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Original Contribution

LIGHT-DEPENDENT GENERATION OF REACTIVE OXYGEN SPECIES IN CELL CULTURE MEDIA

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Abstract—Cell culture media (RPMI 1640, Dulbecco's Minimal Essential Medium and yeast extract-peptone-glucose medium) were found to oxidize dichlorodihydrofluorescein diacetate and dihydrorhodamine 123, and to generate spin adduct of 5,5′-dimethyl-1-pyrroline N-oxide, which indicates formation of reactive oxygen species (ROS). The production of ROS was light dependent. The main component of the media responsible for the generation of ROS was riboflavin, but tryptophan, tyrosine, pyridoxine, and folic acid enhanced the effect of riboflavin. These observations point to exposure of cells to ROS under in vitro culture conditions. © 2001 Elsevier Science Inc.

Keywords—Reactive oxygen species, Oxidative stress, Cell culture, Superoxide, Hydrogen peroxide, Riboflavin, Free radicals

INTRODUCTION

Studies of the effect of oxidative stress on cultured cells are often based on a tacit assumption that, in the absence of exogenous agents, cells in culture are in perfect oxidant-free conditions (except for molecular oxygen from the atmosphere), reactive oxygen species (ROS) being generated only intracellularly from rather well-defined sources. We have been puzzled, however, when finding generation of free radicals in fibroblast culture to be only slightly higher than in control samples containing cell-free medium [1] and measuring generation of hydrogen peroxide in yeast culture not significantly higher than that in the pure medium (unpublished). As this phenomenon may be of a more general importance and concern [2], we examined it in more detail.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium with Glutamax-I, sodium pyruvate and pyridoxine (Cat. No. 31966; DMEM), and DMEM without Phenol Red (Cat. No. 21063), RPMI 1640 medium with Glutamax-I (Cat. No. 31996) and 25 mM HEPES, fetal calf serum (FCS), and yeast extract were from GibcoBRL (manufactured by Life Technologies, Gaithersburg, MD, USA). Gelysate Peptone was from Becton Dickinson (Mountain View, CA, USA). 5,5′-Dimethyl-1-pyrroline N-oxide (DMPO), diethylenetriaminepentaacetic acid (DTPA), deferoxamine mesylate (DFO), superoxide dismutase (from erythrocytes; SOD), horseradish peroxidase (HRP), and catalase (from liver) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 1,4 Diazabicyclo[2.2.2.]octane (DABCO) was from Aldrich Chemical Co. (Milwaukee, WI, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) and dihydrorhodamine 123 (H_2R123) were from Molecular Probes (Eugene, OR, USA). Glucose and other reagents were from POCh (Gliwice, Poland). Yeast culture medium (YPD) contained 1% yeast extract, 1% peptone, and 2% glucose. It was autoclaved for 22 min for sterilization and stored for 1 d at 4°C before use.

Spin trapping

Fifty microliter aliquots of the cell culture media or phosphate-buffered saline (145 mM NaCl in 10 mM sodium phosphate, pH 7.4; PBS), supplemented with

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appropriate additives if needed, were added with DMPO (0.2 M final) and incubated at 37°C (mammalian cell media) or 28°C (yeast cell medium). After appropriate incubation times, ESR spectra of the samples were taken in a Bruker ESP 300E X-band ESR spectrometer (Karlsruhe, Germany).

Oxidation of fluorogens

One milliliter aliquots of the cell culture media without additives or containing necessary additives were supplemented with H_2DCFDA or dihydrorhodamine 123 to a final concentration of 5 μM and incubated as above. Fluorescence was measured in a Perkin Elmer LS-5B spectrofluorimeter (Norwalk, CT, USA) at excitation/emission wavelengths, respectively, of 504 nm/529 nm for H_2DCFDA and at 507 nm/529 nm for dihydrorhodamine.

RESULTS

Incubation of all the cell culture media studied (DMEM, RPMI 1640, and yeast YPD medium) lead to oxidation of H_2DCFDA and dihydrorhodamine 123 introduced into the media, indicative of production of reactive oxygen species. The oxidation rate was higher for DMEM medium than for RPMI 1640 medium and was significantly attenuated in complete media containing 10% FCS as compared with serum-free media (Fig. 1, Table 1). The rate of fluorogen oxidation was dependent on the oxygen access to the media and was higher when the experiments were performed under conditions simulating those of cell culture conditions (broad vessels allowing for oxygen access in the case of mammalian cell culture media, Erlenmeyer flasks incubated in a rotary shaker for yeast medium).

