Original Article

The Expression of NADPH Oxidases and Production of Reactive Oxygen Species by Human Lung Adenocarcinoma Epithelial Cell Line A549

(lung epithelial cells / reactive oxygen species / fluorometry / chemiluminescence)

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Abstract. Controlled production of reactive oxygen species (ROS) by NADPH oxidases in non-phagocytic cells has recently been suggested to participate in the regulation of cellular functions. Due to the role of ROS in control of cellular functions, precise and accurate detection of ROS is of essential importance. However, various methodological approaches currently used for ROS determination vary in sensitivity, specificity, as well as in requirements for specialized equipment. In this study, human lung epithelial cell line A549 was screened for expression of NADPH oxidases NOX1, NOX2, NOX4, NOX5, DUOX1 and DUOX2 by quantitative RT-PCR. Fluorometric, colorimetric, and chemiluminometric methods were applied to determine ROS production. A549 cells were found to significantly express NOX1, NOX2, DUOX1 and DUOX2. ROS production by A549 cells was detected with fluorometric probes 2',7'-dichlorofluorescein-diacetate, dihydroethidium, and amplex red or colorimetric probe nitrobluetetrazolium. The production of ROS detected by these probes was partially reduced by NADPH oxidase inhibitor diphenyleneiodonium. The inhibitory effect of diphenyleneiodonium was the most significant regarding amplex red detection of phorbol myristate acetate-activated ROS production. In contrast to other probes, neither cytochrome c colorimetric determination nor luminol- and L-012-amplified chemiluminescence, regardless of the addition of horseradish peroxidase, exerted sufficient sensitivity to detect ROS production by A549. The results revealed differences among methods used for ROS formation measurement by human lung epithelial cell line A549 and highlighted the sensitivity of fluorometric determination for this purpose.

Introduction

Production of reactive oxygen species (ROS) by eukaryotic cells was believed to be connected with formation of ROS as toxic by-products of various cellular metabolic processes. Deliberate production of ROS by NADPH oxidase was restricted to phagocytic cells as a component of antimicrobial host defence. Recently, the importance of controlled ROS production by non-phagocytic cells, including lung epithelial cells, in the regulation of physiological functions was suggested in a number of studies (Geiszt et al., 2003; Fischer et al., 2007; van der Vliet, 2008). Particular attention has been focused on homologues of NADPH oxidase (NOX), enzymes producing superoxide in response to specific stimuli that have been shown to be expressed in various cell types other than phagocytes (van der Vliet, 2008). The NOX family also includes dual oxidases (DUOX) that contain an additional peroxidase domain and are suggested to have particular importance in epithelial lining cells.

Due to the role of ROS in a wide range of physiological and pathogenic events, it is important to accurately detect and quantify ROS production. Detection of ROS production by non-phagocytic cells, because of their low steady-state concentrations and short half-life, requires more sensitive and elaborate methods compared to the detection of ROS produced by professional phago-

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Abbreviations: CaI – calcium ionophore; CL – chemiluminescence; DCFH-DA – 2',7'-dichlorofluorescein diacetate; DHE – dihydroethidium; DPI – diphenyleneiodonium; DUOX – dual oxidases; HBSS – Hank's Buffered Salt Solution; HRP – horseradish peroxidase; NBT – nitrobluetetrazolium chloride; NOX – NADPH oxidase; PMA – phorbol myristate acetate; RLU – relative light units; ROS – reactive oxygen species.

cytes (Tarpey and Fridovich, 2001). Currently, the most frequently used methodological approaches to ROS detection include fluorometric, colorimetric, and chemiluminometric (CL) determinations (Gallova et al., 2004; Pavelkova and Kubala, 2004; Bartosz, 2006; Freitas et al., 2009). These approaches do not require the laborious preparation of samples and specialized, expensive instruments compared to other methodological approaches, such as electron paramagnetic resonance. However, the sensitivity of these methods for the determination of ROS production by non-phagocytic cells varies.

The most frequently used colorimetric determinations are based on reduction of cytochrome c and nitrotetrazolium blue chloride (NBT). Membrane non-permeable ferricytochrome c molecules are reduced to ferrocytochrome c by reception of an electron from superoxide; the change in absorbance is then determined (Freitas et al., 2009). NBT, a yellow water-soluble compound, is reduced by superoxide to a dark-blue, insoluble formazan product. Formazan is trapped within the cell but can be released by solubilization in a solvent solution and then quantified by measuring the absorbance of the solution (Freitas et al., 2009).

