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ARTICLES

Role of Mitochondria in Ultraviolet-Induced Oxidative Stress

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Abstract The biological effects of ultraviolet radiation (UV), such as DNA damage, mutagenesis, cellular aging, and carcinogenesis, are in part mediated by reactive oxygen species (ROS). The major intracellular ROS intermediate is hydrogen peroxide, which is synthesized from superoxide anion ($^{\bullet}O_2^-$) and further metabolized into the highly reactive hydroxyl radical. In this study, we examined the involvement of mitochondria in the UV-induced H_2O_2 accumulation in a keratinocyte cell line HaCaT. Respiratory chain blockers (cyanide-*p*-trifluoromethoxy-phenylhydrazone and oligomycin) and the complex II inhibitor (theonyltrifluoroacetone) prevented H_2O_2 accumulation after UV. Antimycin A that inhibits electron flow from mitochondrial complex III to complex IV increased the UV-induced H_2O_2 synthesis. The same effect was seen after incubation with rotenone, which blocks electron flow from NADH-reductase (complex I) to ubiquinone. UV irradiation did not affect mitochondrial transmembrane potential ($\Delta\Psi_m$). These data indicate that UV-induced ROS are produced at complex III via complex II (succinate-Q-reductase). J. Cell. Biochem. 80:216–222, 2000.

Key words: free radicals; hydrogen peroxide; respiratory chain

Short- (280–320 nm) and long-waved (320–400 nm) UV is known to stimulate the intracellular synthesis of ROS, which have been implicated in mutagenesis, carcinogenesis, and skin aging [Adelman et al., 1988; Ames, 1988]. On the cellular level, ROS cause oxidative DNA damage, are able to stimulate apoptosis, and mediate cellular aging [Devary et al., 1993; Chen and Ames, 1994; Chen et al., 1995; Hildeman et al., 1999]. Recent research has shown that ROS may also play a physiologic role in the regulation of cellular growth and metabolism, either directly by acting as second messengers [Irani et al., 1997] or by activating other enzymes such as mitogen-activated protein kinase [Peus et al., 1998; Gotoh and Cooper, 1998], stress-activated protein kinases [Shafman et al., 1995], or transcription factors AP-1 and NF-κB [Weichselbaum et al., 1994; Huang et al., 1996].

The most important type of ROS induced by UV is the superoxide anion ($^{\bullet}O_2^-$), which is rapidly converted to H_2O_2 by superoxide dismutase [Fridovich, 1978]. In the presence of iron ions, H_2O_2 may further be metabolized into the highly reactive hydroxyl radical ($^{\bullet}OH^-$), which is the main species responsible for oxidative stress.

A profound insight into cellular ROS metabolism has been gained through the studies on apoptosis. Oxidative stress caused by the treatment with H_2O_2 is lethal and leads to cell cycle block and apoptosis. Many different proapoptotic factors, including UV, have been shown to stimulate H_2O_2 synthesis, a step that triggers the biochemical cascade leading ultimately to oxidative cell death [Larrick and Wright, 1990; Devary et al., 1993; Manome et al., 1993; Quillet-Mary et al., 1996, 1997]. The H_2O_2 synthesis step is often regulated by mitochondria. In the very early phases of apoptosis, opening of the mitochondrial PTP takes place, leading to a decrease in the mitochondrial $\Delta\Psi_m$ [Zamzami et al., 1995, 1996]. This induces the

Abbreviations used: carboxy-FC, 2′,7′-dichlorofluorescein derivatives; carboxy-H_2DCFDA, carboxy-dichlorodihydrofluorescein diacetate; $\Delta\Psi_m$, transmembrane potential; FCCP, cyanide-*p*-trifluoromethoxy-phenylhydrazone; JC1, 5,5′, 6,6′-tetrachloro-1.1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; LSC, laser scanning cytometry; PTP, permeability transition pores; ROS, reactive oxygen species; TTFA, theonyltrifluoroacetone; UV, ultraviolet radiation.

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mitochondrial $^{\bullet}O_2^-$ and H_2O_2 synthesis, which culminates in caspase activation and cell death. In the case of ceramide-induced apoptosis, the mitochondrial $^{\bullet}O_2^-$ production is caused by an increase in a proportion of electrons undergoing a one-electron reduction of oxygen at the ubiquinone site of the mitochondrial respiratory channel [Quillet-Mary et al., 1997].

