DA stock solution + 4 ml 0.01 N NaOH + 20 ml PBS, pH 7.5-8.0, avoid light.

2.2 Methods

2.2.1 Cell cultures

Human immortalized keratinocytes (HaCaT) were kindly provided by Prof. N. E. Fusenig, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany (Boukamp *et al*, 1988). Cells were cultured in Dulbecco's modified essential medium supplemented with 10% fetal calf serum and 100 U/ml penicillin/ 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were subcultured every 5 days. To subculture the cells, medium was removed and the cells were washed twice with PBS. To detach the cells from the flask, 2 ml Trypsin/EDTA mixture was added and the cells were incubated at 37°C for 1 to 2 minutes. After adding 10 ml fresh medium, the cell suspension was collected and centrifuged at 1,000 rpm for 5 minutes. The old medium was aspirated, the cells resuspended in 10 ml fresh medium and splited at 1:5 to 1:10.

2.2.2 UVB irradiation and H_2O_2 treatment

HaCaT cells were irradiated with a PL-S 9W/12 (UV21) UVB light source (Philips, Aachen, Germany), with an emission peak at 313 nm. The UVC content of the emitted

light was 0.15% (Fig 6). The intensity of this UVB source was 0.33 mW/cm^2 as measured using a Waldmann UV-meter (Waldmann, Villingen-Schwenningen, Germany). Prior to UVB-exposure of HaCaT cells, cell culture medium was removed and cells were rinsed twice with PBS, covered with PBS and exposed to UVB doses of 10, 20, and 30 mJ/cm^2 or sham irradiated (controls). After irradiation, cells were incubated again in cell culture medium for indicated time periods. For H_2O_2-treatment, cells were incubated in serum-free medium with different concentrations of H_2O_2 (0.1, 0.5, and 1 mM) for the indicated time periods. Under the conditions used, H_2O_2 is metabolized by mammalian cells within 30-40 minutes after being added to the cell culture medium (Davies, 1999).

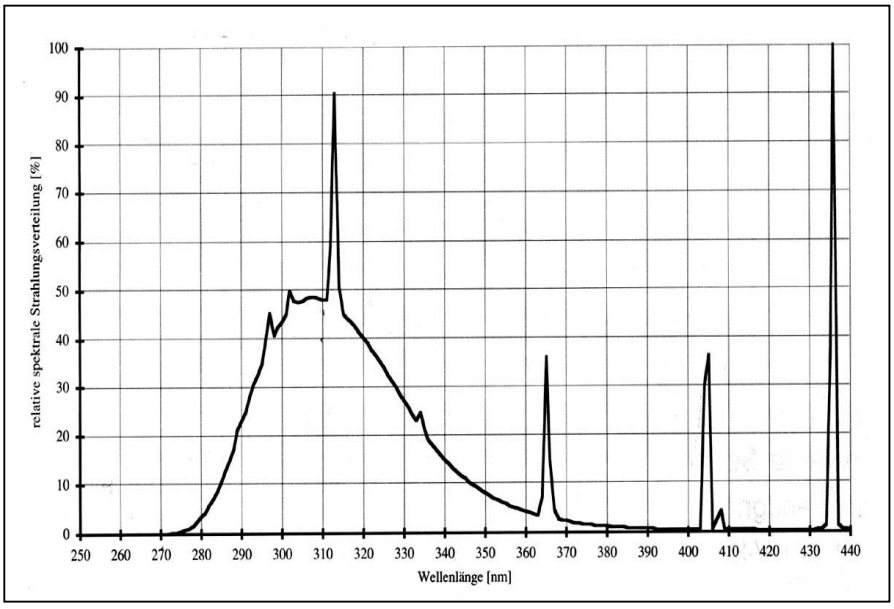

Figure 5. Scheme of UVB light source as supplied by the manufacturer. *UVB:280-315 nm, UVA: 315-400 nm, UVC: < 280 nm. The emission peak of UVB is at 313 nm. The content of UVC is 0.15%,*

2.2.3 Antioxidants treatment

2.2.3.1 Catalase treatment

Prior to UVB or H_2O_2 exposure, HaCaT cells were pre-treated with 100 ng/ml, 1 µg/ml, or 10 µg/ml catalase in DMEM medium for a period of 24 hours as previously described for neutrophils (Aoshiba *et al*, 1999). The medium was subsequently removed and cells were washed with PBS twice. Thereafter, cells were covered with PBS and irradiated with 20

mJ/cm^2 UVB, and post-incubated with catalase (at levels matching the pre-incubation periods) for 16 hours after which PAR immunofluorescence was performed.
For H_2O_2 treatment experiments, cells were pre-incubated with catalase in the same manner as given for UVB experiments. After the catalase pre-incubation, cell culture medium was removed completely, cells were washed twice with PBS, and exposed to 1mM H_2O_2 for 16 hours. For H_2O_2 experiments, no catalase post-incubation was performed, since this would directly lead to extracellular scavenging of H_2O_2.

2.2.3.2 NAC treatment

Prior to UVB or H_2O_2 exposure, HaCaT cells were pre-treated with 0.5, 1, 2.5, 5, and 10 mM N-acetyl-L-cysteine (NAC) in DMEM medium for 1 hour. Then, the medium was removed and cells were washed with PBS twice. Thereafter, cells were covered with PBS and irradiated with 20 mJ/cm^2 UVB, and post-incubated for 16 hours. Afterwards, PAR immunofluorescence was performed.
For H_2O_2 treatment experiments, cells were pre-incubated with NAC in the same manner as given for UVB experiments. After the NAC pre-incubation, cell culture medium was removed completely, cells were washed twice with PBS, and exposed to 1 mM H_2O_2 for 16 hours.

2.2.4 PD98059 treatment

HaCaT cells were pre-treated with 20 μM of the MEK1 inhibitor PD98059 (Calbiochem, Schwalbach, Germany) for 1 hour before exposure to 30 mJ/cm^2 UVB or 1 mM H_2O_2 and were incubated for 15, 30, and 60 minutes. Thereafter Western Blot for detection of phospho-ERK was performed.

2.2.5 Immunofluorescence

2.2.5.1 Immunofluorescence of Poly(ADP-ribose)

HaCaT cells were grown on coverslips (Nunc, Wiesbaden, Germany) for 48 hours, exposed to UVB as described above, and post-incubated for 4, 8, 16, and 24 hours, respectively. For H_2O_2-treatments, cells were incubated in serum-free medium with different concentrations of H_2O_2 (0.1, 0.5, and 1 mM) for 16 hours. After the exposure to

respective treatment, cells were rinsed in PBS and fixed with 10% ice-cold trichloroacetic acid for 10 minutes. After successive washing in ice-cold 70%, 90%, and 96% ethanol for 3 minutes, respectively, the cells were air dried and rehydrated in PBS. The coverslips were subsequently incubated with the monoclonal antibody against poly(ADP-ribose) at a concentration of 2.5 µg/ml, diluted in 1% BSA and 0.05% Tween 20 for 1 hour at 37°C. Thereafter, cells were washed in PBS and subsequently incubated with a Cy3™-conjugated goat anti-mouse secondary antibody for 1 hour at 37°C. The coverslips were washed in PBS, and embedded in Vectashield™ mounting medium. Finally, the percentage of poly(ADP-ribose) positive cells was determined by BX-40 fluorescence microscopy (Olympus, Hamburg, Germany).

2.2.5.2 Immunofluorescence of thymine dimer

Confluent HaCaT cells grown on coverslips were exposed to 0, 10, or 30 mJ/cm^2 of UVB or to 1 mM H_2O_2. Cells were analyzed at 30 minutes, as well as 2, 16, and 24 hours after exposure by immunofluorescence of DNA photoproducts. Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, placed in freshly prepared 70 mM NaOH in 70% ethanol for 2 minutes to denature DNA, followed by neutralization for 1 minute in 100 mM Tris-HCl (pH 7.5). Cells were then washed once in 70% ethanol and twice in PBS for 5 minutes each. After washing, cells were incubated with 10% goat serum in PBS for 30 minutes to prevent non-specific binding. Afterwards the cells were incubated with 50 µg/ml cyclobutane thymine dimer-specific monoclonal antibody. After incubation for 1 hour at 37°C and three washes in PBS, cells were subsequently incubated with a Cy3™-conjugated goat anti-mouse secondary antibody for 1 hour at 37°C. Coverslips were then washed in PBS, embedded in Vectashield™ mounting medium and analyzed by BX-40 fluorescence microscopy and, subsequently, densitometric image analysis. Three areas of each slide were randomly selected and the staining intensity per cell was measured and quantified by Analysis 3.0 Software™ (Soft Imaging System, Muenster, Germany).

2.2.6 Detection of apoptosis by DNA laddering

2.2.6.1 Isolation of DNA

For evaluation of internucleosomal DNA fragmentation, an Apoptotic DNA Ladder Kit (Roche, Mannhein, Germany) was used according to the manufacturer's instructions. Briefly, 2 x 10^6 cells were suspended in a sample volume of 200 µl PBS, and the suspension was incubated for 10 minutes at 15-25°C after addition of 200µl binding buffer. After incubation and addition of 100 µl isopropanol, the suspension was vortexed and centrifuged for 1 minute at 8,000 rpm in a standard table top centrifuge. The supernatant was discarded and 500 µl washing buffer was added. Thereafter, the suspension was centrifuged for 1 minute at 8,000 rpm. The pellet was washed again with 500 µl washing buffer and finally the suspension was centrifuged for 10 seconds at 13,000 rpm to remove residual washing buffer. After addition of 200 µl pre-warmed (70°C) elution buffer, the suspension was centrifuged for 1 minute at 8,000 rpm. The DNA can be stored at –20°C for later analysis.

2.2.6.2 DNA Electrophoresis

1-3µg purified DNA was electrophoretically separated on 1% agarose gel in 1 x TBE containing 0.5 µg/ml of ethidium bromide at 5-10 V/cm for 45 minutes. Gels were illuminated with UV light to visualize the ethidium bromide bound DNA.

2.2.7 Detection of apoptosis by TUNEL assay

For evaluation of apoptosis, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Molecular Biochemicals, Mannheim, Germany). In this assay, terminal deoxynucleotidyl transferase (TdT), which catalyzes polymerization of nucleotides to free 3'-OH DNA ends, is used to label DNA strand breaks. DNA of fixed cells labeled by the addition of fluorescein dUTP at strand beraks by TdT. Fluorescein labels incorporated into nucleotide polymers were detected by fluorescence microscopy. HaCaT cells grown on coverslips were exposed to UVB or H_2O_2 as described above. After treatment, cells were rinsed in PBS and fixed with freshly prepared 4% paraformaldehyde solution (pH 7.4) for 1 hour at 15-25°C. After incubation with permeabilisation solution

for 2 minutes on ice, the cells were washed twice with 1% BSA in PBS, thereafter the cells were incubated with 50 µl TUNEL reaction mixture for 1 hour at 37°C. The coverslips were washed with PBS and embedded in Vectashield™ mounting medium. Finally, the percentage of TUNEL-positive cells was determined by BX-40 fluorescence microscopy.

2.2.8 Fluorescence measurement of intracellular peroxides

H_2O_2 generation was measured using a H_2O_2-sensitive fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCF-DA) as described previously (Yu *et al*, 1997). The non polar character allows DCF-DA to freely penetrate all cells. Once inside the cells, it is cleaved by esterases to the polar molecule, 2,'7'-dichlorofluorescein (DCF). In the presence of peroxides, especially H_2O_2, DCF is oxidized to fluorescent 2',7'-dichlorofluorescein, which emits a fluorescent signal following excitation by blue light (488nm). Confluent HaCaT cells were treated with 30 mJ/cm^2 UVB or 1 mM H_2O_2 for 30 minutes, and then incubated with 20 µM 2',7' dichlorofluorescein diacetate (DCF-DA) for additional 30 minutes at 37°C. After chilling on ice, cells were washed with ice-cold PBS, trypsinized, and resuspended at 5 x 10^5 cells/ml in PBS containing 2% fetal bovine serum and 20 µM DCF-DA. The fluorescence intensities of DCF-DA of 10,000 cells from each sample were analyzed by flow cytometry using a DAKO GALAXY™ flow cytometer (Hamburg, Germany) with excitation and emission settings of 488 and 525 nm, respectively. Data were analyzed using FloMax Software (Partec, Münster, Germany). Results are given as the mean peak (MP) of fluorescence intensity.

2.2.9 Western blot analysis

2.2.9.1 Preparation of protein extracts

HaCaT cells were washed twice with PBS after different treatments, and lysed by scraping in ice-cold lysis buffer. Cellular debris was removed by centrifugation at 14,000 rpm for 20 minutes at 4°C. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, St-Augustin, Germany) according to the manufacturer's instructions.

