

**CALCIUM-INDEPENDENT PHOSPHOLIPASE A<sub>2</sub>-CATALYZED PLASMALOGEN  
HYDROLYSIS IN HYPOXIC HUMAN CORONARY ARTERY ENDOTHELIAL CELLS**

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Running head: PLA<sub>2</sub> activation in hypoxic endothelial cells

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**ABSTRACT**

Thrombin stimulation of human coronary artery endothelial cells (HCAEC) results in activation of a membrane-associated, calcium-independent phospholipase A<sub>2</sub> (iPLA<sub>2</sub>) that selectively hydrolyzes membrane plasmalogen phospholipids. Rupture of an atherosclerotic plaque and occlusion of the coronary vasculature results in a coronary ischemic event in which HCAEC in the ischemic area would be exposed to dramatic decreases in oxygen tension in addition to thrombin exposure.

We exposed HCAEC to hypoxia in the presence or absence of thrombin stimulation and measured iPLA<sub>2</sub> activation, membrane phospholipid hydrolysis and the accumulation of biologically active phospholipid metabolites. HCAEC exposed to hypoxia, thrombin stimulation, or a combination of the two conditions, demonstrated an increase in iPLA<sub>2</sub> activity and an increase in arachidonic acid release from plasmalogen phospholipids. Thrombin stimulation of normoxic HCAEC did not result in an accumulation of choline lysophospholipids, but hypoxia alone and in combination with thrombin stimulation led to a significant accumulation of lysoplasmalogen phospholipids (LPLsCho). We propose that the presence of hypoxia inhibits lysoplasmalogen phospholipid catabolism, at least in part, as a result of the accumulation of long chain acylcarnitines. The combination of increased production and decreased catabolism of LPLsCho is necessary for its accumulation. Pretreatment with bromoenol lactone (BEL) to inhibit iPLA<sub>2</sub>, blocked membrane phospholipid hydrolysis and production of membrane phospholipid-derived metabolites.

The increase in iPLA<sub>2</sub> activity and the subsequent accumulation of membrane phospholipid-derived metabolites in HCAEC exposed to hypoxia or thrombin stimulation alone, and particularly in combination, have important implications in inflammation and arrhythmogenesis in atherosclerosis/thrombosis and subsequent myocardial ischemia.

Keywords: myocardial ischemia, arrhythmogenesis, thrombosis

## INTRODUCTION

Sudden cardiac death in man invariably results from malignant ventricular arrhythmias secondary to acute myocardial ischemia precipitated by the evolution of an intracoronary thrombus (3,7,32). We have demonstrated previously that thrombin stimulation of human coronary artery endothelial cells (HCAEC) results in hydrolysis of membrane plasmalogen phospholipids by a  $\text{Ca}^{2+}$ -independent phospholipase  $\text{A}_2$  (iPLA<sub>2</sub>) that leads to the generation of several phospholipid metabolites that may play an important role in inflammation or arrhythmogenesis in the heart (21).

Once blood flow is interrupted or severely reduced by increased or complete occlusion of a coronary artery precipitating an ischemic event, the endothelial cells in the ischemic area would be exposed to dramatic decreases in oxygen tension in addition to exposure to thrombin. Results from studies involving release of arachidonic acid from hypoxic endothelial cells are conflicting and appear to depend on both the species and site from where endothelial cells are isolated originally (9,27). In human umbilical vein endothelial cells, hypoxia and reoxygenation results in increased platelet-activating factor production, indicating increased PLA<sub>2</sub> activity (9). Additionally, in bovine aortic endothelial cells, hypoxia results in release of free arachidonic acid from plasmenylethanolamine, suggesting that a plasmalogen-selective PLA<sub>2</sub> is involved (27).

