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Irradiation of Cells with Ultraviolet-A (320–400 nm) in the Presence of Cell Culture Medium Elicits Biological Effects Due to Extracellular Generation of Hydrogen Peroxide

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Biological effects of ultraviolet A (UVA) irradiation have been ascribed to the photochemical generation of singlet oxygen. Not all effects described in the literature, however, are explicable solely by the generation of singlet oxygen, but rather resemble effects elicited by hydrogen peroxide (H_2O_2). Here, we show that when cells are kept in cell culture media during exposure to UVA, stress kinases, including ERK 1 and ERK 2 as well as Akt (protein kinase B), are activated, whereas there is no or only minor activation when cells are kept in phosphate-buffered saline during irradiation. Indeed, the exposure of cell culture media to UVA (30 J/cm^2) results in the generation of significant amounts of H_2O_2, with concentrations of about 100 μM. H_2O_2 concentrations are at least three-fold higher in HEPES-buffered culture media after UVA irradiation. From experiments with solutions of riboflavin, tryptophan or HEPES, as well as combinations thereof, it is concluded that riboflavin mediates the photooxidation of either tryptophan or HEPES, resulting in the generation of H_2O_2. Thus, if signaling effects of UVA radiation are to be investigated in cell culture systems, riboflavin and HEPES/tryptophan should be avoided during irradiation because of artificial H_2O_2 generation. It should be taken into account, however, that *in vivo* tryptophan and riboflavin might play an important role in the generation of reactive oxygen species by UVA as both substances are abundant in living tissues.

Keywords: Ultraviolet A; Singlet oxygen; Hydrogen peroxide; Cell culture; Stress signaling; MAP kinases

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; HEPES, *N*-(2-hydroxyethylpiperazine)-*N*′-2-ethanesulfonic acid; HRP, horseradish peroxidase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; RPMI, Roswell Park Memorial Institute; UVA, Ultraviolet A (320–400 nm)

INTRODUCTION

A major part of solar ultraviolet radiation that reaches the surface of the earth is in the ultraviolet A (UVA) spectral region (320–400 nm). Regarding its action on humans, UVA has been connected with skin aging and photocarcinogenesis.[1,2] Both effects are ascribed not only to the destruction of skin cell components, including DNA, but also to effects of UVA on gene expression. A well-known example for a UVA-inducible gene is that of collagenase (matrix-metalloproteinase 1, MMP-1).[3] MMP-1 is known to contribute to the degradation of extracellular matrix, thus favoring skin aging as well as tumor metastasis and invasion. Hence, it is of major importance to identify the cellular signaling pathways activated by UVA that lead to such an induced expression of a gene.

Singlet oxygen, $^1O_2$, an electronically excited form of molecular oxygen (see Ref. [4] for recent review), is generated photochemically by UVA after excitation of cellular photosensitizers, including porphyrins, flavins and certain quinones,[5] and has been demonstrated to be the primary mediator of UVA-induced MMP-1 expression.[6] Similarly, $^1O_2$

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was shown to mediate the activation by UVA of certain signaling pathways involved in the regulation of transcription, including mitogen-activated protein kinase (MAPK) pathways.[7] $^1O_2$, generated chemically or photochemically, activates JNK and $p38^{MAPK}$ but not ERK1 or ERK2 in human skin fibroblasts.[8] The same pattern of MAPK activation is found in human keratinocytes after 5-aminolevulinic acid photodynamic therapy.[9]

However, there are some discrepancies in the literature as to biological effects of UVA: (i) hydrogen peroxide (H_2O_2), rather than singlet oxygen, has been frequently discussed as a major reactive species generated upon UVA irradiation and to be responsible for biological effects such as DNA damage.[10] Furthermore, signaling effects of H_2O_2, such as the inactivation of receptor tyrosine kinase-regulating phosphatases,[11] were demonstrated to be mimicked by UVA. (ii) Regarding MAPK signaling after exposure to UVA, ERK1 and 2 were also shown to be activated by UVA,[12,13] different from the above-mentioned findings.

There are several possible explanations for these discrepancies, including the difference in cell lines and UVA sources used. However, we hypothesize that a major factor influencing the reactive species generated and the biological effects elicited upon irradiation of cells with UVA is the nature of the buffer that the cells are kept in during exposure to UVA.

