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Induction of apoptosis and modulation of production of reactive oxygen species in human endothelial cells by diphenyleneiodonium

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Abstract

Diphenyleneiodonium (DPI) inhibits activity of flavoenzymes like NAD(P)H oxidase, the major source of superoxide anion in cardiovascular system, but affects also other oxidoreductases. Contradictory data have been published concerning the effect of diphenyleneiodonium on the production of reactive oxygen species in cells, both inhibitory and stimulatory action of DPI being reported. We have examined the effect of DPI on the cellular production of reactive oxygen and nitrogen species (ROS/RNS) and on the proliferation and apoptosis of human vascular endothelial cells. We found increased oxidation of ROS-sensitive probes (dihydrorhodamine 123 and 2′,7′-dichlorodihydrofluorescein diacetate) when DPI (20 μM–100 μM) was present in the treated cells. However, oxidation of the fluorogenic probes was inhibited if DPI (20 μM–100 μM) was removed from the reaction medium after cell preincubation. These results suggest an artifactual oxidation of the fluorogenic probes by DPI or its metabolites. A similar pattern of influence of DPI on the production of NO (measured with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) was observed. Modulation of generation of reactive oxygen and nitrogen species in DPI-treated cells influenced the nitration of tyrosine residues of cellular proteins, estimated by Western blotting. Decreased level of nitration generally paralleled the lowered production of ROS. A decreased 3-(4,5-dimethylthiazolyl)-3-3(4-sulphophenyl) tetrazolium (MTT) reducing activity of cells for was observed immediately after 1 h treatment of human endothelial cells with DPI (1 μM–100 μM), in spite of lack of changes in cell viability estimated by other methods. These results point to a next limitation of MTT in estimation of viability of cells treated with oxidoreductase inhibitors. DPI inhibited the proliferation of HUVECs as well as immortalized cell line HUVEC-ST, as assessed by acid phosphatase activity test and measurement of total nucleic acid content. Proapoptotic action of DPI was observed 12 h after incubation with this compound.

Keywords: Diphenyleneiodonium; Apoptosis; Reactive oxygen/nitrogen species; Endothelial cells

1. Introduction

Generation of reactive oxygen (ROS) and nitrogen species (RNS), including superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, nitric oxide and peroxynitrite, is associated with normal cellular metabolism [1]. It influences the physiology and proliferation of endothelial cells [2,3]. While low levels of ROS and RNS stimulate cell growth, increased levels of ROS have been demonstrated to induce apoptosis and necrosis of various

Abbreviations: DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; dHR 123, dihydrorhodamine 123; DPI, diphenyleneiodonium; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; JC-1, 1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; MTT, 3-(4,5-dimethylthiazolyl)-3-3(4-sulphophenyl) tetrazolium; RNS; reactive nitogen species; ROS; reactive oxygen species

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cells, including vascular endothelial cells [4–6]. Oxidative stress has been identified as a pathogenic factor of many cardiovascular diseases, such as hypercholesterolemia, atherosclerosis, hypertension, diabetes and heart failure [7,8]. Regulation of activity of cellular sources of free radicals has a critical importance for the physiology and pathology of endothelial cells.

Diphenyleneiodonium (DPI) affects many enzymes involved in cellular production of ROS/RNS. DPI is a well-known irreversible inhibitor of flavoproteins. It has been shown to inhibit NAD(P)H oxidase [9,10], the major source of superoxide anion in the cardiovascular system, xanthine oxidase [11], nitric oxide synthase [12], cytochrome P-450 reductase [13] and oxidoreductases of the mitochondrial respiratory chain, including NADH:ubiquinone oxidoreductase [14,15]. A result of electron transport through the flavin moieties of these flavoenzymes is the reduction of DPI to its diphenyleneiodonyl radical form and irreversible phenylation of flavin or adjacent amino acid and heme groups of the proteins [16]. Inhibition by iodonium derivatives is a marker of a radical mechanism of function of flavoprotein enzymes, because other flavoproteins, which are not involved in the generation of free radicals, such as glutathione reductase, glucose oxidase and amino acid oxidases, are not inhibited [11]. DPI changes the redox state of cells not only by modulation of the level of ROS and RNS production. It was found that diphenyleneiodonium induces the efflux of GSH from T24 cells to extracellular medium via a specific transport channel. The loss of reduced glutathione is blocked by bromosulfophthalein, an inhibitor of the canalicular GSH transporters [17].

DPI may have a proapoptotic action by modulating the cellular oxidant-antioxidant homeostasis. It has been demonstrated that DPI sensitizes carcinoma cells to Fas-mediated apoptosis [18]. Fas, referred to also as APO-1 and CD95 is known to induce apoptosis by activation of caspase 8 and caspase 3, after binding its ligand, Fas-L. Under physiological conditions Fas is widely expressed on the surface of many cells, mainly on activated T lymphocytes but also on endothelial cells [19,20]. Expression of Fas is up-regulated by hydrogen peroxide [21].

Although DPI, as a flavoprotein inhibitor, can be expected to decrease the cellular production of ROS and RNS, results of studies of this question are controversial. Both stimulation and inhibition of ROS/RNS production have been reported [14,22]. The aim of this study was to resolve this controversy with respect to human vascular endothelial cells. Moreover, we have also examined the effect of DPI on the proliferation and apoptosis of endothelial cells. Our results demonstrate that DPI inhibits ROS generation in endothelial cells and that stimulation of ROS production in the presence of DPI may be an artifact. We provide also evidence that the 3-(4,5-dimethylthiazolyl)-3-3(4-sulphophenyl) tetrazolium (MTT) test cannot be applied to measure the viability of DPI-treated cells and that exposure to even low concentrations of DPI induces apoptosis.