2.2.9.2 Western blot

Protein extracts were resolved by electrophoresis using 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. Proteins were transferred to PVDF membranes (Amersham Pharmacia Biotech, Freiburg, Germany) using semi-dry transfer conditions. In semi-dry blotting, the gels and the membranes were sandwiched between buffer-soaked 3MM Whatmann paper. A constant current of 2.5 mA/cm^2 was applied for one hour. The membranes were then blocked with TBS-Tween buffer containing 5% dry milk for 30 minutes at room temperature. Following blocking, membranes were incubated overnight at 4°C with anti-phospho-ERK antibody diluted 1:2000 in TBS-Tween buffer containing 1% dry milk. The membranes were washed three times with TBS-Tween, and, subsequently incubated for 1 hour at room temperature with HRP-conjugated goat anti-mouse secondary antibody (diluted 1:5,000) in TBS-Tween containing 1% dry milk. After three washes in TBS-Tween, the membranes were submerged for 1 minute in ECL solution (Amersham Pharmacia Biotech), proteins were visualized and exposed to Kodak Biomax films. To ensure the equal loading of proteins, the membranes were analyzed for panERK. Prior to incubation with the panERK antibody, the phospho-ERK antibody and the HRP-conjugated secondary antibody were removed by incubation of the membranes in stripping buffer for 30 minutes at 50°C, and subsequent blocking.

2.2.10 Statistics

All data are expressed as mean ± standard error of the mean (Mean ± SEM). Statistical analysis was carried out using Instat™ (Graphpad, San Diego, CA, USA). Significance test between groups were performed using one-way ANOVA. $P < 0.05$ was considered statistically significant. All experiments were carried out in triplicates and repeated at least twice.

3. Results

3.1 Both, UVB and H_2O_2 induce apoptosis of HaCaT cells

3.1.1 Morphological changes after UVB- and H_2O_2-treatment

Characteristic morphological features of apoptotic cells include detachment of the cells from neighbouring cells, shrinkage and condensation of chromatin and cytoplasm, and membrane blebbing. These morphological changes can be detected by electron or light microscopy. In this work, 8 hours after 20 mJ/cm^2 UVB irradiation, HaCaT cells began to exhibite cell blebbing, a characteristic morphological feature of apoptosis (Fig 6b), which was not observed in sham irradiated cells (Fig 6a). Similar morphological changes were observed when HaCaT cells were incubated with H_2O_2 (Fig 6c).

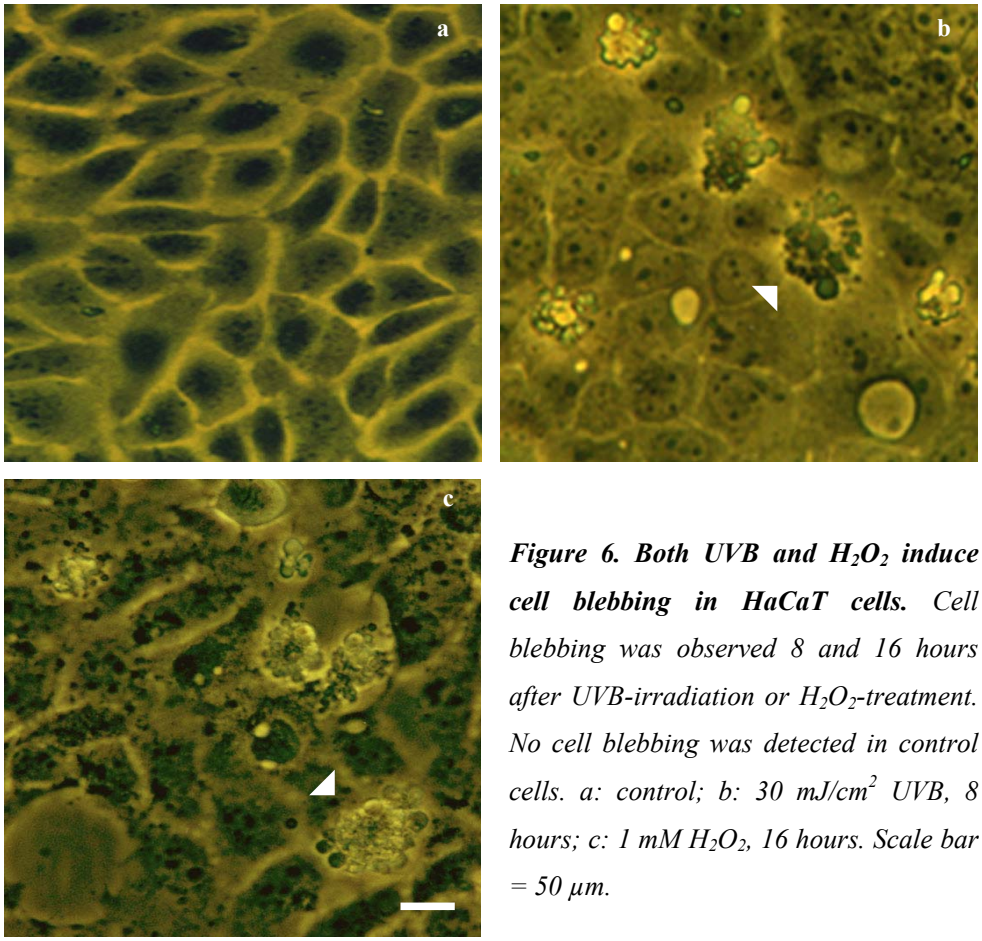

Figure 6. Both UVB and H_2O_2 induce cell blebbing in HaCaT cells. *Cell blebbing was observed 8 and 16 hours after UVB-irradiation or H_2O_2-treatment. No cell blebbing was detected in control cells. a: control; b: 30 mJ/cm^2 UVB, 8 hours; c: 1 mM H_2O_2, 16 hours. Scale bar = 50 µm.*

3.1.2 DNA laddering in UVB-irradiated HaCaT cells

A prominent biochemical event in apoptosis is the cleavage of DNA between nucleosomes by endonucleases (Arends *et al*, 1990) that produces fragments in multiples of approximately 180 bp (Wyllie, 1980). This phenomenon can be analyzed by agarose gel electrophoresis, in which DNA fragmentation can be visualized by separation of DNA fragments forming a typical DNA-ladder. In this study, the morphological features of UVB-induced HaCaT cell apoptosis were accompanied by an enhanced DNA fragmentation into mono- and oligonucleosomes. HaCaT cells were exposed to 10 or 30 mJ/cm^2 UVB irradiation and post-incubated for 4, 8, 16 and 24 hours, respectively. The total cellular DNA was isolated from UVB-irradiated cells. DNA laddering was apparent at 16 and 24 hours after 30 mJ/cm^2 UVB irradiation (Fig 7, lane G and I), no DNA laddering was visible at earlier time points (4 and 8 hours) or lower UVB dose (10 mJ/cm^2) (Fig 7, lane A-F, H).

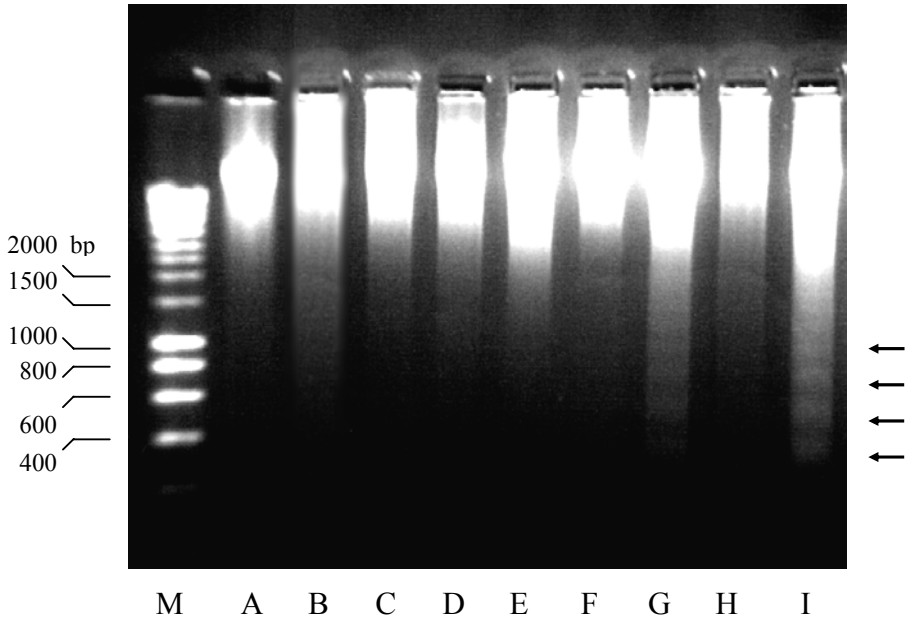

Figure 7. UVB induces DNA laddering in HaCaT cells at 16 and 24 hours after 30 mJ/cm^2 UVB-irradiation. *HaCaT cells were treated with 10 or 30 mJ/cm^2 UVB and harvested at 4, 8, 16, and 24 hours, respectively. M: 200 bp DNA molecular weight marker (bp); A: control; B: 4 hours, 10 mJ/cm^2; C: 4 hours, 30 mJ/cm^2; D: 8 hours, 10 mJ/cm^2; E: 8 hours, 30 mJ/cm^2; F: 16 hours, 10 mJ/cm^2; G: 16 hours, 30 mJ/cm^2; H: 24 hours, 10 mJ/cm^2; I: 24 hours, 30 mJ/cm^2.*

3.1.3 Poly(ADP-ribose) formation in UVB- and H_2O_2-treated cells

Poly(ADP-ribosylation) is a post-translational modification of nuclear proteins catalyzed by poly(ADP-ribose) polymerase (PARP). Recently, several authors have reported a modulation of this process during apoptosis (Negri *et al*, 1997; Scovassi *et al*, 1998; Scovassi and Poirier, 1999) and suggest poly(ADP-ribose) synthesis as a sensitive marker for identifying apoptotic cells (Donzelli *et al*, 1997). In response to both, UVB irradiation and H_2O_2 incubation, nuclei of apoptotic cells stained intensely for PAR (Fig 8). A time- and dose-dependent accumulation of PAR in UVB irradiated cells was observed (Fig 9a); 24 hours after 30 mJ/cm^2 UVB irradiation, 45% of all cells were PAR-positive. Furthermore, PAR formation was detectable as early as 4 and 8 hours after UVB irradiation, whereas DNA fragmentation was only detected at later stages of apoptosis (16 and 24 hours after irradiation). Similarly, it was found that H_2O_2 also induced the formation of PAR in a dose-dependent manner, as assessed 16 hours after addition of H_2O_2 (Fig 9b).

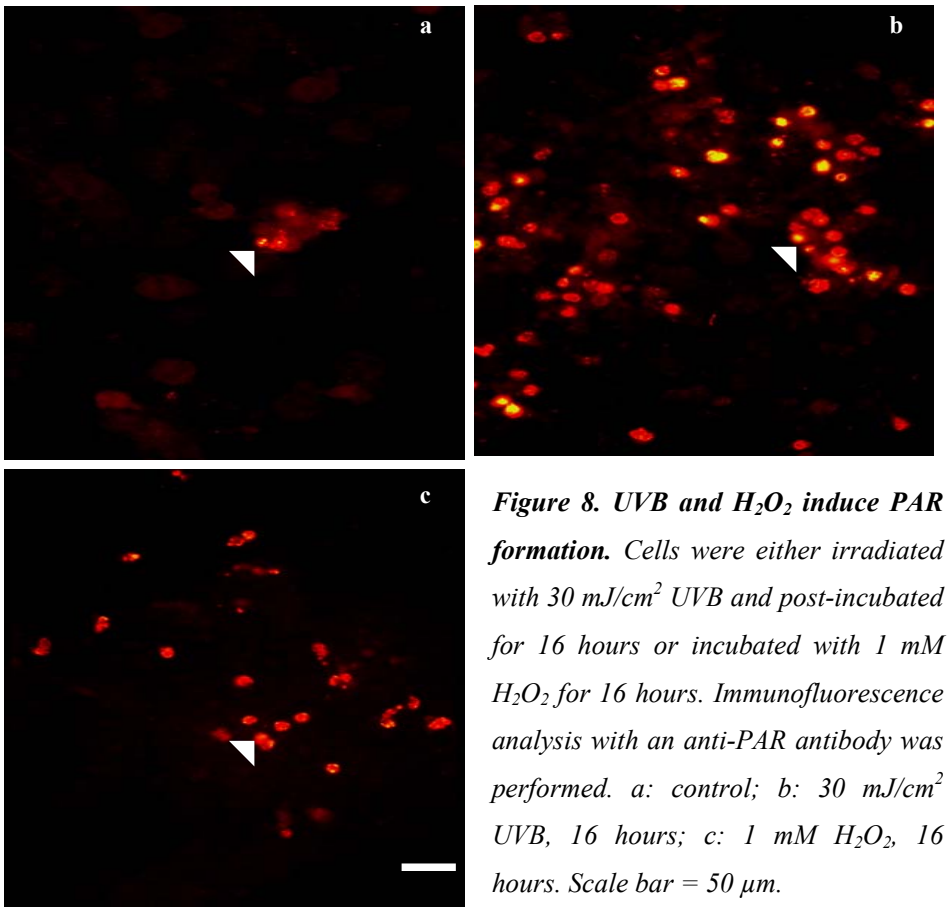

Figure 8. UVB and H_2O_2 induce PAR formation. *Cells were either irradiated with 30 mJ/cm^2 UVB and post-incubated for 16 hours or incubated with 1 mM H_2O_2 for 16 hours. Immunofluorescence analysis with an anti-PAR antibody was performed. a: control; b: 30 mJ/cm^2 UVB, 16 hours; c: 1 mM H_2O_2, 16 hours. Scale bar = 50 µm.*

