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Protective Effects of the Thiophosphate Amifostine (WR 2721) and a Lazaroid (U83836E) on Lipid Peroxidation in Endothelial Cells During Hypoxia/Reoxygenation

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ABSTRACT. Little is known about pharmacological interventions with thiophosphates or lazaroids in endothelial cells injured by hypoxia/reoxygenation with respect to membrane lipid peroxidation (LPO) caused by reactive oxygen species. Therefore, a cell line of bovine aortic endothelial cells was studied after 120-min hypoxia followed by 30-min reoxygenation, resulting in moderate and predominantly reversible injury (energy depression/cytosolic Ca^{2+}-accumulation during hypoxia, which almost normalized during reoxygenation; membrane blebs, an increasing amount of lysosomes, vacuolization, lipofuscin formation, alterations in mitochondria size, some lyzed cells). 18.9 ± 4.3% of the cells died. Radical-induced LPO measured as malondialdehyde continuously increased to 2.18 ± 0.17 nmol/mg of protein after reoxygenation vs control (0.41 ± 0.13, $P < 0.05$). Simultaneously, the content of 4-hydroxynonenal, a novel indicator of LPO, increased from 0.02 ± 0.01 to 0.11 ± 0.02 nmol/mg of protein ($P < 0.01$). The results support the assumption that reoxygenation injury is accompanied by an increase in membrane LPO, causing structural and functional disturbances in the monolayer. The thiophosphate WR 2721 [S-2-(3-aminopropylamino) ethylphosphorothioic acid] and the lazaroid U83836E {(-)-2-[[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl] methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol (dihydrochloride)} were effective scavengers of $^{\cdot}OH$, being more efficient than trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carbon acid) used as standard (EC_{50}: 12, 5 and 15 μM, respectively, measured by electron spin resonance spectroscopy). One mM WR 2721, 10 μM U83836E, and 5 μM trolox C reduced formation of malondialdehyde during hypoxia/reoxygenation to 53 ± 7, 51 ± 10 and 48 ± 6%, respectively ($P < 0.05$ each, versus control). In general, WR 2721 and U83836E prevent radical-induced membrane LPO in a model of endothelial cells injured by hypoxia/reoxygenation. The use of these two agents is a new approach to protect the endothelium against oxidative stress. BIOCHEM PHARMACOL **56**;8:945–954, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. endothelial cells; hypoxia and reoxygenation; lipid peroxidation; radical scavengers; thiophosphate WR 2721; lazaroid U83836E

Vascular endothelial cells are important structural and functional components in all tissues, forming a selectively permeable blood-tissue barrier. They are involved in the regulation of different processes, such as hemostasis, regulation of vessel tone, coagulation or inflammation. Due to their constant exposure to blood and high oxygen tension, arterial endothelial cells are constantly subjected to oxidative stress and the attack of free radicals [1]. Under normal conditions, this load is balanced by an endogenous defense system. However, pathological circumstances, such as hypoxia, may overcome the defense. Among others, free radical-mediated LPO¶ of membrane phospholipids may lead to endothelial injury and dysfunction [2, 3].

Little is known about pharmacological interventions on radical-induced disturbances of endothelium. *In vitro* and *in vivo* investigations with radical scavengers, such as vitamin E and its analogs, have indicated a therapeutic benefit of antioxidants [4]. Thiol compounds are among the most

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¶ *Abbreviations:* DMPO, dimethylpyrroline oxide; DNPH, dinitrophenylhydrazine; F VIII, factor VIII/von Willebrand; FBS, fetal bovine serum; HNE, 4-hydroxynonenal; LDH, lactate dehydrogenase; LPO, lipid peroxidation; MDA, malondialdehyde; trolox C, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carbon acid; U74389G, 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1,4,9(11)-triene-3,20-dione, (Z)-2-butenedioate (1:1); U83836E, (-)-2-[[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl] methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol (dihydrochloride), no synonym; WR 1065, aminopropylamino-ethylmercaptan; and WR 2721, S-2-(3-aminopropylamino) ethylphosphorothioic acid, - synonym: amifostine.

effective radical scavengers. The toxicity of numerous synthetic thiol compounds, however, has forced the synthesis of less toxic derivatives [5]. The thiophosphate WR 2721 belongs to these derivatives. It has been protective against free radical-induced damage in different types of diseases [6] and, in cancer patients, against side-effects of radiation and chemotherapy [7], which are related to radical-mediated deteriorations. Lazaroids are potent inhibitors of LPO in neural tissue which have been developed for the treatment of acute CNS injury. The protective mechanism, for instance of the lazaroid U78517F, has been found to be the reduction of membrane LPO [8]. However, it is not known whether thiophosphates or lazaroids are able to protect endothelial cells against radical-mediated injury caused by hypoxia.

The aim of this paper was to investigate the protective potential of WR 2721 and of the lazaroid U83836E, a stereoisomer of U78517F, in a monolayer of endothelial cells during hypoxia and reoxygenation. The thiophosphate and the lazaroid were studied as to their radical scavenger activity and their effect on radical-induced LPO in membranes. The data obtained were compared to those of trolox C, a water-soluble analogue of α-tocopherol, which has been shown to be a potent antioxidant and to prevent lipid peroxidative damage of endothelial cells in tissue [9].