2. Materials and methods

2.1. Materials

Cell culture medium 199, OptiMEM, fetal bovine serum, collagenase II and antibiotics were purchased from Invitrogen. CyQUANT proliferation assay kit and fluorogenic probes were from Molecular Probes. Sheep anti-nitrotyrosine polyclonal antibodies were from OXIS Research. Immobilon P was purchased from Millipore. Hoechst 33258 was from Calbiochem. DPI, endothelial cell growth supplement (ECGS), secondary antibodies using for Western blotting (mouse anti-goat/anti-ship IgG, clone GT-34) and other reagents were from Sigma.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from veins of freshly collected umbilical cords, by collagenase type II digestion [23] and used for the experiments at passage 2–4. They were cultured in medium 199, containing 20% fetal bovine serum, 10 U/ml penicillin, 50 μg/ml streptomycin, 5 μg/ml heparin sodium and 75 μg/ml ECGS. The cells were grown at 37 °C and 5% CO_2 on plastic flasks coated with 1% gelatin.

Immortalized human umbilical vein endothelial cells (HUVEC-ST) were cultured in OptiMEM, containing 3.5% fetal bovine serum and antibiotics: 10 U/ml penicillin and 50 μg/ml streptomycin. The HUVEC-ST cell line was obtained from Prof. C. Kiéda (Orlèans, France).

The cultured cells showed the typical cobblestone-like appearance, and were identified as endothelial cells by FACS analysis for von Willebrand factor with ECA-4 antibodies [24] (kindly donated by Dr. Monica Spadofora-Fereira, University of Sao Paulo, Brazil). SW620 cells were used as a negative control (data not shown).

2.3. Determination of reactive oxygen/nitrogen species production

Measurement of intracellular ROS and RNS production in human endothelial cells seeded onto gelatin-coated 96-well plates, was recorded by monitoring changes in the fluorescence of 5 μM dihydrorhodamine 123 (dHR 123), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM), immediately after 1-h incubation of cells with DPI or after 6 h, 12 h or 24 h following the DPI treatment. DPI was either washed off or was present in the cell samples. Assays were performed in a modified Hank's buffered salt solution (HBSS), containing 140 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 1 mM

Na_2HPO_4, 10 mM HEPES and 1% glucose, pH 7.0. Fluorescence intensity was monitored with a Fluoroskan Ascent FL microplate reader.

2.4. Western blotting

Cells (4×10^6) were lysed in 300 μl of a hypotonic lysis buffer (20 mM phosphate buffer, pH 7.4) supplemented with a mixture of protease inhibitors (0.3 μM aprotinin, 0.1 mM leupeptin, 1.4 μM E-64, 13 μM bestatin, 1 mM ethylenediaminetetraacetate (EDTA) and 2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBEF)). The cells were harvested on ice, frozen-thawed at −70 °C, and sodium dodecyl sulfate (SDS) was added. Protein concentration was estimated according to Lowry [25]. A total of 20 μg of protein from the cell lysates was separated by SDS–PAGE electrophoresis [26], and transferred to Immobilon polivinylidene difluoride membrane. Nitration of tyrosine residues was detected using ship polyclonal antibodies. The membrane was incubated at room temperature for 1 h with 5% milk and than incubated with the appropriate antibodies for another 2 h. A horseradish peroxidase-conjugated mouse anti-ship/anti-goat antibody (clone GT-34), was used as a secondary antibody. The signal was detected with enhanced chemiluminescence (ECL) detection system.

2.5. MTT assay

Proliferation of cells was estimated by measuring the ability of live cells to metabolize MTT to formazan [27]. Cells were seeded onto gelatin-coated 96-well plates at a density of 3×10^3 cells per well (HUVEC) or 1×10^3 cells per well (HUVEC-ST). After overnight culture the cells were treated for 1 h by diphenyleneiodonium in a range of concentrations (1 μM–100 μM). At the end of treatment the cell monolayers were rinsed with HBSS, fresh medium was added and incubation was continued for 72 h. In the next step, MTT-containing medium (final MTT concentration of 333 μg/ml) was added. After 3 h the medium was removed and formazan crystals were dissolved in DMSO. Absorbance was read at 590 nm.

2.6. Acid phosphatase activity assay

Viability of cells after inhibition of oxidant systems by DPI was checked by measurement of acid phosphatase activity. The procedure of the experiment was the same as in the case of MTT assay. After 1 h incubation of cells with DPI and then 72 h in a complete medium, monolayers of the cells were rinsed with HBSS. Lysis buffer, containing 10 mM *p*-nitrophenyl phosphate, and 0.1% Triton X-100 in 0.1 M sodium acetate, pH 5.5, was added to the cells for 2 h. The samples were alkalized by adding 1 M NaOH and absorbance of *p*-nitrophenol was read at 405 nm.

2.7. Quantification of DNA and RNA

Measurement of nucleic acid content in endothelial cells, treated with DPI for 1 h, was used to estimate cells proliferation. The CyQUANT assay is based on green fluorescence of CyQUANT GR dye emitted after binding