Phospholipase  $\text{A}_2$  are responsible for hydrolyzing the sn-2 ester bonds in membrane plasmalogen phospholipids, such as plasmenylcholine and plasmenylethanolamine, releasing a free fatty acid and a lysophospholipid. We have shown that the majority of HCAEC PLA<sub>2</sub> activity is membrane associated, does not require calcium for activity, and demonstrates a preference for arachidonylated substrates. Calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>) activity has been demonstrated in both the cytosol and membrane fractions of mammalian cells (2,11,12,19,28).

iPLA<sub>2</sub> plays a critical role in the formation of inflammatory mediators arachidonic acid and platelet-activating factor (PAF) which are essential in both the initiation and propagation of the inflammatory response. Once liberated from phospholipids by iPLA<sub>2</sub>, arachidonic acid is converted to prostaglandins by the action of cyclooxygenase (COX) enzymes. Liberated lysophospholipids, such as lysoplasmenecholine and lysophosphatidylcholine, can be rapidly acetylated at the sn-2 position by lyso-PAF:acetyl CoA acetyltransferase to produce PAF. To date, no studies have examined the effects of hypoxia on human endothelial cells from an arterial origin with respect to PLA<sub>2</sub> activation, phospholipid hydrolysis and the production of biologically active phospholipid metabolites.

In this study, we determined whether hypoxia alone resulted in increased iPLA<sub>2</sub> activity and membrane phospholipid hydrolysis and whether hypoxia potentiates the thrombin-stimulated changes that we have described previously.

## MATERIALS AND METHODS

*Cell Isolation and Culture.* Human coronary artery endothelial cells (HCAEC, Cambrex Bio Science, Walkersville, MD) were grown to confluence in MCDB 131 medium with 5% fetal calf serum, 10 ng/ml epidermal growth factor, 1 µg/ml hydrocortisone, 200 µg/ml endothelial cell growth supplement, and 90 µg/ml heparin. Cells were allowed to grow to confluence achieving a contact inhibited monolayer of flattened, closely apposed endothelial cells in 4 to 5 days.

*Induction of Hypoxia.* A glucose-free 1.2 mM Ca-HEPES buffer, pH 7.4, was degassed under vacuum for 1 hour and then bubbled with 100% prepurified N<sub>2</sub> for at least 2 hours to attain a pO<sub>2</sub> of <15 mm Hg. The medium surrounding confluent HCAEC was removed and replaced with glucose-free 1.2 mM Ca-HEPES in a hypoxic environment in which room air had been exchanged with 100% N<sub>2</sub>. The 100% N<sub>2</sub> atmosphere was maintained above the hypoxic solution and cells for the entire hypoxic interval.

*Measurement of ATP Content.* High energy phosphates were separated and quantified using an HPLC system (Waters Chromatography, Milford, MA) consisting of a model 510 HPLC pump, an injector (Rheodyne Inc, Cotati, CA), and a model 484 tunable absorbance detector, set at a wavelength of 214 nm, interfaced to an IBM PC-AT computer using a 900 series interface and 3000 series chromatography data systems software (Nelson Analytical, Cupertino, CA). Samples (10 to 50 µl) were injected onto an Hi-Pore RP-18 reverse-phase HPLC column (Bio-Rad) and eluted with an isocratic mobile phase of 0.1 mol/L ammonium phosphate, pH 5.5, at a flow rate of 0.5 ml/min. Under these conditions, ATP eluted from the column within 20 mins. Quantification of ATP was determined by comparing the integrated peak area to a linear regression curve constructed from the integrated peak areas obtained from injecting 0.2 to 1.0 nmol of ATP standard (Sigma Chemical Co, St. Louis, MO). ATP levels were corrected for

protein content for each cell culture and expressed as nanomoles per milligram of protein.

*Phospholipase A<sub>2</sub> Activity.* Confluent HCAEC were subjected to hypoxia with or without thrombin stimulation or thrombin stimulation alone for the allocated time intervals. For iPLA<sub>2</sub> inhibition experiments, HCAEC were pretreated with 5  $\mu$ M BEL prior to exposure to hypoxia or thrombin stimulation. Additionally, in selected experiments, 10 mM ATP was added to HCAEC prior to exposure to hypoxic conditions. At the end of the stimulation period, the surrounding buffer was removed and immediately replaced with ice cold buffer containing (mmol/liter): Sucrose 250, KCl 10, Imidazole 10, EDTA 5, DTT 2 with 10% glycerol, pH = 7.8. Cells were removed from the tissue culture well using a cell scraper and the suspension was sonicated on ice. Phospholipase A<sub>2</sub> activity was assessed by incubating 50  $\mu$ g cellular protein with 100  $\mu$ M (16:0, [3H]18:1) plasmenylcholine in assay buffer containing 100 mM Tris, 4 mM EGTA, 10% glycerol, pH = 7.0 at 37°C for 5 mins in a total volume of 200  $\mu$ l. Specific enzyme activity was expressed as the rate of radiolabeled fatty acid production determined following separation from the labeled phospholipid substrate using thin layer chromatography and liquid scintillation spectrometry with activity normalized to protein content.