MATERIALS AND METHODS

Cell Cultivation

The bovine aortic endothelial cell line $BKE_{Z\text{-}7}$ [10] was cultivated in modified Eagle's medium with 10% FBS (Sigma), 2 mM glutamine (Biochrom), without antibiotics and under 5% CO_2/95% air at 37° (Incubator). Confluent monolayers were used, if not stated otherwise, at day 4 after seeding of $2 \cdot 10^4$ cells/cm^2 (12th–20th passage), resulting in *ca.* $1.8 \cdot 10^5$ cells/cm^2 (*ca.* 30 μg of protein). The endothelial origin of cells (up to the 25th passage) was confirmed by phase contrast and electron microscopy, factor VIII antigen (Dako), activity of angiotensin-converting enzyme, and alkaline phosphatase [11].

Radical Scavenger Activity

Radical scavenging activity was tested in the presence of 1 mM DMPO (Sigma), 14 mM HCl, 93 mM NaCl, 1.24 mM pyridine, allowing the comparison of lipophilic and hydrophilic compounds [12]. After addition of 50 μM $FeSO_4$ and 100 μM H_2O_2, electron spin resonance spectra of the DMPO-hydroxyl radical adduct (DMPO-OH) were recorded at room temperature (ECS 106 spectrometer, Bruker: scan range, 10 mT; modulation amplitude, 0.1 mT; microwave power, 20 mW). Maximum signal intensity, reached 2 min after initiation of the reaction, was taken to evaluate $^{\cdot}$OH-scavenging activity. The concentration of the following compounds reducing the DMPO-OH signal intensity by 50% (EC_{50}) was detected: WR 2721, WR 1065 (Dr. Tapp, Biopharm, Berlin, Germany), U83836E, U74389G (Dr. Hall, Upjohn, Kalamazoo, MI, USA), trolox C (LaRoche), α-tocopherol (Serva), mannitol (Sigma).

Hypoxia/Reoxygenation

Experiments were carried out on 6- and 24-well plates in a rotating water bath (GFL, 37°, 25 rpm) after washing the cells with PBS (Ca^{2+}, Mg^{2+}, without glucose, pH 7.1, Biochrom). One mL PBS was added to each of the 6 wells (0.5 mL, 24 wells). PBS was chosen to omit radical scavengers from the incubation solution because radical metabolism and radical scavengers were investigated. Plates were gassed with 95% N_2/5% CO_2 for 2 hr (hypoxia) followed by Carbogen (95% O_2/5% CO_2) for 30 min (reoxygenation) to ensure fast gas exchange and sufficient O_2 supply, respectively [11]. For control, plates were gassed with Carbogen (control) or kept in the incubator for 150 min.

Biochemical Analysis

LDH (L-lactate:NAD oxidoreductase, EC 1.1.1.27., Kit 1087592) and lactate (Kit 149993, Boehringer Mannheim) were assayed in the incubation solution. For detection of LPO and nucleotides, cells were scraped off after the experiment and immediately frozen in liquid N_2. Nucleotides were detected by HPLC (Waters; photometric detection at 254 nm, detector RF-10A) [13]. For MDA [14], the cell suspension was boiled in 1.5 mL final volume containing 75 mM H_3PO_4 and 10 mM thiobarbituric acid for 60 min. The reaction was stopped by cooling on ice. Immediately before HPLC analysis (Shimadzu; fluorometric detector RF-10A, 150 × 4 mm of LC-18-S Supelcosil column), an equal volume of 100 mM NaOH in methanol (90%) was added (eluent: 50 mM K-phosphate buffer, pH 6.8, with 40% methanol). Standard was malondialdehydbis(diethylacetal) (Merck) [11]. HNE was measured after derivatization with DNPH, TLC separation and isocratic separation of DNPH-HNE [15, 16] using the same HPLC device as taken for nucleotide analysis (eluent:methanol:water [4:1, v/v]; wavelength 378 nm). The agents studied disturbed neither the assay of MDA nor that of HNE. Protein determination was performed by a Lowry Kit (P5656, Sigma).

Cell Viability

Viability was detected using the neutral red assay (50 μg/mL cultivation medium containing 2% FBS, 2 hr at 37°) [17]. After washing (PBS), the dye absorbed was extracted (50% ethanol/1% glacial acetic acid, 10-min shaking) and measured (540 nm, microtiter plate reader, Cambridge Technology 7500). To select a noncytotoxic concentration of an agent, 7×10^4 cells/cm^2 were seeded (24-well plates). Twenty-four hours later the substance was added for 24 hr. Then, the inhibitory concentration reducing cell viability by 50% (IC_{50}) was calculated (Hill model, 4 parameters,

SigmaStat/SigmaPlot, using at least five different concentrations in the linear part of the concentration-response curve: each concentration was double the previous one).

Microscopy

Phase contrast light microscopy was used. For electron microscopy, cells were prepared by the pop-off method. Cell monolayers grown on cover slides were fixed (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, 1 hr). After postfixation for 30 min (1% osmium tetroxide), the cells still adherent to the slide were dehydrated and embedded in Epon resin. The resin blocks were broken from the glass by focal heating of the briefly frozen slides with a burner. Sections were cut from the blocks (ultramicrotome, diamond knife; LKB), mounted on 200 mesh copper grids, stained (uranyl acetate, lead citrate) and examined on a Zeiss EM 10 transmission electron microscope at 60 kV (Zeiss). Volume density of mitochondria (as a measure of their size) was morphometrically determined for cytosol [18]. For detection of Ca^{++}, cells were grown in 30-mm Petri dishes, incubated with 4.42 μM Fluo3 and 13.3 μM FuraRed (acetomethyl esters in PBS; MoBiTec) for 45 min, washed (2 × 10 min in PBS), and studied with a confocal laser scanning microscope (LSM 410 invert, Zeiss) using a Plan-Neofluar ×63 lens (numerical aperture 1.25). After excitation (Argon–Krypton laser, 488 nm), the fluorescence light of the two dyes was split (beam splitter FT 580) and measured separately (≥515 nm for Fluo3, cut-off filter LP515; ≥590 nm for FuraRed, LP590) [19]. The ratio of the signal intensities (Fluo3/FuraRed) representing the Ca^{2+}-concentration was measured after hypoxia and reoxygenation and compared to the control. The fluorescence ratios were adjusted to the cells kept in incubator and expressed in %. The values of cells gassed with Carbogen and those of the incubator did not differ significantly. Statistical analysis was performed by one-way ANOVA and the Newman–Keuls post hoc test.