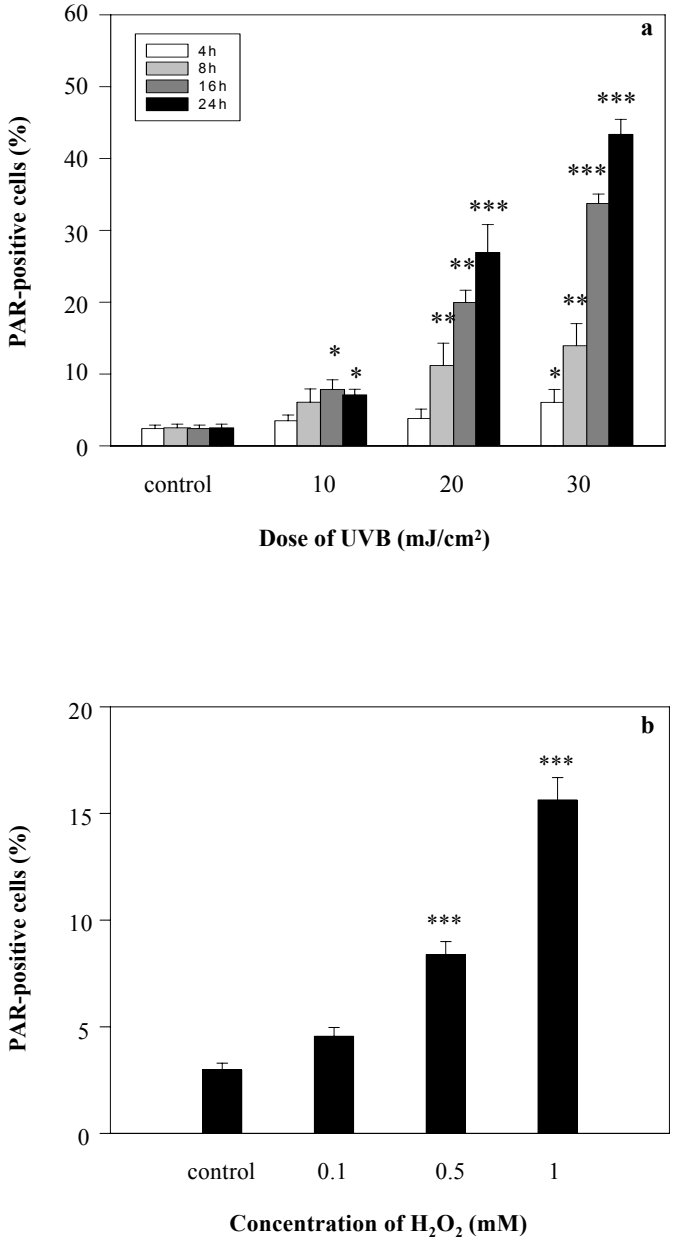

Figure 9. UVB and H_2O_2 induce PAR formation in a time- and dose-dependent manner. *a: Quantitative analysis of PAR-positive cells after UVB-irradiation. HaCaT cells were exposed to 10-30 mJ/cm² UVB and post-incubated for 4-24 hours. b: Quantitative analysis of PAR-positive cells after H_2O_2-treatment. Cells were incubated with 0.1-1 mM H_2O_2 in serum free medium for 16 hours. *p<0.05; **p<0.01; *** p<0.001*

3.1.4 TUNEL assay

To further define the apoptotic cells, UVB-irradiated and H_2O_2-treated cells were examined via a TUNEL assay (Fig 10). As shown in Figure 11a and 11b, an increase in the number of TUNEL-positive cells was observed after UVB irradiation and H_2O_2 treatment. The number of TUNEL-positive cells significantly increased at 16 and 24 hours after 20 and 30 mJ/cm^2 UVB irradiation, and 16 hours after incubation with 1mM H_2O_2. No significant increase was observed at earlier time points (4 and 8 hours) or lower UVB doses (Fig. 11a), or with H_2O_2 concentrations lower than 1mM (Fig. 11b).

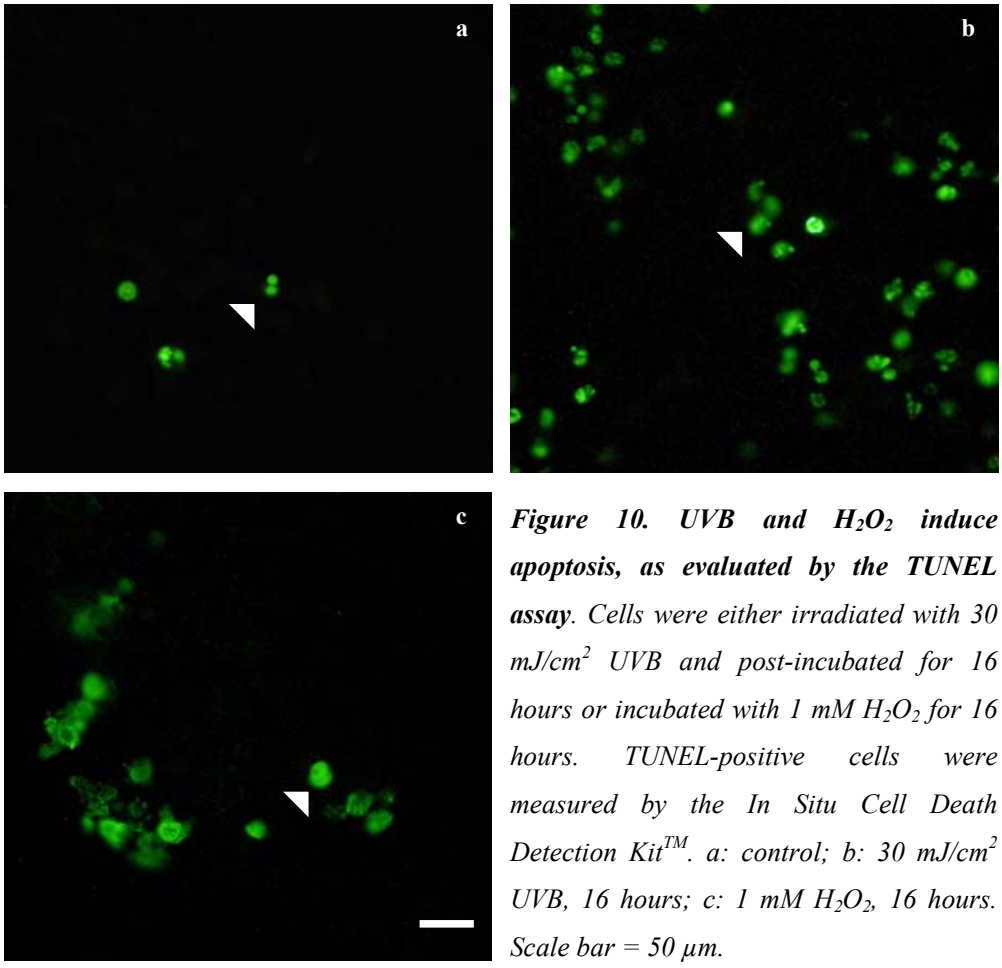

Figure 10. UVB and H_2O_2 induce apoptosis, as evaluated by the TUNEL assay*. Cells were either irradiated with 30 mJ/cm^2 UVB and post-incubated for 16 hours or incubated with 1 mM H_2O_2 for 16 hours. TUNEL-positive cells were measured by the In Situ Cell Death Detection Kit^{TM}. a: control; b: 30 mJ/cm^2 UVB, 16 hours; c: 1 mM H_2O_2, 16 hours. Scale bar = 50 µm.*

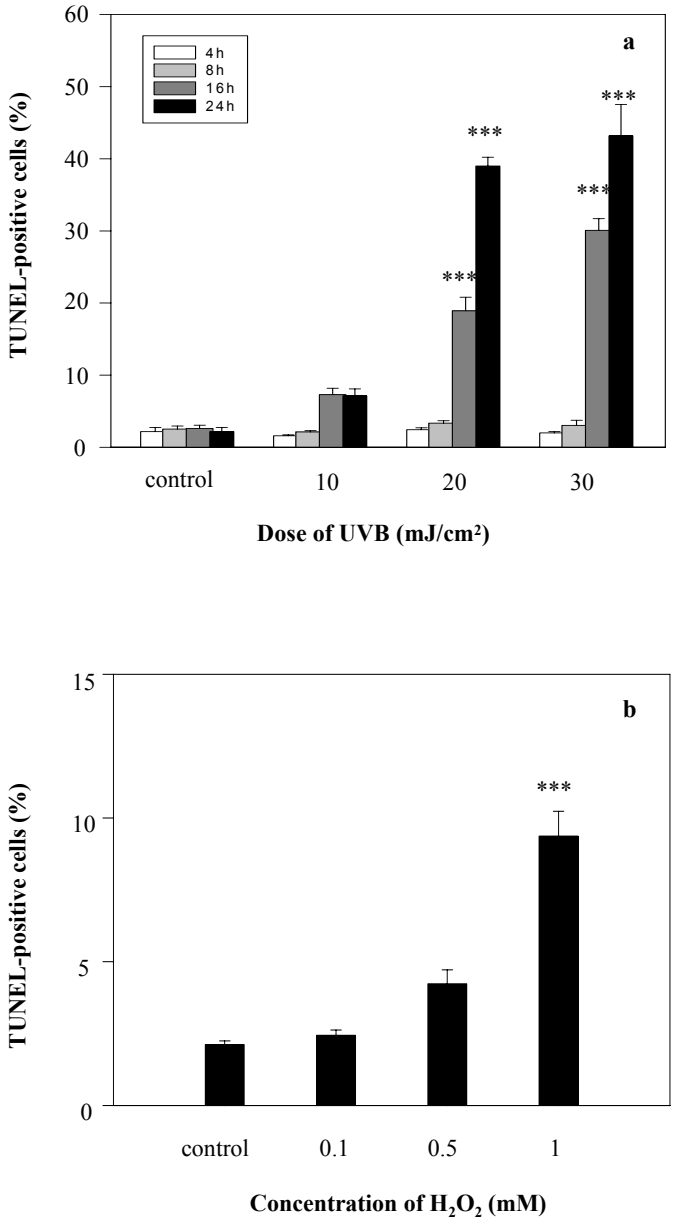

Figure 11. UVB and H_2O_2 induce HaCaT cell apoptosis in a time- and dose-dependent manner. *a: Quantitative analysis of TUNEL-positive cells after UVB-irradiation. HaCaT cells were exposed to 10-30 mJ/cm^2 UVB and post-incubated for 4-24 hours. b: Quantitative analysis of TUNEL-positive cells after H_2O_2 treatment. Cells were incubated with 0.1-1 mM H_2O_2 in serum free medium for 16 hours. *** $p<0.001$.*

3.2 UVB, but not H_2O_2, induces formation of cyclobutane thymine dimers in nuclear DNA of HaCaT cells

It has been reported that mechanisms that are less sensitive to cellular redox control, such as formation of cyclobutane pyrimidine dimers (CPD) and <6-4> photoproducts, may prevail in UVB-induced apoptosis (Kulms and Schwarz, 2000). Therefore, we compared cyclobutane dimer formation in H_2O_2- and UVB-treated cells. As shown in figures 12 and 13, UVB-exposure induced formation of cyclobutane thymine dimers in nuclear DNA of HaCaT cells as early as 30 minutes after UVB-irradiation. Furthermore, even irradiation of the cells with 10 mJ/cm^2 of UVB resulted in detectable levels of thymine dimers formation (Fig 12 and 13a). The dimer formation reached its maximum 30 minutes after 30 mJ/cm^2 UVB-exposure, indicating that DNA damage in the form of cyclobutane thymine dimers appeared in a clearly dose-dependent fashion. However, up to 24 hours after exposure, no significant difference of photoproducts was found between control and H_2O_2-treated cells (Fig 13b).