Another methodological approach to ROS determination is employment of a wide range of fluorescent probes. Currently, the most frequently used fluorescent probes are dihydroethidium (DHE), 2',7'-dichlorofluorescein-diacetate (DCFH-DA), and 10-acetyl-3,7-dihydroxy-phenoxazine (amplex red) (Zhou et al., 1997; Tarpey and Fridovich, 2001; Kubala et al., 2002; Bartosz, 2006; Freitas et al., 2009). DHE is a cell-permeant compound that can undergo two-electron oxidation to form DNA-binding fluorophore ethidium bromide. DCFH-DA is a compound readily diffusing into cells. It can be hydrolysed by intracellular esterases to non-fluorescent 2',7'-dichlorofluorescein. In the presence of ROS the probe is oxidized to a highly fluorescent compound. Amplex red is a colourless and non-fluorescent molecule able to be oxidized to highly fluorescent resorufin (Zhou et al., 1997). For the oxidation of amplex red, the presence of active peroxidase is suggested to be crucial.

CL methods are based on the reaction of a luminophore, such as luminol, lucigenin or L-012, with ROS followed by generation of an unstable product with excited electrons (Nishinaka et al., 1993; Kubala et al., 2002; Daiber et al., 2004; Pavelkova and Kubala, 2004; Freitas et al., 2009). When the excited electrons return to their ground state, visible light is emitted. The key advantage of CL determination is not only sensitivity, but also a high range of CL signal detection.

The aim of this study was to demonstrate the expression of different members of the NADPH oxidase family in human lung epithelial cells and to compare the sensitivity of fluorometric, spectrophotometric, and CL methodological approaches to the determination of ROS in these cells. The results underline the advantages as well as limitations of the application of these methods.

Material and Methods

Cell culture

Human lung carcinoma cell line A549 from The American Type Culture Collection (Manassas, VA) was maintained in Dulbecco's Eagle medium (PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 10% foetal bovine serum (PAN-Biotech) and 0.045 mg/l gentamycin (Sigma-Aldrich, St. Louis, MO) at 37 °C in a humidified atmosphere with 5% CO_2 (Krejcova et al., 2009). For the experiments, cells were detached by trypsinization, centrifuged (250 *g* for 10 min at 4 °C) and resuspended in Hank's Balanced Salt Solution (HBSS) at the concentration 3×10^6 cells/ml. Human Umbilical Vein Endothelial Cells (HUVEC, European Collection of Cell Cultures, Salisbury, UK) were cultivated in Endothelial Basal Medium (Lonza, Allendale, NJ) (Baldus et al., 2006).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from A549 and HUVEC cells using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH), and reverse transcription was performed with 1 μg RNA, according to the manufacturer's instructions (DyNAmo™ cDNA Synthesis Kit, Finnzymes Oy, Espoo, Finland) (Dvorakova et al., 2008). The HUVEC cell line was used to confirm the functionality and specificity of the primers to detect NOX4 and NOX5. qRT-PCR was carried out using FastStart TaqMan Probe Master mix (Roche, Mannheim, Germany), according to the manufacturer's instructions.

The following primers and probes were designed using Universal Probe Library (Roche): human NOX1: forward, 5'-aag gat cct ccg gtt tta cc-3'; reverse, 5'-ttt gga tgg gtg cat aac aa-3'; probe # 3; human NOX2: forward, 5'-ggt ttt ggc gat ctc aac ag-3'; reverse, 5'-cga tgg ttt tga aag ggt ga-3'; probe # 9; human NOX4: forward, 5'-tgg tgt tac tat ctg tat ttt ctc agg-3'; reverse, 5'-agt tga ggg cat tca cca ga-3'; probe # 5; human NOX5: forward, 5'-cgt ctg tgc cgg ctt atc-3'; reverse, 5'-cca att cca gat aca aca tga ctg-3'; probe # 64; human DUOX1: forward, 5'-tgt gcc aga tac cca aag c-3'; reverse, 5'-tga cac ctc aaa ctt atg cca ct-3'; probe # 49; human DUOX2: forward, 5'-tgt atg acc tgg atg aga atg g-3'; reverse, 5'-gtt gga gat ctc gat gaa gga-3'; probe # 55; and human GAPDH (Universal Probe Set, Human Reference Gene Assays, Roche). qRT-PCR was performed in the Applied Biosystems 7300 RT-PCR System (Applied Biosystems, Carlsbad, CA). The reaction consisted of initial denaturation at 95 °C for 15 min, 40 cycles with denaturation at 94 °C for 10 s, annealing at 55 °C for 30 s, and synthesis at 72 °C for 30 s. Relative NOX and DUOX mRNA amounts were standardized against the amount of GAPDH mRNA and expressed as ΔC_t. qRT-PCR products of amplification were separated by 1.7 % agarose gel electrophoresis in Tris buffer and visualized by ethidium bromide staining.

