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Ultrastructural Modifications and Phosphatidylinositol-3-kinase Expression and Activity in Myocardial Tissue Deriving from Rats in Different Experimental Conditions

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ABSTRACT. **Oxygen supply is essential in the maintenance of the physiological cell metabolism.In fact, both lower and higher O_2 concentrations induce modifications of the enzymatic activity of the cell which determine, in turn, morphological changes at nuclear and cytoplasmic level. Among the molecules involved in the maintenance of the cellular homeostasis, the signal transduction pathway PI-3-kinase/AKT-1 should be included. Here we suggest a relationship between the modulation of this pathway and the morphological modifications occurring *"in vivo"* in myocardial tissue upon hypoxic and hyperoxic stress. In particular, down regulation of this pathway, which when activated is known to deliver an anti-apoptotic signal, is concomitant to the maintenance of the apoptotic events occurring in these cells in response to oxidative stresses.**

Key words: PI-3-kinase/apoptosis/myocardial tissue/hypoxia/hyperoxia

Cell growth is regulated by several factors which influence cell metabolism. Among these factors oxygen supply plays a fundamental role. Oxygen, at normal level, keeps the enzymatic machinery in a physiological state, while, when hypoxic and hischemic stress are established, the body tends to react with several modifications in ventilation, cardiocirculatory system and other specific tissue reactions. At cellular level increased anaerobic glycolysis , loss of contractility, changes in lipid and fatty acid metabolism, eventual irreversible membrane damage and cell death occur, resulting in conditions incompatible with life (Webster *et al*.,1993; Franklin and Poyton, 1996). At molecular level, the cellular response to chronic cellular hypoxia involves, besides the reduced enzymatic activity, changes in DNA-protein interaction leading to gene expression alterations (Helfmann and Falanga, 1993; Poyton, 1999). Also 100% of oxygen supply is not a physiological condition and, by producing free radicals, induces tissue damage, resulting in functional alterations acting as a killer for those cells which have a very high metabolism or oxygen consumption (Di Giulio *et al*.,1998). In conclusion, too high or too low oxygen level disturb the homeostasis of metabolism.

Among the molecules involved in the maintenance of the homeostatic state in the cell, phosphatidylinositol-3-kinase (PI-3-kinase) must be included. This is a signalling enzyme, present in mammalian cells, responsible for most of the cellular response to stimuli of different nature, including differentiation and apoptosis (Wymann and Pirola, 1998; Cataldi *et al*., 2000). The response of this enzyme, which is cell type and stimulus dependent, leads to the production of PI(3,4)P_2 and PI(3,4,5)P_3 considered to be signalling molecules (Carpenter and Cantley, 1996). In addition these products can modulate the downstream effector of PI-3-kinase, the serine/threonine AKT-1/Protein Kinase B (Franke *et al*.,1995; Cataldi *et al*., 2001), which has been demonstrated to be a survival factor. In fact, the ability of AKT-1 to promote survival seems to be dependent both on its kinase activity and on the activity of its upstream activator, PI-3-kinase (Kennedy *et al*.,1997). It is still unclear how chronic hyperoxia and hypoxia alter PI-3-kinase expression, but this enzyme is oxygen dependent (Skaper *et al*., 1998; Yin *et al*., 1998).

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Abbreviations: PI-3-Kinase, phosphatidylinositol 3-Kinase; PI(3, 4) P_2, phosphatidylinosipol 3, 4, bisphosphate; PI(3, 4, 5) P_3, phosphati; dylinosipol 3, 4, 5 priphosphate; NGS, normal goat serum.

Here we try to explain the relationship between PI-3-kinase levels and activity and ultrastructural modifications occurring "*in vivo*" in myocardial tissue upon hypoxic and hyperoxic treatment of rats, interpreting these changes as one of the attempts of the organism to react to the homeostasis variations induced by these stresses.