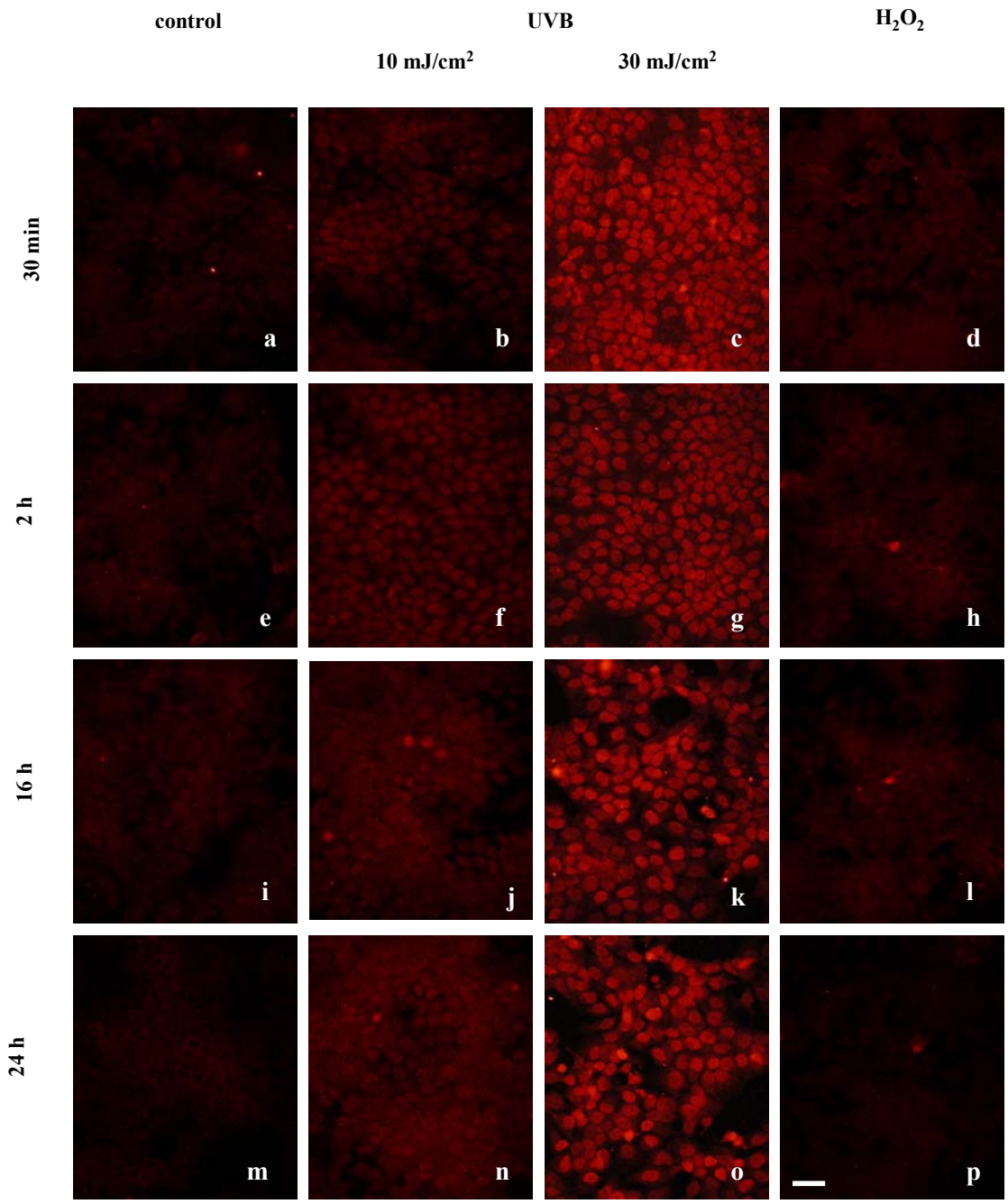

Figure 12. Formation of cyclobutane pyrimidine dimers (CPD) by UVB-irradiation, but not by H_2O_2-treatment. *Cells were either irradiated with 10 and 30 mJ/cm² UVB (b, f, j, n, and c, g, k, o) and post-incubated for 30 minutes, 2, 16, and 24 hours, or incubated with 1 mM H_2O_2 (d, h, l, p) for 30 minutes to 24 hours. After treatments, cells were incubated with an anti-CPD antibody and analyzed by immunofluorescence. Scale bar = 50 µm*

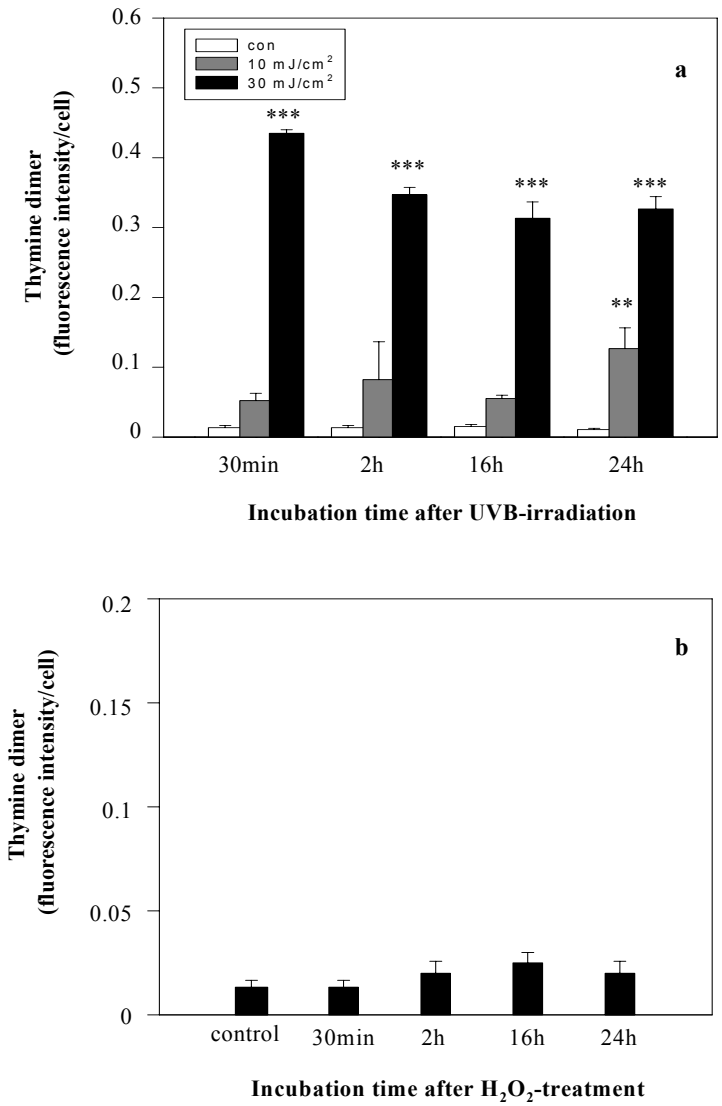

Figure 13. Dose-dependent generation of cyclobutane pyrimidine dimers by UVB-irradiation, but not by H_2O_2-treatment. *a: Quantitative analysis of CPD-positive cells after UVB-irradiation. Cells were irradiated with 10 or 30 mJ/cm² UVB, and post-incubated for 30 minutes to 24 hours. Immunofluorescence analysis with antibody against CPD was performed. b: Quantitative analysis of CPD-positive cells after H_2O_2-treatment. Cells were exposed to 1 mM H_2O_2 and incubated for 30 minutes to 24 hours. No photoproduct formation was detected. Fluorescence intensity was quantified by analysis 3.0 Software™ (Soft Imaging System, Muenster, Germany). ** p<0.01, ***p<0.001.*

3.3 UVB irradiation and H_2O_2 exposure induce production of intracellular H_2O_2

As shown in figure 14, control cells exhibited an intrinsic H_2O_2 concentration (a and d, mean peak (MP) = 34.64). However, in response to both stressors, UVB irradiation and H_2O_2 exposure this baseline level was significantly increased: 30 minutes after 30 mJ/cm^2 UVB irradiation (b and e) and 1mM H_2O_2 incubation (c and f), intracellular H_2O_2 concentration increased rapidly (MP = 58.27 and 80.96, respectively).

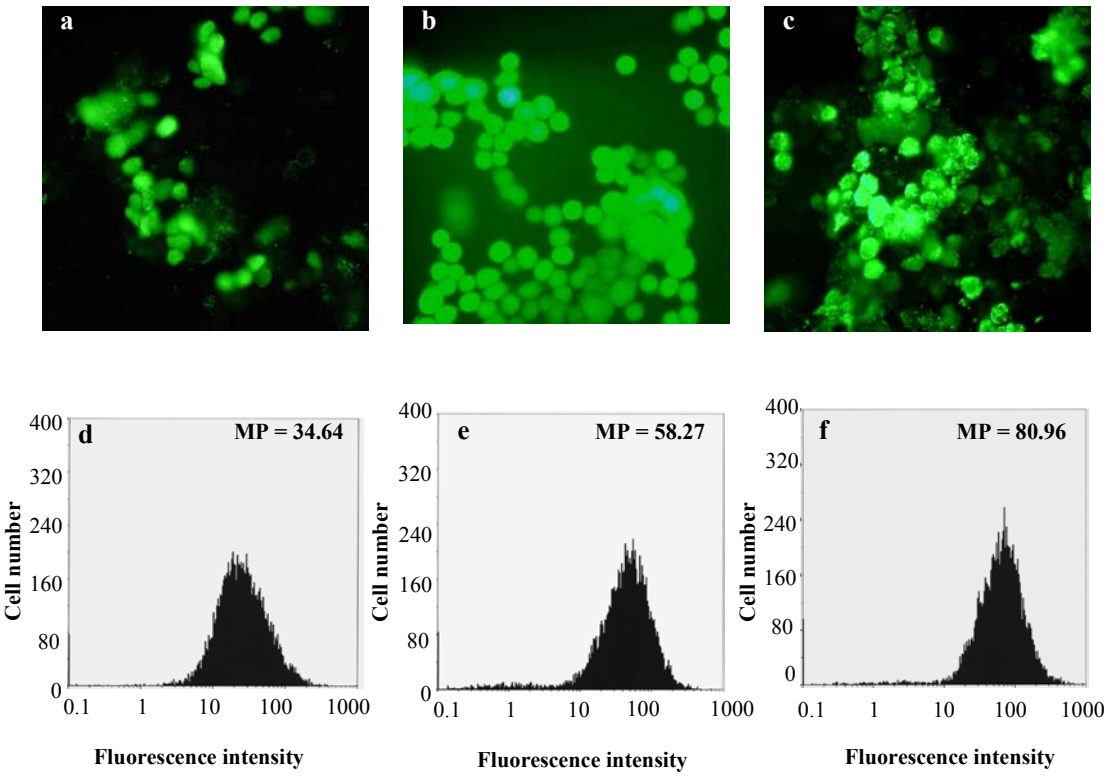

Figure 14. UVB- and H_2O_2-exposure induce generation of intracellular H_2O_2. *HaCaT cells were treated either with 30 mJ/cm^2 of UVB or 1 mM H_2O_2 for 30 minutes, and subsequently incubated with 20 µM 2′,7′-dichlorfluorescencein diacetate (DCF-DA) for 30 minutes at 37°C, and analyzed by fluorescence microscopy (a-c). d-f: Flow cytometric histogram of intracellular H_2O_2 levels. Results are expressed as mean peak of fluorescence intensity and cell number. a and d: control without treatment; b and e: 30 minutes after 30 mJ/cm^2 UVB-irraidiation; c and f: 30 minutes after 1 mM H_2O_2-incubation. MP: mean peak.*

3.4 Antioxidants inhibit H_2O_2-induced, but not UVB-induced apoptosis

3.4.1 Catalase pre-treatment inhibits H_2O_2-induced PAR-formation

Catalase is the most powerful antioxidative enzyme for scavenging H_2O_2. Aoshiba *et al* found that 24 hours pre-incubation with catalase inhibits spontaneous apoptosis in human neutrophils (Aoshiba *et al*, 1999). It was also demonstrated by the present study that both, UVB and H_2O_2, can induce apoptosis and intracellular H_2O_2 generation in HaCaT cells. In order to evaluate if UVB and H_2O_2 induce apoptosis by the same pathway, HaCaT cells were pre-treated with different concentrations of catalase for 24 hours. As shown in figure 15, H_2O_2-induced apoptosis was significantly inhibited by pre-incubation with catalase. Both, morphological features (data not shown) and PAR formation (Fig 15) showed a significant decrease in the number of apoptotic cells. By contrast, pre- and post-incubation with the same concentrations of catalase did not show any protective effect against the UVB-induced apoptosis as measured by PAR formation (Fig 15).