It has been known for decades that tissue culture media containing riboflavin, tyrosine and tryptophan rapidly deteriorate when exposed to light during storage.[14] Moreover, the toxicity of near-UV irradiation of cultured cells is strongly enhanced if irradiation is performed in the presence of cell culture medium containing riboflavin, tryptophan and/or tyrosine,[15] and H_2O_2 was identified as one of the toxic photoproducts generated under these conditions.[16]

It is demonstrated here that indeed H_2O_2 is generated upon irradiation of cell culture media at concentrations sufficient to elicit cellular responses such as the activation of stress signaling pathways. The effects of this artificially generated H_2O_2 will be superimposed to those of other mediators of UVA effects, including $^1O_2$. Further, the role of commonly used components of cell culture media in the generation of H_2O_2 upon exposure to UVA is explored.

MATERIALS AND METHODS

UVA Sources

A UVA700 irradiation device (Waldmann Lichttechnik, Villingen, Germany) was used for UVA (320–400 nm) irradiations. Maximum emission was at 365 nm. For viability tests (see Fig. 6), a Sellas-4000 UVA1 (340–400 nm) irradiation device was used (Sellas Medizinische Geräte, Gevelsberg, Germany) with maximum emission between 370 and 380 nm.

Assay of H_2O_2

H_2O_2 was assayed in cell culture media or buffer solutions immediately after exposure to UVA with the 7-hydroxy-6-methoxycoumarin (scopoletin)/horseradish peroxidase (HRP) assay according to Ref. [17]. Scopoletin is oxidized by H_2O_2 in the presence of HRP, and loss of fluorescence correlates with H_2O_2 concentrations. A 300 μl of irradiated or control medium or of irradiated or control buffer solution, respectively, was added to 2690 μl of scopoletin (Sigma-Aldrich; 22.3 μM in PBS), followed by the addition of 10 μl (5 U) of HRP (Sigma-Aldrich). DMSO/PBS was used instead of scopoletin/PBS and water added instead of HRP in control experiments. Ten minutes after addition of HRP, fluorescence of the mixture at 460 nm was determined after excitation at 350 nm in a Perkin–Elmer LS-5 luminescence spectrometer. H_2O_2 concentrations were calculated from the decrease in fluorescence in this sample relative to (i) control samples and to the *same* sample *before* addition of HRP as well as (ii) to a sample mixture that 100 U of catalase were added to 1 min prior to the addition of HRP, all in relation to fluorescence decreases induced by H_2O_2 standards. Ten minutes were chosen for the time of incubation because oxidation of scopoletin was complete with H_2O_2 concentrations in the range of interest at that time.

The concentration of H_2O_2 generated as determined in some of the solutions tested was further controlled for, and verified by, oxygraphic determination of oxygen released upon addition of catalase (Oxygenmeter 781; Strathkelvin Instruments, Glasgow, UK).

Cell Culture, Cytotoxicity Assay and Western Blotting

Human skin fibroblasts derived from plastic surgery biopsies and WB-F344 rat liver epithelial cells (kindly provided by Dr John Trosko, East Lansing, MI) were cultured at 37°C in the dark in a humidified atmosphere with 5% (v/v) CO_2 and kept in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Deisenhofen, Germany) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. Alternatively, human skin fibroblasts were also cultured in RPMI 1640 supplemented with 10% FCS.

For cell viability analysis, cells were seeded in 12-well dishes at a density of 10,000 cells per well.

After attachment, cells were washed and covered with 500 µl/well of 0.9 % (v/v) NaCl with or without additions, followed by UVA irradiation. After exposure to UVA, cells were washed and kept in RPMI 1640 supplemented with 10% FCS for another 24 h. Cells were then incubated for 90 min with 0.03% (w/v) neutral red in complete medium, washed twice with PBS and lysed with 500 µl/well of 2-propanol containing 1% (v/v) of 1 N HCl. Absorbance at 530 nm linearly correlated with the number of viable cells.