Cytoprotection Assay

During hypoxia/reoxygenation, the antioxidants were administered to the cells after washing the monolayer with PBS. The agents were added in 1 mL of PBS to each well of the 6-well plate. In the case of U83836E, BSA was applied as a vehicle, which was without any effect both under control conditions and during hypoxia/reoxygenation when used alone at the concentration given. The effect of a substance was compared to cell cultures containing all agents except the test substance (in the control group and the hypoxia/reoxygenation group). The assays (influence on MDA and HNE) were performed in PBS to exclude the radical scavenging activity of FBS and medium.

TABLE 1. Radical scavenger activity of tocopherol derivatives (α-tocopherol, trolox C), of lazaroids (U74389G, U83836E), and of thiocompounds (WR 1065, WR 2721) compared to the ·OH-specific radical scavenger mannitol

	·OH scavenging activity		
Substance	Concentration range* (μM)	EC_{50}† (μM)	Relative to mannitol
Mannitol	≥200	1300	—
α-Tocophereol	≥500	1650	0.78
Trolox C	≥2	15	86.7
WR 1065	≥1	8	163
WR 2721	≥5	12	108
U74389G	≥20	130	10
U83836E	≥1	5	260

Radical scavenger activity of a compound was expressed as inhibition of the DMPO-hydroxyl radical adduct (DMPO-OH) formation measured ESR spectroscopically (spin trapping technique) in the presence of the ·OH-generating Fenton system. Data represent mean values calculated from three independent concentration-response curves.

*Concentration reducing the signal intensity of DMPO-OH by at least 10%.

†Concentration reducing the signal intensity of DMPO-OH by 50%.

Statistics

Data represent means ± SEM. If not stated otherwise, significance values were calculated by using the *U*-test for independent random samples (two sites of variance).

RESULTS

Characterization of Aortic Endothelial Cells

Factor VIII, alkaline phosphatase (0.8 ± 0.2 U/mg of protein) and angiotensin-converting enzyme (362 ± 70 pmol/min × mg of protein) were expressed, comparable with other reports [10, 20]. The extent of the markers was similar in the passages used (and detectable at least up to passage 25). The monolayers were characterized by a cobblestone appearance and were free of α-smooth muscle actin; vimentin was positive, as expected for mesenchymal cells. The cell line showed normal growth characteristics, contact-inhibited proliferation, maintenance of their endothelial morphology, and differentiated phenotype at least up to the 25th passage. As reported earlier, the cell line also preserved other differentiated properties, such as β-glucuronidase, leucineaminopeptidase, cyclooxygenase, lipoxygenase, binding and metabolism of norepinephrine [10].

Radical Scavenger Activity and Cytotoxicity of Antioxidants

The ·OH scavenger activity of various compounds is shown in Table 1. Compared to mannitol, all agents, except α-tocopherol, showed excellent scavenger activity. Trolox C was two orders of magnitude more effective than α-tocopherol. Thus, trolox C was applied as standard in cytoprotection studies (against hypoxia/reoxygenation injury), using 5 μM as reported to prevent oxidative injury in cellular systems [21]. As far as trolox C was soluble in the test system, it did not show any cytotoxicity up to 4 mM.

TABLE 2. Cytotoxicity of thiol derivatives (WR 1065, WR2721) and lazaroids (U74389G, U83836E), compared to that of the standard radical scavenger trolox C

Substance	Cytotoxicity (mM)	
	Concentration range*	IC_{50}†
α-Tocophereol	Not detectable‡	
Trolox C	>4	Not detectable‡
WR 1065	≥1	3.25 ± 0.10
WR 2721	≥10	16.02 ± 0.52§
U74389G	Not detectable‡	
U83836E	≥0.020	0.028 ± 0.001

Cytotoxicity, expressed as inhibition of viability of aortic endothelial cell cultures, was measured by means of the neutral red assay. IC_{50} is the concentration reducing cell viability by 50%. Data represent estimated parameter values ± approximative standard error from nonlinear curve fitting (Hill model); 4–11 independent experiments were performed with at least 3 replicates for each compound and concentration.

*Concentrations showing cytotoxic effects.

†Concentration reducing cell viability by 50%.

‡Not soluble in cultivation medium.

§$P < 0.001$ compared to WR 1065.

The thiophosphate WR 2721 exhibited similar radical scavenger activity when compared to the free thiol WR 1065. However, the thiophosphate was less cytotoxic than the free thiol. Consequently, WR 2721 was chosen for the protection experiments, using a 1 mM final concentration which was not cytotoxic. The sufficiently hydrophilic U83836E was *ca.* 25 times more effective as radical scavenger than the more lipophilic U74389G. Ten micromolar U83836E (bound to BSA, 0.05% w/v) were not cytotoxic and were used for further cell experiments (Table 2). U74389G was not studied in cell cultures; it was not soluble under the experimental conditions and showed weak radical scavenger activity.