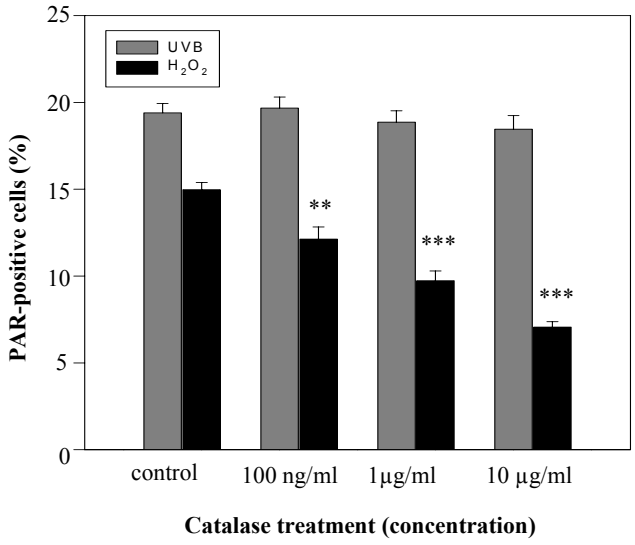

Figure 15. Catalase inhibites PAR formation in H_2O_2-treated, but not in UVB-irradiated cells. *HaCaT cells were pre-incubated with different concentrations of catalase, exposed to 20 mJ/cm^2 UVB, and post-incubated with catalase for 16 hours prior to PAR immunofluorescence. For the H_2O_2-treatment experiment, HaCaT cells were pre-treated with catalase for 24 hours and incubated with 1 mM H_2O_2 for 16 hours prior to PAR immunofluorescence. **: $p<0.01$, ***: $p<0.001$.*

3.4.2 NAC pre-treatment inhibits H_2O_2-induced PAR formation

Figure 16 shows that pre-incubation with NAC at 2.5 and 5 mM for 1 hour protected the cells from H_2O_2-induced apoptosis. PAR formation was reduced by 50%, while pre-treatment with 20 mM NAC increased PAR-positive cells, suggesting that high concentration of NAC is toxic. However, similar to catalase, pre-treatment with same concentrations of NAC did not show any protective effect against UVB-induced apoptosis (Fig 16).

These findings indicated that UVB-induced intracellular H_2O_2 generation may not exert a major effect on HaCaT cell apoptosis.

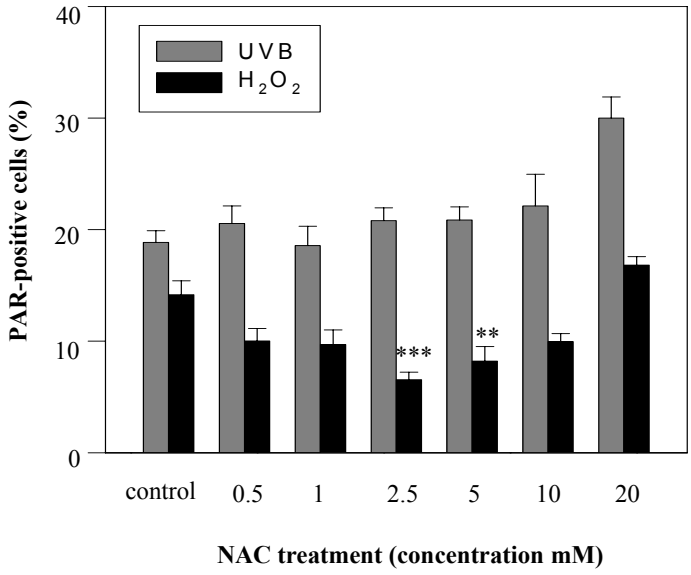

Figure 16. NAC inhibites PAR formation in H_2O_2-treated, but not in UVB-irradiated cells. *HaCaT cells were pre-incubated with different concentrations of NAC for 1 hour, exposed to 20 mJ/cm^2 UVB, and post-incubated for 16 hours prior to PAR immunofluorescence. For the H_2O_2-treatment experiment, HaCaT cells were pre-treated with NAC for 1 hour and incubated with 1 mM H_2O_2 for 16 hours prior to PAR immunofluorescence. **: p<0.01, ***: p<0.001.*

3.5 Both UVB and H_2O_2 induce ERK phosphorylation in HaCaT cells

It was previously described that ERK is highly activated by treatment with oxidants such as H_2O_2 (Guyton *et al*, 1996; Wang *et al*, 1998) and ERK activity is a key factor in regulating apoptosis (Xia *et al*, 1995). To characterize specific cellular responses mediated by UVB and H_2O_2 in HaCaT cells, the activation of ERK after treatment with UVB and H_2O_2 was evaluated. Levels of phosphorylated ERK were examined from 15 to 60 minutes after exposure to 10-30 mJ/cm^2 UVB or 0.1-1 mM H_2O_2. Within 15 minutes of both UVB and H_2O_2 treatment, ERK phosphorylation was maximally upregulated (Fig 17 and 18). By 30 minutes after treatment, the activity declined. Furthermore, increasing doses of UVB (10-30 mJ/cm^2) or concentrations of H_2O_2 (0.1-1 mM) stimulated ERK phosphorylation in a dose- or concentration-dependent manner. Total ERK protein levels at all time points were equivalent as measured by immunoblot of the same stripped membranes using a panERK antibody.

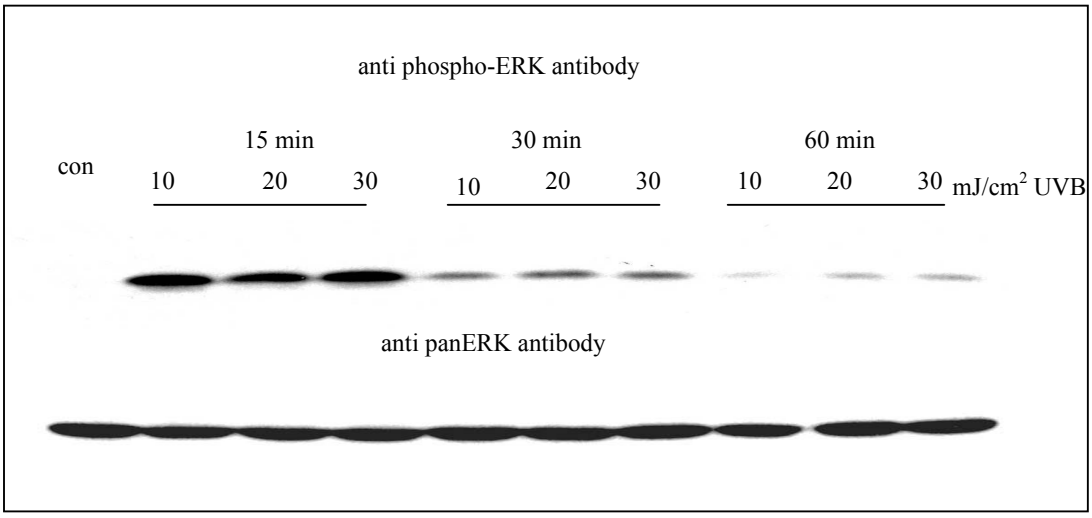

Figure 17. UVB induces ERK phosphorylation in HaCaT cells. *Cells were irradiated with 10-30 mJ/cm^2 UVB, and incubated for 15 to 60 minutes. Total cell proteins were extracted and seperated by SDS-PAGE gel electrophoresis, transferred to PVDF membrane and visualized with anti phospho-ERK antibody. Equal loadding of protein was monitored by immunoblot with anti panERK antibody using the same stripped membrane. ERK phosphorylation was maximally activated 15 minutes after irradiation.*

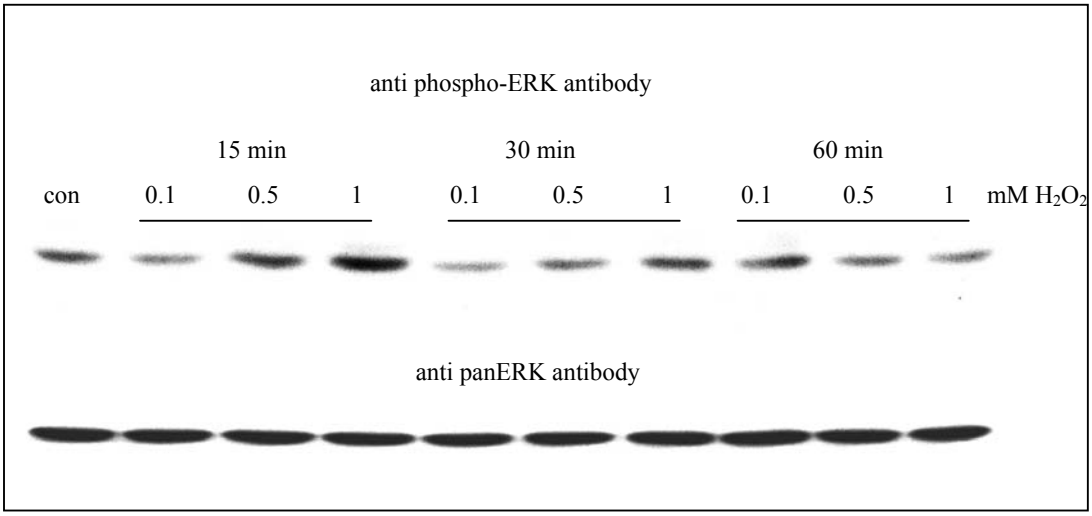

Figure 18. Concentration dependent ERK phosphorylation by H_2O_2. *Cells were incubated with 0.1-1 mM H_2O_2 for 15 to 60 minutes. Immunoblotting with anti phospho-ERK was performed. ERK phosphorylation was maximally activated 15 minutes after 1 mM H_2O_2 incubation.*

3.6 Inhibition of ERK phosphorylation results in enhanced apoptosis after H_2O_2-exposure

3.6.1 PD98059 inhibites ERK phosphorylation induced by UVB and H_2O_2

MEK1 inhibitor PD98059 has been reported to inhibit specifically ERK phosphorylation in different cell types (Alessi *et al*, 1995; Dudley *et al*, 1995). The inhibition of ERK activation induced by UVB or H_2O_2 by this compound was determined. HaCaT cells were pre-incubated with 20 μM PD98059 for 1 hour, and exposed to 30 mJ/cm^2 UVB or 1 mM H_2O_2 for 15 to 60 minutes. Both UVB- and H_2O_2-stimulated ERK phosphorylation were inhibited by PD98059 (Fig 19 and 20), indicating that ERK avtivation was mediated via classical Raf-MEK-ERK pathway.

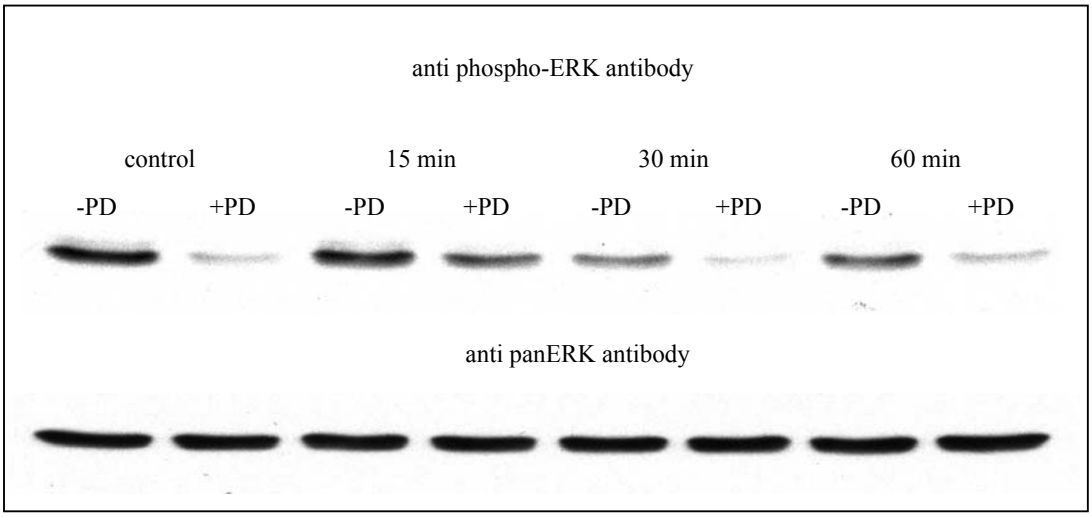

Figure 19. Inhibition of ERK phosphorylation by PD98059 after UVB irradiation. *HaCaT cells were pre.-treated with 20 µM PD98059 for 1 hour, exposed to 30 mJ/cm^2 UVB and post-incubated for 15 to 60 minutes. Immunoblotting with anti-phospho-ERK antibody was performed. ERK phosphorylation was inhibited by PD98059. Equal loadding of protein was monitored by immunoblotting with anti-panERK antibody using the same stripped membrane.*