The central question is the molecular mechanism of UV-dependent induction of ROS. It is virtually unknown whether ROS synthesis is an actively regulated metabolic process involving flavin-containing oxidases [Hockberger et al., 1999] or instead a passive photochemical reaction independent of the metabolic status of the cell. Recently, we observed that blocking of mitochondrial metabolism resulted in a decrease in H_2O_2 synthesis (unpublished data). We hypothesized that mitochondrial activity is essential for ROS induction by UV, in a similar manner to that documented for apoptosis. In this study, we investigated this issue in detail and provide evidence that ROS synthesis after UV irradiation maps to the complex III of the respiratory chain.

MATERIALS AND METHODS

Reagents

Carboxy-H_2DCFDA and JC1 were obtained from Molecular Probes (Eugene, OR), dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mM and stored at 100 μl aliquots at −20°C for not longer than 24 h before an experiment. Mitochondrial blockers were purchased from Sigma (St. Louis, MO).

Cell Culture

A HaCaT keratinocyte cell line [Boukamp et al., 1988] was used in this study. The cells were seeded at 7×10^3 cells per cm^2 and cultured in Dulbecco's minimal essential medium (Gibco BRL, Life Technologies, Rockville, MD) supplemented with 10 % (vol/vol) heat-inactivated fetal calf serum (Gibco) at 37°C in an atmosphere of 100% humidity and 5% CO_2. Cell viability assayed by trypan blue exclusion test was not affected by trypsinization, UVA irradiation, and/or treatment with mitochondrial blockers and exceeded 95%.

UVA Irradiation

The UVA source consisted of an array of Philips TL 10R tubes (Philips, Eindhoven, Netherlands) with an intensity of 3.7 mW/cm^2. The TL 10R tubes emit 99.9 % of the total UV radiation in the spectral region between 341and 400 nm (UVA1), with the peak at 367 nm. The emitted intensities were measured using an International Light (IL) 1700 research radiometer with a SED 400 detector as described previously [Bech-Thomsen et al., 1991]. The HaCaT cell cultures were irradiated from above through a 0.5-cm layer of phosphate-buffered saline (PBS).

Laser Scanning Cytometry

The laser scanner cytometer (CompuCyte Corp., Cambridge, MA) equipped with a 488 nm line argon laser excitation source was used. The principle of LSC is analogous to flow cytometry and allows quantitative measurement of fluorescence from single cells [Kamentsky and Kamentsky, 1991]. A suspension of labeled HaCaT cells was coverslipped, and fluorescence was excited with the argon laser focused on the sample through a 20× objective of Olympus BX microscope. The cells were interrogated by a 5-μm-diameter laser beam scanning along a line as the specimen was moved by a computer-controlled motorized stage. The cells were contoured using forward scatter, and events corresponding to single cells were gated on a plot of scatter area versus its maximal pixel (the value of the brightest pixel within the contour). Integral fluorescence (the sum of values of all pixels within the contour) was measured for each applied fluorochrome and shown as histograms or dot plots using the proprietary WinCyte software (CompuCyte Corp.). Seven thousand to 10,000 cells were scanned for each histogram. The scale of the y axis of the histograms has been equalized to the highest peak of the curve to remove the differences caused by different amounts of cells analyzed in particular experiments.

Determination of Intracellular H_2O_2 Concentrations

Intracellular levels of H_2O_2 were analyzed using carboxy-H_2DCFDA, which is irreversibly oxidized and converted to the green fluorescent 2′,7′-dichlorofluorescein derivatives (carboxy-FC) as previously described [Kehrer and Paraidathathu, 1992]. The aqueous solution of this probe is not directly oxidized by UVA doses used in this study (Molecular Probes: personal communication and unpublished own data).

The HaCaT cells were loaded with 10 μM carboxy-H_2DCFDA in PBS for 30 min at 37°C. We found that basal fluorescence remained stable for at least 2 h with the continuous presence of carboxy-H_2DCFDA at 37°C in the dark [Garland and Halestrap, 1997]. Carboxy-H_2DCFDA-loaded cells were exposed to an appropriate mitochondrial blocker and subjected to UVA irradiation, as indicated, without washing out the chemical. After irradiation, the cells were released by trypsination, centrifuged, and the integrated fluorescence of intracellular FC was measured by LSC using a green (530 ± 30-nm) filter. In each experiment with mitochondrial blockers, three controls were included: 1) sham-irradiated cells treated with DMSO (solvent for mitochondrial blockers); 2) DMSO-treated cells irradiated with UVA, and 3) sham-irradiated cells treated with an appropriate blocker. To quantify the effect of mitochondrial blockers on UVA-induced ROS production, an integral FC fluorescence was calculated for the control and experimental groups and the following formula was used:

$$\text{Change in fluorescence (\%)} = \left[\frac{(\text{UV} + \text{blocker}) - (\text{sham irradiation} + \text{blocker})}{(\text{UV} + \text{DMSO}) - (\text{sham irradiation} + \text{DMSO})}\right] \cdot 100\%.$$