*Separation and Quantification of Individual Choline and Ethanolamine Glycerophospholipid Molecular Species.* Cellular phospholipids were extracted from HCAEC by the method of Bligh and Dyer (4). The chloroform layer was dried under N<sub>2</sub> and the lipid residue resuspended in 1 ml of chloroform/methanol [1:1 (v/v)]. Phospholipids were separated into different classes by HPLC using gradient elution with a mobile phase comprised of hexane/isopropanol/water (20). Individual choline and ethanolamine glycerophospholipid molecular species were separated by reverse-phase HPLC using a gradient elution system with a mobile phase comprised of acetonitrile/methanol/water with 20 mM choline chloride (20). Quantification of individual

phospholipid molecular species was achieved by determination of lipid phosphorus in reverse phase HPLC column effluents (20).

*Arachidonic Acid Release.* The extent of arachidonic acid release was determined by measuring the amount of [ $^3\text{H}$ ] arachidonic acid released into the surrounding medium from HCAEC prelabeled with  $3\mu\text{Ci}$  of [ $^3\text{H}$ ] arachidonic acid per 35 mm culture dish for 18 h. Following incubation, HCAEC were washed three times with Tyrode's solution containing 3.6% bovine serum albumin to remove unincorporated [ $^3\text{H}$ ] arachidonic acid. Endothelial cells were incubated at  $37^\circ\text{C}$  for 15 mins prior to implementation of the experimental conditions. At the end of the stimulation period, radioactivity in both the surrounding medium and endothelial cells was quantified by liquid scintillation spectrometry.

*Measurement of Phosphatidate Phosphohydrolase (PAPH) Activity.* Magnesium-dependent PAPH activity in the cytosolic fraction of HCAEC was measured by incubating cytosolic protein with  $600\mu\text{M}$  [ $^{14}\text{C}$ ] glycerol labeled phosphatidic acid (specific activity, 1 mCi/mmol) at  $37^\circ\text{C}$  for 10 min in a final volume of  $100\mu\text{l}$  containing 100 mM Tris-HCl buffer (pH 7.0), 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 2 mM  $\text{MgCl}_2$  and 0.5% Triton X-100. The reaction was stopped by the addition of  $\text{CHCl}_3/\text{CH}_3\text{OH}$  (2:1, v/v) and 0.5 ml of 0.1 M KCl. The tubes were mixed vigorously for 2 min and the phases separated by centrifugation. [ $^{14}\text{C}$ ] phosphatidic acid and released [ $^{14}\text{C}$ ] diacylglycerol were separated by thin layer chromatography on silica gel plates using petroleum ether/ether/acetic acid (60:40:1). Released [ $^{14}\text{C}$ ] diacylglycerol was quantified by liquid scintillation spectrometry.

*Choline Lysophospholipid Production.* Lysophosphatidylcholine (LPtdCho) and lysoplasmerylcholine (LPIsCho) were measured using a modification of a radiometric assay method published previously (22). Lipids were extracted from HCAEC and the surrounding medium by the method of Bligh and Dyer (4), and lysophospholipids were

separated from other phospholipids by HPLC. The purified LPtdCho and LPIsCho fractions were acetylated with [<sup>3</sup>H]acetic anhydride and the acetylated lysophospholipids separated by thin-layer chromatography and radioactivity quantified by liquid scintillation spectrometry. Standard curves were constructed, and the LPtdCho and LPIsCho content was derived for all samples and normalized according to the protein content of HCAEC. [<sup>14</sup>C]LPtdCho was added as an internal standard to all samples to correct for the loss of sample that occurred during extraction, purification, and acetylation (22).