Spin trapping demonstrated formation of an $^{\bullet}$OH-type adduct in mammalian cell culture media, again attenuated in the presence of FCS. The spectra originated from decomposition of $O_2^{-\bullet}$ adducts as their intensity was significantly reduced in the presence of SOD (Fig. 2).

Addition of superoxide dismutase and DTPA decreased also the rate of H_2DCFDA oxidation. DFO decreased the rate of H_2DCFDA oxidation in DMEM medium but slightly increased it in RPMI 1640 medium. SOD decreased, while horseradish peroxidase considerably stimulated the H_2DCFDA oxidation rate (Fig. 3).

Production of ROS was not due to the presence of Phenol Red present in the medium. DMEM medium devoid of Phenol Red oxidized H_2DCFDA at a rate not significantly different than Phenol-Red containing medium. Oxidation of the fluorogens was considerably light dependent as samples protected from light showed much lower DCFDA production (Table 1).

We tested possible sources of photogeneration of ROS in mammalian cell culture media by measuring generation of ROS in PBS supplemented with various compounds present in the media, at concentrations corresponding to those in a DMEM medium. Only riboflavin was able to generate significant amounts of ROS in the absence of other compounds, while several compounds (tryptophan, tyrosine, pyridoxine, and folic acid) were able to enhance the amount of ROS generated by riboflavin. On the other hand, some compounds, among them Phenol Red, decreased the generation of ROS (Fig. 4), due apparently to quenching photoexcited riboflavin.

In order to identify the ROS responsible for oxidation of the fluorogens, effect of addition of hydrogen peroxide, catalase, singlet oxygen quenchers azide, histidine, and DABCO, as well as mannitol as a hydroxyl radical scavenger, were studied. Oxidation of H_2DCFDA was augmented by exogenous hydrogen peroxide and attenuated by catalase in both the media studied (Fig. 5), while oxidation of H_2R123 was not affected significantly by either hydrogen peroxide or catalase (Fig. 6). A concentration-dependent decrease of oxidation of both the fluorogens by azide was observed for both media. DABCO decreased H_2R123 oxidation in DMEM medium but higher concentrations of this compound alkalized the media and were not studied. Histidine and mannitol did not affect oxidation of the fluorogens significantly, a slight protective effect of histidine being visible only at the lower concentration studied (2 mM) (Figs. 5 and 6).

DISCUSSION

Time-dependent oxidation of H_2DCFDA and dihydrorhodamine 123 in cell culture media, proceeding at a much higher rate with respect to PBS, points to formation of ROS. Both fluorogens can be oxidized by various oxygen species [3,4]. Formation of a fluorescent oxidation product from H_2DCFDA in a cell culture medium, in the absence of esterases, is somewhat surprising as the common belief is that H_2DCFDA must be first hydrolyzed by intracellular esterases before the H_2DCF can be oxidized. However, hydrolysis of H_2DCFDA at pH 7.4 is not negligible and can be sufficient for formation of a fluorescent product (manuscript in preparation). In fact, a method of estimation of total antioxidant activity is based on oxidation of H_2DCFDA by 2,2′-azobis(2-amidopropane) in a cell-free medium [4] and this compound, is used for the detection of peroxynitrite [5,6] .

The formation of ROS in cell culture media was light dependent (Table 1, Figs. 4–6) and riboflavin was identified as the main source of species responsible for oxidation of fluorogens (Fig. 4). Most probable intermediates are therefore products of type 1 or type 2