Fluorometry assays

A549 cells (0.3×10^6 per well) were incubated with 24 μM dihydroethidium; 2,7-diamino-10-ethyl-9--phenyl-9,10-dihydrophenanthridine (DHE), 67 μM 2',7'-dichlorofluorescein diacetate (DCFH-DA), or a combination of 3 μM amplex red (10-acetyl-3,7-dihydroxyphenoxazine) (Invitrogen, Carlsbad, CA) with 0.1 U horseradish peroxidase (HRP) (Sigma-Aldrich) in a total volume of 100 μl in 96-well microplates for 1 h at 37 °C. Selected samples were treated by one of the activators 1.16 μM phorbol myristate acetate (PMA) or 9.55 μM calcium inophore A23187 (CaI) and/or by inhibitor 10 μM diphenyleneiodonium (DPI). Fluorescence was measured by microplate reader Infinite M200 (Tecan Trading AC, Männedorf, Switzerland) with an excitation maximum at 480 nm (DCFH-DA), 563 nm (amplex red), 490 nm (DHE) and emission maximum at 565 nm (DCFH-DA), 587 nm (amplex red), 600 nm (DHE) after 1 h, 2 h, 3 h and 4 h. The wavelengths were optimized to get the best signal-to-noise ratio with the employed fluorometer (data not shown). Samples containing the probe and other components without cells were determined as the background values.

Spectrophotometry assays

A549 cells (0.3×10^6 per well) were incubated with 0.48 mM nitrobluetetrazolium chloride (NBT, 2,2-bis(4--nitrophenyl)-5,5-diphenyl-3,3-(3,3-dimethoxy-4,4--diphenylene) (Sigma-Aldrich) in a total volume of 100 μl in 96-well microplates for 1 h at 37 °C. Selected samples were treated by one of the activators 0.81 μM PMA or 9.55 μM CaI and/or by 10 μM DPI. The reaction was stopped, and intracellularly accumulated formazan was solubilized by 10 % Triton X-100 in PBS. The cytochrome c (Sigma-Aldrich) determination was performed with cells (0.313×10^6, 0.156×10^6, 0.078×10^6, 0.039×10^6 per well) incubated with 10 μM cytochrome c in a total volume of 100 μl in 96-well microplates for 2 h at 37 °C and/or activated by 1.16 μM PMA or 9.55 μM CaI. The absorbance was quantified by microtitre plate reader SLT Rainbow (Tecan) at 570 nm (NBT) and 542 nm (cytochrome c). Samples containing the probe without cells were used to determine the background values.

CL assays

The reaction mixture consisted of cells (0.125×10^6, 0.062×10^6, 0.031×10^6 cells per well), one of the concentrations of HRP (10 U, 2.5 U, 0.625 U, 0.156 U, 0.039 U, 0.01 U per well) or HBSS, one of the luminophores – 1 mM luminol (5-amino-2,3-dihydro-1,4--phthalazinedione) (Sigma-Aldrich) or 40 μM L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine--1,4(2H,3H)dione) (Wako Pure Chemical Industries, Osaka, Japan), and in selected samples one of the activators 0.81 μM PMA or 9.55 μM CaI. The CL emission was measured with an Orion microplate luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany). Light emission, expressed as relative light units (RLU), was recorded continuously at 37 °C for 1 h (Gallova et al., 2004). Samples containing the reaction mixture without cells were used to determine the background values.

Statistical analysis

All the data are expressed as the means (±SEM) of at least three independent experiments. Statistical analyses were performed with STATISTICA for Windows 7.0 (StatSoft, Inc.,Tulsa, OK).