*Long Chain Acylcarnitine Production.* Long chain acylcarnitine (LCAC) content in confluent HCAEC exposed to hypoxia was determined using the method of McGarry and Foster (17) that was modified as previously described in detail (18,33).

## RESULTS

In order to determine the most appropriate conditions for thrombin stimulation and hypoxia exposure, a time course of  $\text{Ca}^{2+}$ -independent  $\text{PLA}_2$  (i $\text{PLA}_2$ ) activation following each condition was performed. As shown in Figure 1A, maximal i $\text{PLA}_2$  activity occurs after 2 mins of thrombin stimulation (0.1IU/ml, filled circles). Pretreatment with bromoenol lactone (BEL), a selective i $\text{PLA}_2$  inhibitor (12), completely inhibited the increase in i $\text{PLA}_2$  activity measured in thrombin stimulated HCAEC (Figure 1A, open circles).

Confluent monolayers of HCAEC were also incubated under hypoxic conditions for increasing time with or without 30mins reoxygenation and i $\text{PLA}_2$  activity was measured. Increases in i $\text{PLA}_2$  activity are maximal after 10mins of hypoxia (Figure 1B, filled circles), and stay elevated for up to 40mins. Hypoxic conditioning for up to 20 mins followed by 30 mins of reoxygenation returns i $\text{PLA}_2$  activity to normoxic control levels (Figure 1B, open circles). Since 10mins of hypoxia resulted in maximal and reversible  $\text{PLA}_2$  activity, we chose this time point for all further studies.

Previously our laboratory has shown that the presence of ATP significantly inhibits membrane-associated  $\text{PLA}_2$  activity in both normoxic and hypoxic myocytes, (22). Thus, we examined whether decreased ATP levels, which occur early after the onset of oxygen depletion, serve as a potential mechanism for the activation of i $\text{PLA}_2$  in hypoxic HCAEC. ATP content was measured in HCAEC after exposure to hypoxic conditions for up to 60 mins (Figure 2). ATP levels decrease significantly over time (Figure 2). Additionally,  $\text{PLA}_2$  activity was measured in normoxic HCAEC and HCAEC exposed to hypoxic conditions in the presence or absence of ATP (10nm). Hypoxic conditions in the absence of ATP lead to significant increases in  $\text{PLA}_2$  activity (Figure 3, filled bars). The presence of ATP significantly inhibits the hypoxia induced increase in  $\text{PLA}_2$  activity (Figure 3, open bars). These results suggest that the decrease in ATP

content observed early in hypoxia may, at least in part, be responsible for the increase in iPLA<sub>2</sub> activity.

In order to determine whether the effects of hypoxia and thrombin stimulation on PLA<sub>2</sub> activity were additive, the effects of these conditions alone or in combination in either the absence or presence of BEL pretreatment were compared. Incubation of confluent monolayers of HCAEC under either hypoxic conditions (10 mins, filled bars) or thrombin stimulation (0.1 IU/ml, 2 mins, filled bars) alone led to a significant increase in iPLA<sub>2</sub> activity (Figure 4). These increases in iPLA<sub>2</sub> activity were completely inhibited by BEL pretreatment (Figure 4, open bars). Thrombin stimulation in the presence of hypoxia increased iPLA<sub>2</sub> activity (filled bars) significantly more than either treatment alone. This increase in iPLA<sub>2</sub> activity was also inhibited by BEL pretreatment (Figure 4, open bars).

To determine whether the increase in iPLA<sub>2</sub> activity resulted in accelerated membrane phospholipid hydrolysis, we measured the individual membrane phospholipid molecular species in choline and ethanolamine glycerophospholipid classes. A significant decrease in plasmenylcholine molecular species with arachidonic (20:4) or linoleic (18:2) acid at the *sn*-2 position was observed following exposure to hypoxia that was potentiated by thrombin stimulation (Figure 5, filled bars). There was no significant change in phosphatidylcholine (data not shown), suggesting preferential plasmalogen phospholipid hydrolysis. A significant decrease in arachidonylated plasmenyethanolamine was observed in hypoxic HCAEC that were stimulated with thrombin (Figure 6, filled bars), but no change in ethanolamine phospholipids was observed with hypoxia or thrombin stimulation alone. Pretreatment with BEL completely inhibited iPLA<sub>2</sub>-catalyzed hydrolysis of all membrane phospholipids tested (Figures 5 and 6, open bars).