Significance of this parameter was established with the *t*-test versus 100% (no effect of an inhibitor), $P < 0.05$ was considered significant.

Assessment of $\Delta\Psi_m$

A selective fluorescent probe JC1 (Molecular Probes) was used to determine differences in transmembrane electrical potential of mitochondria ($\Delta\Psi_m$) [Cossarizza et al., 1993; Salvioli et al., 1997]. At low $\Delta\Psi_m$, JC1 monomers (JC_M) emit green fluorescence; however, at higher $\Delta\Psi$, JC1 forms orange fluorescent JC1-aggregates (JC_A). For JC1 staining, the HaCaT cells were released by trypsination, centrifuged, and resuspended in the culture medium containing freshly prepared 10 μM JC1 [Cossarizza et al., 1993; Garland and Halestrap, 1997]. The cells were incubated for 20 min at 37°C and immediately subjected to LSC, gathering emitted fluorescence in the green (530 ± 30 nm) and orange (580 ± 30 nm) channels.

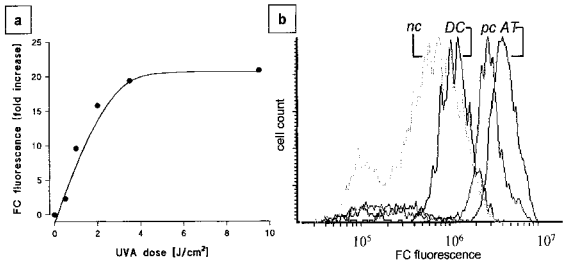

Fig. 1. UVA-induced H_2O_2 synthesis in HaCaT cells. Nonconfluent HaCaT cells were loaded with carboxy-H_2DCFDA for 30 min and (**a**) irradiated with different doses of UVA, or (**b**) incubated with diethyldithiocarbamate (5 mM, 3 h before irradiation) or aminotriazol (5 mM, 40 min before irradiation) and irradiated with 3.7 J/cm² UVA. After irradiation, the cells were trypsinized and the FC fluorescence was determined by LSC. The changes in FC fluorescence in (**a**) are relative to the sham-irradiated controls. In (**b**), nc represents basal fluorescence histogram for sham-irradiated control cells (negative control), pc: cell irradiated with UVA (positive control), DC: cells pre-treated with diethyldithiocarbamate, AT: cells pre-treated with aminotriazol. The experiment was reproduced three times with identical results.

RESULTS

UVA Irradiation Causes an Intracellular Accumulation of H_2O_2

Carboxy-FC fluorescence of the control, sham-treated cells defined the background staining dependent of H_2O_2 production in the course of normal cellular metabolism. Irradiation with UVA caused a dose-dependent increase in fluorescence, indicating H_2O_2 accumulation (Fig. 1a). Figure 2 shows that there was some variability in the shape and peak position of histograms representing UVA-irradiated cells. Sometimes, an additional peak or shoulder of lower fluorescence was observed. This phenomenon depends on the degree of confluence of the culture because cells establishing extensive cell–cell contacts produce lower amounts of ROS than single cells (J. Vicanova et al., unpublished communication). Specific blocking of $^{\bullet}O_2^{-} \rightarrow H_2O_2$ conversion with diethyldithiocarbamate, an inhibitor of superoxide dismutase, decreased carboxy-FC fluorescence, whereas a catalase inhibitor, aminotriazol, increased fluorescence, showing that carboxy-H_2DCFDA is specifically oxidized by H_2O_2 (Fig. 1b).