Biochemical Characterization of Hypoxia/Reoxygenation Injury

The cellular release of LDH activity (97 ± 24 mU/mg of protein, $P < 0.05$) after 2-hr hypoxia and 30-min reoxygenation was higher than that after 150 min of control incubation (18 ± 11 mU/mg of protein). Lactate production was enhanced throughout the experiment (2.21 ± 0.47 μmol/mg of protein after reoxygenation compared to 1.54 ± 0.13 μmol/mg of protein of the control). However, the changes were not statistically significant, due to the absence of any substrate in the incubation solution. The content of ATP was lowered after 2 hr of hypoxia compared to the control and was restored within 30 min of reoxygenation. ADP changed in an opposite manner. The ATP:ADP ratio was reduced after hypoxia compared to the control and was almost normalized during reoxygenation, indicating predominantly reversible disturbances of adenine nucleotide metabolism (Table 3). Energy depression and lactate release were consistent with other studies [22, 23]. The content of MDA continuously increased to 0.96 ± 0.32 nmol/mg of protein after hypoxia and to 2.18 ± 0.17 after reoxygenation, compared to 0.41 ± 0.13 after control incubation (Fig. 1). In the same manner, the content of HNE increased to 0.085 ± 0.010 during hypoxia and to 0.112 ± 0.020 after reoxygenation, compared to 0.022 ± 0.003 nmol/mg of protein of the control (Fig. 2).

Morphology and Viability after Hypoxia/Reoxygenation

After 2 hr of hypoxia, a few cells experienced autolysosomal destruction. Most cells were intact, the glycocalix and cell structure were not influenced significantly, and some blebs appeared at cell membranes (Fig. 3b). Thirty minutes after reoxygenation, two populations of cells were observed: the

TABLE 3. Content of ATP and ADP and the ATP:ADP ratio in confluent monolayers of bovine aortic endothelial cell cultures after 2 hr of hypoxia followed by 30 min of reoxygenation, compared to 150 min of control incubation

	ATP (nmol/mg protein)	ADP (nmol/mg protein)	ATP:ADP
Incubator	28.83 ± 2.80	6.22 ± 0.76	4.69 ± 1.44
Control	32.42 ± 4.00	5.86 ± 1.17	5.51 ± 1.36
Hypoxia	11.63 ± 1.67†	12.38 ± 1.13*	0.93 ± 0.24†
15-min reoxygenation	18.76 ± 1.75*	3.50 ± 0.83	5.46 ± 1.05
30-min reoxygenation	35.62 ± 6.95	8.75 ± 3.63	4.08 ± 2.57

Hypoxia: hypoxic gassing with 95% N_2/5% CO_2; control and reoxygenation: gassing with 95% O_2/5% CO_2; incubator: air/5% CO_2. Data represent means ± SEM, N = 6 in each group; *, † $P < 0.01$ and 0.001, respectively, significantly different compared to the control.

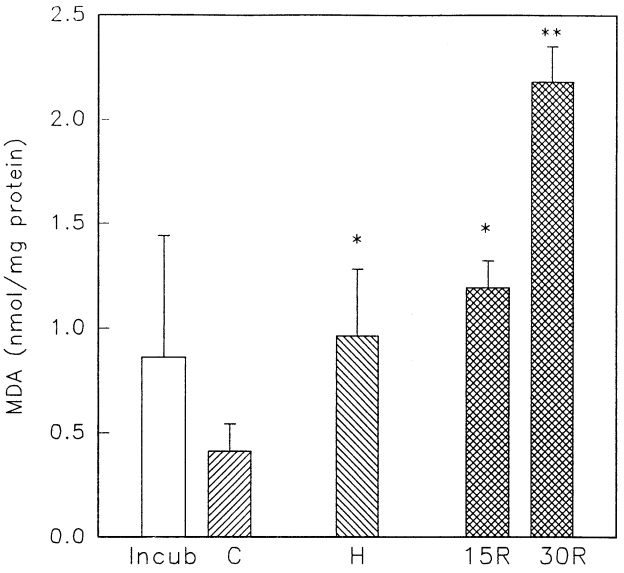

FIG. 1. Formation of MDA in bovine aortic endothelial cells during hypoxia (H) and reoxygenation (R; 15 and 30 min, respectively, after hypoxia), compared to the normoxic control (C). Incub: cells kept in incubator using comparable conditions. Means ± SEM, N = 6 in each group. *P < 0.05 and **P < 0.01, respectively, significantly different compared to the control. For further details, see Table 3.

majority showed moderate alterations similar to hypoxic injury (Fig. 3C) while the minority manifested severe cellular injury (Fig. 3D), as compared to control (Fig. 3A). In the moderately injured population, the number of pinocytotic vesicles and blebs increased; vacuolization, increasing amount of lysosomes with lipofuscin granules, and autolytic cells appeared. Bleb formation related to modification of cytoskeleton is known to be an early event for hypoxia-related injury in isolated, intact cells and cell monolayers [24]. In the severely injured population, completely damaged cells were observed. Similar results were obtained in vivo with few aortic endothelial cells exhibiting severe signs of injury or lysis after 2 hr of hypoxia while being surrounded by virtually normal-looking cells [24]. However, functional alterations may already occur without any evidence of cytolysis [25]. In our studies, mitochondrial volume density decreased during hypoxia (0.073 ± 0.007, N = 25, $P < 0.05$) compared to the control (0.089 ± 0.007, N = 25). During reoxygenation, the moderately affected population showed mitochondria similar to hypoxic injury (Fig. 3c; volume density 0.071 ± 0.004, N = 10, $P < 0.05$). The severely damaged population showed increased volume density (0.106 ± 0.023, N = 8); obviously, mitochondria were swollen and disrupted (Fig. 3D). Hypoxic injury and, to some extent, reoxygenation were accompanied by an increase in cytosolic Ca^{++}-concentration (Fig. 4). The highly active nuclei and nucleoli of the endothelial cells were not influenced by reoxygenation. Cell viability was 92.8 ± 4.3% (N = 14) after 2 hr of hypoxia and decreased to 81.1 ± 4.3% (N = 11, $P < 0.05$) 30 min after reoxygenation, compared to the control, 97.7 ± 6.3% (N = 7).