Arachidonic acid release from HCAEC was significantly increased by exposure to hypoxia or thrombin stimulation and was further potentiated when hypoxia was combined with thrombin stimulation (Figure 7, filled bars). Pretreatment with BEL completely inhibited any increase in arachidonic acid release (Figure 7, open bars).

Studies by other laboratories have shown that in addition to inhibiting iPLA<sub>2</sub>, BEL inhibits phosphatidate phosphohydrolase (PAPH) activity, an enzyme whose activation also results in arachidonic acid release. PAPH converts phosphatidic acid, released from membrane phospholipids following hydrolysis by phospholipase D, to diacylglycerol (DAG). Subsequent activation of protein kinase C by DAG can lead to the activation of iPLA<sub>2</sub>, releasing arachidonic acid as a product of plasmalogen phospholipid hydrolysis. To ensure the decrease in arachidonic acid seen upon BEL pretreatment of thrombin stimulated cells is not a result of inhibition of PAPH, we examined PAPH activity in the presence and absence of BEL. PAPH activity is only significantly decreased at concentrations of BEL greater than 10  $\mu$ M (Figure 8), 2-fold higher than the concentration utilized to directly inhibit iPLA<sub>2</sub> activity. These results indicate that HCAEC PAPH activity is not inhibited by 5  $\mu$ M BEL pretreatment.

The increased iPLA<sub>2</sub>-catalyzed hydrolysis of membrane phospholipids was accompanied by an increase in choline lysophospholipid accumulation that was significant when hypoxic HCAEC were stimulated with thrombin (Figure 9, filled bars). HCAEC exposed to hypoxia alone demonstrated a significant increase in LPIsCho accumulation (Figure 9, left, filled bars). While hypoxia led to an increase in LPtdCho (Figure 9, right, filled bars), this increase was not significant. BEL pretreatment completely inhibited any increase in choline lysophospholipid production.

We propose that under hypoxic conditions, the majority of lysoplasmalogen that is hydrolyzed by iPLA<sub>2</sub> is further catabolized, thus precluding large increases in choline lysophospholipids. Under normoxic conditions, the capacity of the myocardium for

lysophosphatidyl-choline (LPtdCho) catabolism is approximately 100-fold its capacity for LPtdCho production via PLA<sub>2</sub> activity (23). However, in the ischemic myocardium, LPtdCho catabolism is significantly inhibited by both the accumulation of long-chain acylcarnitines (LCAC) and the presence of acidosis. To determine whether the increase in choline lysophospholipids in hypoxic HCAEC may be a result of decreased catabolism, we measured LCAC content in HCAEC subjected to increased time intervals of hypoxia. As demonstrated in Figure 10, LCAC content was significantly increased in HCAEC exposed to 10 mins hypoxia and was maximal after 20 mins. These data suggest that the accumulation of LCAC could contribute to a decrease in the rate of catabolism of choline lysophospholipids under hypoxic conditions and may be responsible for their accumulation in hypoxic HCAEC.

## DISCUSSION

Thrombotic coronary occlusion has been shown to contribute directly to the incidence of malignant ventricular arrhythmias (5,10), suggesting that products released from, or associated with, a thrombotic occlusion either directly or indirectly influence the electrophysiologic behavior of ischemic cardiac myocytes. In previous studies, we have demonstrated that thrombin stimulation of endothelial cells results in hydrolysis of membrane phospholipids by iPLA<sub>2</sub> that results in the generation of several phospholipid metabolites that may play an important role in arrhythmogenesis either directly or indirectly (14,21). An evolving thrombus in a coronary vessel is associated with increased amounts of thrombin (26), some of which will be carried downstream by residual blood flow and come in contact with endothelial cells distal to the thrombus, activating endothelial cell thrombin receptors. Once blood flow is interrupted or severely reduced by increased or complete occlusion of the coronary vessel precipitating an ischemic event, the endothelial cells in the ischemic area would be exposed to dramatic decreases in oxygen tension in addition to exposure to thrombin. In this study, we demonstrate that a decrease in oxygen such as would occur in myocardial ischemia can potentiate the thrombin-stimulated production of membrane phospholipid metabolites via activation of iPLA<sub>2</sub> in HCAEC.