Effect of Mitochondrial Inhibitors on H_2O_2 Production

In preliminary studies, we demonstrated that FCCP, an uncoupler of mitochondrial F_0-

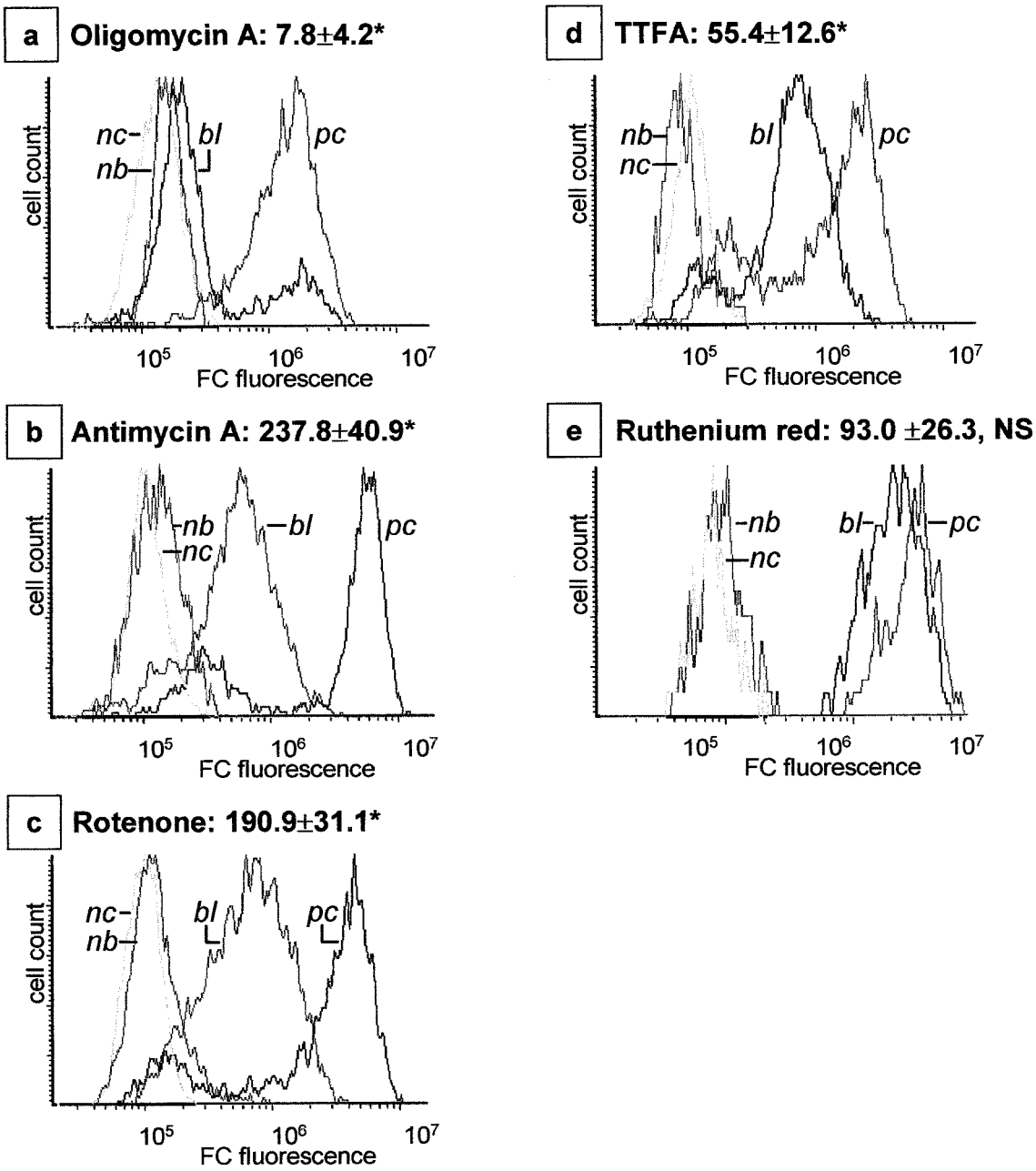

Fig. 2. Effect of mitochondria blockers on intracellular H_2O_2 levels after UVA irradiation. Nonconfluent HaCaT cells were loaded with carboxy-H_2DCFDA and (**a**) 50 μM oligomycin, (**b**) 10 μg/ml antimycin A, (**c**) 50 μM rotenone, (**d**) 50 μM TTFA, or (**e**) 50 μM ruthenium red, irradiated with 3.7 J/cm^2 UVA for 30 min, released by trypsinization and scanned for FC fluorescence. Control cells were sham-irradiated and/or treated with DMSO as described in Methods. Basal fluorescence histograms from sham-irradiated DMSO-treated cells and sham-irradiated cells treated with mitochondria blockers are marked with nc and nb, respectively; pc: UVA irradiated, DMSO-treated cells (positive control); bl: UVA irradiated cells pre-treated with mitochondria blockers. The experiment was reproduced three times with identical results. Numbers represent a mean change [(n = 3) ± standard deviation] in integral FC fluorescence caused by mitochondria blockers, and were calculated as described in Methods. Significant values are marked with *, NS – not significant versus 100%.