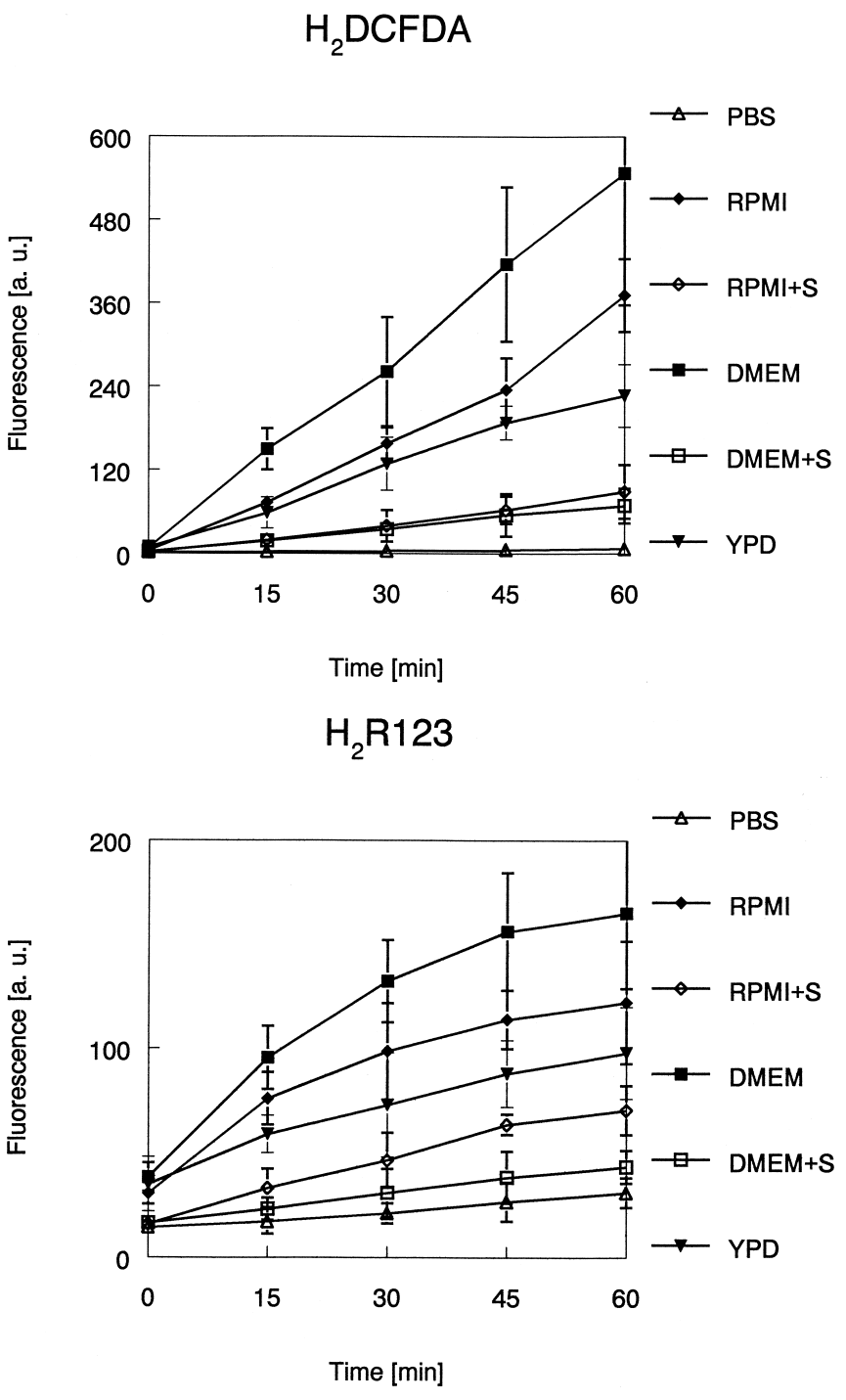

Fig. 1. Oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and dihydrorhodamine 123 (H_2R123) by cell culture media. H_2DCFDA and H_2R123 were present in the media at concentrations of 5 and 10 μM, respectively. Mammalian cell culture media were incubated at 37°C and YPD medium at 28°C on a rotary shaker under daylight. RPMI + S, DMEM + S, media containing 10% FCS. Results are means ± SD from five independent experiments.

photoreactions of riboflavin, i.e., superoxide and hydrogen peroxide as product of its dismutation, and singlet oxygen. Riboflavin has been identified as one of the intracellular sources of superoxide when irradiated by solar light [7]. In fact, superoxide generation by photoirradiated riboflavin underlies the common method of gel staining for superoxide dismutase activity [8]. Exposure to room fluorescent light was identified as a major cause of tissue culture medium deterioration. The latter effect was ascribed to generation of phototoxic products by

Table 1. Dihydrorhodamine 123 Oxidation in Cell Culture Media in a Herasafe HS12 Laminar Flow Cabinet (Heraeus) Illuminated (≥800 lux) and in the Darkness

Rhodamine 125 production (μM/h)	Light	Darkness
DMEM w/o Phenol Red	1.341 ± 0.045	0.056 ± 0.004
DMEM + Phenol Red	1.352 ± 0.005	0.050 ± 0.006
DMEM + Phenol Red + 10% FCS	0.054 ± 0.006	0.004 ± 0.001
RPMI + Phenol Red	0.545 ± 0.002	0.016 ± 0.002

The media were incubated for 1 h.

light-exposed riboflavin and tryptophan [9,10], among them hydrogen peroxide, which was estimated to be responsible for about 40% of the toxicity of Dulbecco's modified Eagle's tissue culture medium when exposed to "daylight" fluorescent light [11].