Previous studies by our laboratory have focused on determining the mechanism of iPLA<sub>2</sub> activation by thrombin (24) with results suggesting that thrombin-stimulated iPLA<sub>2</sub> activation is mediated by novel protein kinase C isoform. Studies detailed in this manuscript suggest the decrease in ATP levels which accompany ischemia may be a possible mechanism for the activation of iPLA<sub>2</sub> seen in hypoxic conditions.

The accumulation of amphiphilic metabolites, such as lysophospholipids, in the ischemic myocardium can elicit electrophysiologic derangements in normoxic myocytes as a result of incorporation into the phospholipid bilayer of the sarcolemma (23). More

importantly, prevention of sarcolemmal accumulation of amphiphilic metabolites markedly reduces the incidence of ischemia-induced ventricular arrhythmias (6). LPTdCho has been shown to associate preferentially with the outer leaflet of the myocyte sarcolemma and it has been suggested that this type of association may be responsible for LPTdCho-induced electrophysiologic alterations in the heart (16). Akita et al have demonstrated that intracellular microinjection of LPTdCho into isolated myocytes at concentrations as high as 500  $\mu\text{M}$  did not alter electrophysiologic properties of the myocytes (1), whereas extracellular concentrations as low as 5-10  $\mu\text{M}$  have been shown to have profound effects (1,23). These data, together with the finding that LPTdCho is elevated in the venous and lymphatic effluent from the ischemic myocardium of both animals and man (29,30) with no evidence of irreversible myocyte damage, suggests that LPTdCho from an extramyocytic source plays an important role in arrhythmogenesis.

We have more recently examined the effect of including lysoplasmenylcholine (LPIsCho) in the perfusate of isolated normoxic cardiac myocytes and have observed profound alterations in the action potential of these myocytes, leading to the production of afterdepolarizations (15,22). These changes were reversible upon removal of LPIsCho from the perfusate. Although the changes in cardiac myocyte action potential parameters were similar to those observed previously using other amphiphilic compounds such as LPTdCho and LCAC (see 23 for review), they occurred at much lower concentrations, suggesting that LPIsCho may interact specifically with ion channel proteins in the membrane in addition to its ability to compromise the biophysical properties of the phospholipid bilayer. The action potential derangements caused by LPIsCho occur as a result of the action of this amphiphilic compound on multiple membrane currents and on myocytic calcium handling (15).

In addition to the production of choline lysophospholipids, activation of HCAEC iPLA<sub>2</sub> results in the production of a free fatty acid. When the membrane phospholipid

that is hydrolyzed is arachidonylated, the resultant free arachidonic acid can be further metabolized to eicosanoids. The synthesis of arachidonic acid and eicosanoids in the heart has been implicated in several pathological conditions and may have direct inotropic or chronotropic effects (13). Finally, acetylation of choline lysophospholipids to produce platelet-activating factor in HCAEC may also play an important role in heart disease. Although PAF remains endothelial cell-associated (21), increased PAF production results in increased binding of neutrophils to the endothelial cell surface (25,31) and may play a role in the adhesive interaction of other cells such as eosinophils (8), thus contributing to progressing atherothrombosis and inflammatory processes in the coronary vasculature.

The increased LPtdCho observed in HCAEC stimulated with thrombin in the presence of hypoxia was not accompanied by a significant decrease in phosphatidylcholine. Under ischemic conditions in the myocardium, LPtdCho catabolism is significantly inhibited by the presence of acidosis and LCAC accumulation (see 23 for review). Since we detected significant LCAC accumulation in hypoxic HCAEC, we propose that there may be little, if any, LPtdCho catabolism in these cells and that the PLA<sub>2</sub>-catalyzed hydrolysis of phosphatidylcholine was too small to determine a significant decrease in mass. In contrast, we detected a significant decrease in plasmenylcholine (approximately 8 nmol PO<sub>4</sub>/mg protein) in hypoxic HCAEC stimulated with thrombin that was accompanied by an increase in LPIsCho of only 0.3 nmol/mg protein. These data suggest that even under hypoxic conditions, the majority of LPIsCho can be further catabolized. We did not detect an increase in choline phospholipids that would indicate reacylation of LPIsCho, suggesting that the majority of LPIsCho may be catabolized via lysoplasmalogenase or lysophospholipase D. To date, there are no data available regarding the catabolic pathways for LPIsCho in endothelial cells, however, these are clearly future studies.