For Western analysis, cells were grown to near confluence in cell culture dishes with a diameter of 3 cm. Before exposure to UVA cells were washed with PBS once, followed by addition 2 ml/dish of the respective buffer or medium and irradiation. After irradiation, medium or buffer was replaced by serum-free medium and the cells incubated for another 30 min, followed by lysis in 100 µl/dish of 2 × SDS-PAGE sample buffer [125 mM Tris/HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 100 mM DTT, 0.02% (w/v) bromophenol blue, pH 6.8], collection of lysates with a cell scraper and, if needed, brief sonication to lower viscosity. Samples were boiled for 5 min, cooled on ice and applied to SDS-polyacrylamide gels of 10% (w/v) acrylamide, followed by electrophoresis and blotting according to standard procedures. Immunodetections were performed using the following antibodies at dilutions recommended by the suppliers: rabbit polyclonal anti-phospho-Akt (Ser^{473}), anti-total Akt, and anti-phospho-ERK antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Monoclonal anti-GAPDH was from Chemicon (Temecula, CA).

RESULTS AND DISCUSSION

Generation of H_2O_2 in Cell Culture Media Exposed to UVA

To test the hypothesis that H_2O_2 is a photoproduct in cell culture media exposed to UVA that may influence cellular responses, H_2O_2 generation was determined in DMEM exposed to UVA (30 J/cm^2). DMEM of various formulations and from different suppliers were used (Table I). Indeed, about 120 µM of H_2O_2 were found in DMEM without phenol red ("DMEM A") after irradiation, and concentrations were even three-fold higher in the presence of HEPES ("DMEM D"). Phenol red appeared to attenuate rather than enhance H_2O_2 generation and thus acted like a filter rather than like a photosensitizer ("DMEM B" vs. "DMEM A"). Similarly, the presence of FCS seemed to hamper the formation of high steady-state concentrations of H_2O_2, perhaps due to peroxidase activity in the serum, or due to the presence of a UVA-filtering component.

TABLE I Generation of hydrogen peroxide by irradiation of media with UVA (30 J/cm^2)

Medium*	Supplements†				H_2O_2 (µM)‡
	PR	HEPES	FCS	Gln/Pen/Strep	
PBS	−	−	−	−	3 ± 2
DMEM (A)	−	−	−	−	116 ± 10
DMEM (B)	+	−	−	−	66 ± 4
DMEM (B)	+	−	+	+	58 ± 6
DMEM (C)	+	+	−	−	151 ± 14
DMEM (C)	+	+	+	+	99 ± 16
DMEM (D)	−	+	−	−	375 ± 16
DMEM (D)	−	+	+	+	87 ± 2

*DMEM (A): Sigma (Deisenhofen, Germany) #D5921; DMEM (B): Sigma #D5546; DMEM (C): PAA (Cölbe, Germany) #E15-007; DMEM (D): modified DMEM with HEPES and low bicarbonate, Biochrom KG, Germany. †PR, phenol red; HEPES, *N*-(2-hydroxyethylpiperazine)-*N*′-2-ethanesulfonic acid; FCS, fetal calf serum; Gln/Pen/Strep, Glutamine, penicilline, streptomycin; for concentrations, see "Materials and methods" section. ‡Data are means of three independent experiments ± SD.

What medium components could H_2O_2 generation be attributable to? Components absorbing in the UVA region include phenol red and riboflavin. The former can be excluded as a sensitizer for reasons detailed above, whereas cytotoxicity of riboflavin solutions irradiated with UVA was described to be due to H_2O_2 generation,[18] which had already been noted before to be even more pronounced in solutions of riboflavin in combination with tryptophan or tyrosine.[15,16] Interestingly, the spectral changes in DMEM without phenol red observed after exposure to UVA (Fig. 1A) are very similar to those in a mixture of riboflavin and tryptophan (Fig. 1B), which is further reflected by the difference spectra (Fig. 1C). In order to define the roles of riboflavin, tryptophan as well as HEPES (*vide supra*) in UVA-induced H_2O_2 production, solutions of these compounds in PBS at concentrations usually found in tissue culture media were exposed to UVA (30 J/cm^2) and H_2O_2 concentrations determined (Table II). No enhanced generation of H_2O_2 was seen in solutions of either of the mentioned compounds (entries 1–3) or of tryptophan plus