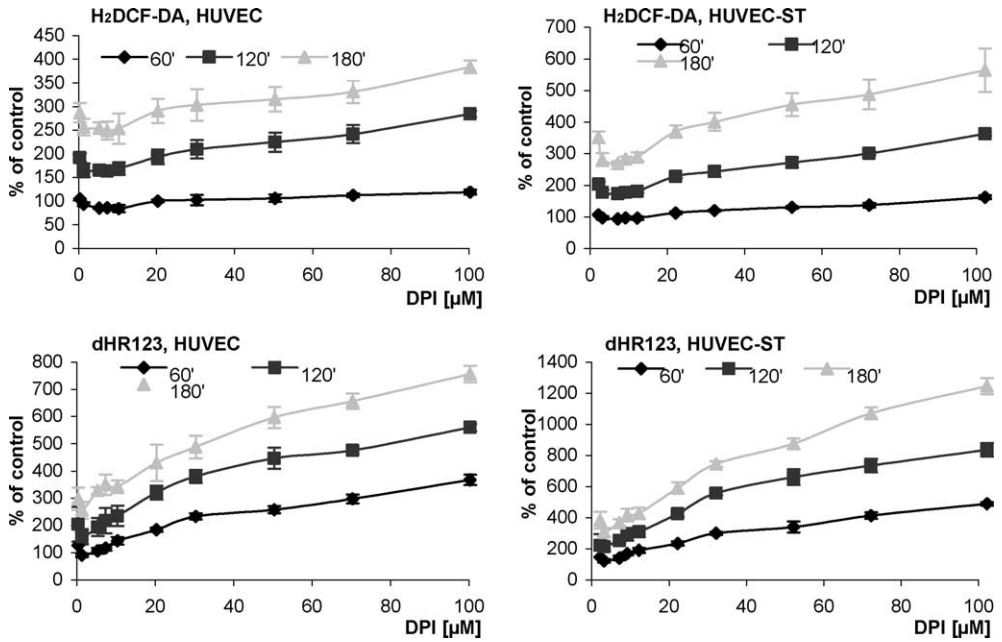

Fig. 1. Production of ROS in HUVEC and HUVEC-ST during the 3 h treatment with DPI. ROS production was estimated with H_2DCF-DA and DHR123. After 10 min pretreatment of cells by DPI (1 μM–100 μM) supernatants were removed, and fresh solutions, containing the inhibitor and a fluorescent probe (5 μM) were added. The measurement of reactive oxygen species production was performed for 3 h. Results are presented as mean ± S.D. of three independent experiments.

the probe to cellular nucleic acids. Similar to MTT assay and acid phosphatase assay, proliferation was checked after 72 h. The culture medium was removed and cells on the 96-well plates were frozen at −70 °C. Then the cells were thawed and lysed by addition of the CyQUANT cell buffer. Fluorescence intensity was measured (λ_{ex} = 485 nm, λ_{em} = 538 nm) in a Fluoroskan Ascent FL microplate reader.

2.8. Mitochondrial membrane potential

Changes in mitochondrial membrane potential were assayed by means of 1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) fluorescence intensity ratio measurements. This carbocyanine dye accumulates in the mitochondrial membrane in a membrane potential-dependent manner. Negative potential of the inner mitochondrial