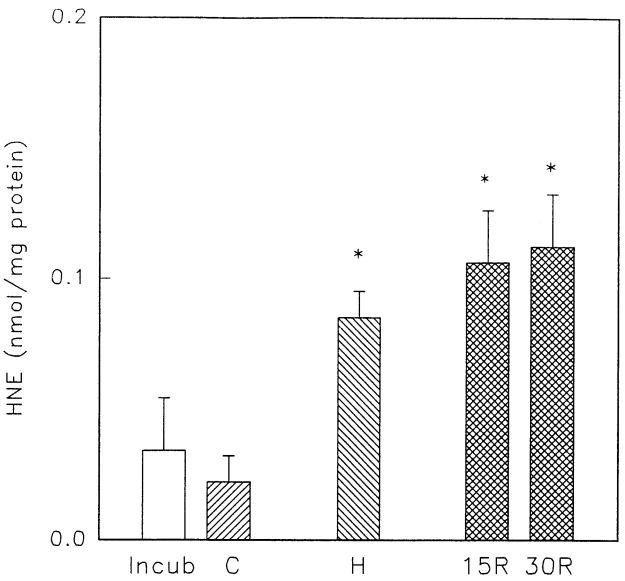

FIG. 2. Formation of HNE in bovine aortic endothelial cells during hypoxia (H, N = 14) and reoxygenation (R; 15 and 30 min, respectively, after hypoxia; N = 9, each), compared to the normoxic control (C, N = 5). Incub: cells kept in incubator using comparable conditions (N = 5). Means ± SEM; *P < 0.05, significantly different compared to the control. For further details, see Table 3.

Cytoprotection Studies

The compounds investigated (nontoxic concentrations) did not show any effect on the morphology or on LPO when added under control incubation conditions; the viability was 100.0 ± 1.6% (N = 12), 98.9 ± 3.7% (N = 7) and 99.1 ± 2.7% (N = 14) after addition of 10 μM U83836E, 1 mM WR2721 and 5 μM trolox C, respectively, compared to 101.2 ± 2.1% (N = 12) in the absence of these compounds. After hypoxia and reoxygenation, the viability was improved by U83836E, WR2721 and trolox C to 98.4 ± 2.6% (N = 7), 96.1 ± 2.8% (N = 7) and 87.7 + 2.0% (N = 14), respectively, compared to hypoxia/reoxygenation without treatment (93.2 ± 2.5%, N = 7; 86.0 ± 4.1%, N = 7; 84.2 ± 2.1%, N = 14). However, these improvements were statistically not significant. After 2-hr hypoxia and 30-min reoxygenation, the MDA contents of cells treated with U83836E, WR 2721 or trolox C were lower than those after the respective control treatment (without the agents; Fig. 5). The HNE content in endothelial cells which underwent hypoxia/reoxygenation was lowered in the group treated with WR 2721 compared to the nontreated hypoxia/reoxygenation group. U83836E and trolox C slightly prevented HNE accumulation. However, the changes were statistically not significant at the concentrations tested (Fig. 6).