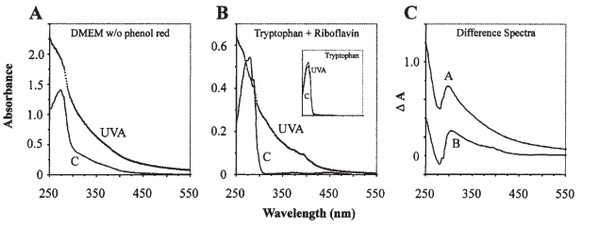

FIGURE 1 Spectral changes in cell culture medium and in riboflavin/tryptophan after exposure to UVA. UV/Vis spectra of DMEM without phenol red (A) or tryptophan (B, insert) or of tryptophan plus riboflavin (B) were recorded before ("C") and after ("UVA") exposure to UVA (30 J/cm^2). Changes between "C" and "UVA" of (A) and (B) are shown as difference spectra in (C). Tryptophan (78 µM) and riboflavin (1 µM) were used at concentrations usually found in cell culture media.

TABLE II Generation of hydrogen peroxide by irradiation of mixtures of riboflavin, HEPES and tryptophan with UVA at 30 J/cm^2

	Supplements*			
Solution/entry	Riboflavin	HEPES	Trp	H_2O_2 (μM)†
PBS	−	−	−	3 ± 2
1	+	−	−	4 ± 4
2	−	+	−	4 ± 4
3	−	−	+	3 ± 3
4	−	+	+	4 ± 4
5	+	−	+	55 ± 13
6	+	+	−	476 ± 54
7	+	+	+	461 ± 32

* Supplements were dissolved in PBS at the concentrations usually found in cell culture media formulations: riboflavin, 1 μM (0.4 mg/l); HEPES, 25 mM (5958 mg/l); L-tryptophan, 78 μM (16 mg/l).
† Data are means of three independent experiments ± SD.

HEPES (entry 4). As soon as riboflavin was present in addition to any of the other compounds, H_2O_2 was generated (entries 5–7). The mixture of riboflavin plus tryptophan can account for 47–83% of the H_2O_2 generated in DMEM without HEPES (see Table I), and in the presence of HEPES, H_2O_2 concentrations (about 470 μM) even exceed those determined in irradiated media, implying that either other UVA filters are present in those media or that some peroxidase activity diminishes H_2O_2 concentrations. As riboflavin is required to initiate the sequence of events leading to the generation of H_2O_2, it can be hypothesized to be the initial UVA absorbing species. Upon absorption, riboflavin will undergo transition into an excited state (RF*) and then be able to react with tryptophan or HEPES (Fig. 2). The concomitant photoreduction of riboflavin is commonly proposed to be initiated from its triplet excited state (^{3}RF*) rather than its excited singlet state (^{1}RF).[19] Upon excitation, the redox potential of the flavin is drastically increased (see discussion in Ref. [20]), followed by oxidation of the tryptophan indole moiety or of HEPES. The resulting riboflavin anion may then reduce oxygen to generate superoxide[20] which rapidly dismutates spontaneously to form H_2O_2. A second way of generating superoxide in this scenario has been described for HEPES and starts with the radical cations formed from photoreduction. HEPES$^{\bullet+}$, after deprotonation, may rapidly react with oxygen to form an intermediate peroxyl radical which, in turn, will rearrange and release superoxide. This mechanism has been proposed for the generation of H_2O_2 resulting from the oxidation of HEPES by peroxynitrite.[21]

Biological Consequences of H_2O_2 Generated upon Irradiation of Media

As has been shown by Halliwell and coworkers,[22,23] H_2O_2 artificially generated under cell culture conditions may account for biological effects erroneously ascribed to other factors. H_2O_2 is known to cause a variety of cellular stress responses at rather low concentrations, such as the activation of transcription factor AP-1,[24] the activation of MAPK pathways, including ERK1 and ERK2,[25] or of the PI3K/Akt pathway.[26] In order to test for such effects of H_2O_2 in the case of UVA, human skin fibroblasts were irradiated with UVA (30 J/cm^2) and kept in PBS or in media during irradiation. Akt (protein kinase B) phosphorylation was barely, if at all, enhanced by UVA, if irradiation was in