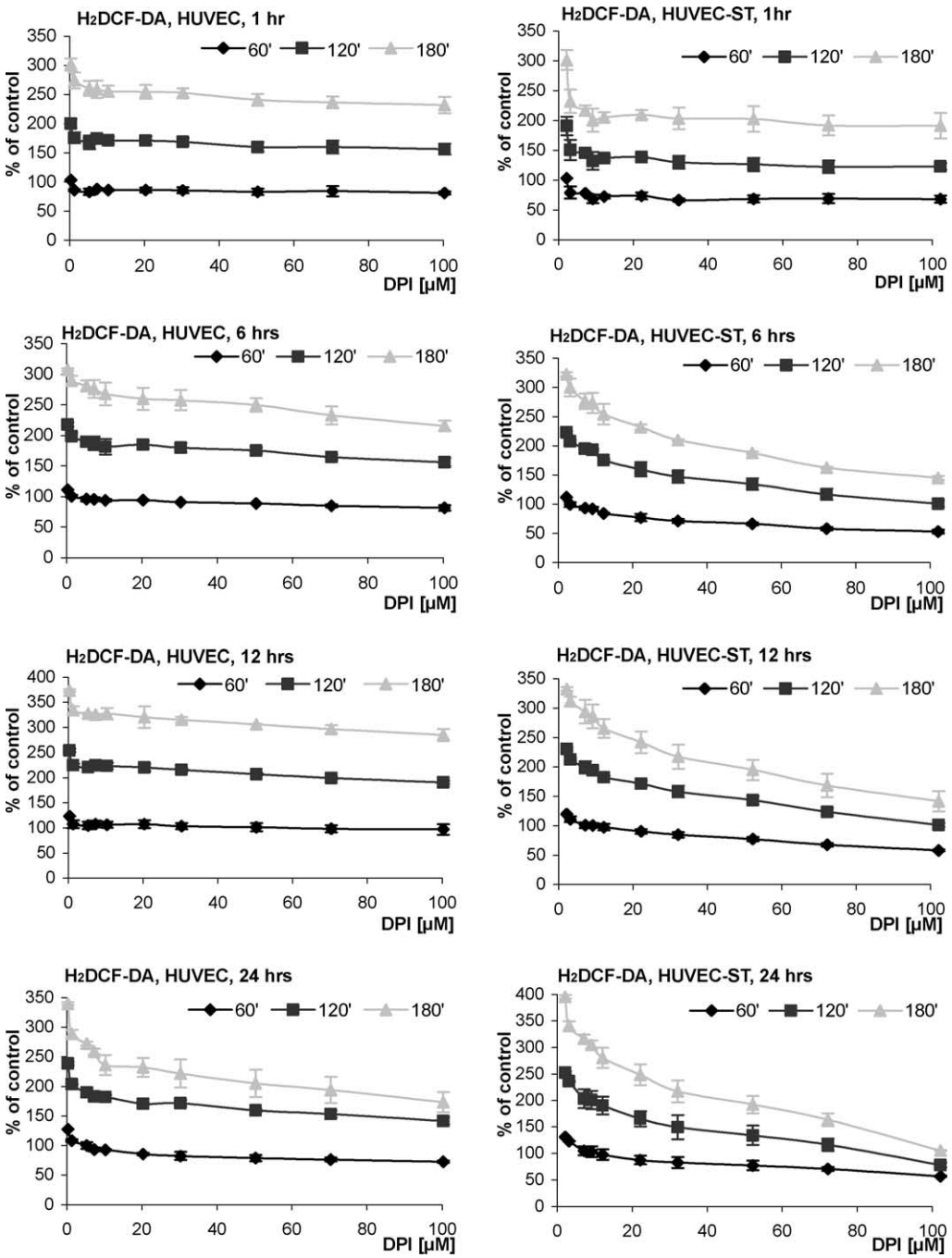

Fig. 2. Production of ROS in HUVEC and HUVEC-ST immediately and 6, 12 and 24 h after the incubation with DPI. ROS production was estimated with H_2DCF-DA. Cells were seeded onto gelatin-coated plates (20×10^5 cells per well), incubated overnight, and treated by DPI, in a complete medium (HUVEC in M199; HUVEC-ST in OptiMEM), for 1 h in a range of concentrations (1 μM–100 μM). Then the cell monolayers were rinsed with HBSS and a 5 μM probe was added. Oxidation of fluorescence probe was monitored by 3 h. Results are presented as mean ± S.D. of three independent experiments.

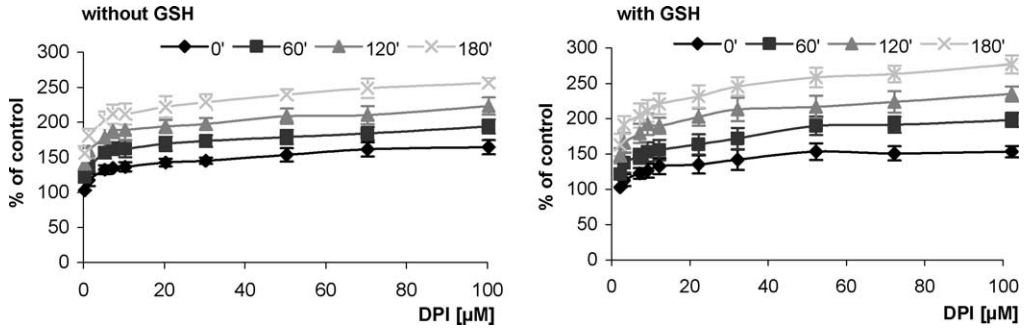

Fig. 3. Fluorescence of 5 μM DHR123 incubated with different concentrations of DPI in HBSS in the absence (A) and in the presence (B) of 1 mM glutathione. Results are presented as mean ± S.D. of four independent experiments.

membrane facilitates formation of the dye aggregates (J-aggregates) with both excitation and emission peaks shifted towards red (530 nm/590 nm) when compared with that for monomers (485 nm/538 nm) [28].

The cells were incubated with 5 μM JC-1 in the HBSS for 30 min. Prior to measurements, the cells were washed twice with the HBSS. The fluorescence was measured in a Fluoroscan Ascent plate reader with the filter pairs of 530 nm/590 nm and 485 nm/538 nm. Results are shown as a ratio of fluorescence measured at 530 nm/590 nm to that measured at 485 nm/538 nm (dimer to monomer fluorescence).

2.9. Apoptosis assay using nucleic acids stains

Acridine orange exhibits metachromatic fluorescence that is sensitive to DNA conformation. Green fluorescence is emitted when acridine orange binds to double-stranded

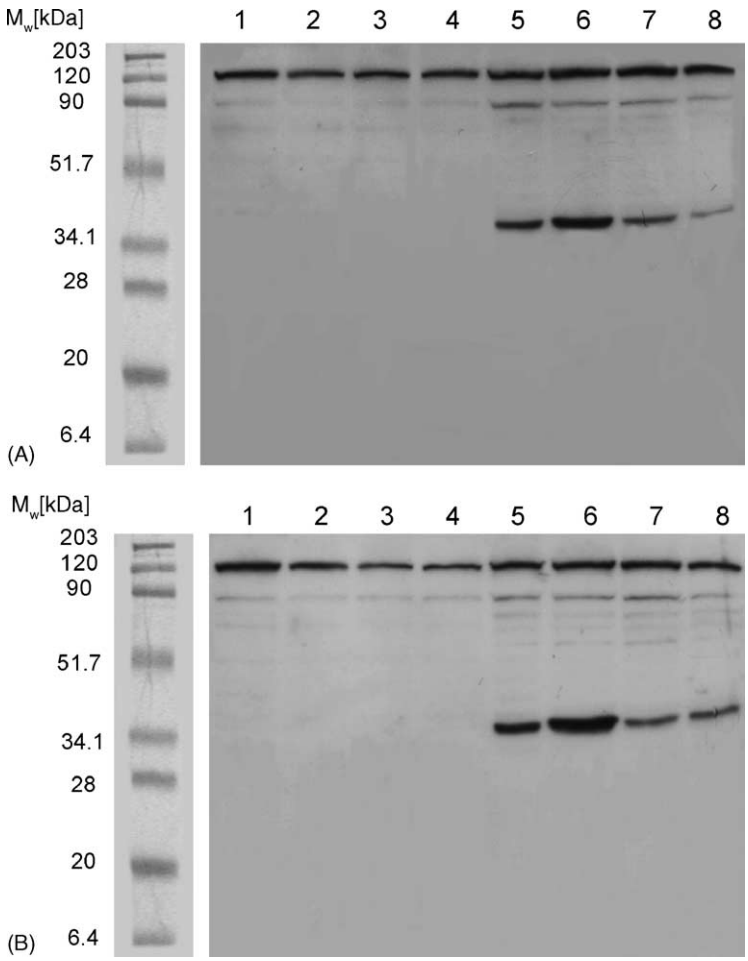

Fig. 4. Western blot analysis of tyrosine residues of proteins of DPI-treated endothelial cells. (A) Twelve hour after incubation with DPI; (B) 24 h after incubation with DPI. Lanes are as follows: (1) control HUVEC; (2–4) HUVEC treated with: 10 μM, 50 μM and 100 μM DPI; (5) control HUVEC-ST; (6–8) HUVEC-ST treated with: 10 μM, 50 μM and 100 μM DPI, respectively. Results are representative of three separate experiments.

nucleic acids (λ_{ex} = 500 nm, λ_{em} = 538 nm) and red fluorescence when dye is bound to single-stranded nucleic acids (λ_{ex} = 485 nm, λ_{em} = 620 nm). Apoptotic cells stained by acridine orange show reduced green fluorescence and enhanced red fluorescence in comparison to normal cells.