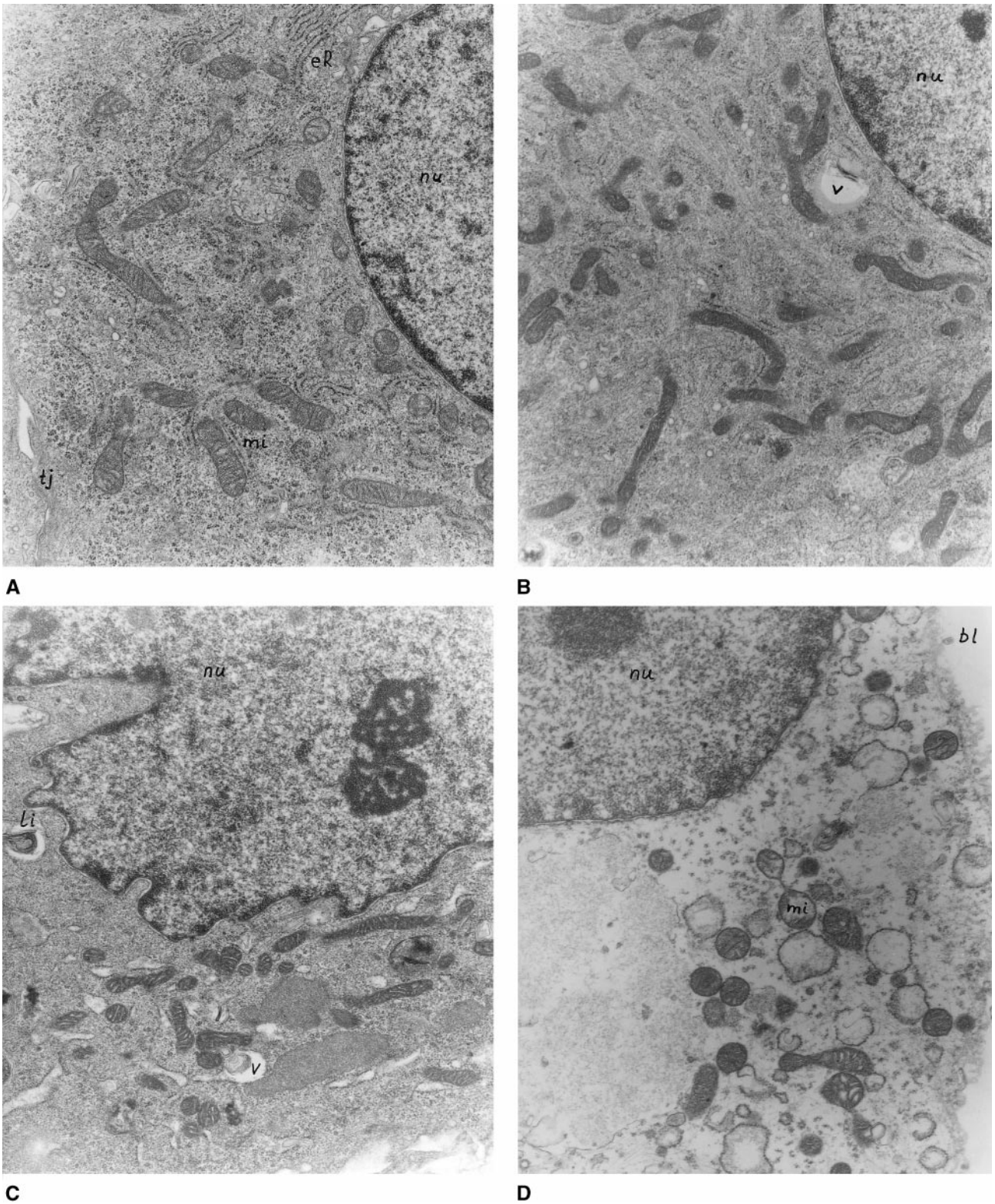

FIG. 3. Representative electron micrographs of bovine aortic endothelial cells (magnification ×20,000): (A) control conditions, tight junctions (tj), nucleus (nu), intact glycocalix, mitochondria (mi), rough endoplasmic reticulum (eR); (B) after 2 hr of hypoxia: vacuoles (v); after 2 hr of hypoxia and 30 min of reoxygenation moderately injured cells (C) and severely damaged cells (D) were observed: vacuoles, lysosomes with lipofuscin (li), blebs (bl), swollen and disrupted mitochondria. For further details, see Table 3.

DISCUSSION

The results of the present study showed biochemical and ultrastructural alterations in an endothelial cell line during 2 hr of hypoxia and 30 min of the following reoxygenation, with only a few cells being completely damaged. This corresponds with the fact that *ca.* 81% of the cells survived

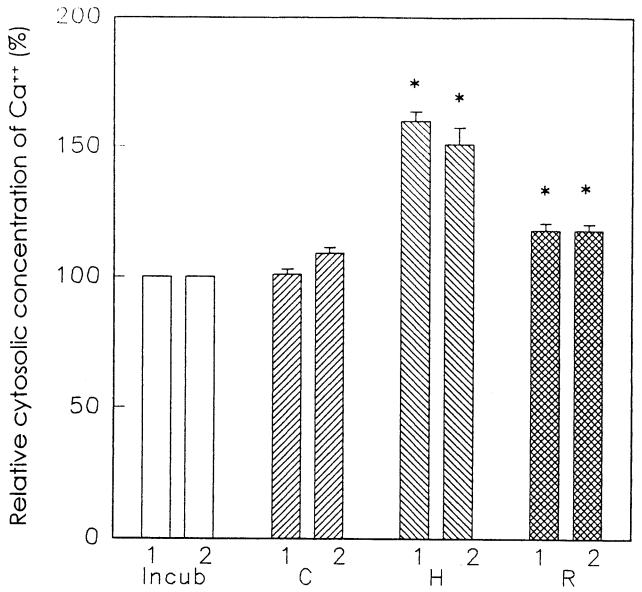

FIG. 4. Effect of hypoxia (H) and reoxygenation (R) on the cytosolic Ca^{2+}-concentration of bovine aortic endothelial cells, compared to normoxic control (C). The Ca^{2+}-concentration was measured by laser scanning microscopy as the ratio of Fluo3/FuraRed. The values were related to those of cells kept in the incubator (Incub) and were expressed in %. The cells were analyzed near to the nucleus (1, absolute ratio 0.68 ± 0.22) and at the periphery (2, absolute ratio 0.83 ± 0.12). Means ± SEM of at least 200 cells per group; *P < 0.05, significantly different compared to controls 1 and 2, respectively. For further details, see Table 3.