Riboflavin photoxidation in Fischer's medium exposed to ultraviolet or visible light was reported to have cystostatic effects on L5178Y cells [12]. Another study identified HEPES and riboflavin as sources of cytotoxicity of light-exposed cell culture media and ascribed their effects to the production of hydrogen peroxide [13]. The relative contribution of the singlet oxygen and superoxide/hydrogen peroxide pathways to the damage of biological targets by photosensitized riboflavin has been, however, a subject of controversy. While hydrogen peroxide was found to be largely responsible for the damage to cultured rat lenses by photosensitized riboflavin [14], singlet oxygen was found to be the main intermediate in the riboflavin-sensitized photodegradation of the guanine base of DNA and RNA [15], hemolysis of erythrocytes [16], and crosslinking of lens crystallins [17].

In our study, formation of superoxide has been demonstrated by spin trapping (Fig. 2). However, superoxide was reported to be incapable of direct oxidation of the both probes [6,18,19]. Indirect oxidation of the probes by hydrogen peroxide is rather inefficient but this reaction can be catalyzed by transition ion metals present in the medium [19]. Augmentation of H_2DCFDA oxidation by HRP (Fig. 3) supports the involvement of hydrogen peroxide in the oxidation of the fluorogens. Stimulation rather than inhibition of fluorogen oxidation by catalase in RPMI-1640 medium (Figs. 5A and 6A) is somewhat surprising and can be ascribed to the peroxidative activity of catalase at low substrate concentration.

Concentration-dependent diminution of the fluorogen oxidation by azide (and DABCO for H_2R123 in DMEM medium; Figs. 5 and 6) points to the involvement of singlet oxygen. The lack of a concentration-dependent effect of histidine, another quencher of singlet oxygen, does not support this conclusion. However, histidine is present in the unsupplemented media; moreover, after reaction with singlet oxygen, this amino acid may gain new reactivity. It has been demonstrated that histidine, when present in a photoirradiated riboflavin-containing