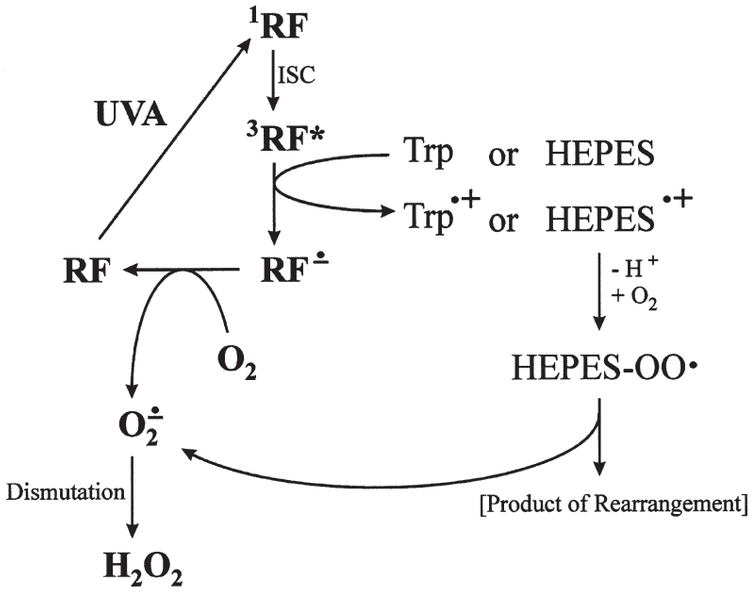

FIGURE 2 Schematic representation of riboflavin (RF)-dependent generation of hydrogen peroxide in the presence of tryptophan or HEPES. See text for details. ISC, intersystem crossing.

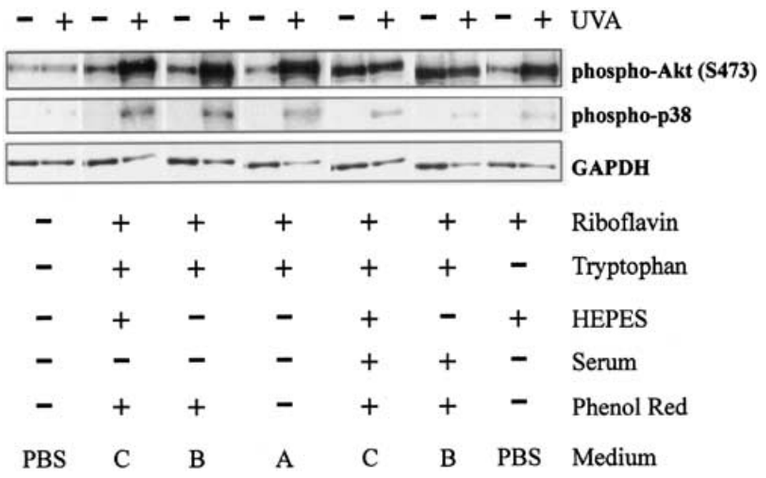

FIGURE 3 Phosphorylation of Akt and activation of $p38^{MAPK}$ after exposure to UVA. Human skin fibroblasts were kept in PBS, various formulations of DMEM or PBS with riboflavin (1 μM) plus HEPES (25 mM) during exposure to UVA (30 J/cm^2). Phosphorylation of Akt and $p38^{MAPK}$ as well as the presence of GAPDH was analyzed by Western blotting. GAPDH served as gel loading control. For formulations of DMEM A, B, and C, see footnotes of Table I. Data are representative of at least three (for medium "A": two) independent experiments with similar results.

PBS. However, in the presence of media during irradiation, or if riboflavin and HEPES were added to the PBS, Akt phosphorylation at Ser473, which is one of the two phosphorylation sites regulating Akt activation, was strongly enhanced (Fig. 3). It did not matter whether or not HEPES or phenol red were present in the respective medium. In media supplemented with FCS, however, there was no enhanced phosphorylation of Akt or ERK because basal phosphorylation levels, due to growth factors and other serum components activating these pathways, were already very high, thus masking any induced phosphorylation (Fig. 3). PI3K (and to some extent Akt) as well as $p70^{S6kinase}$ was shown to be activated by UVA,[27] although here (Fig. 3) Akt was not activated by UVA when exposure was in the presence of PBS whereas activation was significant if cells were kept in medium. This raises the question whether H_2O_2, which is known to activate both PI3K/Akt[26] and $p70^{S6kinase}$ (Ref. [28]), might have mediated these effects described by Zhang *et al.*[27]

UVA and $^1O_2$ are known, however, to activate $p38^{MAPK}$ (Ref. [8]), and indeed this can be seen in Fig. 3 as well: the kinase is always activated regardless of whether irradiation was in PBS or medium. Interestingly, the dual phosphorylation (and activation) of $p38^{MAPK}$ was stronger when exposure of cells to UVA was in medium, perhaps due to the additional activation of the kinase by H_2O_2.