Materials and Methods

Animals

Three groups, each composed by 10 Wistar rats, 4 months old, weighing 200–250 g were used according to the Declaration of Helsinki. One group was kept in room air (21% O_2) as control group, the second was kept in a Plexiglas chamber for 12 days in chronic hypoxia (10–11 % inspired oxygen) and the third was kept for 60–65 hr in 98–100% inspired O_2, according to literature (Padmanabhan *et al*., 1985). Chamber temperature (25±2°C) and CO_2 concentration (23%) were maintained in physiological ranges. The chamber air was recirculated by the use of a pump and CO_2 was removed from the chamber air with Baralyme and was continuously monitored with a capnograph. Boric acid was mixed with the litter to minimize emission of urinary ammonia. The rats were anaesthetized with Nembutal (40 mg/kg) and dissected. Left ventricles were excised from each rat in the different experimental conditions and processed for experiments.

Electron microscopy analysis

Samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.6 for 4 hr at 4°C. After washing in the same buffer, they were post-fixed in 1% OsO_4 in cacodylate buffer for 1 hr at 4°C. Samples were then alcohol dehydrated and embedded in Spurr. Semithin sections were stained with 1% toluidine blue and observed at light microscopy. Images were obtained using Hewlett Packard Scanner 5200 and diameter fibers were measured by using LEICA Quantimet 500 plus software. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Zeiss 109 electron microscope (Germany). Printed images were obatained by using Hewlett Packard Scanner 5200 and mitochondrial area was evaluated by using Leica Quantimet 500 plus software.

TUNEL method

TUNEL (terminal deoxynucleotidyl transferase [TdT] mediated deoxyuridine triphosphate [d-UTP]nick end-labelling) is based on the preferential binding of TdT to the 3'-hydroxil ends of DNA (Negoescu, 1996). Fresh tissue sections of heart were used . Slides were fixed in 4% paraformaldehyde in PBS pH.7.4. After washes in PBS, slides were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. Tissue sections were stained with 5 units of TdT (Boehringer Mannheim GmbH, Germany), 2.5 mM $CoCl_2$ for 5 min at room temperature. Residues of biotinylated dUTP (Boehringer Mannheim GmbH, Germany) were catalitically added to the ends of DNA fragments with the enzyme TdT for 60 min at 37°C in an humified chamber. After the end labelling, sections were incubated with streptavidin biotinylated horseradish peroxidase complex (Amersham Ltd, Life Science, Buckinghamshire, England) for 45 min at room temperature, stained with 50mM 3-3'diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayers hematoxylin (Merck, Darmstadt, Germany) to detect the biotin-labeled nuclei. For negative controls, deionized water was used instead of TdT. Apoptotic cells were brown stained.The extent of DNA fragmentation was quantified by direct visual counting of peroxidase labeled nuclei at 200×magnification.

Immunohistochemical analysis

Unfrozen sections were rinsed in 50 mM Tris-HCl buffer pH 7.4 for 5 min at room temperature, placed in a Petri dish and blocked in NGS (normal goat serum). After rinsing in 50 mM Tris-HCl buffer, slides were incubated with rabbit p85 α polyclonal antibody (Santa Cruz Lab., Santa Cruz, CA, USA) (1:100) for 30 min at room temperature. After several washes in Tris-HCl, slides were incubated with rabbit IgG Biotin conjugated secondary antibody (SIGMA Che. Co. St. Louis, USA) for 30 min and washed in 50 mM Tris-HCl. They were then incubated with ExtrAvidin Peroxidase conjugated (SIGMA Che. Co. St. Louis, USA) for 30 min, washed in 50 mM Tris-HCl and peroxidase was developed by using diaminobenzidine chromogen diluted in imidazolic buffer, pH 7.6, for 6–10 min. After alcohol dehydration and clarification, slides were mounted with Permount and observed. Negative control was performed by omitting the primary antibody.