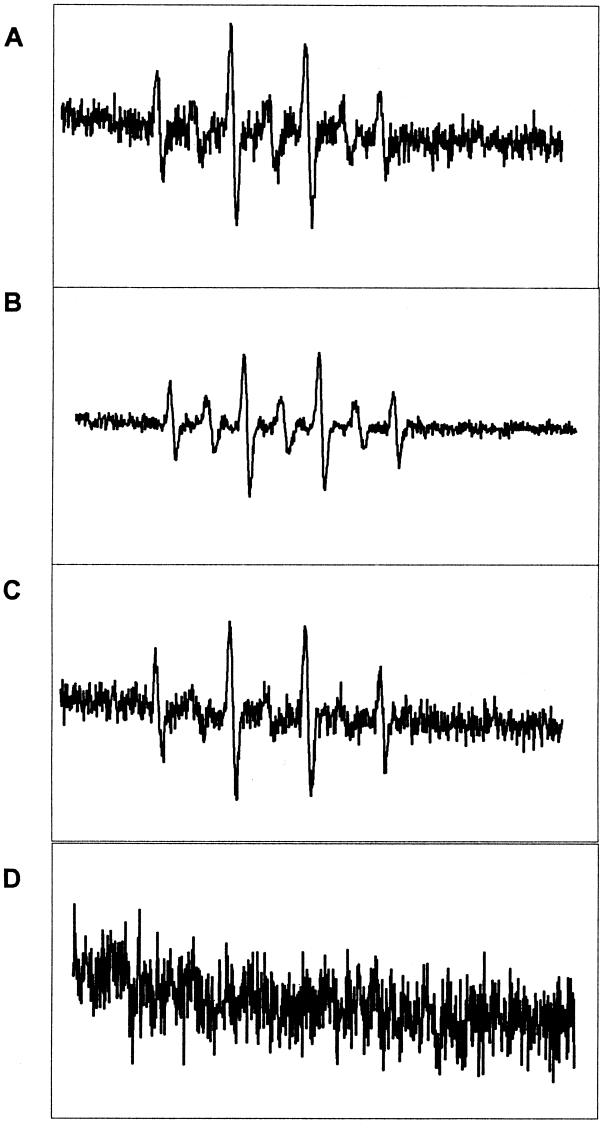

Fig. 2. ESR spectra of cell culture media incubated for 60 min at 37°C with 0.2 M DMPO under daylight: (A) RMPI 1640 medium; (B) DMEM medium; (C) DMEM medium containing 10% FCS; (D) DMEM medium (without FCS) added with 50 μg/ml SOD. The spectra are not in scale; relative peak heights are A:B:C = 1.14: 1.64: 1.00 as determined by peak-picking procedure. Measurement conditions: scan range 100 G, modulation frequency 1 kHz, modulation amplitude 1.0 G, time constant 20.48 ms, conversion time 81.92 ms, attenuation 15 dB.

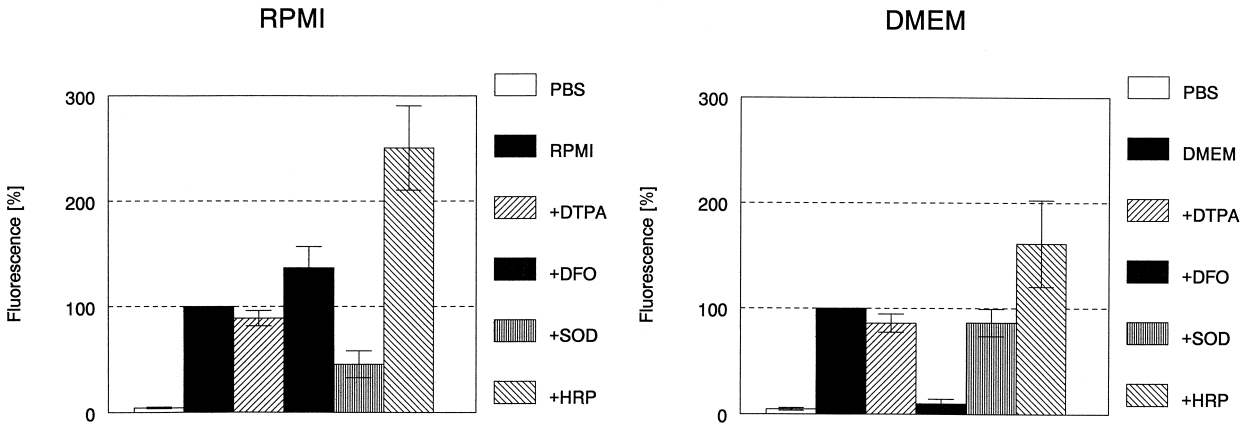

Fig. 3. Effect of additives on the oxidation of H_2DCFDA by RPMI 1640 and DMEM media. The media were incubated at 37°C for 1 h under daylight. RPMI = samples without additives; + DTPA = medium with 500 μM DTPA; + DFO = medium with 500 μM DFO; + SOD = medium + 50 μg/ml SOD; + CAT = medium + 50 μg/ml catalase; + HRP = medium 50 μg/ml horseradish peroxidase. Mean ± SD, $n = 5$.

tissue medium, becomes an inducer of benzo[a]pyrene-3-mono-oxygenase [20]. Azide and DABCO are also good scavengers of the hydroxyl radical; in order to take into account this possible action of singlet oxygen quenchers, the effect of another hydroxyl radical scavenger, mannitol, was also studied and found to be insignificant (Figs. 5 and 6). Therefore, the presented results point to the involvement of products of both type 1 and type 2 photoreactions of riboflavin in the oxidation of H_2DCFDA and H_2R123 in cell culture media exposed to light. The contribution of both these pathways seems to be different for various fluorogens and different media.

The rate of dihydrorhodamine 123 oxidation in a Herasafe 12 laminar flow cabinet illuminated at ≥ 800 lux was of an order of 1 μM per hour (Table 1). However, in this experiment the culture media were placed in Eppendorf tubes so exposure of cell culture media to light under usual conditions allowing for a better penetration of light and oxygen may result in a much higher generation of ROS in the media. The riboflavin concen-