Results

The expression of selected members of the NADPH oxidase family in A549 cells was evaluated by qRT-PCR. As shown in Fig. 1, significant expression of NOX1, NOX2, DUOX1 and DUOX2 was detected. In contrast, the expression of NOX4 and NOX5 in A549 cells was under the detection limit. The HUVEC cell line was employed to confirm the functionality of the primers and probes to detect specific NOX4 and NOX5. These cells expressed mRNA for NOX4 ($\Delta C_t = 6.1$) and NOX5 ($\Delta C_t = 12.2$). Visualization by agarose gel electrophoresis approved specific products for each qRT-PCR reaction (Fig. 2).

Fluorometric determinations revealed significant DCFH-DA and DHE fluorescence in unstimulated A549 cells, compared to the background (Fig. 3A and 3B). The activation of the cells with CaI or PMA did not increase the fluorescence of these probes. In contrast, fluorescence of amplex red was not significant, compared to the background in non-activated and CaI-activated cells. Interestingly, the activation of A549 cells with PMA induced significant oxidation of amplex red (Fig. 3C). To determine the involvement of NADPH oxidase in generation of ROS detected by these methods, DPI (10 μM),

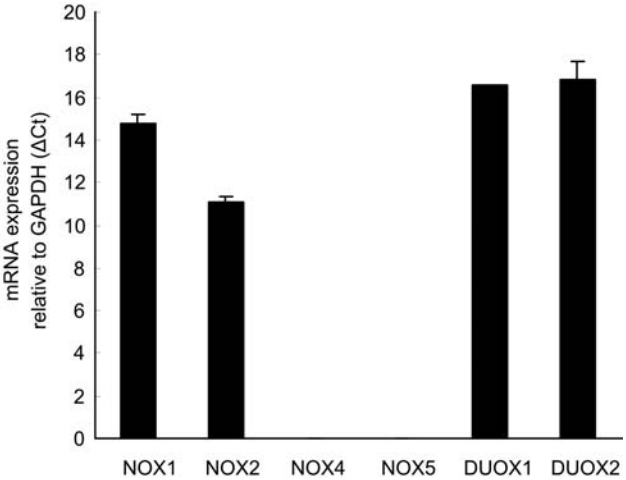

Fig. 1. Expression of NOX1, NOX2, NOX4, NOX5, DUOX1 and DUOX2 in A549 cells determined by qRT-PCR. Data are expressed as levels of mRNA for particular NADPH oxidase relative to housekeeping gene *GAPDH*. Data present the mean ± SEM (N = 4).

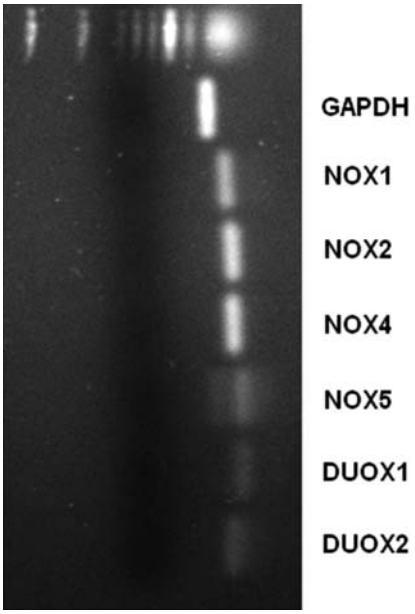

Fig. 2. Agarose gel electrophoresis of qRT-PCR products for GAPDH, NOX1, NOX2, NOX4, NOX5, DUOX1, and DUOX2. GAPDH, NOX1, NOX2, DUOX1, and DUOX2 are products of qRT-PCR reaction from A549 cells and NOX4 and NOX5 are products of qRT-PCR reaction from HUVEC cells.

an inhibitor of NADPH oxidases, was employed. The level of ROS production was decreased by incubation with DPI (10 μM for 1 h) to 87 ± 3 % (DHE), 86 ± 3 % (DCFH-DA), 87 ± 10 % (amplex red) of the signal obtained with cells without DPI treatment (N = 3). Interestingly, the PMA-stimulated ROS production by A549 cells in the presence of DPI detected by amplex red revealed a considerably higher ratio of inhibition 74 ± 6 % of PMA-activated cells without DPI treatment (N = 3). The DPI in aforementioned concentration did not reveal any significant effects on cell viability in the period of these experiments (data not shown).