Protein extraction, PI-3-kinase and AKT-1 immunoprecipitation and western blot analysis

Left ventricles were excised from each rat, washed in PBS, cut in small pieces, resuspended in RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 1% SDS, 100 mM sodium vanadate, 0.2 mM PMSF, 30 µl /ml aprotinin) and homogenized in a Polytron homogenizer. Samples were then centrifuged twice at 15000×g for 30 min at 4°C. Supernatants were recovered and considered total cellular lysates. Aliquots of these lysates (500 µg) were incubated for 30 min at 4°C in the presence of 0.25 µg normal rabbit IgG together with 20 µl Protein G-Agarose. Beads were centrifuged at 1000×g for 5 min at 4°C. Cell lysates were incubated with 2 µg rabbit p85 α or goat AKT-1 polyclonal antibody for 1 hr at 4°C under constant agitation. Twenty µl Protein G-Agarose were added and the incubation was allowed to proceed for 1 hr at 4°C. Immunoprecipitates were then lysed in RIPA buffer, separated by SDS polyacrilamide gels (8%) and then transferred to nitrocellulose membranes. Nitrocelluloses were blocked in 5% non fat milk in wash buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 hr at room temperature and probed with rabbit p85 α polyclonal antibody or goat AKT-1 polyclonal antibody (Santa Cruz Lab., Santa Cruz, CA, USA). After washes, nitrocelluloses were incubated with enzyme conjugated anti-rabbit or anti-goat IgG horseradish peroxidase for 1 hr at room temperature in wash buffer, non fat milk and bands were detected by ECL detection system (Amersham Intl., Buckinghamshire,U.K.). When required, blots of

immunoprecipitated PI-3-kinase or AKT-1 were stripped of bound antibodies by incubating membranes in wash buffer containing 2% SDS at 50°C for 30 min, blocked and reprobed with anti-phosphotyrosine monoclonal antibody (PY-99) or Phospho AKT (Ser 473) 4 E 2 MoAb (New England Biolabs ltd, Hertfordshire, England,U.K.).

"In vitro" PI-3-kinase activity

In vitro PI-3-kinase activity was performed by using aliquots of immunoprecipitated PI-3-kinase. In particular, 10 μg of sonicated PI(4,5)P_2, resuspended in 20 mm Hepes, pH 7.4, were added to PI-3-kinase immunoprecipitates and incubated for 10 min at 4°C. Then 10 mM $MgCl_2$, 2 mM adenosine, which inhibits PI-4-kinase, 1 mM ATP and [γ32 P] ATP (10 Ci /mmol) were added and incubation was performed for 15' at room temperature. The reaction was stopped by addition of chloroform/methanol/HCl (200:100: 0.75 v/v) followed by two washes in chloroform/methanol/ 0.6N HCl (3:48:47 v/v). The organic phase was dried, resuspended in chloroform and resolved in oxalate activated thin layer chromatography plates developed in isopropanol/acetic acid/H_2O (65:1:34 v/v). After autoradiography spots of PI(3,4,5)P_3, were excised and quantified by scintillation counting. Values are expressed as incorporated dpm/mg protein.

Image processing and analysis system

Quantitative analysis was performed using a Sony videocamera connected to a Leica Quantimet 500 plus (Leica Cambridge Ltd., Cambridge, U.K.) determining the change in Integrated Optical Intensity (I.O.I.) using ISO transmission density Kodak CAT 152-3406 (Eastman Kodak Company, Rochester, USA) as standard. Results were expressed as mean±S.D. Statistical analysis was performed using the analysis of variance (ANOVA). Probability of null hypothesis of 0.1 % ($p<0.001$) was considered statistically significant.

