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Hypoxia-Induced Modifications in Plasma Membranes and Lipid Microdomains in A549 Cells and Primary Human Alveolar Cells

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ABSTRACT

We evaluated the response to mild hypoxia exposure of A549 alveolar human cells and of a continuous alveolar cell line from human excised lungs (A30) exposed to 5% O_2 for 5 and 24 h. No signs of increased peroxidation and of early apoptosis were detected. After 24 h of hypoxia total cell proteins/DNA ratio decreased significantly by about 20%. Similarly, we found a decrease in membrane phospholipid and cholesterol content. The membrane fluidity assessed by fluorescence anisotropy measurements was unchanged. We also prepared the detergent resistant membrane fraction (DRM) to analyze the distribution of the two types of lipid microdomains, caveolae and lipid rafts. The DRM content of Cav-1, marker of caveolae, was decreased, while CD55, marker of lipid rafts, increased in both cell lines. Total content of these markers in the membranes was unchanged indicating remodelling of their distribution between detergent-resistant and detergent-soluble fraction of the cellular membrane. The changes in protein markers distribution did not imply changes in the corresponding mRNA, except in the case of Cav-1 for A30 line. In the latter case we found a parallel decrease in Cav-1 and in the corresponding mRNA. We conclude that an exposure to a mild degree of hypoxia triggers a significant remodelling of the lipid microdomains expression, confirming that they are highly dynamic structures providing a prompt signalling platform to changes of the pericellular microenvironment. J. Cell. Biochem. 105: 503–513, 2008.

KEY WORDS: ALVEOLAR CELLS; HYPOXIA; MEMBRANE; CAVEOLAE; LIPID RAFTS

The alveolar epithelium is normally exposed after birth to a mean alveolar oxygen pressure of 100 mm Hg, but alveolar hypoxia may occur in many physiological or pathological conditions, such as ascent to high altitude, alveolar hypoventilation, or pulmonary edema from heart failure or acute lung injury. We have recently studied the early in situ lung cellular response in anesthetized rabbits exposed to hypoxia (12% oxygen) for 3 and 5 h and we found considerable changes in lipid and protein composition of plasma membranes as well as a rearrangement of lipid microdomains expression [Botto et al., 2006]. This study suggested a prompt lung cellular response to hypoxia although we had no indications on which cell type was involved in the response. It appears conceivable to hypothesize that alveolar cells, representing a functional interface between the exterior and the interior compartments, might respond to hypoxia and this could be of relevance in the mechanisms of hypoxia tolerance. To substantiate this hypothesis, we evaluated the response of A549 alveolar human cells in culture, and of a continuous alveolar cell line from human excised lungs (named A30) to 5% oxygen providing a degree of hypoxia similar to that occurring in vivo with 12% oxygen exposure. We focused our study on the structure of plasma membranes and, in particular, of lipid microdomains. The consequence of exposure to hypoxia on alveolar cellular cultures focussed primarily on membrane ion transport systems [Mairbaurl et al., 1997; Wodopia et al., 2000], without paying attention to the chemical and physical perturbations of plasma membrane structure/composition, in particular as far as specific membrane sites involved in signalling transduction are concerned. The lipid microdomains are regions of cell membrane resulting from selective affinities between specific lipids and membrane proteins. In particular, microdomains are enriched in glycosphingolipids and cholesterol that act to compartmentalize membrane proteins performing different biochemical functions [Anderson, 1998; Oh and Schnitzer, 2001; Fielding and Fielding, 2003]. At present, two common types of

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microdomains have been proposed: caveolae, 70–80 nm flask-like microdomains stabilized by structural proteins (caveolin) [Anderson, 1998] and planar lipid rafts, enriched in glycosylphosphatidylinositol (GPI)-anchored proteins, and deficient in caveolin [Oh and Schnitzer, 2001]. Many evidences strongly suggest that caveolae and lipid rafts represent a heterogeneous population of specific signalling platforms playing different roles in cell biology [Sprenger et al., 2006; Allen et al., 2007; Parton and Simons, 2007]. They are considered highly dynamic entities subject to rapid changes [Sergey and Smart, 2002; Fielding and Fielding, 2003; Legler et al., 2003], a feature that appears important for a prompt signalling response. For this reason, we addressed the specific hypoxia-induced modification to the distribution on plasma membrane of the two types of lipid microdomains.

MATERIALS AND METHODS

CHEMICAL

The reagents used (analytical grade) and HPTLC plates (Kieselgel 60) were purchased from Merck GmbH (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin/streptomycin, CAPS, MES, PMSF, anti-protease, HRP-CTB were from Sigma Chem. Co. (Milano, Italy). Antibody against caveolin-1 (C2297) was from Transduction Labs (Lexington, KY, USA). Antibodies against γENaC (sc-21014), AQP5 (sc-9890), CD55 (sc-9156) and PKC (sc-80) were from Santa Cruz Biotechnology (CA). Antibody against actin (A2066) was from Sigma, antibody against HO-1 was from QED Bioscience Inc. (CA), antibody against GM130 (golgi marker), clone-35 was from Becton Dickinson and antibody against transferrin receptor was Zymed Laboratories Inc. (CA). All the material for the electrophoresis was from BioRad (Milano, Italy). Autoradiography films were from Amersham Pharmacia Biotech (Uppsala, Sweden).

CELL CULTURES

Two human cell lines were used: A549 cells (adenocarcinoma, American type culture collection) and continuous alveolar cell line (A30) obtained from lung specimens from patients who underwent lobectomy in the Thoracic Surgery Unit of S. Gerardo Hospital, Monza. These cells were isolated from the apparently healthy portion of the excised lung lobe using the methods of Dobbs et al. [1986], with modifications. A30 cells were fixed in 4% paraformaldehyde for 20 min at room temperature in Petri dishes, then scraped from the surface, collected in Eppendorf vial, spinned at 1,500 rpm for 10 min in order to obtain a pellet of cells, and post-fixed in Bouin liquid for 60 min. The cells were then dehydrated and included in paraffin. From the block, 3-μm thick paraffin sections were obtained and stained in a Benchmark XT Ventana automated immunostainer for cytokeratin pool (clone AE1-AE3-PCK26) using Ultraview universal DAB detection kit. Haematoxylin was used as nuclear counterstain. All the reagents were purchased from Ventana Medical System Inc. The positive control consisted in A549 cells, obtained from a line of lung carcinoma. The negative control was performed by omitting the primary antibody in the immunostaining procedure or using mouse monoclonal antibodies unrelated to an epithelial cell type (e.g. anti-CD3, smooth muscle specific actin-SMA).