Endothelial cells were seeded onto gelatin-coated 96-well plates, and incubated for 24 h at 37 °C. After that time DPI was added for 1 h. When the inhibitor was removed, monolayers of the cells were rinsed with HBSS and complete medium was added. After 6 h, 12 h and 24 h nucleic acids staining with acridine orange was performed.

In the first step, cells were fixed by 15 min incubation with 1% formaldehyde in PBS, containing Ca^{2+} and Mg^{2+} ions at 4 °C. After that time formaldehyde was removed, cells were rinsed with PBS, containing Ca^{2+} and Mg^{2+}, cold 70% ethanol was added, and incubation was continued for next 3 h. In the next step, after ethanol removal, RNase (200 μg/ml) was added for eliminating fluorescence resulting from the dye bound to RNA. The samples were incubated for 30 min at 37 °C. Then the solution of RNase was removed, 0.1 M HCl was added for 45 s, and finally, the staining solution (6 μg/ml acridine orange in

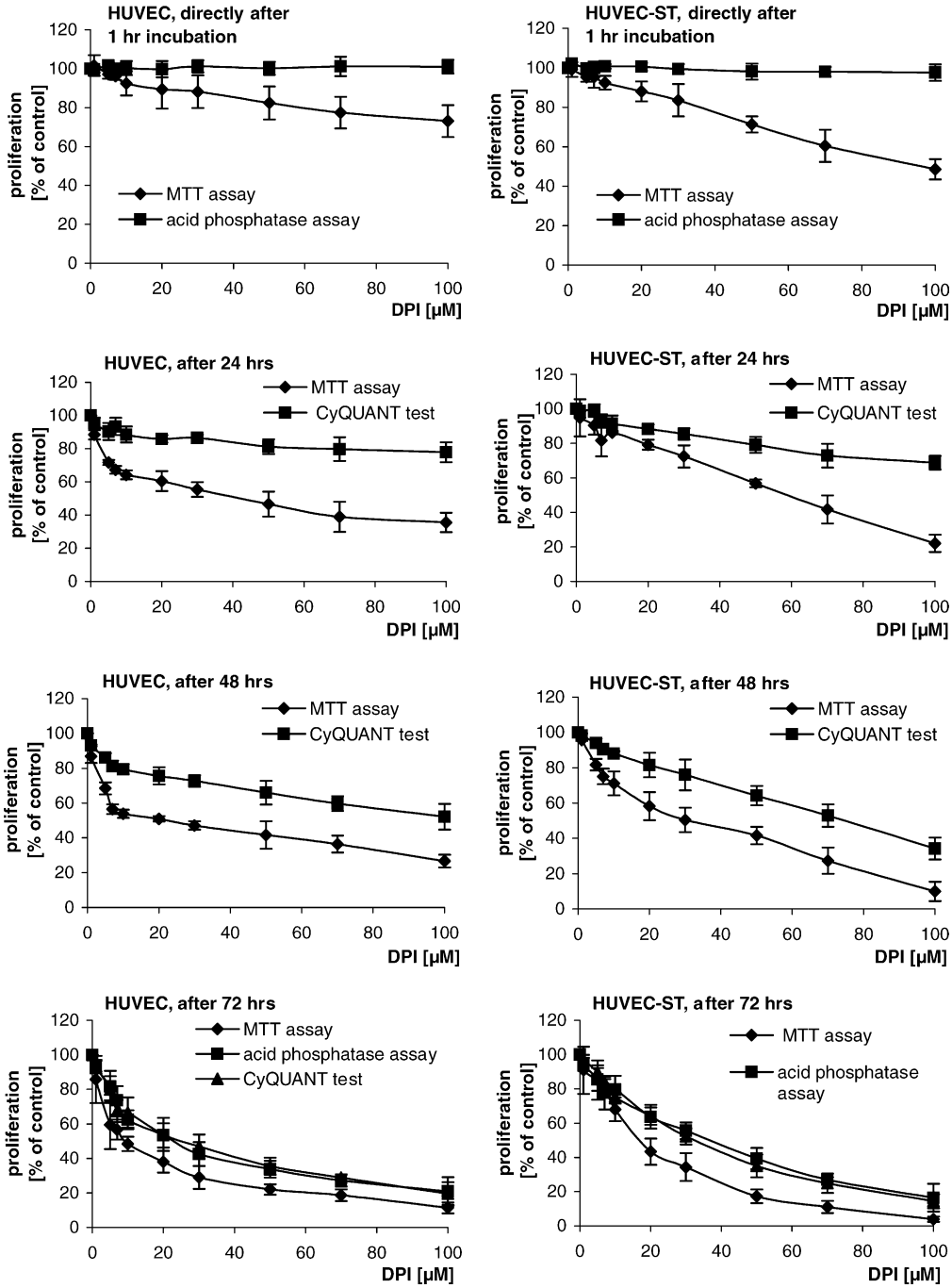

Fig. 5. Effect of DPI treatment on the viability of HUVEC and HUVEC-ST estimated with the MTT assay, acid phosphatase assay and the CyQuant test. Results are presented as mean ± S.D. of four independent experiments.