Results

Semithin sections, observed at light microscopy, disclosed a reduction in diameter of myocardial fibers upon moification of O_2 concentration, when measured by using a Leica Quantimet 500 plus software. In fact, myocardial fibers deriving from rats kept in room air (21% O_2), measured medially 8.2±1.6 μ ($p<0.002$) in diameter, while those deriving from rats kept in chronic hypoxia (10–11% O_2 for 12 days) measured 5.2±1.5 μ ($p<0.002$). In hyperoxic rats (98–100% O_2 for 60–65 hr) these fibers measured medially 4.2 μ ±1.6 μ ($p<0.002$) (Fig.1 a,b,c). In parallel, in these sections, we noticed an increased space between myofibrils in both hypoxic and hyperoxic rats and, only in the hypoxic, an augmented vascularization. No cardiac hypertrophy was evidenced upon hypoxic and hyperoxic stress.When an ultrastructural analysis was performed by electron microscopy, we observed a reduced thickness of the fibers concomitant to a different morphology, distribution and number of

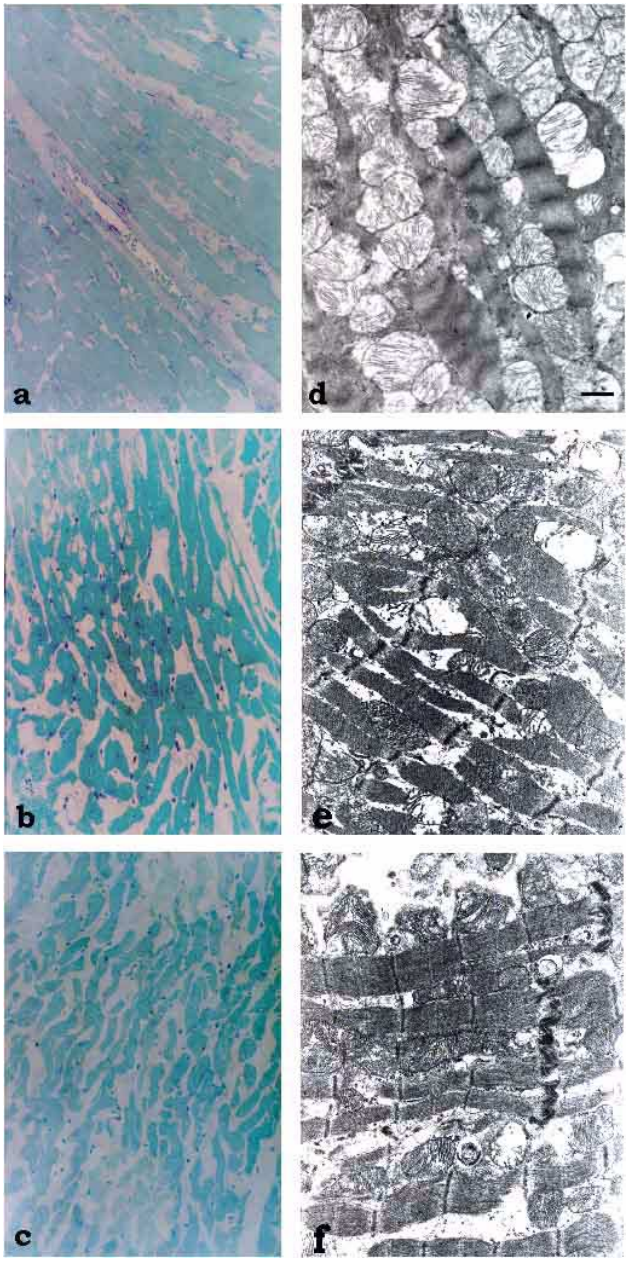

Fig. 1. **(a,b,c)** Semithin sections of myocardial tissue deriving from rats in different experimental conditions. Magnification: 300×. **a**. Control; **b**. Hypoxic condition; **c**. Hyperoxic condition. **(d,e,f)** Transmission electron microscopy of myocardial tissue deriving from rats in different experimental conditions. Magnification: 7000×. Bar: 1.5 μm. **d**. Control; **e**. Hypoxic condition; **f**. Hyperoxic condition.