Cells were grown in 100 mm dishes on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% penicillin/streptomycin and 1% glutamine. Cells were maintained in normoxia (21% O_2, 5% CO_2, 74% N_2 at 37°C) until they reached confluence (typically on 3 days after plating). For exposure to hypoxia, after replacing the culture medium, confluent monolayer were placed in a acrylic glass box that was flushed with sterile-filtered humidified gas of 5% O_2, 5% CO_2 in N_2 at 4 L/min for 20 min and then the glass box was sealed and kept at 37°C for 5 or 24 h. Control normoxic cells were maintained in 21% O_2, 5% CO_2, 74% N_2 humidified incubator for the same period of time. pH in culture medium measured at the end of exposure was not significantly different under normoxic and hypoxic conditions.

CELL VIABILITY

Viability of cells was assed by Trypan blue exclusion (0.4%, Sigma). Lactate dehydrogenase (LDH) released into the culture medium was determined in according to Omodeo-Sale' [Omodeo-Sale' et al., 1991]. Apoptosis test was performed by annexin V-FITC apoptosis detection kit (Medical &Biological Laboratories Co. Ltd.).

MEMBRANE-ENRICHED FRACTION (MEF)

At the end of the treatments, cells kept in control and hypoxic conditions were washed twice with PBS, then scraped in 2.5 ml of a solution containing 250 mM sucrose, 0.1 mM EDTA, 40 μg/ml of the protease inhibitor cocktail (aprotinin, chymostatin, leupeptin and antipapain) and butylate hydroxytoluene (BHT, 0.2 mM) in 1 mM potassium phosphate buffer, pH 7.4, and finally centrifuged (8000 g, 10 min). BHT is antioxidant [Botto et al., 2006; Coatrieuex et al., 2007] and therefore its use is aimed to maintain intact membrane structure. The pellets were homogenized and centrifuged (1,000*g* for 10 min) for three times in the same buffer, and the pooled supernatants centrifuged at 100,000*g* for 1 h. The pellets from the last centrifugation represent "membrane-enriched fraction" (MEF) referred to as MEFc (membranes of control cells), MEF5 and MEF24 (membranes of cells exposed for 5 or 24 h to hypoxia, respectively). MEF were prepared according to Preti et al. [1980] and included a fraction from endocellular membranes. The enrichment of γENaC, as membrane markers, respect to homogenate, was about threefold.

PREPARATION AND CHARACTERIZATION OF DETERGENT-RESISTANT MEMBRANE FRACTIONS (DRM)

Cells kept in control and exposed to hypoxia were washed twice, harvested in PBS solution and centrifuged. The pellets were used to prepare the detergent-resistant membrane fraction (DRM) [Palestini et al., 2003; Botto et al., 2004]. All the procedure was carried on ice to maintain the integrity of lipid rafts and in order to maintain a constant protein/detergent ratio, cell pellets, each containing about 2.5 mg of cellular proteins, were incubated in 2 ml of 1%Triton X-100 in 25 mM MES buffer, pH 6.5, containing 150 mM NaCl, 1 mM PMSF and 75 units/ml aprotinin (MBS buffer), for 20 min on ice. Finally, the 2 ml of cell lysate, were diluted with an equal volume of 80% (w/v) sucrose in MBS lacking Triton X-100 and placed at the

bottom of a tube where a discontinuous sucrose density gradient was created (40, 30, 5% sucrose, from bottom to up) in MBS lacking Triton X-100. After centrifugation at 250,000*g* for 18 h at 4°C with a SW-41 rotor (Beckman Instruments), 1 ml fractions were collected and submitted to further analysis. The detergent-resistant membrane fraction (DRM) corresponds to fraction 5 from the top while the high density-soluble fractions are 8–12 [Palestini et al., 2003; Botto et al., 2004].

PROTEIN ANALYSIS

Aliquots of MEFc, MEF5, MEF24 and all fractions collected from the gradient, were submitted to trichloroacetic acid precipitation. The pellets, washed with acetone, were suspended in water and the protein quantity determined by BCA method (SIGMA). Thereafter, 50 μg of MEFc, MEF5, MEF24 and 15 μg of proteins collected from the gradient, respectively, were loaded on SDS-PAGE (10%-polyacrylamide gel) and submitted to electrophoresis and Western blotting. With this method, proteins are allowed to run in absence of sucrose and/or detergent that hinder a good separation of proteins in the electrophoresis. The enrichment of a specific protein in the various fractions could be calculated by knowing the total amount of proteins in each fraction [Palestini et al., 2003; Botto et al., 2004]. Subsequently, the proteins transferred to membranes were stained with Ponceau S to assess protein loading. The protein loading was assessed by densitometry (BIORAD Densitometry 710, program Quantity one) as described [Moore and Viselli, 2000; Daffara et al., 2004] and normalized, in the case of MEF proteins, by actin content.

After blocking, blots were incubated for 2 h with the primary antibodies diluted in PBS-T/milk (anti-Cav-1 1:1,000, anti-HO-1 1:1,000, anti-CD55 1:100, anti-AQP5 1:100, anti-PKC 1:200, anti-γENaC 1:200, anti-GM130 1: 250, anti-transferrin receptor 1:750, anti-actin 1:1,000). Then, blots were incubated for 2 h with horseradish peroxidase-conjugated anti-mouse/goat IgG (5,000-2,000-fold diluted in PBS-T/milk). Protein analysis was repeated three times for control and hypoxia at different times. Proteins were detected by ECL using the SuperSignal detection kit (Pierce, Rockford, IL). We performed immunoblot analysis of samples from control and treated cells for proteins from total plasma membrane and all gradient fractions. Immunoblot bands were analyzed by BIORAD Densitometry 710 and quantified as described [Botto et al., 2004].

PHOSPHORUS AND FLUIDITY ANALYSIS

Aliquots of MEFc, MEF5 and MEF24 were used for phospholipid phosphorus determination by the Bartlett procedure [Bartlett, 1959]. Data were expressed as μmol/mg of DNA. The membrane fluidity of different samples was assessed by fluorescence anisotropy measurements of the fluorescent probe 1,6-diphenyl-1,2,5-hexatriene (DPH) as described [Harris and Schroeder, 1982; Palestini et al., 2002; Botto et al., 2006]. A suspension of MEFc, MEF5 and MEF24 containing ~200 nmol of phosphorus per 1.5 ml of PBS was used. The fluorescent probe molecule DPH was added to membrane suspension at a final concentration of 2 μM. Light scattering was corrected by using a blank containing the sample but not DPH. Membranes with and without fluorescent probe were incubated in the dark under stirring for 45 min at 37°C and were used for fluorescence polarization studies immediately after preparation. A polarization spectrofluorimeter (Cary Eclipse, Varian) with fixed excitation and emission polarization filters was used to measure fluorescence intensity parallel (I_{pa}) and perpendicular (I_{per}) to the polarization plane of the exciting light [Harris and Schroeder, 1982; Palestini et al., 2002]. Excitation and emission wavelengths were 360 and 430 nm, respectively. Fluorescence anisotropy was calculated as $r = [(I_{pa} - I_{per})/(I_{pa} + I_{per})]$. The sample was continuously stirred with a microstirrer, and the temperature (37°C) was monitored by a thermistor in the cuvette.