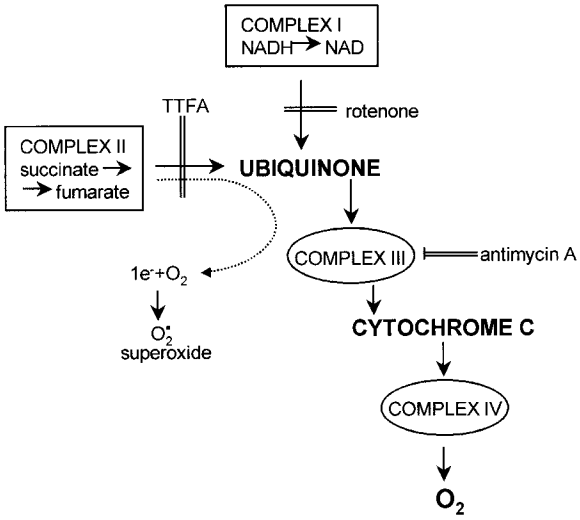

Fig. 3. A simplified diagram of electron flow in the mitochondria. The sites of actions of rotenone, TTFA, and antimycin A are indicated. Oligomycin is an ATP synthase (complex IV) inhibitor but, as described in the Discussion section, inhibits electron flow in the entire respiratory chain. In the normal situation, there is a coordinated four-electron reduction of O_2 into two water molecules (solid arrows). However, after UVA radiation, superoxide is produced because of one-electron reduction of O_2 into superoxide via a TTFA-inhibitable pathway (dotted arrow).

F_1-ATPase, reduced the UVA-dependent carboxy-FC signal (unpublished). However, we could not exclude the possibility that the disruption of $\Delta\Psi_m$ rather than inhibition of the respiratory chain was responsible for ROS production. Therefore, we treated the cells with oligomycin, an ATP synthase inhibitor that does not disrupt $\Delta\Psi_m$. This treatment before UVA irradiation resulted in a decrease in H_2O_2 synthesis (Fig. 2a).

To further investigate the role of mitochondrial electron flow in H_2O_2 generation, we treated the cells with a panel of specific respiratory chain inhibitors before UVA irradiation.

Antimycin A inhibits specifically complex III by interfering with electron flow from ubiquinone to complex IV [Garland and Halestrap, 1997] (Fig. 3). This compound increased the UVA-induced H_2O_2 synthesis (Fig. 2b). To block the respiratory chain proximally to complex III, we used rotenone (a specific inhibitor of complex I that interferes with electron flow from NADH-linked substrates and NADH dehydrogenase to the ubiquinone pool) and TTFA, which interferes with the electron transport from succinate dehydrogenase to the ubiquinone pool (a complex II inhibitor) (Fig. 3). TTFA prevented H_2O_2 induction by UVA, whereas rotenone enhanced H_2O_2 accumulation (Fig. 2c,d).

To elucidate the involvement of mitochondrial calcium signaling in UVA-induced H_2O_2 synthesis, we treated the cells with ruthenium red – a specific inhibitor of the mitochondrial calcium uniport [Chance et al., 1979, Broekemeier et al., 1994]. This compound did not affect the UVA-dependent carboxy-FC generation (Fig. 2e).

UVA Irradiation Does Not Alter $\Delta\Psi_m$

Treatment of HaCaT cells with FCCP caused an expected decrease in the JC_A population from 89 ± 7% to 36.2 ± 5%, indicating the disruption of $\Delta\Psi_m$. UVA doses up to 9.5 J/cm^2 did not change $\Delta\Psi_m$.

DISCUSSION

The mitochondria constitute the most important source of endogenous ROS in the cells. Two sites of the mitochondrial respiratory chain have been identified as sources of ROS: complex I (NADH-Q-reductase) and the ubiquinone reductase site (complex III) [Chance et al., 1979; Hansford et al., 1997] (Fig. 3). Superoxide anions that escape dismutation by mitochondrial superoxide dismutase may further be metabolized into other ROS such as H_2O_2 and $^{\bullet}OH$. The oxidative stress caused by the endogenous, mitochondrial $^{\bullet}O_2^-$ production has been shown to play a role in the pathogenesis of degenerative diseases, diabetes, and aging [Beckman and Ames, 1998; Esposito et al., 1999; Nishikawa et al., 2000].