As mentioned before, ERKs are not activated by UVA and $^1O_2$ in human skin fibroblasts. However, if cells were irradiated in media, there was a tendency toward an activation of ERKs, which is not seen in PBS-irradiated cells (data not shown). In order to further establish this observation and test for an effect of H_2O_2 generated during irradiation of media on ERK phosphorylation we employed a cell line apparently more sensitive towards changes in H_2O_2 concentrations than skin fibroblasts, the rat liver epithelial cell line WB-F344, which was merely used as an "indicator" for signaling effects of low concentrations of H_2O_2. As shown in Fig. 4, ERK phosphorylation was slightly induced by UVA/PBS in these cells, but very strongly enhanced if cells were in medium during irradiation. To test the hypothesis that this effect was due to the formation of H_2O_2, catalase was added to the medium during irradiation, but only minor inhibitory effects were seen (data not shown), which is probably due to the well-known inactivation of catalase during exposure to UVA in the presence of riboflavin and tryptophan.[29] To circumvent the direct inactivation of

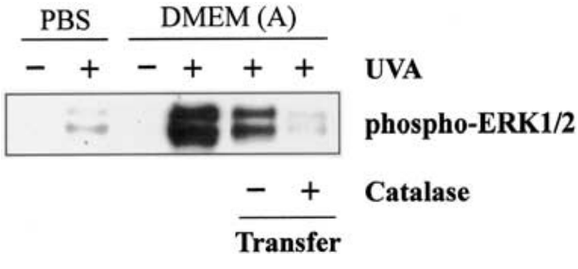

FIGURE 4 Activation of ERKs after exposure to UVA in the presence of medium. Rat liver epithelial cells were kept in PBS or in DMEM without phenol red (medium "A" in Fig. 3 and Table I) during exposure to UVA (30 J/cm^2). Activation of ERK 1 and ERK 2 was analyzed by Western blotting employing antibodies specific for the dually phosphorylated (activated) forms of the kinases. For the transfer experiment DMEM without phenol red was irradiated with UVA (30 J/cm^2) and treated with catalase (40 U/ml) before addition to cells and incubation of cells for 15 min. If catalase was heat-inactivated before addition to irradiated medium, ERK activation was still seen (not shown). Data are representative of at least three independent experiments with similar results.

catalase, medium was irradiated (30 J/cm^2), treated with catalase (40 units/ml) for 30 s and then added to cells for 15 min before lysis. The mere fact that medium irradiated in the absence of cells, once transferred onto cells, may activate ERKs implies that this activation cannot be attributable to the formation of $^1O_2$, which is very short lived (4 μs in water)—different from H_2O_2. In fact, catalase treatment of irradiated medium prior to transfer onto cells diminishes ERK activation (Fig. 4). This abrogation of ERK activation is not seen with heat-inactivated catalase (not shown).

In line with the above findings (Tables I and II) that riboflavin plus tryptophan or riboflavin plus HEPES are the components of media that H_2O_2 generation upon UVA irradiation can be largely attributed to, irradiation of cells kept in riboflavin/tryptophan or in riboflavin/HEPES solution in PBS resulted in activation of ERKs (Fig. 5). This activation of ERKs was not seen when cells were irradiated in PBS or in PBS containing either of the above compounds alone.

Artificially generated H_2O_2 not only activates signaling pathways but might also be toxic. Irradiation of riboflavin/HEPES mixtures resulted in substantial H_2O_2 generation (Table II), and exposure of cells to UVA while kept in 0.9% (w/v) NaCl plus riboflavin/HEPES indeed was significantly more toxic to cells than exposure in the absence of riboflavin (Fig. 6A). Interestingly, however, this toxicity was not due to the interaction between riboflavin and HEPES but rather due to the presence of riboflavin alone (Fig. 6B). HEPES may be regarded as a protectant rather than an enhancer of riboflavin-mediated UVA-toxicity. Hence, UVA/riboflavin toxicity is suggested here to be due to the interaction of the (triplet) excited riboflavin (Fig. 2) with cellular components rather than with HEPES.