LIPID PEROXIDATION

Lipid peroxidation was assessed on total MEF by a colorimetric assay (Bioxytech LPO-586, OxisResearch) of malondialdehyde (MDA) as indicator of peroxidation. Data were expressed as nmol/mg of DNA.

LIPID ANALYSIS

Aliquots of MEFc, MEF5 and MEF24 were submitted to lipid extraction [Palestini et al., 2002; Botto et al., 2006]. The lipids were separated on HPTLC plates. The phospholipids from MEFc, MEF5 and MEF24 were chromatographed in *solution A* (chloroform:-methanol:acetic acid:water, 60:45:4:2 v/v/v/v) and the cholesterol was chromatographed in *solution B* (hexane:diethylether:acetic acid, 20:35:1 v/v/v). Phospholipids and cholesterol were visualized with anisaldehyde [Svennerholm, 1956]. The plates were scanned with Bio-Rad system; spot identification and quantification was accomplished by comparison with authentic standard lipids. In the case of the glycosphingolipid GM1, HPTLC separation, blotting with HRP-CTB, enhanced chemiluminescence (ECL) detection and quantification were performed as described [Palestini et al., 2003].

TOTAL CELLULAR DNA

Total cellular DNA equivalent to $10–20 \times 10^5$ cells was extracted by TRIzol Reagent®, using data sheet instructions and quantified by spectrophotometer at 260 nm.

RT-PCR

Total RNA was extracted from A549 or A30 cells in control and after hypoxia exposure (5 or 24 h) using TRIzol Reagent (Invitrogen) allowing the manufactured instruction. RNA concentration was determined by absorbance at 260 nm, and integrity was confirmed by means of electrophoresis on 1% agarose gels, in presence of 0.1 μg/ml ethidium bromide. After DNase treatment, cDNA was synthesized using 1.5 μg RNA and Super Script III First-Strand Synthesis System for RT-PCR (Invitrogen). PCR reactions were performed using *Taq* DNA Polymerase (Invitrogen), specific primers and PCR conditions are indicated below: CD55 upper 5′-GCATCCCTCAAACAGCCTTA-3′, lower 5′-GCCACTCCACTCTCCTT-CAT-3′ to obtain a fragment of 516 bp, annealing temperature 55°C, 30 cycles; caveolin-1 upper 5′-CTACAAGCCCAACAACAAGGC-3′, lower 5′-AGGAAGCTCTTGATGCACGGT-3′ to obtain a fragment of 340 bp, annealing temperature 58°C, 30 cycles [Podar et al., 2003]. Expression of β-actin was used as a control to measure integrity of the RNA samples, primers and PCR condition are indicated below: upper 5′-GATTACTGCTCTGGCTCCTA-3′, lower 5′-CAGTAACA-GTCCGCCTAGAA-3′ to obtain a fragment of 230 bp, annealing

temperature 55°C, 28 cycles. Finally, 10 μl of PCR product were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. Image analysis and quantification were performed using KODAK Image Station 2000R. Densitometric data relative to mRNAs of CD55 and Cav-1 were normalized to the mRNA of β-actin determined in control and after 5 and 24 h of hypoxia.

IMMUNOFLUORESCENCE

Confluent cells were fixed with methanol at −20°C for 10 min, permeabilized with glycin for 10 min in PBS, washed twice with GDB buffer and treated with blocking buffer (BSA 1% in GDB) at RT for 10 min. Fixed cells were incubated with primary antibody rabbit polyclonal anti-Cav-1 (BD, Italy) diluted 1:500 in blocking buffer for 2 h at RT. After washing three times with HS buffer, cells were incubated with anti-rabbit Alexa 488-Conjugated secondary antibody (Molecular Probes) diluted 1:100 in blocking buffer at RT for 1 h. Cells were then washed three times with HS buffer and twice with LS buffer, incubated with DAPI for 5 min and mounted with glycerol 95% in PBS. This protocol was applied in control condition and after 6 and 24 h of hypoxia exposure.

VIDEO CONFOCAL MICROSCOPY

Video confocal microscopy was performed using ViCo system on an inverted Nikon ECLIPSE TE2000E microscope with 60× objective.

STATISTICAL ANALYSIS

Biochemical determinations were repeated three to four times for each experimental condition (control cells and exposed to hypoxia for 5 and 24 h). Biochemical results were expressed as mean ± SD, averaging data from the three to four independent experiments. The significance of the differences among groups was determined using one-way ANOVA and *t*-test.

RESULTS

The aim of this work is to study the response of alveolar cells to mild hypoxia exposure, in particular the modification of plasma membrane and lipid microdomains expression.

A30 cells, as A549 cells, after immunostaining showed strong cytoplasmic immunoreactivity for cytokeratin, while no immunolabeling was observed on negative controls, treated by omission of primary antibody or his substitution by anti-CD3 or anti-SMA (Fig. 1).

We checked whether hypoxia influenced cells viability assessed by the Trypan blue and the LDH tests (Table I). Trypan blue, a molecule of about 960 Da, enters cells when permeability of the plasma membrane is increased by stress agents; therefore, Trypan blue test is negative in healthy cells. As Table I shows, this test proved negative in both cell lines after hypoxia exposure. The release of LDH, a molecule of about 140 kDa, in the medium occurs for relatively large damage to the plasma membrane. Table I shows that a significant increase of LDH in the medium, though of only 40%, occurred in A30 cells at 24 h of hypoxia exposure (in severe membrane lesion LDH increases by as much as 25 times [Beretta et al., 2007]). Early signs of apoptosis were not detected by annexin V-FITC test in both cell lines after either 5 or 24 h of hypoxia exposure; similarly, no signs of necrosis were revealed using the propidium iodide labeling (data not shown). Finally, Table I shows