0.1 M citric acid and 0.2 M Na_2HPO_4, pH 6.0) was added for 5 min. Last incubation with the dye was performed at room temperature in the dark. Fluorescence intensity was monitored in a Fluoroskan Ascent FL microplate reader.

2.10. Hoechst 33258/PI staining

Apoptotic/necrotic cell death was analyzed by Hoechst 33258/propidium iodide staining. Endothelial cells were plated onto chambered cover glasses (1×10^4 cells per well). After 24 h, medium was removed and fresh DPI-containing medium, at concentrations of 10 μM, 50 μM, 100 μM, was added for 1 h. Then monolayers of cells were rinsed by HBSS, and incubation at 37 °C was continued in complete culture medium by 12 h, 24 h, and 48 h. Cells with condensed and fragmented nuclei, typical morphologic changes of apoptosis, were distinguishable from intact nuclei and red necrotic cells under Nikon Optiphot-2 fluorescence microscope.

3. Results

3.1. ROS/RNS production

Measurements with both H_2DCF-DA and dHR123 showed an apparent DPI-concentration dependent increase of ROS production when DPI was present in the cell samples during the measurements, practically identical in HUVEC and in the HUVEC-ST cell line (Fig. 1). However, an inhibition of ROS and RNS production was found when the measurements were made after washing off DPI. The inhibition of ROS (Fig. 2) and RNS production (not shown) was dose- and time-dependent and more expressed in HUVEC-ST cells than in HUVEC. This disparity of results suggests that the augmented ROS production observed in the presence of excess DPI is an artifact due to a direct reaction between DPI and the fluorogenic probe.

Indeed, incubation of DPI with dHR123 alone in the buffer, in the absence of cells, lead to DPI-concentration dependent increase in the fluorescence of the oxidation product of the probe. The presence of glutathione in the incubation medium did not prevent and even augmented the rise in fluorescence (Fig. 3), which suggests that the same effect can occur inside the cells.

3.2. Protein nitration

Electrophoresis and immunoblotting of endothelial cell proteins with anti-tyrosine antibodies revealed preferential nitration of several protein bands of molecular weight in the range of 34 kDa–120 kDa. The level of nitration decreased in cells treated with high concentrations of DPI as it could be expected for an agent inhibiting enzymes producing both superoxide and nitric oxide. However, a transient increase in the level of nitration was noted for HUVEC at the DPI concentration of 10 μM (Fig. 4), perhaps, indicating optimization of the ratio of superoxide: nitric oxide fluxes necessary for effective nitration [29] due to differential inhibition of superoxide-producing enzymes and nitric oxide synthase at this concentration.

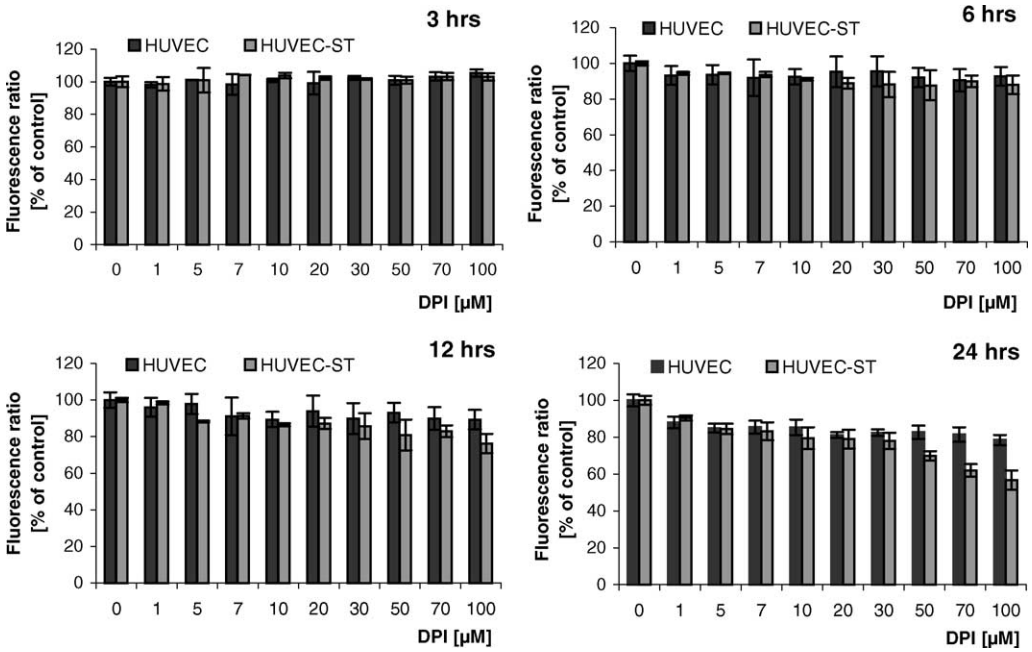

Fig. 6. Apoptosis of DPI-treated HUVEC and HUVEC-ST estimated by cell staining with acridine orange 3 h, 6 h, 12 h and 24 h after the treatment with DPI. Results are presented as a ratio of fluorescence of acridine orange bound to double-stranded DNA to that of acridine orange bound to single-stranded DNA and represent mean ± S.D. of three independent experiments. Fluorescence ratio of control cells was assumed as 100%.