Table I. MORPHOMETRIC ANALYSIS OF MITOCHONDRIAL AREA IN MYOCARDIAL TISSUE DERIVING FROM RATS IN DIFFERENT EXPERIMENTAL CONDITIONS±S.D.(P<0.001).VALUES ARE EXPRESSED IN PIXELS

Control	Hypoxic	Hyperoxic
633.85±256.29	472.35±246	660.75±196.25

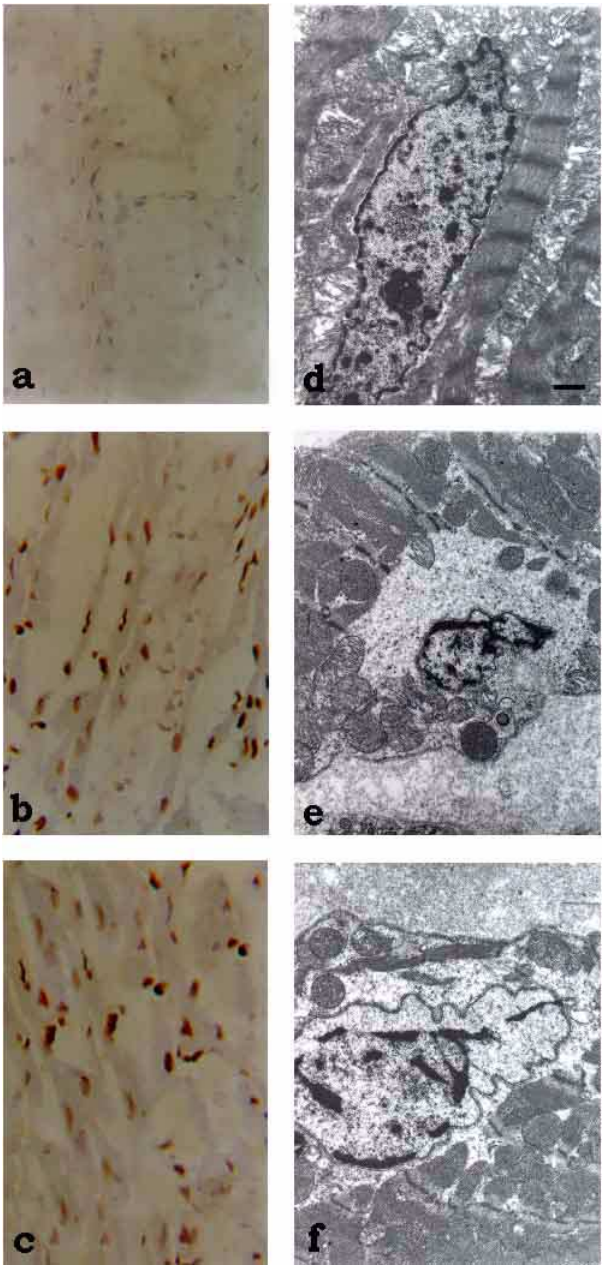

Fig. 2. **(a,b,c)** TUNEL detection of apoptotic cells in myocardial tissue deriving from rats in different experimental conditions. Five slides were examined per sample. The extent of DNA fragmentation was quantified by direct visual counting of peroxidase labeled nuclei at 200× magnification. **a**. Control; **b**. Hypoxic condition; **c**. Hyperoxic condition **(d,e,f)** Transmission electron microscopy of myocardial tissue deriving from rats in different experimental conditions. Magnification: 7000×. Bar: 1.5 μm. **d**. Control; **e**. Hypoxic condition; **f**. Hyperoxic condition. Note chromatin reorganization and nuclear envelope shrinkage in the hypoxic and hyperoxic conditions indicating the occurrence of an apoptotic state.

mitochondria (Fig.1 d,e,f). In control samples these organelles were clustered around the myofibrils and well preserved. In hypoxic samples they appeared reduced in