In this study, we present evidence that mitochondria play a role in ROS formation triggered by a physiologically relevant exogenous factor, UV. Oxidative stress induced by longwave UV may cause DNA and protein damage and have a pathogenic role in skin aging, the development of skin cancer, and probably lymphomas [Setlow et al., 1993; Adami et al., 1995; Bernerd and Asselineau, 1998].

Our data provide the first evidence that UVA-triggered H_2O_2 synthesis is an active metabolic process that depends on the mitochondrial complex III activity. The experiments using complex I–IV inhibitors showed that respiratory chain blocking between succinate-Q-reductase and complex III decreased the H_2O_2 synthesis, whereas an inhibition of electron flow distally to the ubiquinone pool by antimycin A, or between NADH

reductase and complex III by rotenone, caused a significant potentiation of H_2O_2 synthesis after UVA irradiation. This suggests that succinate-Q-reductase (complex II) serves as an electron donor in this reaction (Fig. 3).

Oligomycin (a blocker of the F_0 subcomplex and thus an inhibitor of proton flow) decreased the UVA-induced radical production. We speculate that this effect of oligomycin was caused by a secondary inhibition of electron flow in the respiratory chain because of a block in ATP synthesis, an effect analogous to the respiratory "state 4" in isolated, oligomycin-treated mitochondria. Surprisingly, no increase in FC fluorescence was seen after the treatment of cells with oligomycin alone, despite earlier observations that one-electron oxygen reduction to $^{\bullet}O_2^-$ may be elevated when the activity of complex V is decreased [Boveris and Chance, 1973]. This suggests a tight coupling between ATP synthesis and electron transport in mitochondria in keratinocytes.

We considered a possibility that carboxy-H_2DCFDA oxidation may be directly caused by UVA rather than by the UVA-induced $^{\bullet}O_2^- \rightarrow H_2O_2$ conversion. Several lines of evidence argue against such a possibility. First, carboxy-H_2DCFDA is not oxidized in aqueous solutions by UVA. Second, carboxy-FC fluorescence was enhanced in the presence of the catalase inhibitor, which blocks hydrogen peroxide decomposition. Third, an inhibitor of superoxide dismutase caused a decrease in carboxy-FC, indicating that superoxide is a major substrate for the UV-induced intracellular H_2O_2 pool.

The exact biochemical mechanism responsible for UVA-induced H_2O_2 at complex III could not be elucidated. It seems to be different from the recently described pathway of growth factor–induced ROS formation, which is rotenone insensitive and depends on NADPH oxidase activity [Irani et al., 1997]. It also does not resemble the situation seen in apoptosis, where increased mitochondrial ROS synthesis is accompanied by $\Delta\Psi_m$ collapse because of opening of PTP [Kroemer et al., 1995; Zamzami et al., 1995, 1996] because no $\Delta\Psi_m$ alterations could be observed after UVA irradiation. Another possibility is involvement of calcium signaling [Pralong et al., 1994; Hajnoczky et al., 1995], a mechanism that seems to play a role in the process of ceramide-induced oxidative stress [Quillet-Mary et al., 1997]. We considered this pathway unlikely in view of the fact that ruthenium red, an inhibitor of mitochondrial calcium signaling [Broekemeier et al., 1994], did not influence the UVA-induced H_2O_2 production.

While this article was in review, another research group reported that mitochondrial complex II is involved in ROS production in diabetes [Nishikawa et al., 2000]. It has been postulated that the mitochondria-derived ROS is responsible for some important diabetes complications, such as formation of advanced glycation endproducts or sorbitol accumulation. It is conceivable that complex II is responsible for ROS production in different pathological conditions and that this pathway is not specific for UVA. An important question is the biological significance of mitochondria-derived H_2O_2 with respect to protein and DNA damage in the mitochondria and in the cells. Because mitochondrial blockers were not able to completely inhibit cellular H_2O_2 production in response to UVA, it is conceivable that a mitochondria-independent ROS synthesis pathway exists in keratinocytes. The relationship between these two pools of ROS and their contribution to cellular oxidative stress is an area of current research in this laboratory.

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