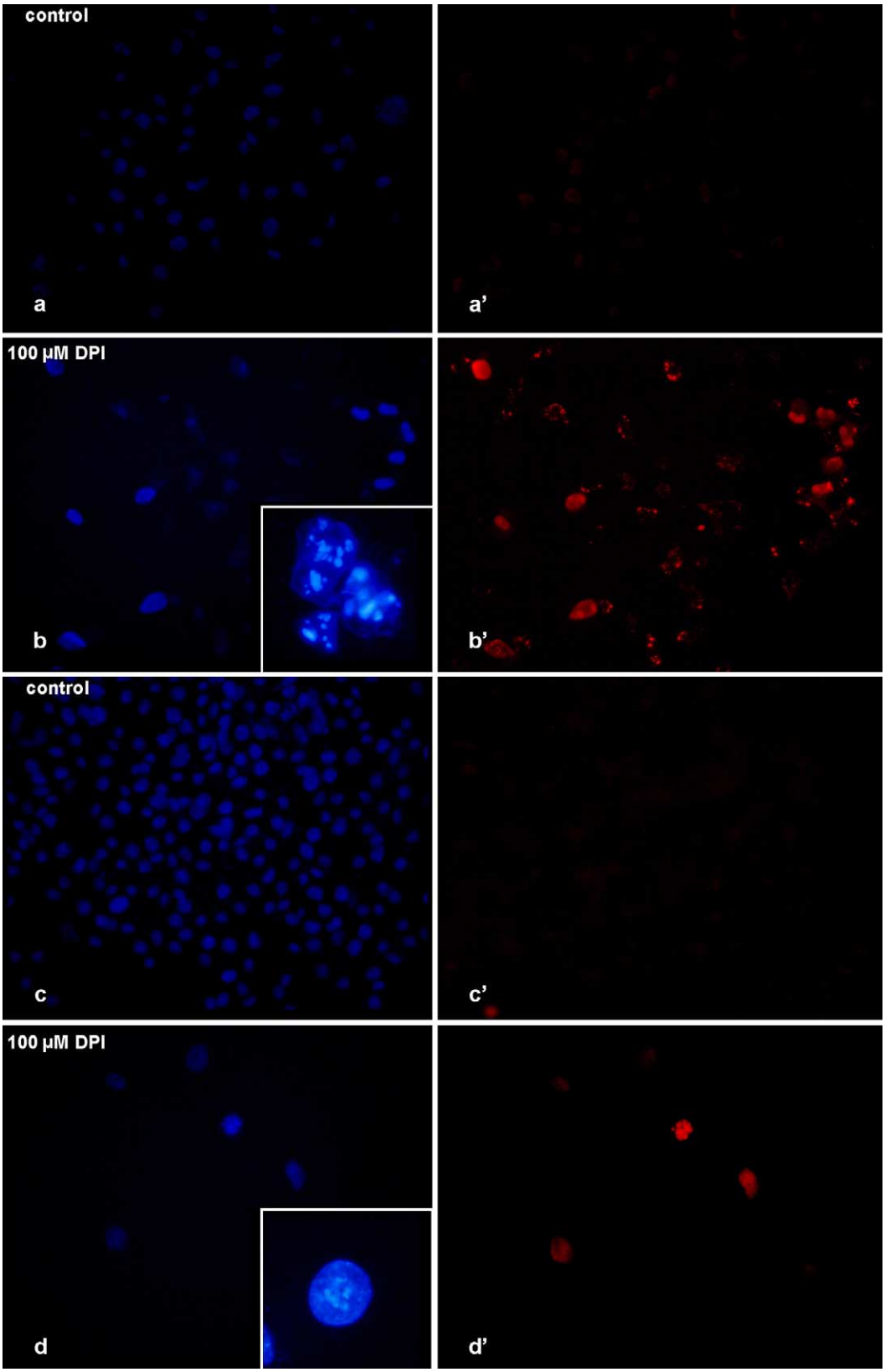

Fig. 7. DPI-induced cell death in human endothelial cells HUVEC and HUVEC-ST. Cells were stained by 15-min incubation in a solution of 7.5 μg/ml Hoechst 33258 and 2.5 μg/ml propidium iodide in PBS–Mg^{2+}/Ca^{2+}. The presented panel of pictures is representative for three independent experiments. Magnification: 400×.

3.3. Cell viability

We compared the quantification of DPI-treated cells using three assays: one based on MTT reduction, another based on estimation of acid phosphatase activity in cell lysates, and the last one, the CyQuant assay, based on estimation of total nucleic acid amount. A striking observation was the considerable decrease of apparent cell viability estimated with MTT, already 1 h after the end of incubation with DPI. This effect was manifested especially in HUVEC-ST cells which, according to the results of the MTT assay, lost their viability in 50% at 100 μM DPI, while showing 100% viability in the acid phosphatase test. Longer incubation time after exposure to DPI brought about a progressive viability loss of the cells but always the viability estimated with MTT was considerably lower than that probed by acid phosphatase test. The measurements made 72 h after exposure demonstrated consistency of results of cell viability estimation by the acid phosphatase and CyQuant assay and significantly lower values obtained with MTT (Fig. 5).

3.4. Mitochondrial potential

DPI treatment brought about a dose-dependent drop in the mitochondrial potential observed immediately after exposure, with some recovery after 3 h, more expressed in HUVEC-ST. The dimer-to-monomer fluorescence ratio decreased by about 20%–30% for HUVEC and by 30%–40% in HUVEC-ST for all DPI concentrations in the range of 5 μM–100 μM (not shown). This deterioration of the energy status of the mitochondria may be linked to the decreased potency of the cells to reduce MTT.

3.5. Apoptosis

ROS have been shown to play an important role in the induction of apoptosis. We found that inhibition of reactive oxygen/nitrogen species production by diphenyleneiodonium results in progressive dose-dependent apoptosis in DPI-treated cells, again more visible in HUVEC-ST. After 24 h, about 10% of both HUVEC and HUVEC-ST were apoptotic after treatment with the lowest DPI dose applied (1 μM), while close to 20% and 40% of HUVEC and HUVEC-ST, respectively, were apoptotic after treatment with 100 μM DPI (Fig. 6).