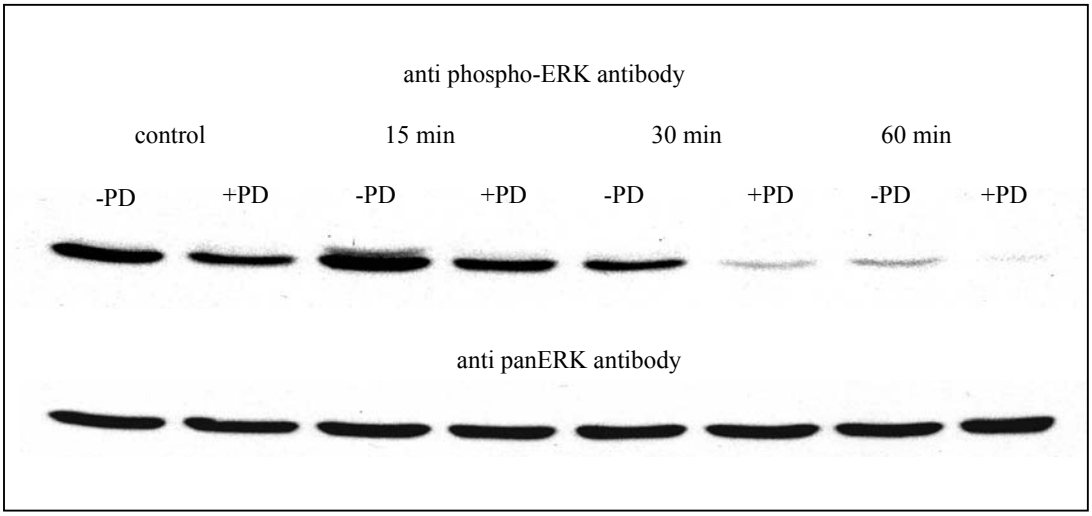

Figure 20. Inhibition of ERK phosphorylation by PD98059 after H_2O_2 treatment. *HaCaT cells were pretreated with 20 µM PD98059 for 1 hour, exposed to 1 mM H_2O_2 and post-incubated for 15 to 60 minutes. Immunoblotting with anti-phospho-ERK antibody was performed. ERK phosphorylation was inhibited by PD98059.*

3.6.2 Inhibition of ERK phosphorylation results in enhanced apoptosis after H_2O_2-exposure, but not after UVB-irradiation

The role of ERK activation in cell survial and apoptosis remains controversial among different cell lines and different treatments (Aikawa *et al*, ; Gunn-Moore *et al*, 1997; Sheng *et al*, 1997). In this study with HaCaT cells, it was demonstrated that PD98059 inhibited ERK activation elicited by UVB and H_2O_2. To further investigate the potential physiological role of ERK phosphorylation in UVB- and H_2O_2-induced apoptosis, the effect of PD98059 on PAR formation was analyzed. After pre-treatment with 20 μM PD98059 for 1 hour, HaCaT cells were exposed to 10 to 30 mJ/cm^2 UVB or 0.1-1 mM H_2O_2. As shown in figure 21b, 16 hours after incubation with 0.5 and 1 mM H_2O_2, PAR formation increased significantly in cells treated with PD98059. However, no significant difference in PAR formation was observed between cells with and without PD98059 pre-treatment, followed by UVB irradiation (Fig 21a).

The result indicated that ERK activation plays an important part in protecting HaCaT cells from apoptosis induced by H_2O_2-exposure, but had no significant effect on UVB-induced HaCaT cell apoptosis.

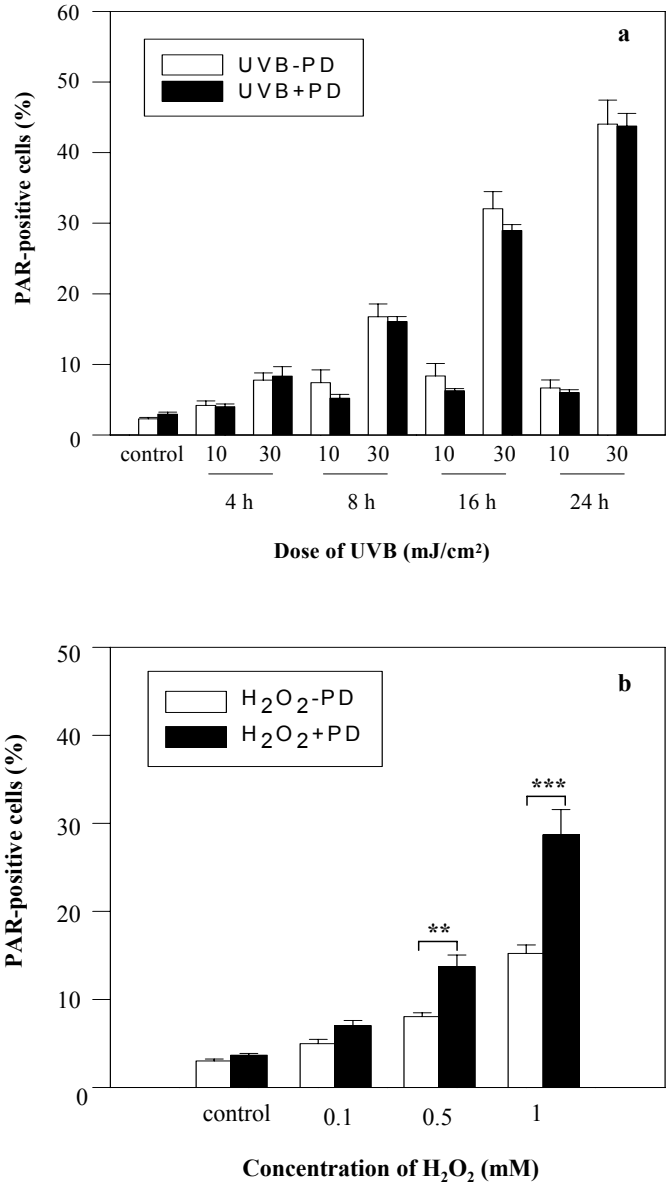

Figure 21. Pre-treatment of HaCaT cells with PD98059 enhances H_2O_2-induced apoptosis, but has no effect on UVB-induced apoptosis. *a: Cells were pre-incubated with 20 µM PD98059 for 1 hour before exposure to 10 or 30 mJ/cm² UVB. Apoptosis was assessed by PAR immunofluorescence at 4, 8, 16, and 24 hours after the irradiation. b: Cells were pre-treated with 20 µM PD98059 for 1 hour before the addition of different concentrations of H_2O_2 (0.1, 0.5, and 1 mM). PAR formation was detected 16 hours after the exposure. **p<0.01; ***p<0.001.*

4. Discussion

Apoptosis is an active form of cell death and is initiated by a variety of stimuli, including UVB and ROS. A number of methods have been developed for the detection of apoptotic cells. In this study, we were able to establish a sensitive new method for the early detection of apoptosis induced by UVB and H_2O_2. Furthermore, the role of H_2O_2 in UVB induced apoptosis in HaCaT cells was investigated. The present study demonstrated that: 1) The formation of poly(ADP-ribose) is an early and sensitive marker for the detection of UVB- and H_2O_2-induced apoptosis in HaCaT cells. 2) Both UVB-irradiation and H_2O_2-treatment induce HaCaT cell apoptosis in a time- and dose-dependent manner. 3) Cyclobutane thymine dimers are formed upon exposure to UVB, but not upon H_2O_2-treatment. 4) Both UVB and H_2O_2 lead to increased levels of intracellular H_2O_2. 5) Antioxidants catalase and NAC dose-dependently inhibit apoptosis when induced by H_2O_2, not by UVB. 6) Both UVB and H_2O_2 induce ERK phosphorylation. 7) MEK1 inhibitor PD98059 inhibits ERK phosphorylation induced by UVB, as well as by H_2O_2. 8) Inhibition of ERK phosphorylation increases apoptosis induced by H_2O_2, not by UVB.

4.1 PAR formation is a sensitive marker for the early detection of UVB- and H_2O_2-induced apoptosis

Several methods have been developed for the detection of apoptosis, including morphological changes, DNA laddering and TUNEL assay. Recently, poly(ADP-ribose) synthesis was suggested as a useful tool for identifying apoptotic cells. Poly(ADP-ribosylation) is a post-translational modification of nuclear proteins controlled mainly by the action of two enzymes: poly(ADP-ribose) polymerase (PARP), which synthesizes the polymer, and poly(ADP-ribose) glycohydrolase (PARG), which is responsible for polymer degradation. PARP, a Zn-finger nuclear protein activated by DNA breaks, utilizes β-NAD^+ as a substrate to catalyze the synthesis of (ADP-ribose) polymers on nuclear proteins, including PARP itself. Apoptotic DNA cleavage was suggested to be the stimulus of PARP enzyme activity and the presence of DNA fragments during apoptosis could regulate the ADP-ribosylation process (Scovassi and Poirier, 1999).

The synthesis of poly(ADP-ribose), as a consequence of DNA fragmentation, can be used to identify apoptotic cells. Negri and Donzelli (1997) developed a new “tricolour” assay

allowing the simultaneous analysis of three parameters: (i) the changes in nuclear morphology after Hoechst 33258 staining; (ii) the appearance of DNA breaks, evaluated by the TUNEL assay; (iii) the presence of endogenous cellular poly(ADP-ribose) synthesis which can be detected by immunofluorescence. It was demonstrated that endogenous poly(ADP-ribose) production is indeed stimulated in HeLa cells undergoing apoptosis, and the detection of PAR formation, combined with morphological and other biochemical parameters, facilitated the detection of apoptotic cells.

In the study, PAR synthesis was used for the first time as a parameter for identification of apoptosis in keratinocytes after UVB and H_2O_2 challenge. It was found that both UVB- and H_2O_2 induced PAR formation, which correlated well with conventional markers of apoptotis, such as cell blebbing, DNA laddering and TUNEL assay (Figs. 6, 7 and 10). Furthermore, a significant increase in PAR synthesis was detected as early as 4 hours after 20 mJ/cm^2 UVB-irradiation, while the TUNEL assay revealed significantly elevated levels of apoptosis only at 16 and 24 hours after 20 and 30 mJ/cm^2 UVB-irradiation. By analogy, DNA laddering and cell blebbing were observed at later time points than PAR formation was detectable. Likewise, Rosenthal *et al* described synthesis of poly(ADP-ribose) in human osteosarcoma cells at a very early stage of apoptosis when morphological characteristics of apoptosis, such as nuclear condensation and cell shrinkage, were not yet apparent (Rosenthal *et al*, 1997).

During apoptosis, DNA fragmentation is crucial for activating PARP, which reacts to the presence of DNA free ends by synthesizing polymers of ADP-ribose on itself (Alvarez-Gonzalez *et al*, 1999). PARP proteolysis has become a hallmark for apoptosis in different cell types treated with various inducers of apoptosis. From reports on different apoptotic systems, it can be argued that, as a first response to apoptotic DNA fragmentation, PARP activity is increased (Bernardi *et al*, 1995). In the presence of a limited amount of DNA damage or DNA free ends, PARP remains intact. However, when there is too much DNA damage or degradation, PARP is cleaved by caspase and thus inactivated (Scovassi *et al*, 1998). This is supported by the phenomenon that PARP proteolysis occurs when some other apoptotic changes (such as DNA laddering) within cells are already evident. Aragane *et al* reported that 30 mJ/cm^2 UVB induced significant PARP cleavage only at 8 hours, but not at 4 hours after irradiation (Aragane *et al*, 1998). Under the same conditions used in this study, a significant increase of PAR formation was detected already at 4 hours after irradiation (Fig 9a).

4.2 UVB-induced HaCaT cell apoptosis is not mediated by H_2O_2

It has been demonstrated that ROS, and the resulting cellular redox changes, regulate signal transduction pathways during apoptosis, and that antioxidants such as N-acetylcysteine (NAC), as well as overexpression of manganese superoxide dismutase (MnSOD) can block or delay apoptosis (Shindo and Hashimoto, 1998). Several studies have shown that at the early stage after UV exposure, production of ROS occurs in irradiated tissues (Dixit *et al*, 1983; Pelle *et al*, 1990), and accumulation of ROS within UVB-treated keratinocytes may promote cell death (Lawley *et al*, 2000). Thus, ROS were regarded as the second messagers to regulate biological effects in response to UV irradiation, such as NF-kappa B activation (Flohe *et al*, 1997) and phosphorylation of EGF recoptors (Peus *et al*, 1998). The present study demonstrated that H_2O_2, similar to UVB-irradiation, also induced apoptosis of HaCaT cells in a time- and dose-dependent manner. Furthermore, we found that both UVB-irradiation and H_2O_2-incubation led to increased intracellular levels of H_2O_2. It has also been reported previously that UVB-irradiation of keratinocytes leads to dose-dependent intracellular production of H_2O_2 (Peus *et al*, 1998), and that the effects of UVB-irradiation may be mediated, at least in part, by H_2O_2 (Peus *et al*, 1999). Based on these findings, it was hypothesized that UVB-induced apoptosis of keratinocytes is mediated by H_2O_2 generation. However, neither pre- nor post-exposure incubation of keratinocytes with catalase, the most powerful H_2O_2-degrading enzyme, inhibited UVB-induced PAR formation (Fig 15). By contrast, apoptosis induced by H_2O_2 could be counteracted by pretreatment with catalase, and H_2O_2-induced PAR formation was reduced by almost 50% (Fig 15). The effect of another antioxidant NAC was also investigated. The thiol-compound NAC has antioxidant activities both as a direct scavenger of oxidant radicals and as a precursor of glutathione (GSH) synthesis, a key antioxidant in cells (De Vries and De Flora, 1993). In the present study, NAC was found to be effective in protecting cells from H_2O_2-induced apoptosis, but had no effect on UVB-induced PAR formation. Thus, these findings indicated although both UVB and H_2O_2 are capable of inducing increased intracellular H_2O_2 levels and apoptosis, UVB-induced apoptosis seems to be mediated mainly by mechanisms independent of H_2O_2 in keratinocytes. Similarly, this was supported by another study examming the effect of antioxidants on UVB-induced caspase activation in HaCaT cells. Pre-treatments of the cells with various antioxidants, such as 1,10-phenanthroline, pyrrolidinedithiocarbamate,

and trolox showed no effect on the UVB-induced caspase-3 activation (Shimizu *et al*, 1999). Bush *et al* also reported that pre-treatment with non-toxic doses of NAC showed no significant protection against UVB-induced apoptosis in keratinocytes (Bush *et al*, 1999). These data suggest that oxidative stress may only play a minor role in UVB-induced apoptosis of keratinocytes.