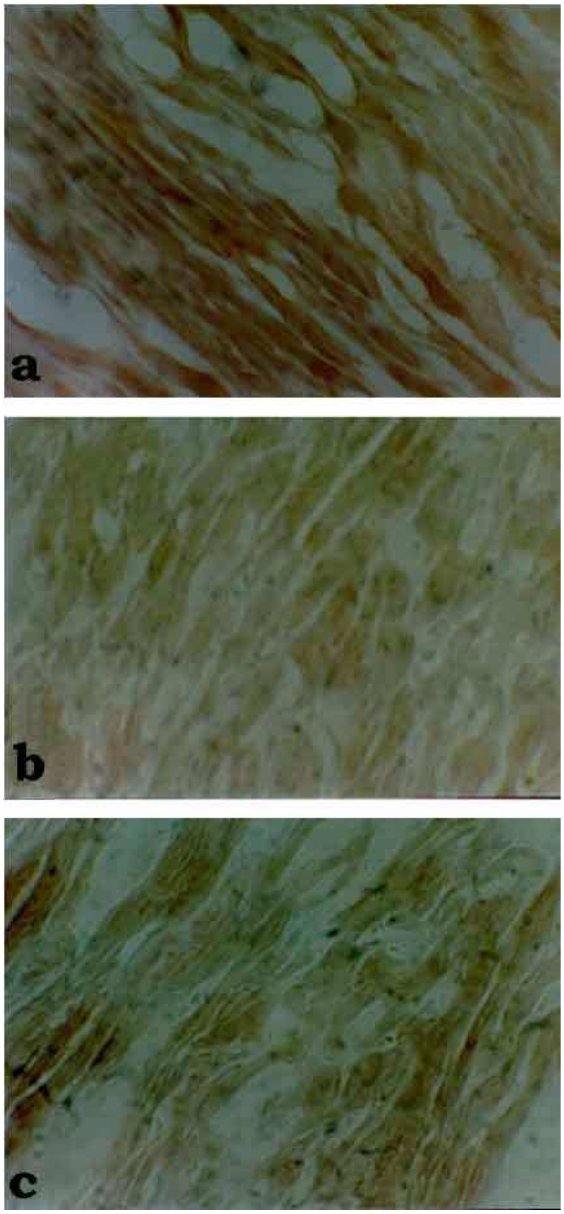

Fig. 3. Immunohistochemical expression of p85 α subunit of phosphatidylinositol-3-kinase in myocardial tissue deriving from rats in different experimental conditions. **a**. Control; **b**. Hypoxic condition; **c**. Hyperoxic condition.

number, in extension, uniformly distributed and in many cases without cristae. In hyperoxic samples they were reduced in number, often well preserved, while the extension remained unchanged, with respect to control, as revealed by densitometric analysis (Table. I). At this level the space between myofibrils appeared to be more represented in both stressed samples, with respect to control. In particular, in the hypoxic the compartment between myofibrils seemed to be occupied by augmented vascularization, while in the hyperoxic it seemed to be occupied by a larger amount of

exudate. In addition, we had results which showed the number of apoptotic cells increasing up to 25% of total upon hypoxic and hyperoxic treatment as determined by the TUNEL method (Fig.2 a,b,c), even though no DNA ladder was evident (not shown). Electron microscopy confirmed this evidence disclosing, at nuclear level, in both the experimental conditions, chromatin reorganization with clustering on one side of the nucleus and nuclear envelope shrinkage, suggesting an initial stage of apoptosis (Fig.2 d,e,f). As we had such evidence showing an increased number of apoptosis upon hypoxic and hyperoxic treatment, and knowing the involvement of PI-3-kinase and, in particular, of the pathway PI-3-kinase/AKT-1 in delivering an antiapoptotic signal (Kennedy *et al*.,1997), we checked the expression of both PI-3-kinase and AKT-1 in these samples in order to establish a relationship between these morphological features and a possible molecular event which could justify these effects and to understand if the myocardial cell could have a chance to recover from these stresses.