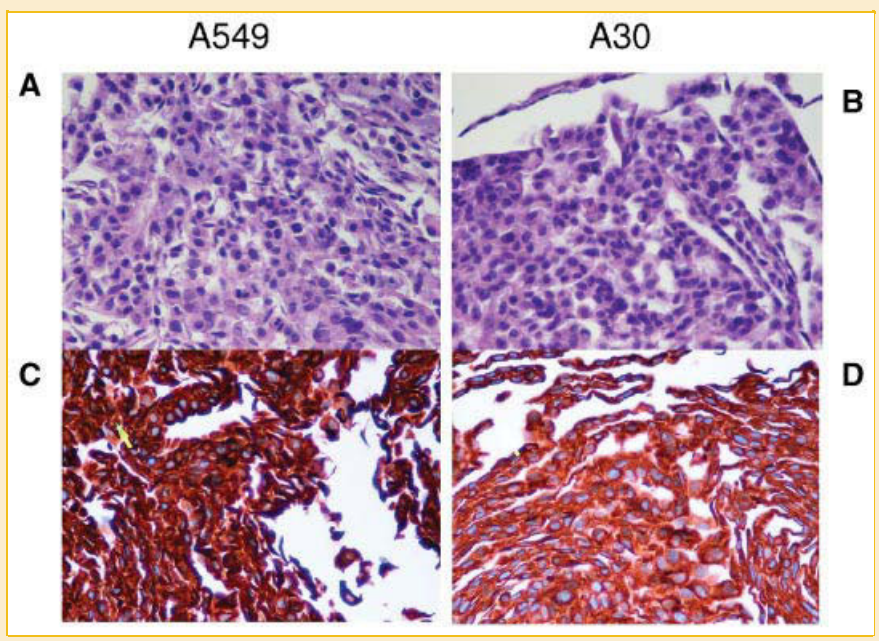


Fig. 1. A30 characterization. Strong and diffuse cytoplasmatic immunoreactivity for cytokeratin (brown colour) is seen in A30 cells (D), as in A549 cells (C). Negative controls are also showed (A and B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Vitality of A30 and A549 Cells in Control Condition and After Hypoxia Exposure (Trypan Blue Exclusion Test), LDH Activity Determination and Index of Lipid Peroxidation

	% Trypan negative cells		LDH activity (UI/ml)		Index of peroxidation (nmol MDA/mg DNA)	
	A30	A549	A30	A549	A30	A549
Control	91.2 ± 2.3	92.8 ± 1.7	0.102 ± 0.02	0.110 ± 0.03	1.03 ± 0.07	0.93 ± 0.04
5 h Hypoxia	94.1 ± 2.7	93.3 ± 0.9	0.096 ± 0.01	0.100 ± 0.02	0.98 ± 0.01	0.90 ± 0.06
24 h Hypoxia	93.8 ± 1.8	93.9 ± 1.7	0.140* ± 0.03	0.112 ± 0.04	1.58* ± 0.01	1.03 ± 0.04

Values are means ± SD. N = 6.
*$P < 0.05$ versus control.

that the index of peroxidation only increased significantly in A30 cells after 24 h of hypoxia.

Since hypoxia exposure did not cause severe damage to plasma membrane, we went to study the perturbations induced by hypoxia on structure/composition, chemical-physical properties and, in particular, lipid microdomains structure/expression.

PROTEINS OF MEF AND DRM

In normoxic A549 and A30 cells, the ratio of MEF proteins to DNA (Table II) was 5 to 1 and did not change significantly after exposure to hypoxia for 5 h. On the contrary, after 24 h of hypoxia, this ratio decreased significantly to 4.3 in A549 and 3.6 in A30 (−14 and −28%, respectively) suggesting a decrease of total membrane proteins.

We performed Western blots to evaluate whether hypoxia exposure altered the expression of some cellular proteins in MEF. In particular, we estimated the amount of proteins considered as markers of lipid microdomains, namely Cav-1 marker of caveolae and CD55 marker of lipid rafts. In parallel, we also evaluated the MEF content of PKC, γENaC, HO-1 and AQP5. This choice is justified because, on one side activation/translocation of PKC occurs after hypoxia exposure [Long et al., 2001; Cataldi et al., 2004] and, on the other correlates with the expression of lipid microdomains [Bécart et al., 2003; Botto et al., 2007]. Furthermore, an inhibition in the expression of γENaC has been described in response to extreme hypoxia [Planes et al., 2002]; HO-1 expression is implicated in antioxidant response to hypoxia [Long et al., 2001; Sato et al., 2006]; finally, AQP5 is the specific protein for water transport in alveolar cells [Verkaman et al., 2000; Johnson, 2007]. Figure 2 shows the total content of the above mentioned proteins in MEF in the two cell lines. It appears that in A30 cells, hypoxia exposure after 24 h, caused a decrease in total membrane content of HO-1, AQP5 and Cav-1 (about 85%, 34%, and 86%, respectively) but no change in the other proteins. In A549 cells we found only a reduction of

TABLE II. Plasma Membrane (MEF) Proteins Content in Control and After Hypoxia Exposure

	mg protein of MEF/mg DNA	
	A30	A49
Control	5.0 ± 0.2	5.0 ± 0.2
5 h Hypoxia	4.8 ± 0.1	5.3 ± 0.3
24 h Hypoxia	3.6* ± 0.2	4.3* ± 0.1

Data normalized to DNA. Values are means ± SD. N = 6.
*$P < 0.02$ versus control.

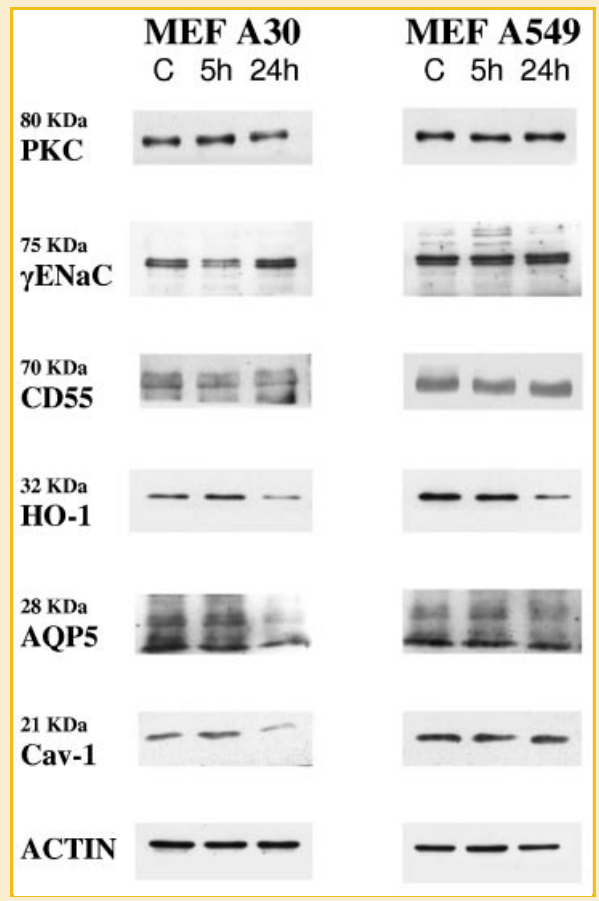


Fig. 2. Effect of hypoxia on the content of some proteins in membrane enriched fraction (MEF). Representative immunoblots of PKC, γENaC, CD55, heme-oxygenase 1 (HO-1), AQP5, caveolin (Cav-1) and actin in "membrane-enriched fraction" (MEF) obtained from A30 and A549 in control condition and after hypoxia exposure (5 and 24 h). Fifty micrograms of proteins obtained from MEF in control condition and after 5 and 24 h of hypoxia, were loaded on SDS-PAGE (10%-polyacrylamide gel), submitted to electrophoresis and Western blotting. Immunoblot bands were analyzed by BIORAD Densitometry 710 and quantified as described [Daffara et al., 2004; Botto et al., 2007]. At 24 h, Cav-1 and AQP5 decreased in A30 line; at 24 h HO-1 decreased in both cells lines.