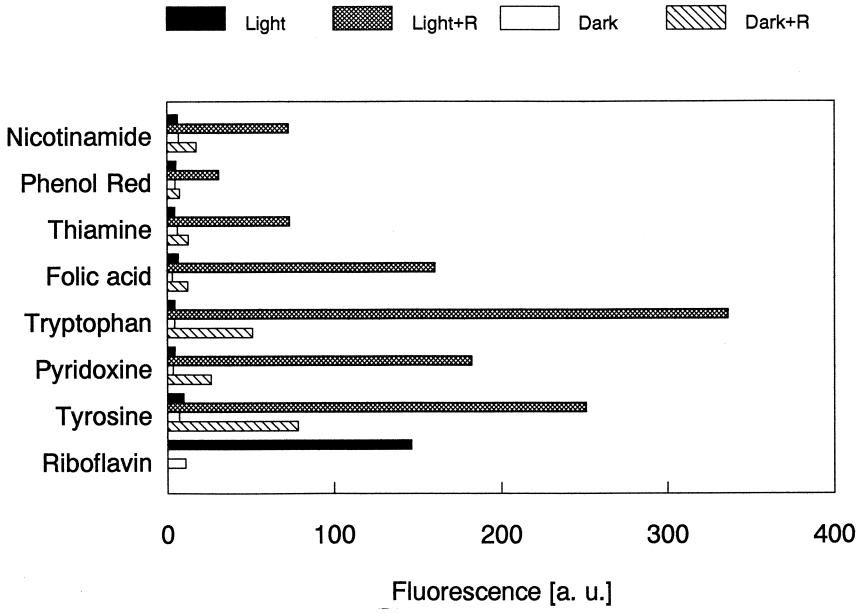

Fig. 4. Oxidation of H_2R123 in PBS supplemented with various components of cell culture media at concentrations used in DMEM medium (GibcoBRL, Cat. No. 31966; Grand Island, NY, USA): nicotinamide, 4 mg/l; Phenol Red, 15 mg/l; thiamine • HCl, 4 mg/l; folic acid, 4 mg/l; L-tryptophan, 16 mg/l; pyridoxine • HCl, 4 mg/l; L-tyrosine, 72 mg/l; riboflavin, 0.40 mg/l) in a Herasafe HS12 laminar flow cabinet at (≥ 800 lux) for 1 h. R = riboflavin. The samples were incubated in Eppendorf tubes. Results of a typical reproducible experiment.