Immunohistochemical analysis evidenced a reduced expression of this enzyme upon oxidative stress (Fig.3 a,b,c), as confirmed by densitometric analysis (Table II), leading us to hypothesize that an oxygen supply change induces the cell to modify the synthesis of this protein. Western blotting analysis of immunoprecipitated PI-3-kinase confirmed this observation (Fig.4) and when the same blot was stripped of the primary antibody and reprobed with PY-99, which recognizes proteins phosphorylated on tyrosine residues, the p85 α subunit of PI-3-kinase resulted less phosphorylated upon hypoxic and hyperoxic treatment (Fig.5). In addition, when an "*in vitro*" PI-3-kinase activity was performed, $PI(3,4,5)P_3$ production resulted reduced in hypoxic and hyperoxic samples, with respect to control (Table III). Finally Fig. 6 shows that also the expression and activation of AKT-1, a downstream effector of PI-3-kinase, decreased upon hypoxic and hyperoxic stress in accordance with the increased number of found apoptotic cells.

Table II. DENSITOMETRIC ANALYSIS OF THE EXPRESSION OF P85 α SUBUNIT OF PHOSPHATIDYLINOSITOL-3-KINASE IN MYOCARDIAL TISSUE DERIVING FROM RATS IN DIFFERENT EXPERIMENTAL CONDITIONS±S.D. (P<0.001). VALUE EXPRESS THE INTEGRATED OPTICAL INTENSITY (I.O.I.)

Control	Hypoxic	Hyperoxic
1.12±0.27	0.71±0.07	0.78±0.09

Table III. "*IN VITRO*" ACTIVITY OF PI-3-KINASE IMMUNOPRECIPITATED FROM MYOCARDIAL TISSUE DERIVING FROM RATS IN DIFFERENT EXPERIMENTAL CONDITIONS. VALUES INDICATE DPM/MG PROTEIN. RESULTS ARE THE MEAN ± S.D. OF THREE SEPARATE EXPERIMENTS (P<0.001)

Control	Hypoxic	Hyperoxic
63546±6201	11381±1133	6226±595

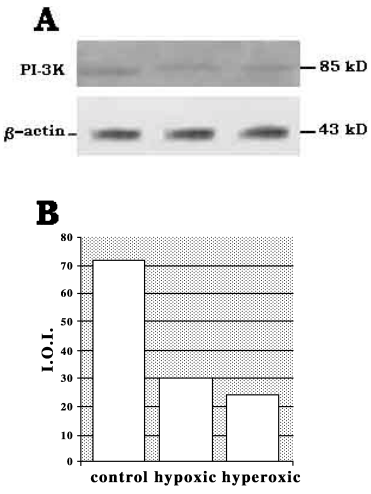

Fig. 4.

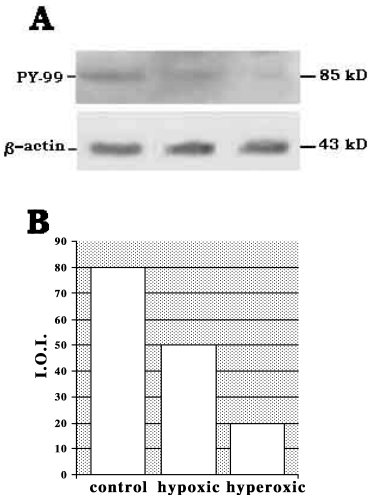

Fig. 5.

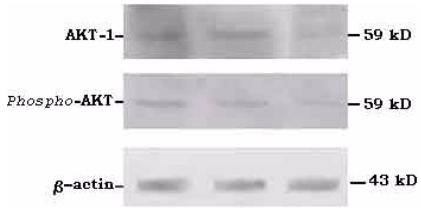

Fig. 6.

Fig. 4. **(A)** Western blotting analysis of immunoprecipitated p85 α subunit of phosphatidylinositol-3-kinase in myocardial tissue deriving from rats in different experimental conditions. Each electrophoretic lane was loaded with equal amount of protein as shown by β actin polyclonal antibody incubation. **(B)** Quantitative analysis of the expression of p85 α subunit of phosphatidylinositol-3-kinase determining the change in Integrated Optical Intensity (I.O.I.).