TABLE III. Total Protein Content, Expressed in μg, in the Different Fractions of Discontinuous Sucrose Gradient (40%, 30%, 5% Sucrose, From Bottom to Up)

	Fraction									
	1, 2, 3	4	5	6	7	8	9	10	11	12
A30 (control)	2.3	3.8	18.8	3	7.5	85.4	190.8	250.6	470.8	1380.8
5 h Hypoxia	1.8	2.5	16.8	3.33	5.8	75.8	183	290.8	468.3	1280.8
24 h Hypoxia	2.6	4.4	17.6	2.7	7.4	80.1	241	394.5	491.3	1133.3
A549 (control)	1.75	4.1	19.95	3	3.75	54	184.5	355	612.5	980
5 h Hypoxia	2.5	6.3	20.2	6	6.5	73.3	270	313.3	633.5	1046.7
24 h Hypoxia	3.6	4.4	19.3	3	10	96	201	325	537	1,106

The detergent resistant membrane (DRM) fraction corresponds to fraction 5 from the top while the high detergent soluble membrane fractions are 8–12 [Palestini et al., 2003; Botto et al., 2004]. The value are the mean of three separated gradient and SD average was about 10%.

HO-1 (about 77%) in response to 24 h of hypoxia. PKC and γENaC, were unchanged following hypoxia exposure in both cell lines.

Next, we analyzed whether hypoxia exposure affected the distribution of these proteins in the sucrose gradient fractions, in particular in the detergent-resistant membrane fraction (fraction 5, DRM) that is known to contain caveolae and lipid rafts.

We assessed that DRMs were adequately obtained by checking that the majority of proteins recovered in the sucrose gradient (>80%) was present in the high detergent-soluble fractions (fractions 8–12) in both cell lines (Table III). On the whole, hypoxia exposure did not modify the total protein content of DRM, amounting to 0.7% ± 0.1 in A30 and 0.9% ± 0.1 in A549, of total cell proteins (Table III).

Next, we analyzed the distribution of Golgi marker (GM130) and transferrin receptor in the sucrose gradient fractions by Western blotting analysis. In Figure 3 we reported a representative immunoblot obtained from A30 cells. The data show that these proteins were largely present in the high detergent-soluble fractions and their distribution was unaffected by hypoxia exposure in both cell lines (data not shown). Note also that Golgi membranes are absent in fraction 5 (DRM).

Figure 4, shows the Western blotting data for Cav-1, CD55 and HO-1 in the gradient fractions obtained from A549 and A30 cells in control conditions and after hypoxia exposure. Immunoblot bands of fraction 5 (DRM) were quantified and the densitometric data, relative to control, are reported in Figure 5. In both cell lines, hypoxia caused an increase in CD55 and a decrease in Cav-1, while HO-1 increased only in A459 line. The insert of Figure 5 also reports a significant inverse correlation when plotting the changes in CD55 versus the changes in Cav-1, upon exposure to hypoxia (data refer to both cell lines and are expressed as a ratio to control values).

PKC and AQP5, were only present in the high detergent-soluble fractions and this distribution was not modified by hypoxia exposure; γENaC was found in prevalence in fractions 8–12 and such distribution remained unchanged after hypoxia exposure in both cells lines (data not shown).

To investigate whether hypoxia exposure induced modification of mRNA levels of CD55 and Cav-1, PCR-RT experiments were performed. Figure 6 presents the effect of hypoxia exposure on the expression of mRNAs for CD55 and Cav-1 in both cell lines. The data

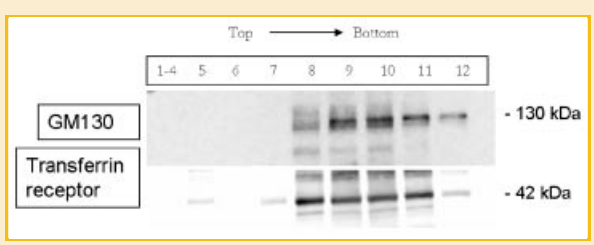


Fig. 3. Characterization of DRM preparation obtained from alveolar cells. A30 cells in control condition and after 5 and 24 h of hypoxia exposure were treated with 1% Triton X-100-containing buffer for 30 min on ice. The suspension was submitted to discontinuous sucrose density gradient centrifugation. One-milliliter fractions were withdrawn from the gradient and submitted to protein assay. Fifteen micrograms of protein of each fraction were submitted to 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies against GM130 and against transferrin receptor. Results in A549 cells were comparable.

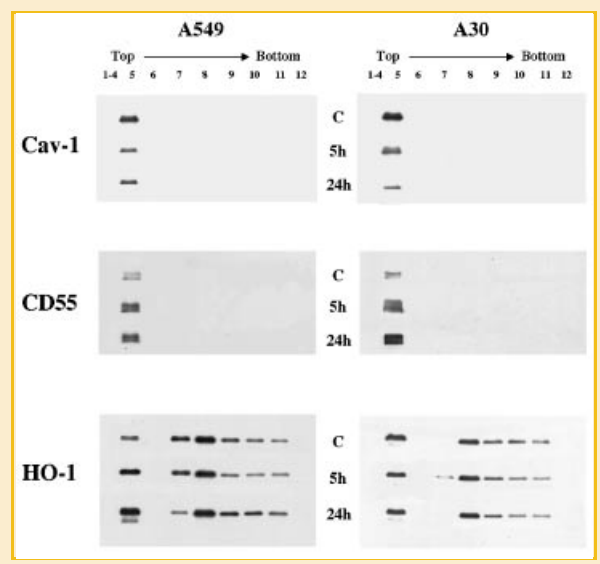


Fig. 4. Effects of hypoxia on the content of Cav-1, CD55 and HO-1 in cellular gradient fractions. Representative immunoblots of Cav-1, CD55 and HO-1 in the various fractions obtained from discontinuous sucrose density gradient centrifugation of A549 and A30 cells in control condition, C and after 5 and 24 h hypoxia exposure. Fifteen micrograms of proteins collected from all the gradient fractions were loaded on SDS-PAGE (10%-polyacrylamide gel), submitted to electrophoresis and Western blotting. Immunoblot bands were analyzed by BIORAD Densitometry 710 and quantified as described [Daffara et al., 2004; Botto et al., 2007].