Morphological changes DPI-treated (10 μM, 50 μM and 100 μM) endothelial cells were determined by fluorescence microscopy. We performed Hoechst 33258/propidium iodide staining to distinguish apoptotic from necrotic cells (Fig. 7). Endothelial cells, HUVEC as well as immortalized cell line HUVEC-ST, in the negative control exhibited a round shape, clear edge and homogenous staining. However, 1 h exposure cells to diphenyleneiodonium turned out to be sufficient to induce apoptosis. After 12 h cells began to show morphologic features of apoptosis, such as cell shrinkage and nuclear chromatin condensation, which was visible most easily after 48 h (Fig. 8).

4. Discussion

It has been reported that DPI enhances the production of superoxide in HL-60 cells by inducing apoptosis, and thus, augmentation of ROS release [22]. The mechanism of the apoptotic action of DPI involves impairment of cyclin B1 accumulation and cell arrest at G(2) [30]. Our results confirm the apoptotic action of DPI but not the augmentation of ROS production. We observed increased production of ROS in cells treated with DPI but ascribe this phenomenon to the direct interaction of fluorescent probes with this compound. Such an effect was observed in the absence of DPI if cells and was enhanced by glutathione. After washing off the excess of DPI we observed diminished production of ROS by the treated

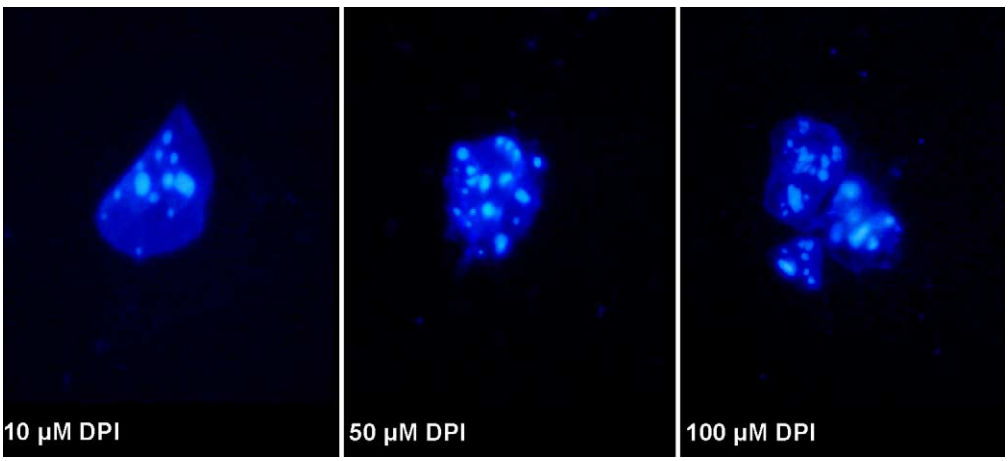

Fig. 8. Effect of DPI on the induction of apoptosis in HUVEC. Morphology of cells was examined under a microscope with Hoechst 33258, 48 h after the incubation with DPI. Magnification: 1400×.

cells, as it could be expected taking into account the role of flavoenzymes in the cellular production of ROS. A similar phenomenon has been reported for respiratory chain inhibitors, antimycin and 2-heptyl-4-hydroxy-quinoline-*N*-oxide [31]. This finding is a next indication for the care which should be taken when interpreting results of measurements of ROS formation with fluorescent probes It should be mentioned that, apart from xenobiotics, also endogenous cell components may directly oxidize H_2DCF-DA without the involvement of ROS, as demonstrated for cytochrome c [32,33].

We confirmed the proapoptotic action of DPI in the whole range of concentrations applied. It is noteworthy that even the lowest concentration studied (1 μM) induced significant increase in apoptosis, suggesting the need of care with long-term cellular experiments involving this compound. Numerous studies have established an important role of ROS in cell proliferation, the rule of thumb being that low doses of ROS are necessary for cell proliferation, while high doses are inhibitory and apoptotic. In endothelial cells, ROS serve as second messengers to activate multiple intracellular proteins and enzymes, including the epidermal growth factor receptor, c-Src, p38 mitogen-activated protein kinase, Ras, and Akt/protein kinase B [4,5,34,35]. It seems that the apoptosis induced by DPI observed by us is due to the disturbance of the generation of ROS necessary for cell proliferation.

A striking phenomenon observed by us, was the strong inhibition of MTT reduction by endothelial cells induced by DPI treatment. The MTT assay is considered to be a measure of activity of mitochondrial oxidoreductases but, in fact, the precise localization of the enzymes responsible for reduction of MTT to the formazan is still unknown [36]. We assessed the impact of diphenyleneiodonium on the activity of oxidoreductases involved in MTT reduction and found significant inhibition of MTT reduction directly after the treatment when cell viability was not compromised when studied by other means. Later on, the viability of cells treated with DPI decreased but still, the values shown with DPI did not correspond to those obtained by acid phosphatase test and CyQuant. Inhibition of MTT reduction was better visible in HUVEC-ST than in the primary cells. The differences between the cell lines probably are due to their different rates of metabolism. Our results show that DPI disrupts MTT test, and MTT should not be applied to estimation of cell viability in case of DPI-treated cells. A reverse effect, viz. increased MTT reduction and apparent increase in cell viability has been reported for mouse mammary tumor (EMT6) and Chinese hamster ovary (CHO) cells treated with dicumarol [37]. Another complication studied recently is the increased extrusion (and thus decreased reduction) of MTT by cells overexpressing multidrug transporters [38]. The present finding is a next indication for the need of a careful interpretation of results of MTT reduction by cells treated with agents affecting the function of mitochondria.

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