CONCLUSION

Many of the effects of UVA radiation in human biology have been ascribed to the photochemical generation of $^1O_2$. These effects include the toxicity of UVA,[30] the activation of signal transduction pathways[4] and the induced expression of genes.[31,32] It is demonstrated here that under certain conditions H_2O_2 may be generated during irradiation of cultured cells with UVA, and that this H_2O_2 activates stress signaling pathways. Although under cell culture conditions such artificial generation of a reactive species with significant biological impact is not desired, it cannot be excluded that under *in vivo*-conditions H_2O_2 indeed is generated as well upon exposure of the respective tissue to UVA, as the presence of both riboflavin and tryptophan should suffice to generate H_2O_2.

In order to minimize artificial H_2O_2 generation along with the biological effects entailed during UVA irradiation of cultured cells, the cells should be held in simple buffers such as PBS during irradiation. When there is no other way than to always keep cells in medium, e.g. in certain artificial skin models, HEPES should be avoided.

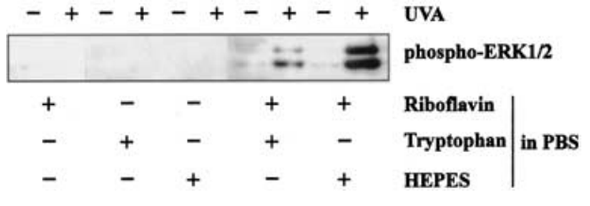

FIGURE 5 Activation of ERKs after exposure to UVA in the presence of PBS with cell culture medium constitutents. Rat liver epithelial cells were kept in PBS containing riboflavin (1 μM), tryptophan (78 μM), HEPES (25 mM) alone or in combinations during exposure to UVA (30 J/cm^2). Activation of ERK 1 and ERK 2 was analyzed by Western blotting employing antibodies specific for the dually phosphorylated (activated) forms of the kinases. Data are representative of 2–6 independent experiments with similar results.

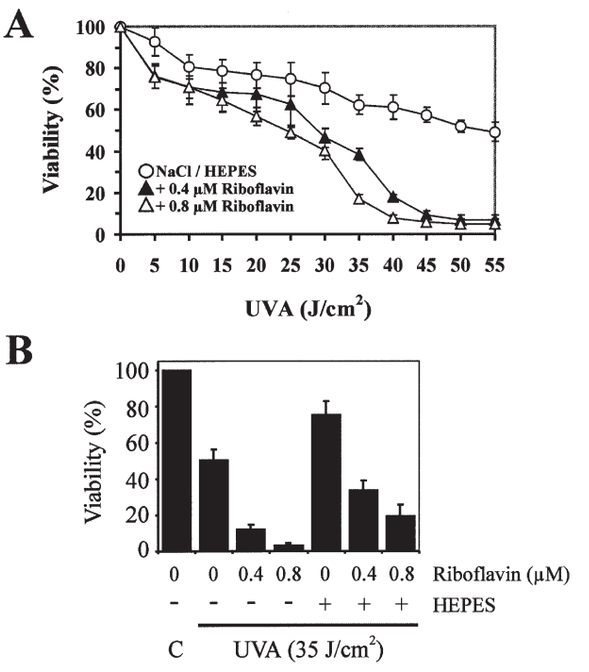

FIGURE 6 Viability of human skin fibroblasts exposed to UVA in the presence of HEPES and riboflavin. (A) Human skin fibroblasts were covered with 0.9% (w/v) NaCl/25 mM HEPES or NaCl/HEPES containing riboflavin at the given concentrations during irradiation with UVA at the respective dose. Cell viabilities were determined 24 h after treatment. Data are means of four different experiments ±SD. (B) Human skin fibroblasts were exposed to 35 J/cm^2 of UVA in the presence of riboflavin and/or HEPES. Cell viabilities were determined 24 h after treatment. Data are means of four different experiments ± SD. All mean values are significantly different from all other data, as determined by ANOVA, followed by multiple comparison according to Student–Newman–Keuls, with the level of statistical significance set to $p < 0.05$. C, control.

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