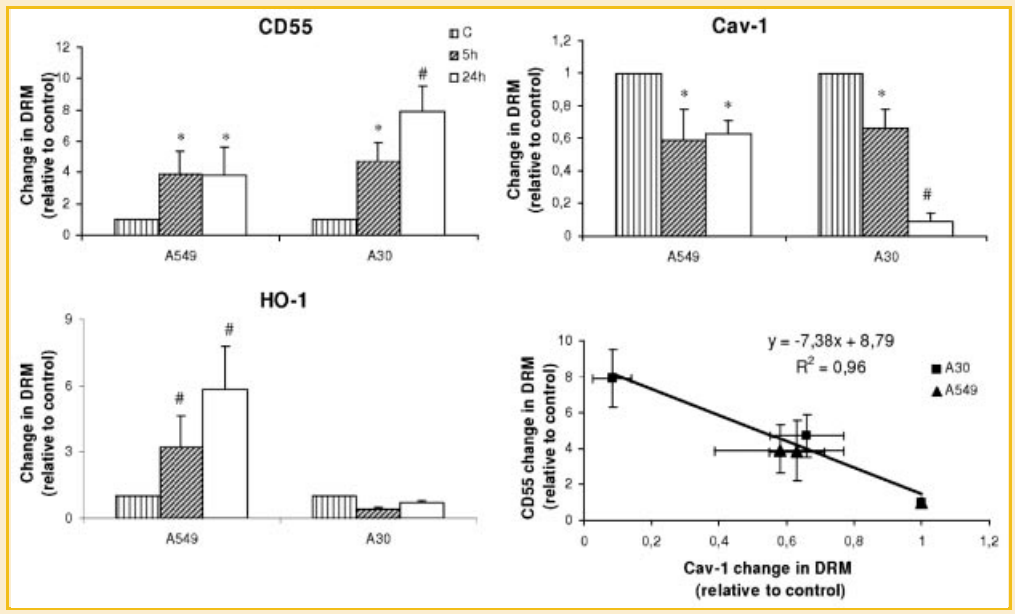


Fig. 5. Effects of hypoxia on the content of Cav-1, CD55 and HO-1 in detergent resistant membrane fraction (DRM). Densitometry data of Western blotting for CD55, Cav-1 and HO-1 content in DRM (fraction 5), expressed as percentage change relative to control value after 5 and 24 h of hypoxia exposure in A549 and A30 cells. CD55, protein marker of lipid rafts, increased in both cell lines; Cav-1, protein marker of caveolae, decreased in both cell lines; HO-1 increased in A459 line while it remained unchanged in A30 line. The insert also reports the inverse correlation between CD55 and Cav-1 in both cell lines. Values are means ± SD for at least three separate experiments performed in triplicate. $^{*}P < 0.05$ versus control; $^{\#}P < 0.02$ versus control.

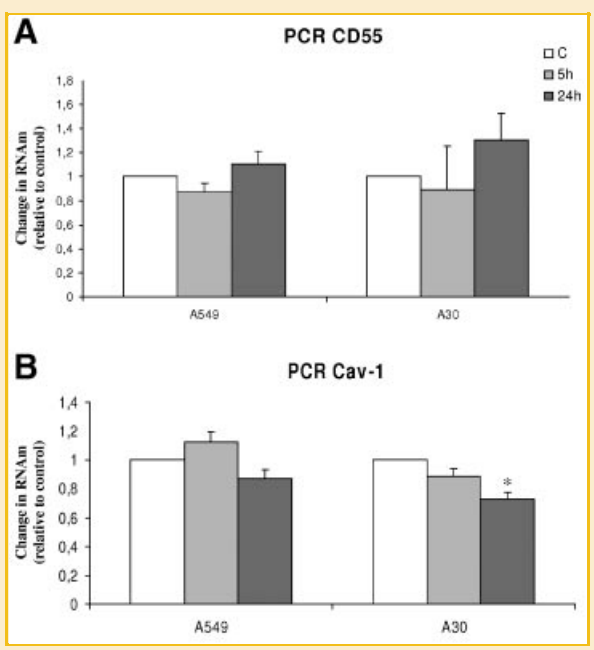


Fig. 6. Effects of hypoxia on CD55 and Cav-1 genes transcription. The figure represents the effect of hypoxia exposure on the mRNAs expression of CD55 (A) and Cav-1 (B) in A549 and A30 cells. The data were normalized for the corresponding actin signal in each lane. The results are expressed as the ratios of CD55 and Cav-1 mRNA/β-actin mRNA. Data are means ± SD for at least three separate experiments performed in triplicate. $^{*}P < 0.05$.

were normalized to the corresponding actin signal in each lane and the results were expressed as the ratios of CD55 and Cav-1 mRNA/β-actin mRNA. We performed PCR experiments using also GAPDH (glyceraldehydes-3-phosphate dehydrogenase) as housekeeping genes (data not shown) that confirmed the results obtained using β-actin; therefore our experimental conditions did not affect mRNA expression of the two housekeeping genes. No significant changes in mRNA for CD55 were detected following hypoxia exposure in both cell lines. Concerning mRNA of Cav-1, no significant change was found in A549 cells, while a significant decrease was found at 24 h for A30 cells.

Finally, since we found that hypoxia-induced changes in DRM content for Cav-1, we investigated by immunofluorescence, the cellular distribution of this marker. Figure 7 shows representative confocal images of Cav-1 distribution in A30 cells in control and after 5 and 24 h of hypoxia exposure. In control condition (A), the immunofluorescence of Cav-1 is distributed along cell profiles of confluent cells, with no intracellular labeling, thus supporting a plasma membrane localization. After 5 h of hypoxia (B), a less evident cellular profile is shown with corresponding increase in intracellular labeling. Finally, after 24 h of hypoxia exposure (C), all labeling is intracellular with clustering of the Cav-1, mostly perinuclear (as evident on colors images with nuclear labeling). Similar pattern was observed for A549 cells.

LIPIDS OF MEF AND DRM

We also analyzed whether hypoxia exposure also affected membrane lipid composition and, in turn, its fluidity.

The total MEF content of phospholipidic phosphorus normalized to DNA (Fig. 8A), decreased at 24 h in both cells lines, reflecting an