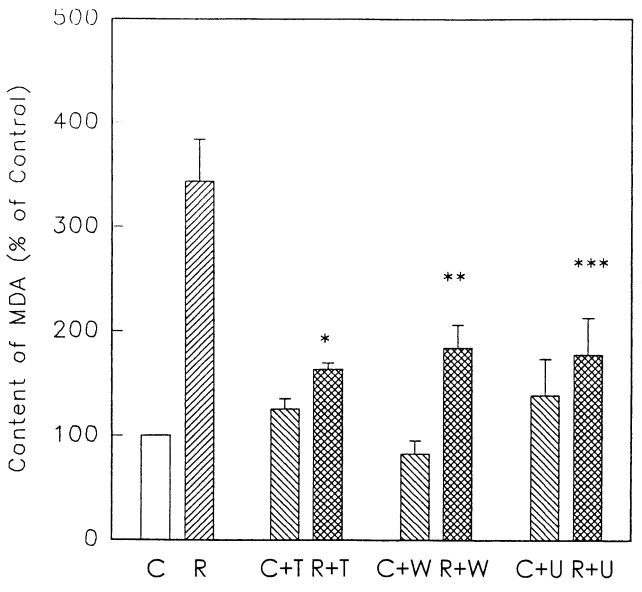

FIG. 5. Protective effects of trolox C (R+T, 5 μM, N = 9), WR 2721 (R+W, 1 mM, N = 5) and U83836E (R+U, 10 μM, N = 8) on MDA content of bovine aortic endothelial cells after hypoxia and reoxygenation compared to reoxygenation without the effectors (R, N = 13). Under control conditions (C), the effectors did not significantly influence the cellular content of MDA (abbreviations as above; C, N = 21; C+T, N = 9; C+W, N = 5; C+U, N = 8). Data were related to the normoxic control group (absolute values 0.35 ± 0.18 nmol/mg of protein) and are expressed in %. Means ± SEM; *P < 0.05, **P < 0.01 and ***P < 0.001, significantly different compared to R. For further details, see Table 3.

hypoxia/reoxygenation, which is in agreement with previous results [11]. Moreover, adenine nucleotide metabolism and cytosolic Ca^{++}-concentration were largely restored after reoxygenation. This indicates that the disturbances resulting from hypoxia/reoxygenation represent a moderate and reversible injury in the majority of the cells and that this model system is suitable for pharmacological studies, as confirmed by the agents tested. In general, these changes are comparable with earlier investigations using brain [11, 16], aortic [26] or venular [27] endothelial cells. However, the aortic endothelial cells used here showed fewer alterations during hypoxia/reoygenation than observed in primary and secondary endothelial cells from porcine brain [11, 16], bovine [27] or human venules [28]. It is possible that the lower sensitivity of our cell line was due to the higher radical defense potential of endothelial cells from aorta as compared to other tissues [29], or to the higher passage of our cells.

Recent studies have suggested radical-related endothelial cell injury under hypoxic conditions [27, 28, 30]. It has been demonstrated that radical-induced LPO is one important causal component of hypoxic cell damage [3, 31, 32]. In the experiments performed here, radical metabolism was investigated by means of aldehydic LPO products analyzed by HPLC, such as MDA (measured as MDA bound to thiobarbituric acid) and HNE (measured as DNPH-derivative). The content of MDA increased continuously up to approximately six times the initial value during 2 hr of hypoxia followed by 30 min of reoxygenation. The time dependence of the HNE content was similar to that of MDA, but the content enhancement was only 3-fold. This difference could be due to different sources; HNE is formed from ω-6-polyene fatty acids only, MDA is formed both from ω-6-polyene fatty acids and ω-3-polyene fatty acids [33]. The occurrence of lipofuscin granules also indicates cellular injury caused by free radicals. Lipofuscin represents end products of cell constituents after radical attack [11]. Lipofuscin is formed only after reoxygenation. This is consistent with the result that LPO showed maximum values during reoxygenation and continuously increased in content during that period.

In general, MDA and HNE are sensitive indicators of radical metabolism and membrane injury in endothelial cells during hypoxia/reoxygenation and, hence, appear suitable probes for pharmacological studies. Intensification of LPO in cells and tissues under pathological circumstances can be prevented by antioxidants and radical scavengers [2, 21]. Vitamin E and its synthetic derivative trolox C, which contains the chromanol ring of α-tocopherol without the isoprenoid side-chain, are potent antioxidants [4]. They are known to prevent LPO damage by interrupting LPO chain reactions initiated by free radicals at the cell membrane. The advantages of trolox C over the

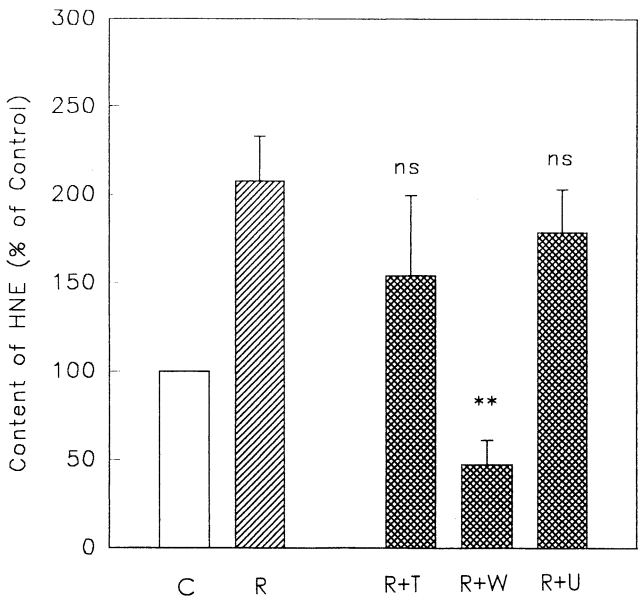

FIG. 6. Effects of trolox C (R+T, 5 μM, N = 4), WR 2721 (R+W, 1 mM, N = 4) and U83836E (R+U, 10 μM, N = 4) on HNE content of bovine aortic endothelial cells after hypoxia and reoxygenation compared to reoxygenation without the effectors (R, N = 8). Data were related to the normoxic control group (C, N = 7, absolute values 0.019 ± 0.005 nmol/mg of protein) and are expressed in %. Means ± SEM; **Significantly different ($P < 0.01$) and [ns]not significantly different, compared to R. For further details, see Table 3.