Colorimetric determinations of the generation of superoxide anion by NBT indicated that both unstimulated and CaI- or PMA-activated cells showed significant formation of reduced NBT over time (Fig. 4). However, the activation of cells with CaI or PMA did not bring a significant increase in reduced NBT formation in comparison with the unstimulated cells. In the case of NBT determination, DPI reduced absorbance to 87 ± 10 % of the signal obtained with cells without DPI treatment (N = 3).

Generation of superoxide anion was also quantified by cytochrome c; however, there was no significant increase in the absorbance. The absorbance of background samples containing cytochrome c without cells was even higher than the absorbance of samples with cytochrome c and different concentrations of cells (data not shown).

CL determinations employing luminol- and L-012-enhanced CL alone or in combination with HRP did not show any significant signal, either in non-activated, CaI- and PMA-activated cells compared to the background (data not shown).

Discussion

In this study, the expression of NOX1, NOX2, DUOX1 and DUOX2 was observed in A549 cells. In contrast, the expression of NOX4 and NOX5 enzymes was not detected. The ROS production in A549 cells could be detected with fluorometric probes DCFH-DA, DHE, and amplex red and colorimetric probe NBT. An inhibitory effect of DPI on ROS production by these probes was observed. Surprisingly, neither the CL probes luminol and L-012 alone or in combination with various concentrations of HRP nor cytochrome c revealed sufficient sensitivity to detect ROS produced by A549.

Studies over the last decades have documented significant generation of NADPH oxidase-dependent ROS in a variety of non-phagocytic cells, including airway epithelial cells (van der Vliet, 2008). Similar to our study, NOX1 expression (Malec et al., 2010), NOX2 expression (Fink et al., 2008) and DUOX2 expression was also found in A549 by other authors (Fink et al., 2008; Luxen et al., 2008; Pacquelet et al., 2008). However, these authors did not detect the expression of DUOX1 (Fink et al., 2008; Pacquelet et al., 2008), or the expression of DUOX1 was only detectable in cells treated by an inhibitor of cell proliferation (Luxen et al., 2008). Thus, confluence of the cell monolayer and the proliferation rate of A549 cells may be suggested to affect DUOX1 expression. Further, some authors also found, in contrast to our study, the expression of NOX4 (Goyal et al., 2005) in A549 cells. These data in general confirmed the expression of NADPH oxidases in A549 cells. The observed discrepancies could be due to variations in the differentiation status of this cell line and proliferation rate in particular experiments of various authors.

Fluorescent probes DHE, used for the measurement of intracellular superoxide production, and DCFH-DA, used for the measurement of a wide range of ROS (Tarpey and Fridovich, 2001; Bartosz, 2006; Freitas et al., 2009), revealed significant fluorescence in A549 cells compared to the background; however, the activation of the cells did not increase this fluorescence signal. Similar results were obtained with the use of NBT for the measurement of intracellular superoxide production. In contrast, the fluorescent probe amplex red, used for the measurement of extracellular hydrogen peroxide production (Zhou et al., 1997), revealed significant fluorescence, compared to the background, only in PMA-stimulated A549 cells. The lack of cell response to stimulation by CaI in our experiments could be caused by disturbance of calcium-dependent intracellular signalling pathways due to harvesting of cells using EDTA/trypsin. However, the release of cells and formation of single-cell suspension is an inseparable part of the method. Other methodological approaches lacking the harvest of adherent cells suffer from other significant problems, such as correction to equal number of cells per sample.

To confirm the involvement of NADPH oxidase in ROS generation, the effect of DPI, used as an inhibitor