4.3 Activation of ERK signaling pathway prevents H_2O_2-induced apoptosis of HaCaT cell

Activation of ERK through different pathways leads to fundamentally different cellular responses (Kurada and White, 1998). Signaling through ERK activation can be anti-apoptotic or pro-apoptotic depending on the cell type and the apoptotic stimulus. Survival through ERK activation against a diverse range of apoptotic signals has been reported, including UV-induced apoptosis of human primary neutrophils (Frasch *et al*, 1998), and tumor necrosis factor α-induced apoptosis of L929 cells (Gardner and Johnson, 1996). By contrast, apoptotic signaling through ERK activation has also been described. Fas-mediated apoptosis in a neuroblastoma cell line was blocked by interference with the ERK pathway (Goillot *et al*, 1997). In this study, the role of the ERK pathway activation in apoptosis was investigated by blocking MAPK kinase (MEK), an upstream activator of ERK, controlling its activity by phosphorylation. It was found that inhibition of ERK phosphorylation by the MEK1 inhibitor PD98059 resulted in an increased apoptosis induced by H_2O_2-incubation. This indicated that ERK phosphorylation functions as an anti-apoptotic factor in HaCaT cells upon H_2O_2-treatment.

Downstream events of ERK activation that are crucial for its ability to suppress apoptosis are still not well understood. Several authors have suggested its activity is closely associated with cell proliferation and inhibition of apoptosis is secondary to its role in proliferation. ERK is translocated to the nucleus upon activation and this is required for induction of gene expression and regulation of cell cycle. Recently, some potential ERK targets that are directly involved in apoptosis have also been identified. Erhardt *et al* have shown that ERK, acting downstream of B-Raf, inhibited cytosolic caspase activation following release of cytochrome *c* from the mitochondria (Erhardt *et al*, 1999). Caspase 9 is activated by cytochrome *c* release (Li and Sedivy, 1993) and will, in turn, activate the key cytosolic downstream caspase, caspase 3. Further possible targets of the ERK

pathway are the pro-apoptotic Bcl-2 family protein Bad (Scheid and Duronio, 1998) and the transcription factor CREB (cAMP response element binding portein) (Ballif and Blenis, 2001), which played a critical role in cell survival. Recently there have been several reports suggesting that ERK activation supressed apoptosis by activating the serine/threonine protein kinase p90/rsk2 (Bonni *et al*, 1999) (Fig 22). The pro-apoptotic protein Bad and transcription factor CREB were implicated as p90/rsk2 substrates important to supress apoptosis (Bonni *et al*, 1999). Bad is known to indirectly influence mitochondrial membrane integrity and the release of cytochrome *c* from mitochondria, by associating with Bcl-2 and Bcl-X_L and inhibiting their anti-apoptotic function (Yang *et al*, 1995). Phosphorylation of Bad at two sites, serine 112 and serine 136 induces its association with 14-3-3 protein, thus sequestering it in the cytosol and away from the mitochondria (Zha *et al*, 1996). Phosphorylation of Bad has been shown to occur during cytokine mediated inhibition of apoptosis in haematopoietic cells and this signaling pathway requires activation of MEK (Scheid and Duronio, 1998). The relatively slower mechanism of protection by regulating transcription may be the result of increasing the transcription of pro-survival proteins or interfering with the transcription of pro-death proteins. Phosphorylation of CREB at serine 133 can lead to the MEK-dependent transcriptional up-regulation of pro-survival Bcl-2 family members such as Bcl-2, Bcl-X_L (Liu *et al*, 1999; Boucher *et al*, 2000; Jost *et al*, 2001) (Fig 22).

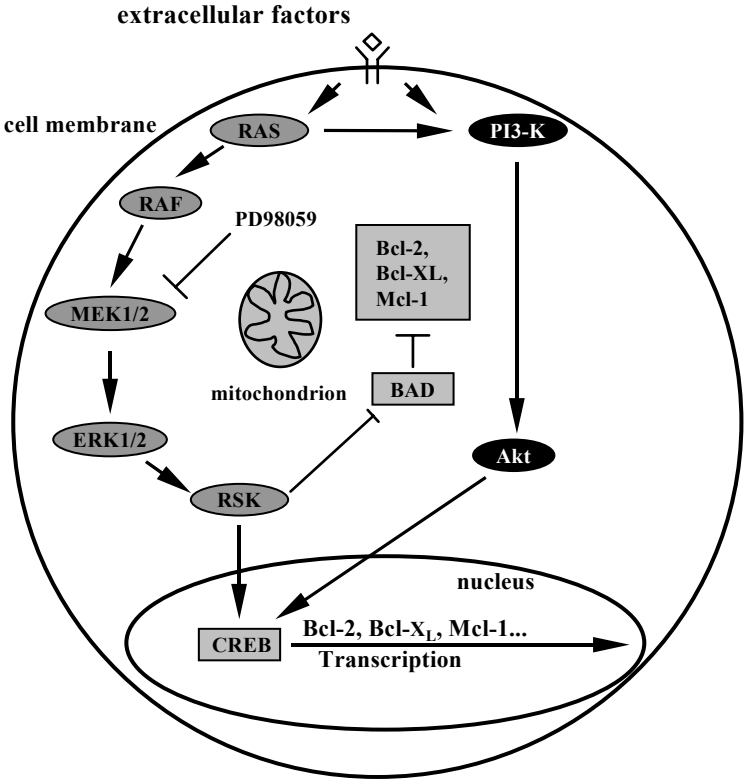

Figure 22. Scheme of MEK-ERK-RSK pathway involved in the regulation of apoptosis. *Extracellular factors induce activation of MEK-ERK-RSK pathway, which control apoptosis by neutralizing the pro-apoptotic effects of BAD and upregulating the transcriptional activity of CREB. Phosphorylated BAD is kept in the cytosol and thus is impaired in its ability to antagonize the mitochondrial-protective functions of a number of Bcl-2 family members. Activated CREB can positively influence the transcription of pro-survival genes including the Bcl-2 family members, Bcl-2, Bcl-X_L, and Mcl-1. (modified from Ballif et al (Ballif et al, 2001)).*

4.4 ERK phosphorylation is not involved in UVB-induced apoptosis in HaCaT cells

In this study, it was demonstrated that both UVB- and H_2O_2-treatment resulted in ERK phosphorylation, and that the potent kinase inhibitor PD98059 inhibited ERK activation upon both UVB-irradiation and H_2O_2-incubation. It has been reported previously that in PC12 and HeLa cells, ERK phosphorylation can be induced by H_2O_2-exposure (Guyton *et al*, 1996; Wang *et al*, 1998). Recently, studies provided evidence that ROS, and in

particular H_2O_2, function as prominent mediators in the activation and regulation of UVB-induced ERK and p38 signal transduction pathways in normal human keratinocytes. Inhibition of ERK activation enhanced apoptosis induced by H_2O_2, as well as by UVB (Peus *et al*, 1999). Our results also demonstrated that UVB-irradiation induced intracellular H_2O_2 generation. Based on these findings, we had hypothesized that UVB-induced ERK phosphorylation is involved in HaCaT cells apoptosis, and that this effect is mediated by intracellular H_2O_2 generation. However, in this study, we found PD98059 treatment increased H_2O_2-induced apoptosis, but had no effect on UVB-induced PAR formation (Fig 21). These results are consistent with another report by Gunn-Moore *et al*, in which it was described that ERK inhibition had no significant effect on the insulin- or brain-derived neurotrophic factor-induced survival of cerebellar granule cells (Gunn-Moore *et al*, 1997). Similarly, it was reported by Nakamura *et al* (Nakamura *et al*, 2001) that UVB-irradiation induced significant increase in the phosphorylation of p38 MAPK, but only a slight increase of ERK phosphorylation, moreover, UVB-induced cell death was not significantly affected by PD98059. These results are in accordance with the findings described here and suggest that UVB-induced apoptosis in keratinocytes is not mediated by the activation of ERK signaling pathway. However, Peus *et al* reported inhibition of ERK phosphorylation by PD98059 resulted in enhanced cell death 24 hours after 40 mJ/cm^2 UVB irradiation in normal human keratinocytes, indicating the role of ERK activation in cell survival remains controversial, and it may be cell type and stimulus dependent.

4.5 UVB-induced apoptosis is triggered by DNA damage

UVB is a hazard to human health by inducing cancer, premature skin aging, immunosuppression, inflammation, and cell death (Fisher *et al*, 1996; Kraemer, 1997). To exert its biological effects, UVB must be absorbed by a cellular chromophore, which transfers the energy into a biochemical signal. Among a number of UVB absorbing molecules (porphyrins, aromatic amino acids, urocanic acid), DNA is regarded as the most important chromophore for several reasons: i) The wavelength dependency of some UVB effects is similar to that for DNA absorption (Petit-Frere *et al*, 1998). ii) Acceleration of DNA repair inhibits particular biological UVB effects (Kripke *et al*, 1992; Nishigori *et al*, 1996; Kibitel *et al*, 1998). iii) Lower UVB doses are necessary to achieve the same

biological effects in DNA repair-deficient cells (Krutmann *et al*, 1994). Thus, these data support the hypothesis that DNA is the most important molecular target for UVB and that DNA damage is crucially involved in mediating the biological effects of UVB. A hallmark event of UV exposure is the induction of apoptotic cell death of keratinocytes (Young, 1987). It is generally accepted that the severity of DNA damage determines whether programmed cells death is initiated and the cells which did not repair UV-induced DNA damage sufficiently initiate apoptosis (Brash *et al*, 1996).

Mechanisms that are less sensitive to cellular redox control, such as UVB-induced formation of cyclobutane pyrimidine dimers and <6-4> photoproducts, may prevail in UVB-induced apoptosis (Kulms and Schwarz, 2000). Therefore, cyclobutane dimer formation, a major DNA photoproduct upon UVB-irradiation, was compared in H_2O_2- and UVB-treated cells. DNA damage in the form of cyclobutane thymine dimers appeared as early as 30 minutes after UVB-irradiation in a clearly dose-dependent fashion, and thus preceded detection of apoptosis (Fig 13a). Importantly, no cyclobutane thymine dimers were detected in H_2O_2-treated cells up to 24 hours after exposure. UVB-induced apoptosis in keratinocytes may be mediated by rapidly formed DNA photoproducts, rather than by H_2O_2. This was confirmed by i*n vivo* studies which showed that enhancement of DNA repair by topical application of the repair enzyme T4 endonuclease V in liposomes reduced apoptosis (Wolf *et al*, 1995). Kulms *et al* reported that HeLa cells exposed to UVB showed significantly reduced levels of both cyclobutane pyrimidine dimers and apoptosis when incubated with the DNA repair enzyme photolyase immediately after UVB-exposure (Kulms *et al*, 1999). The photolyase binds to a UV-induced cyclobutane pyrimidine dimer in DNA and catalyzes its splitting by electron transfer from absorbing wavelengths above 320 nm (photoreactivating light). Similarly, it was reported that photoreactivation of OCP13 cells, a cell line exhibiting high level expression of the gene for CPD photolyase, almost completely reverses UV-induced pyrimidine dimer formation (Nishigaki *et al*, 1998). All these data indicated that DNA damage is crucially involved in UV-induced apoptosis. Activation of caspases appears to be crucial for executing apoptosis induced by UVB-induced DNA damage. Kulms *et al.* (Kulms *et al*, 1999) found enhancement of DNA repair reduced cleavage of caspase 3 into its active form, and furthermore, inhibition of caspase 3 resulted in decreased apoptosis induced by UVB. These findings suggested that UVB induced DNA damage, which initiated the apoptosis via activation of caspase 3. However, UVB induced apoptosis is a highly complex process

in which some other different pathways such as activation of the tumor suppressor gene p53, triggering of cell death receptors either directly by UV or by autocrine release of death ligands, are involved.