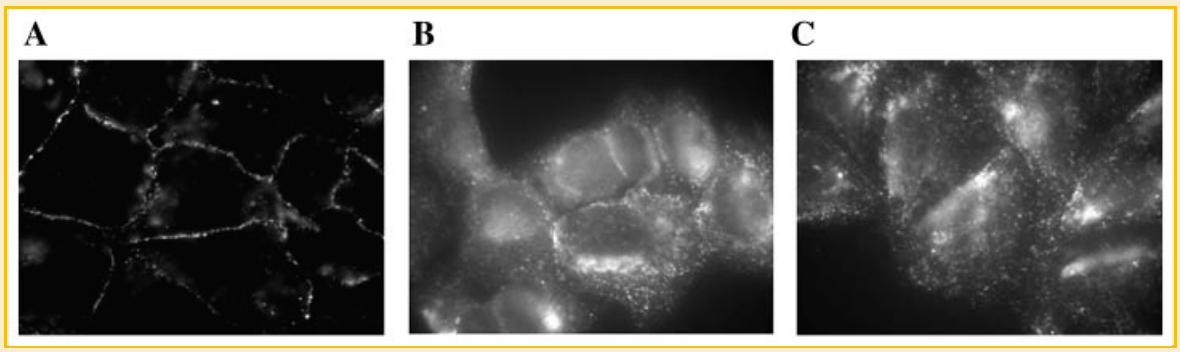
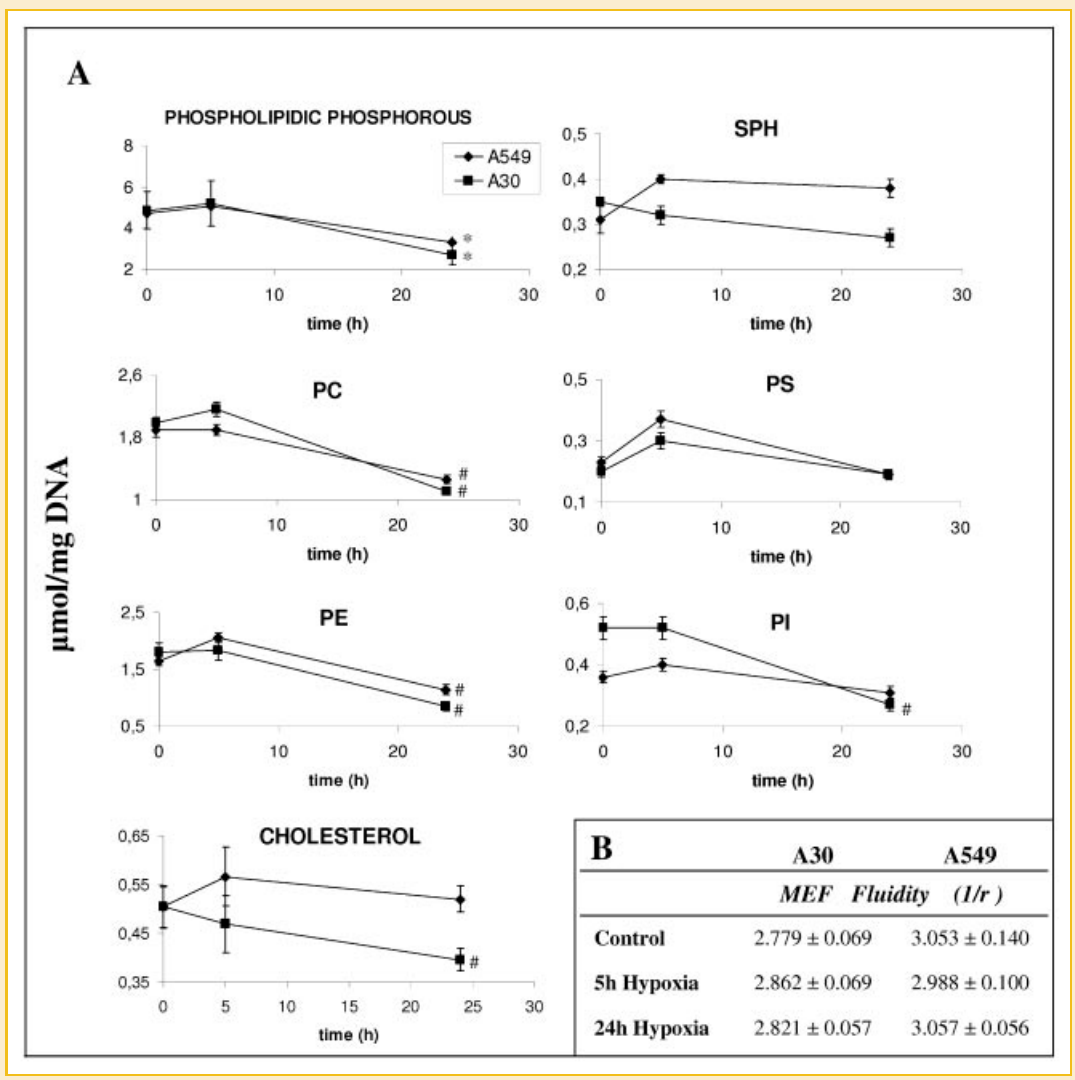


Fig. 7. Cav-1 immunofluorescence staining—Representative confocal images of A30 cells in control condition (A) and after 5 h (B) and 24 h (C) of hypoxia exposure. The detection of Cav-1 was made with secondary antibody conjugated with Alexa 488. In control condition, the fluorescent signal of Cav-1 was mainly distributed to the plasma membrane; after 5 h of hypoxia the signal did not outline the cell surface and showed an intracellular labeling; at 24 h, intracellular distribution revealed clustering of the Cav-1 labeling. Results in A549 cells were comparable.



B

	A30	A549
	MEF Fluidity (1/r)	
Control	2.779 ± 0.069	3.053 ± 0.140
5h Hypoxia	2.862 ± 0.069	2.988 ± 0.100
24h Hypoxia	2.821 ± 0.057	3.057 ± 0.056

Fig. 8. Effects of hypoxia on lipid content of membrane enriched fraction (MEF). A: Content of phospholipidic phosphorous, phospholipids and cholesterol in membrane-enriched fraction (MEF), obtained from A549 and A30 cells in control (time 0 on the abscissa) and after hypoxia exposure (5 and 24 h). SPH: sphingomyelin; PC: phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol; PE: phosphatidylethanolamine. Data are means ± SD from at least three separate experiments performed in triplicate. $^{*}P < 0.05$ versus control; $^{\#}P < 0.02$ versus control. B: Fluidity of membrane enriched fraction (MEF), expressed as the reciprocal of the coefficient of anisotropy (*r*), in control and after hypoxia exposure.

TABLE IV. Cholesterol and GM1 Content in DRM in Control Condition and After Hypoxia Exposure

	nmol of cholesterol/mg proteins		pmol of GM1/mg proteins	
	A30	A549	A30	A549
Control	146 ± 11.68	219 ± 17.52	7.2 ± 0.64	7.5 ± 0.67
5 h Hypoxia	127 ± 8.89	224 ± 15.68	10.8 ± 0.54	9 ± 0.45
24 h Hypoxia	106* ± 6.36	201 ± 12.06	9 ± 0.54	11.25* ± 0.67

Data normalized to protein in DRM. Values are means ± SD. N = 4.
*$P < 0.05$ versus control.

equal percentage wise decrease of the more representative phospholipids, namely phosphatidylcholine and phosphatidylethanolamine. Total membrane cholesterol, decreased significantly only in A30 cells after 24 h of exposure. These changes, did not influence membrane fluidity, assessed by fluorescence anisotropy (Fig. 8B).

We also analyzed the cholesterol and glycosphingolipid (GM1) in DRM, whose concentration is about four and three fold higher, respectively, relative to high detergent-soluble fractions (data not shown). Table IV shows that the content of these lipids, normalized to DRM protein content, remained unchanged up to 5 h of hypoxia exposure in both cell lines, while, at 24 h, we observed a decrease of cholesterol in A30 cells, and an increase of GM1 in A549.