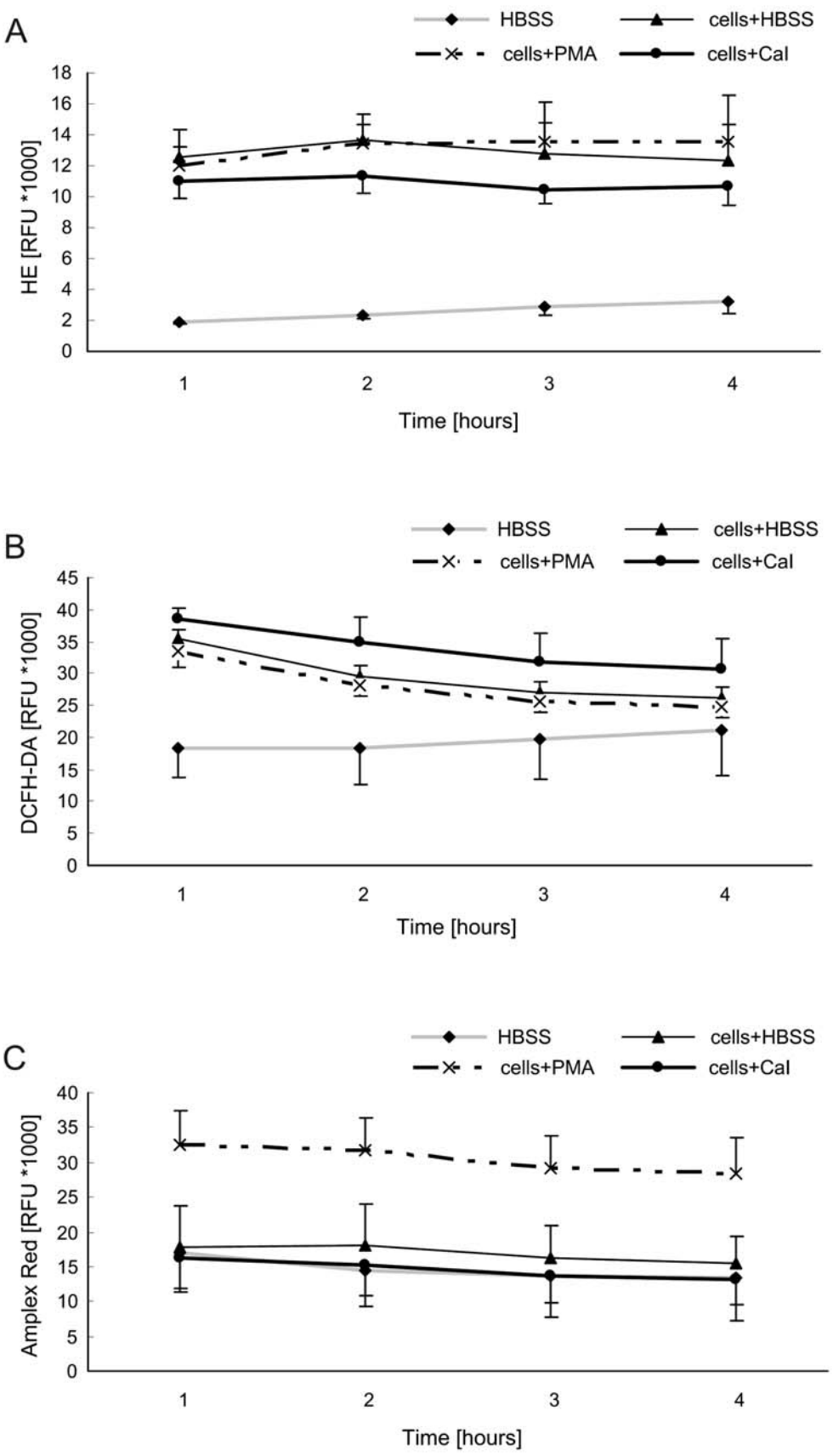

Fig. 3. Fluorometric determination of ROS production in A549 cells using DHE (A), DCFH-DA (B) or amplex red (C). A549 cells were stimulated by activators CaI or PMA or incubated with HBSS in the presence of DHE, DCFH-DA or amplex red. Fluorescence was measured every hour. Samples without cells were used as a background. Data present the mean ± SEM (N = 4).