4.6 Conclusion and Outlook

In this study, a new and sensitive method for the detection of apoptosis induced by UVB and H_2O_2 was developed. Using this method, the following were investigated:

- Influence of UVB and H_2O_2 on HaCaT cells apoptosis
- Effect of antioxidants catalase and NAC on UVB- and H_2O_2-induced apoptosis
- Involvement of ERK phosphorylation in UVB and H_2O_2-induced apoptosis

It was found in this study that 1) PAR formation is an early and sensitive marker for the detection of apoptosis in HaCaT cells after UVB- and H_2O_2-treatment; 2) antioxidants catalase and NAC inhibit H_2O_2-induced HaCaT cells apoptosis, but have no effect on UVB-induced apoptosis; 3) Both UVB and H_2O_2 induce ERK activation; 4) ERK phosphorylation protects HaCaT cells from apoptosis induced only by H_2O_2, but not by UVB.

These findings suggest although both UVB and H_2O_2 induce HaCaT cell apoptosis, different pathways are involved. While apoptosis was demonstrated to be redox sensitive in the H_2O_2 system, redox regulation does not appear to be responsible for UVB-induced apoptosis in HaCaT cells.

Based on these findings, further studies will focus on 1) elucidating UVB- and H_2O_2-induced signaling pathways leading to the DNA damage; 2) screening of UVB protection compounds; 3) evaluation of the use of PAR formation as a sensitive and early marker of UVA- and UVB-induced apoptosis in human skin tissue sections.

5 Summary

Apoptosis is an active and biochemically distinct form of cell death that is initiated by a variety of physiological or pharmacological stimuli, including UVB radiation and ROS. A number of methods have been developed for the detection of apoptotic cells. Poly(ADP-ribose) (PAR) is formed upon activation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), and therefore was suggested as a new marker of apoptosis. Since DNA of epidermal cells represents a well known chromophore for UVB irradiation, and UVB is known to generate H_2O_2 in keratinocytes, it was hypothesized, that PAR is a very early and sensitive marker of UVB- and H_2O_2-induced apoptosis in keratinocytes. To test this, human immortalized keratinocytes (HaCaT) were UVB-irradiated or H_2O_2-treated and, thereafter, apoptosis was identified by comparing conventional parameters, such as morphological analysis, DNA laddering, and TUNEL assay, with PAR formation. Both, UVB- and H_2O_2-treatment induced PAR formation in HaCaT cells in a dose-dependent manner, and its formation was detected as early as 4 hours after irradiation, and at lower UVB doses (10 mJ/cm^2) than was observed by DNA laddering and the TUNEL assay. These findings indicated that detection of PAR formation is a very sensitive and early method for the identification of apoptotic cells in UVB- and H_2O_2-induced apoptosis of human keratinocytes. Previously, it has been reported that UVB-irradiation of keratinocytes leads to intracellular generation of H_2O_2 and that antioxidants can inhibit ROS-induced apoptosis. The current study demonstrated both UVB-irradiation and H_2O_2-incubation resulted in increased intracellular H_2O_2 level. It was hypothesized that UVB-induced apoptosis is mediated by H_2O_2. However, the antioxidants catalase, a H_2O_2-degrading enzyme, and NAC, a direct scavenger of ROS and a precursor of GSH, inhibited apoptosis only when induced by H_2O_2, and treatment of cells with catalase or NAC did not alter cellular sensitivity to UVB, pointing to a H_2O_2-independent pathway of UVB-induced apoptosis. Mechanisms that are less sensitive to cellular redox control, such as UVB-induced formation of cyclobutane pyrimidine dimers and <6-4> photoproducts may prevail in UVB-induced apoptosis. Therefore, we compared cyclobutane dimer formation in H_2O_2- and UVB-treated cells. DNA damage in the form of cyclobutane thymine dimers appeared as early as 30 minutes after UVB-irradiation in a clearly dose-dependent fashion, and thus preceded detection of apoptosis. Importantly, no cyclobutane thymine dimers were detected in H_2O_2-treated cells up to 24 hours after exposure. These

results indicated that direct DNA damage, such as pyrimidine dimer formation, rather than H_2O_2 generation, appears to be a relevant initiating event in UVB-induced apoptosis in HaCaT cells.

The cellular response to diverse external stimuli is controlled via a complex array of phosphorylation cascades. The ERK cascade is a prominent component of the MAPK family that in particular plays an important role in the control of gene expression, cell survial and programmed cell death. ERK activation is believed to have different functions depending on the cell type and stimulus. In this study, it was demonstrated that both UVB and H_2O_2 could activate ERK pathway. Inhibition of ERK phosphorylation by an upstream inhibitor PD98059 increased the number of apoptosis only induced by H_2O_2. However, ERK phosphorylation appeared not to be involved in UVB-induced apoptosis in HaCaT cells.

The findings in the present study suggest that PAR is an early and sensitive marker for the detection of UVB- and H_2O_2-induced apoptosis and can be used for identifying apoptotic keratinocytes. Furthermore, the presented results indicate that two different mechanisms are involved in UVB and ROS induced apoptosis. These results contribute to better understanding of different signaling pathways induced by UVB and oxidative stress in keratatinocytes.

6 Zusammenfassung

Apoptose ist eine aktive, streng regulierte Form des Zelltodes, die durch physiologische, pharmakologische und Umwelteinflüsse, wie zum Beispiel UVB Strahlung oder reaktive Sauerstoff-Verbindungen (ROS), ausgelöst werden kann. Für die Detektion apoptotischer Zellen werden verschiedene Methoden und Marker genutzt. Poly(ADP-ribose) (PAR) wird nach dem Auftreten von DNA-Schäden von der Poly(ADP-ribose) Polymerase (PARP) synthetisiert und wird hier als neuer Marker für die Identifizierung apoptotischer Zellen diskutiert. Die DNA epidermaler Zellen stellt ein Chromophor für UVB Strahlung dar und UVB Strahlung generiert in Keratinozyten H_2O_2. Aus diesem Grund wurde in der vorliegenden Arbeit die Hypothese getestet, daß PAR-Bildung als früher, sensitiver Marker für Apoptose in Keratinozyten benutzt werden kann.

Um diese Hypothese zu überprüfen, wurden immortalisierte, humane Keratinozyten (HaCaT) mit UVB-Strahlung bzw. H_2O_2 behandelt. Im Anschluß an diese Behandlung wurde die Zahl apoptotischer Zellen anhand verschiedener, etablierter Parameter, wie der Änderung der Zellmorphologie, DNA Laddering und TUNEL Assays, bestimmt. Die Ergebnisse wurden mit der Synthese von PAR unter denselben Versuchsbedingungen verglichen. Sowohl UVB- als auch H_2O_2-Behandlung führten zu einer Bildung von PAR. Diese konnte bei einer niedrigen Dosis von 10mJ/cm^2 UVB bereits nach vier Stunden nachgewiesen werden. Die Versuche zeigten, daß PAR-Bildung eine, im Vergleich zu den etablierten Apoptose-Detektions-methoden, sensitivere und frühere Detektion apoptotischer Keratinozyten erlaubt. In der Literatur wird diskutiert, daß UVB-Strahlung über die Bildung von H_2O_2 Apoptose auslösen könnte. ROS-vermittelte Apoptose kann durch Vorbehandlung der Zellen mit Antioxidantien verhindert werden. In der vorliegenden Arbeit konnte gezeigt werden, daß sowohl H_2O_2-Inkubation von HaCaT-Zellen, als auch UVB-Bestrahlung der Zellen eine Erhöhung des intrazellulär messbaren H_2O_2 bedingen. Es konnte daher vermutet werden, daß die UVB-Strahlung induzierbaren, apoptotischen Vorgänge durch das gebildete H_2O_2 hervorgerufen werden. Die durchgeführten Versuche zeigten jedoch, daß verschiedene Antioxidantien, wie NAC, und Glutathionester, sowie eine Behandlung der Zellen mit dem H_2O_2-abbauenden Enzym Catalase, keinen Einfluß auf UVB-vermittelte Apoptose hatten. Im Gegensatz dazu konnte durch H_2O_2-Behandlung induzierte Apoptose durch die beschriebenen antioxidativen Substanzen verhindert werden. Diese Ergebnisse deuten darauf hin, daß

UVB-Strahlung und H_2O_2 verschiedene Mechanismen und Signalwege benötigen, um Apoptose auszulösen. Es kann daher vermutet werden, daß die Induktion UVB-bedingter Apoptose durch Prozesse, die weniger der zellulären Redox-Kontrolle unterliegen, erfolgt. Z.B. induziert UVB-Strahlung direkte DNA-Schäden durch die Bildung von Cyclobutan-Pyrimidin Dimeren oder <6-4> Photoprodukten. In der vorliegenden Arbeit wurde deshalb die Pyrimidin-Dimer Bildung nach H_2O_2- und UVB-Behandlung der HaCaT-Zellen untersucht. Es konnte ein Dosis-abhängiger Anstieg an Dimeren in UVB-, nicht aber in H_2O_2-behandelten Zellen detektiert werden. Die Dimerbildung konnte bereits 30 Minuten nach Bestrahlung nachgewiesen werden, wohingegen in den H_2O_2-behandelten Zellen selbst nach 24 Stunden keine DNA-Dimere detektierbar waren. In den UVB-behandelten Zellen trat die Pyrimidin-Dimerbildung zeitlich vor der Apoptose auf. Diese Ergebnisse zeigen, daß UVB-vermittelte Apoptose in HaCaT-Zellen maßgeblich durch direkte Schädigung der DNA, z.B. durch die Bildung von Pyrimidin-Dimeren, und weniger durch die Bildung von H_2O_2 ausgelöst wird.

Die Antwort einer Zelle auf extrazelluläre Stimuli wird durch Signalübertragungsproteine reguliert, die in komplexen Kaskaden miteinander interagieren. Die ERK-Kinase ist eines der am besten untersuchten Mitglieder einer solchen Signalkaskade, die für die Regulation der Genexpression in Differenzierung und Proliferation von Zellen, sowie der Regulation des programmierten Zelltodes eine wichtige Rolle spielt. In der vorliegenden Arbeit konnte gezeigt werden, daß sowohl UVB-Strahlung als auch H_2O_2 den ERK-Signalweg aktivieren. Die Inhibierung der ERK Aktivierung durch den Inhibitior PD98059 führte zu einem Anstieg der Zahl apoptotischer Zellen in Folge einer H_2O_2-Behandlung. Dagegen hatte eine Inhibierung der ERK-Aktivität keinen Einfluß auf die durch UVB-Strahlung induzierte Apoptose.

Die hier vorgestellten Ergebnisse zeigen, daß PAR als früher und sensitiver Marker für die Detektion von UVB- und H_2O_2–induzierter Apoptose in Keratinozyten verwendet werden kann. Weiterhin zeigt die vorliegende Arbeit, daß verschiedene Signaltransduktionswege an der Induktion UVB- und H_2O_2-induzierter Apoptose beteiligt sind. Die gezeigten Daten tragen daher zu einer genaueren Aufklärung von durch UVB-Strahlung und oxidativen Stress induzierten Prozessen und Signalwegen in Keratinozyten bei.

7. Literature

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8. Appendix

8.1 Abbreviations

BSA: Bovine Serum Albumin

CPD: cyclobutane pyrimidine dimer

CREB: cAMP response element binding portein

DCF: 2',7'-dichlorofluorescein

DCF-DA: 2',7'-dichlorofluorescein-diacetate

DMEM: Dulbecco's Modified Eagle's Medium

ERK: extracellular signal-regulated kinase

ECL: enhanced chemiluminescence

FCS: fetal calf serum

JNK: c-Jun N-terminal protein kinase

H_2O_2: hydrogen peroxide

HRP: horseradish peroxidase

MAPK: mitogen-activated protein kinase

NAC: N-acetyl-cysteine

PAR: poly(ADP-ribose)

PARP: poly(ADP-ribose) polymerase

PBS: phosphate-buffered saline

PD98059: AMF, 2'-amino-3'-methoxy-flavone

ROS: reactive oxygen species

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick labeling

UVA: ultraviolet A

UVB: ultraviolet B

UVB: ultraviolet C

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8.4 Ehrenwörtliche Erklärung

Hiermit erkläre ich, daß mir die Promotionsordnung der Medizinischen Fakultät der Friedrich- Schiller- Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskriptes unterstützt haben: Herr Prof. Dr. med. Peter Elsner, Dr. med. Jens Thiele, Dr. med. Christina Sander, Frau Dr. rer. nat. U.-Ch. Hipler, Frau Brunhilde Knöll, Dr. rer. nat. Wolf Oehrl, Dr. Udo Markert.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und daß Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

daß ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

daß ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, den