In general, A30 cells reveal a greater sensitivity to hypoxia, compared to A549 tumor epithelial cells, considering vitality tests, peroxidation index, changes in MEF proteins and DRM CD55/Cav-1 ratio and lipids contents.

DISCUSSION

In situ alveolar cells are exposed to considerable modifications in their extracellular microenvironment, such as changes in temperature and metabolic substrate fluctuations, exposure to toxic agents and modification of oxygen tension. One can hypothesize that these stressing agents, in particular the last one, could induce modifications of the cell signalling platforms at plasma membrane surface thus eliciting a specific cellular response. This study evaluates the effects of mild hypoxia exposure on plasma membrane composition and lipid microdomains expression in tumor human epithelial cells and in continuous epithelial cells obtained from human lungs. The use of two cells lines allows to compare the response of commercial tumor epithelial cells (A549), notably resistant to hypoxia [Heerlein et al., 2004] to that of continuous cells from native tissue.

Regarding the characterization of A30 cells, keratins are the essential intermediate filament proteins of epithelial cells and epithelial neoplasms, and are considered an antigenic marker characterized by a high degree of specificity and sensitivity for the epithelial origin of normal and tumoral cells [Moll et al., 1982; Miettinen, 1992]. The strong and diffuse immunolabeling for cytokeratin in A30 cells, observed by using a wide-spectrum cytokeratin pool of monoclonal antibodies (see Materials and Methods Section), testifies their epithelial nature and their origin from the epithelial component of lung parenchyma.

In general, the vitality of cells was preserved up to 24 h of hypoxia exposure. Indeed, the percentage of Trypan blue positive cells remained unchanged after hypoxia; however LDH activity in the medium only increased in A30 cells after 24 h, revealing a limited pre-necrotic state. These data were confirmed also by the annexin V-FITC test, indicating that the degree of hypoxia exposure was borderline between the physiological and the pathological condition, as previously noted [Krick et al., 2005].

Both cell lines, after 24 h of hypoxia exposure, showed a decrease in membrane lipid and protein content in according with others [Mairbaurl et al., 1997; Wodopia et al., 2000]. In the case of lipid component, a similar percentage decrease in total phospholipids and cholesterol content was found; as a consequence, the cholesterol/phospholipids and PC/PE ratios remained unchanged, in line with no change in membrane fluidity as previously reported [Palestini et al., 2002; Botto et al., 2006].

We also considered membrane key proteins involved in transports, signalling, anti-inflammatory and anti-apoptotic response. The expression of PKC, was unchanged up 24 h of mild hypoxia in both cell lines, and the expression of amiloride sensitive channels γENaC, was also unchanged, in line with previous observations [Planes et al., 2002].

The total membrane content of HO-1, a protective agent against ROS-induced airway damage [Sato et al., 2006], was slightly decreased in the two cell lines and was markedly increased in DRM of A549, while it was essentially unaffected in A30. The increase in HO-1 in DRM in A549 can be interpreted as a greater anti-inflammatory action buffering the increase in ROS. By the same reasoning, in A30 cells no increase in HO-1 was found in DRM and, in fact, the level of lipid peroxidation was increased suggesting that the anti-oxidative activity of HO-1 is enhanced when this protein migrates to lipid microdomains.

Lipid microdomains represent specialized portions of the plasma membrane covering some 15–25% (depending upon the cell type) of total plasma membrane surface [Pike, 2003; Mayor and Rao, 2004], involved in signalling [Fielding and Fielding, 2003; Allen et al., 2007; Parton and Simons, 2007]. The isolation of the detergent-resistant membrane fraction (DRM) allowed to evaluate the modifications of lipid microdomains expression after hypoxia exposure. We found that hypoxia induced a considerable reshaping of the type of lipid microdomains present in DRM, based on the expression and identification of specific markers for caveolae (Cav-1) and for lipid rafts (CD55), respectively. In both cell lines, we found a decrease in caveolar expression and a corresponding increase in lipid rafts already after 5 h of hypoxia exposure, becoming more marked at 24 h in A30 cells (insert in Fig. 5).

In A549 cells, we found no change in mRNA for Cav-1 as well as in the total content of this protein in MEF. In these cells, the decrease in Cav-1 in DRM, suggest a shift of this protein to non-detergent-resistant membrane fractions and/or to intracellular membrane compartments.

A pre-transcriptional effect can be invoked for the observed decrease of Cav-1 in MEF and DRM in A30 cells due to the decrease in mRNA of Cav-1. The decrease of Cav-1 in DRM of A30 is also compatible with a shift of Cav-1 to intracellular compartments. Immunofluorescence microscopy supports this last hypothesis.

Indeed, confocal video images (Fig. 7) reveal that Cav-1 moves away from plasma membrane surface towards the intracellular compartment at 5 h of hypoxia exposure reaching the perinuclear zone at 24 h. A similar process of internalization for caveolae has been described in response to physic-chemical stimuli [Kang et al., 2000; Barth et al., 2005].

Unlike Cav-1, we found an increase in GPI-anchored protein (CD55) in the DRM with no change in total CD55 content in MEF and no change in mRNA. These results are at variance with those obtained in SVARECs cells by Ledoux [Ledoux et al., 2003] concerning Ecto-5′Nu a GPI-anchored protein, after exposure to a more severe degree of hypoxia. In fact, this author found no change in Ecto-5′Nu in DRM but an increase in its expression at plasma membrane level, with no change in the corresponding mRNA.

The mobility of lipid microdomains has been proposed as a mechanism to promote a rapid receptor response as well as turnover in the cellular signalling in response to exogenous agents [Sergey and Smart, 2002; Fielding and Fielding, 2003; Legler et al., 2003; Sprenger et al., 2006]. Recent reports [Wiliams and Lisanti, 2005] suggest that caveolar may represent "mobile platforms" for specific signalling events. Indeed, Cav-1 controls signalling along the Ras-p42/44 MAP kinase cascade including growth factor receptors (such as Neu/Erb-B2 and EGFR) and also plays a central role as it serves a scaffolding function for the "caveolar release complex" G_i and *Src*, that represents the signalling machinery responsible for endocytosis [Wiliams and Lisanti, 2005]. Thus, caveolae may act as storage containers for preassembled signalling pathways. A similar interpretation may involve lipid rafts, that can also represent "mobile signalling platforms". In fact, the coalescence mediate by ceramide of microscopic rafts into large membrane macrodomains, is essential for the transmission of apoptosis signals across the plasma membrane [Gulbins and Kolesnick, 2003].

Sprenger et al. [2006], demonstrated, in endothelial cells, that lipid rafts and caveolae proteomes are biochemically separated, establishing the importance of a balanced rafts-caveolae interaction and/or ratio in membrane trafficking, transduction and growth. Therefore, the inverse correlation between caveolae and lipid rafts, observed in response to hypoxia, can be interpreted as a relatively fast response of signalling system, necessary to face the cellular/extracellular microenvironment perturbation induced by chemical or physical stress-related stimuli.

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