natural vitamin E are its higher hydrophilicity and its 100-fold higher ·OH scavenger activity as demonstrated by electron spin resonance. The electron spin resonance test solution used contained molar amounts of pyridine. Pyridine shows moderate polarity, allowing the comparison of polar and nonpolar agents, such as trolox C or α-tocopherol, both soluble in this test system. Nevertheless, in tissue, α-tocopherol may act protectively in lipophilic membrane phase and against radicals other than ·OH. Because of the higher radical scavenger activity of trolox C, similar to that of WR 2721 and U83836E, this derivative was applied as standard in this study. Five μM trolox C were shown to reduce the accumulation of MDA, following hypoxia and reoxygenation, by *ca.* 50%. It has been reported to protect myocytes from injury by radicals, but not in saphenous vein endothelial cells and fibroblasts [21]. The protective effect of trolox C on rat aortic endothelial cells exposed to $\cdot O_2^-$-generating xanthine oxidase is negligible [34]. From our data and those of others, it may be concluded that trolox C exhibits a wide cytoprotective concentration range, that it is cytotoxic in high concentrations only, and that its protective ability may vary according to cell type. In our study, an effect of the standard radical scavenger trolox C on hypoxia-related formation of HNE was not found. It thus appears that HNE is less suitable for protection studies than MDA. Comparing our results and data in the literature, trolox C is less cytoprotective than α-tocopherol. The latter effectively already protects primary endothelial cells of human umbilical vein against hypoxia-reoxygenation toxicity at 1 μM [35]. Another reason for the better protection might be that our cell line is less sensitive to hypoxia than primary cells [35] and, hence, that it is more difficult to demonstrate statistically significant differences.

Agents with free thiol groups are usually very effective antioxidants [6] but exert high cytotoxicity [5]. In WR 2721, the thiol residue is phosphorylated, but nonetheless, its radical scavenger potential is estimated to be similar to that of the free thiol WR 1065. Moreover, WR 2721 has been found to be less cytotoxic than WR 1065. Measuring cellular contents of MDA and HNE shows that the thiophosphate reduces radical-induced LPO, following hypoxia and reoxygenation, by at least 50%. This is possible because WR 2721 is accumulated in tissues from extracellular fluid [36]. Intracellularly, the free thiol function is achieved by cleaving the phosphate group, a reaction which is easily catalyzed by intracellular esterases. The resulting free thiol together with WR 2721 [37] are able to preserve cellular thiol residues occurring in many essential proteins and glutathione. This effect would also protect cell structure and function against oxidative stress. Glutathione is a key intermediate of the intracellular radical defense. Its preservation or regeneration would also prevent radical-induced LPO, as observed in our studies. In summary, WR 2721 is a powerful, low-toxicity cytoprotector, as it reduces not only the posthypoxic accumulation of MDA but also that of HNE. In this respect, WR 2721 is a better protector than trolox C, which abolishes MDA formation only. However, trolox C has been shown to be effective at lower concentrations.

In the present study, the ·OH scavenging potency of the relatively hydrophilic lazaroid U83836E is estimated to be 26 times higher than that of the less water-soluble U74389G. U83836E is a steroid with the antioxidative chromanol ring of α-tocopherol. It shows 330-fold higher radical scavenger activity than the tocopherol. Similar differences in antioxidative capacity are found with U78517F, an enantiomer of U83836E, which inhibits iron-catalyzed LPO in rat brain homogenates 10 times more effectively than U74006F and 100 times more effectively than vitamin E. The enantiomer U78517F also inhibits LPO induced by $\cdot O_2^-$-generating xanthine oxidase two orders of magnitude more effectively than U74006F and three orders of magnitude more effectively than vitamin E [8]. In our experiments, U83836E protected aortic endothelial cells from posthypoxic formation of MDA. Stereoisomers exhibit equal radical scavenger activity. This argues against a specific (receptor-mediated) mechanism of protective action. Therefore, the chromanol moiety significantly determines the antioxidative potency of each stereoisomer. It has to be considered that, during ischemia/reperfusion, lazaroids inhibit generation and degranulation of leukotrienes. Moreover, the protective action may be related to the prevention of tissue LPO by scavenging oxygen radicals [38]. This means that lazaroids may inhibit the lipoxygenase pathway, which in turn would reduce the content of MDA and not that of HNE [33] as found in our

studies. Although U83836E is an excellent antioxidant, reducing MDA formation during hypoxia/reoxygenation by approximately 50% in micromolar concentrations, it is highly cytotoxic compared to the other compounds investigated in this study. Thus, its applicability is limited to very low dosage and a high therapeutic risk can be expected.

An early pharmacological intervention for the protection of endothelial cells from free radical damage due to hypoxic disturbances may provide an additional approach for the maintenance of endothelial cell integrity in different tissues and organs [35]. The lazaroid investigated and, especially, the thiophosphate studied seem to be potential new candidates for prevention and treatment of radical-mediated injury in endothelial cells. However, our model studies have been limited to a single cell system *in vitro*, which does not encompass the blood system. For instance, erythrocytes carry a powerful antioxidant system. On the other hand, leukocytes may form reactive species which also interfere with the radical metabolism of endothelial cells [27]. Therefore, investigations should be continued *in vivo*.

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