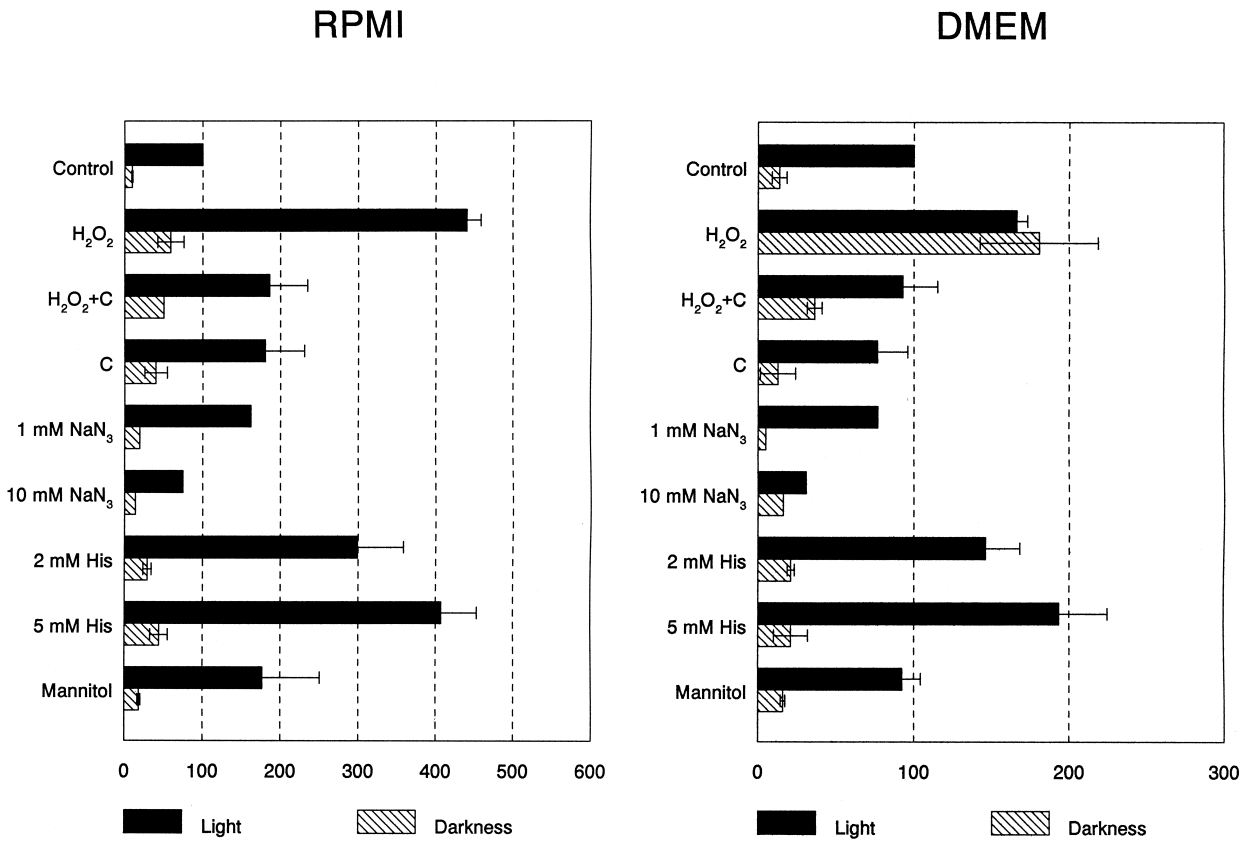

Fig. 5. Effect of hydrogen peroxide and scavengers of ROS on the oxidation of H_2DCFDA by RPMI 1640 and DMEM media. The media were incubated at 37°C in a Herasafe HS12 laminar flow cabinet for 1 h. H_2O_2, 1 mM; C + 50 μg/ml catalase; His = histidine; mannitol, 10 mM.

tration in different cell culture media ranges within rather broad limits, from 0.0038 mg dm^{-3} in MCDB 131 Medium up to 1 mg dm^{-3} in Waymouth's media, and was 0.40 mg dm^{-3} in DMEM and 0.20 mg dm^{-3}in RPMI 1640 Medium. It can not be excluded that the amount of this compound in some media actually exceeds cellular needs.

It has been reported previously that riboflavin present in the media interferes with cytotoxicity assays [21] and leads to neuronal degeneration during in vitro culturing [22]. This action seems to be largely mediated by generation of ROS. Similarly, *Salmonella* cells were reported to be subjected to oxidative stress in culture media due, i.a., to photo-oxidation of riboflavin [23,24].

Another source of ROS in cell culture media, responsible for their light-independent formation, may be autoxidation of reduced components of the media such as thiols and metal ions. DMEM media contain ferric ions and cystine, but reduction of these compounds by other components of the media may form an autoxidizing system. Other components of the media like methionine (or Mn^{2+} present in some media) may also autoxidize [25,26]. RPMI media contain reduced glutathione, which is able of autoxidation. Autoxidation of glucose, especially in the presence of trace amounts of metal ions, is another source of superoxide and other ROS [27–29]. Yeast medium contains numerous autoxidizable compounds present in yeast extract and peptone, as well as a high concentration of glucose.

Formation of superoxide and hydrogen peroxide in microbiological media exposed to atmospheric oxygen has been reported years ago and demonstrated to affect the viability of bacteria [30]. Formation of ROS tended to saturate during longer incubation, perhaps due to the consumption of autoxidizable substrates. However, one can expect that the presence of cells releasing reducing compounds to the media and able to reduce extracellular compounds may lead to redox cycling and a further augmentation of generation of ROS in the medium. We would like to emphasize that a similar situation occurs in media used for culturing mammalian cells. The presence of serum in cell culture media significantly diminishes but does not eliminate the level of ROS, whereas the procedure of serum deprivation used, e.g., in studies of