Fig. 5. **(A)** Western blotting analysis of tyrosine phosphorylation of immunoprecipitated p85 α subunit of phosphatidy1inositol-3-kinase in myocardial tissue deriving from rats in different experimental conditions. **(B)** Quantitative analysis of tyrosine phosphorylation of immunoprecipitated p 85 α subunit of phosphatidylinositol-3-kinase determining the change in Integrated Optical Intesity (I.O.I.).

Fig. 6. Western blotting analysis of the expression (A) and of serine phosphorylation (B) of immunoprecipitated AKT-1/ Protein Kinase B in myocardial tissue deriving from rats in different experimental conditions.

Discussion

Oxygen supply is essential for life. Moreover, oxygen concentration must be stable because if it is over or above physiological levels the cell can undergo substantial modifications. Interestingly, at molecular level, hypoxia induces regulation of intermediary metabolism affecting genes encoding enzymes responsible for glucose transport (Ebert *et al*., 1995), glycolysis (Semenza *et al*., 1994) and gluconeogenesis (Kietzmann *et al*.,1992). Hyperoxia, at the same time, induces expression of genes encoding a number of cytokines, vascular endothelial growth factors (VEGF) (Goldberg and Schneider, 1994), which appear to play an important role in wound healing, response to hischemic injury and tumour pathogenesis. In addition, enzymatic adaptation to hyperoxia depends on the upregulation of appropriate genes that detoxify reactive oxygen species (Fevreau and Pickett, 1993). At structural and ultrastructural level, oxygen supply modifications could imply increased vascularity of the skeletal muscle (Bigard *et al*.,1991), changes in the carotid body (Torbati *et al*.,1993), induced apoptosis in lung (Otterbein, 1998) and in the central nervous system (Taglialatela *et al*.,1998), damage at the mitochondrial level (Jones *et al*., 1991; Miquel *et al*.,1992). Obviously, the morphological modifications induced by low or high oxygen tension are sustained by a modified pattern of synthesized proteins which could justify the maintenance of the apoptotic state.

In this work we report the structural and ultrastructural modifications occurring "*in vivo*" in myocardial tissue upon hypoxic and hyperoxic treatment paralleled to the behaviour of the enzyme PI-3-kinase, enzyme responsible for most of the cellular response to stimuli of different nature including cell growth (Schluter *et al*., 1998), differentiation (Kim *et al*., 1999; Klinz *et al*., 1999; Cataldi *et al*., 2000) and apoptosis (Kennedy *et al*., 1997; Stephens *et al*., 1998). In fact, the myocardial nuclei underwent rearrangement of chromatin, nuclear envelope shrinkage, which could justify the occurrence of apoptosis, even though DNA fragmentation was not observed. The absence of DNA fragmentation, on the other hand, is not considered indicator of intact DNA structure, as elsewhere reported (Umansky and Tomei,1997). At molecular level we observed that the pathway PI-3-kinase/AKT-1 was not activated, as disclosed by a reduced amount of $PI(3,4,5)P_3$ produced in hypoxic and hyperoxic samples with respect to control. Since as elsewhere reported (Webster *et al*.,1993) the cells in hypoxic conditions store diacylglycerol, deriving from Phospholipase C mediated hydrolysis of $PI(4,5)P_2$, the well known substrate for both Phospholipase C and PI-3-kinase, it could be suggested that, in these experimental conditions, the pathway leading to diacylglycerol and inositol-3-phosphate production could be preferred, with respect to that leading to $PI(3,4,5)P_3$ synthesis, justifiyng the reduced expression, activation and activity of PI-3-kinase and of its downstream effector, Akt-1. At the same time, since PI-3-kinase inhibition is related to the occurrence of the apoptotic state as well necrotic for D3 ES cells (Klinz *et al*., 1999) and wortmannin, the well known inhibitor of PI-3-kinase abolished the cytoprotective effect of insulin in an "*in vitro*" similar system (Aikawa *et al*., 2000), it could be suggested that a down regulation of the PI-3-kinase/AKT-1 pathway is involved in the maintenance of the apoptotic events which occur in these cells in response to hypoxic and hyperoxic stress.

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