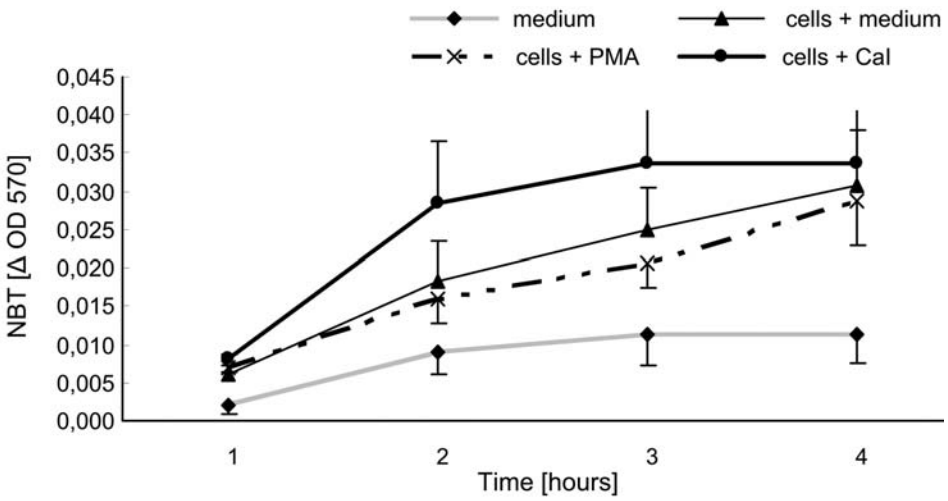

Fig. 4. Colorimetric determination of ROS production in A549 cells based on reduction of NBT. A549 cells were stimulated by activators CaI or PMA or incubated with HBSS in the presence of NBT, and absorbance was measured every hour. Samples without cells were used as a background. Data present the mean ± SEM (N = 4).

of flavin-containing oxidase enzymes including NADPH oxidases, was tested (Serrander et al., 2007). It could be assumed that 10–15 % of DPI-inhibitable signal from fluorescent probes and NBT could originate from NADPH oxidase-dependent ROS production. However, DPI-mediated non-specific inhibition of other flavin-containing enzymes producing ROS cannot be excluded. The rest of the ROS production detected by these probes generated by sources insensitive to DPI could originate from the basic cellular metabolism, such as mitochondria or cytochromes P450. Interestingly, the DPI-mediated decrease of amplex red fluorescence signal in PMA-stimulated cells (around 25 %) suggests an increased contribution of DPI-inhibitable enzymes to ROS formation detected by this probe in stimulated cells. Our data revealed the expression of DUOX2 that is suggested to produce ROS extracellularly, particularly hydrogen peroxide, in response to lung epithelial cell stimulation (van der Vliet, 2008). Thus it could be assumed that amplex red, a probe originally proposed for hydrogen peroxide detection, can detect extracellularly produced ROS by DUOX enzymes in response to A549 cell stimulation by PMA.

Other authors have shown the possibility to determine increased levels of ROS by DCFH-DA and by DHE in vanadate-stimulated A549 cells (Zhang et al., 2001), increased levels of ROS by DCFH-DA in hypoxia-stimulated A549 cells (Jia et al., 2008), and increased levels of superoxide by DHE in cyclically stretched A549 cells (Chapman et al., 2005). Moreover, hydrogen peroxide generation was successfully determined in A549 cells by measuring the oxidation of homovanillic acid to its fluorescent derivative in the presence of HRP (Pacquelet et al., 2008). In agreement with our results these authors have suggested that the mechanism of ROS generation involves both NADPH oxidases and the mitochondrial electron transport chain.

The failure to determine any significant signal employing cytochrome c, used frequently to measure superoxide production, could be due to the low sensitivity of this colorimetric probe. Additionally, reduced cytochrome c can be re-oxidized by cytochrome oxidases and cellular peroxidases. Because of the apparent decrease of cytochrome c reduction in the presence of cells, these peroxidation reactions can overbalance the rate of superoxide formation (Tarpey and Fridovich, 2001). This effect could explain the fact that in our measurements the absorbance of cell-free samples (background) was higher than the absorbance of samples with cells. Therefore, this method is not applicable in the case when peroxidase activity is expected to be significantly higher compared to direct production of superoxide anion.

CL assays are a frequently used alternative to photometric assays. Nevertheless, when using non-leukocyte cells, the obtained light signals are relatively low. Possibly, due to low sensitivity, we did not demonstrate any production of ROS using luminol- or L-012-enhanced CL. The difference in sensitivity of the CL method compared to fluorescent probes could be explained by cumulative determination of ROS formation during the time frame of the particular measurement in the case of fluorometric methods compared to only split of second actual determination of CL signal at a particular time point. However, the failure to observe any significant signal with L-012 was surprising. L-012 was suggested to possess superior properties for the detection of NADPH oxidase-derived ROS in inflammatory cells as well as vascular tissue without undergoing redox cycling (Nishinaka et al., 1993; Daiber et al., 2004).

In conclusion, the data provided in this study suggest a stable level of intracellular ROS production in A549 detectable by DCFH-DA, DHE, and NBT. The activation of cells by PMA induces extracellular ROS production, detectable by amplex red in the presence of HRP.

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