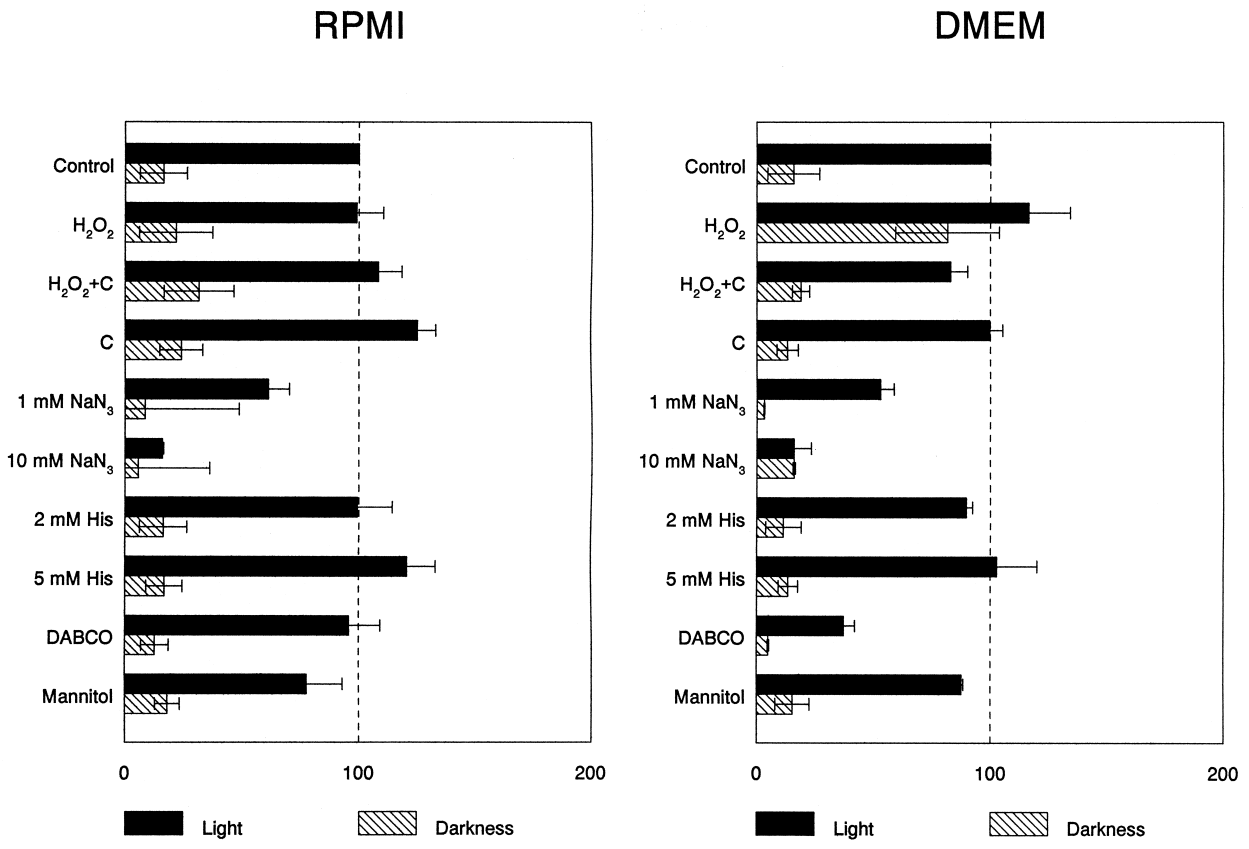

Fig. 6. Effect of hydrogen peroxide and scavengers of ROS on the oxidation of H_2R123 by RPMI 1640 and DMEM media. The media were incubated at 37°C in a Herasafe HS12 laminar flow cabinet for 1 h. H_2O_2, 1 mM; C + 50 μg/ml catalase; His = histidine; DABCO, 1 mM DABCO; mannitol, 10 mM.

apoptosis [31,32], must lead to an increase in the level of production of ROS in the medium of cultured cells.

Superoxide hardly permeates cell membranes in non-dissociated form but hydrogen peroxide penetrates easily into the cells. Cell growth in vitro may be stimulated by low concentrations of hydrogen peroxide but higher H_2O_2 concentrations are cytocidal [33–35]. Moreover, studies of the effects of oxidative stress on cultured cells may be biased by the fact that in the absence of external agents cells in culture are already exposed to oxidative stress due to ROS generated in the medium. Interaction of culture medium with xenobiotics may enhance production of ROS [1]. One should also be careful to include reference samples when determining production of ROS in cultured cells. Obviously, limitation of exposure of cell cultures to light is highly recommended.

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ABBREVIATIONS

DABCO—1,4 diazabicyclo[2.2.2.]octane
DFO—deferoxamine mesylate
DMEM—Dulbecco's Modified Eagle Medium
DMPO—5,5′-dimethyl-1-pyrroline N-oxide
DTPA—diethylenetriaminepentaacetic acid
FCS—fetal calf serum
H_2DCFDA—dichlorodihydrofluorescein diacetate
H_2R123—dihydrorhodamine 123
HRP—horseradish peroxidase
ROS—reactive oxygen species
SOD—superoxide dismutase
YPD—yeast extract-peptone-glucose medium