These findings suggest that the presence of decreased oxygen tension and thrombin activated HCAEC iPLA<sub>2</sub> resulting in increased membrane plasmalogen hydrolysis and the accumulation of phospholipid metabolites that may have important implications in arrhythmogenesis and inflammation following myocardial ischemia.

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**FIGURE LEGENDS**

**FIGURE 1. A.** Time course of thrombin stimulated PLA<sub>2</sub> activity in the absence (filled circles) or presence (open circles) of BEL pretreatment (5 μM, 10 mins). **B.** PLA<sub>2</sub> activity in HCAEC incubated under hypoxic conditions for the indicated time points with (open circles) or without (filled circles) 30mins of reoxygenation. PLA<sub>2</sub> activity was measured using 100 μM (16:0, [<sup>3</sup>H]18:1) plasmenylcholine substrate in the absence of Ca (4 mM EGTA). Data are means + SEM for results from 6 separate cell cultures. \*p<0.05, \*\*p<0.01 when compared to control cell cultures.

**FIGURE 2.** ATP content in HCAEC cultures exposed to hypoxia for increasing intervals. Data represent means +/- SEM for 3 separate cell cultures. \*p<0.05, \*\*p<0.01 when compared to normoxic ATP content.

**FIGURE 3.** The effect of the presence of ATP on membrane-associated PLA<sub>2</sub> activity in normoxic or hypoxic HCAEC. PLA<sub>2</sub> activity was measured using (16:0, [3H]18:1) plasmenylcholine in the absence of Ca (4 mM EGTA), with or without 10 mM ATP. Values represent means + SEM for 4 separate cell cultures. \*p<0.05 when comparing with corresponding values in the absence of ATP. ++ p<0.01 when comparing hypoxic and normoxic PLA<sub>2</sub> activity.

**FIGURE 4.** PLA<sub>2</sub> activity measured in HCAEC following exposure to hypoxia (10 mins), thrombin stimulation (0.1 IU/ml, 2 mins) or hypoxia with thrombin stimulation. PLA<sub>2</sub> activity was measured in HCAEC that were incubated with (open bars) or without (filled bars) bromoenol lactone (BEL, 5 μM, 10 mins) pretreatment. PLA<sub>2</sub> activity was measured using 100 μM (16:0, [<sup>3</sup>H]18:1) plasmenylcholine substrate in the absence of

Ca. (4 mM EGTA). Data are means + SEM for results from 6 separate cell cultures. \* $p < 0.05$ , \*\* $p < 0.01$  when compared to untreated control cell cultures. ++ $p < 0.01$  when comparing hypoxia plus thrombin to either condition alone.

**FIGURE 5.** Changes in plasmenylcholine molecular species containing arachidonic (20:4) or linoleic (18:2) acid at the *sn*-2 position measured in HCAEC following to hypoxia (Hyp, 10mins), thrombin (Thr, 0.1 IU/ml, 2mins) or both (Hyp & Thr). HCAEC were pretreated with (open bars) or without (filled bars) bromoenol lactone (BEL, 5  $\mu$ M, 10 mins) prior to hypoxia and/or thrombin experimental conditions. Data are means + SEM for 6 separate cell cultures. \*\*  $p < 0.01$  when compared with control values. ++ $p < 0.01$  when comparing hypoxia plus thrombin to either condition alone.

**FIGURE 6.** Changes in plasmenylethanolamine molecular species containing arachidonic (20:4) or linoleic (18:2) acid at the *sn*-2 position measured in HCAEC following exposure to hypoxia (Hyp, 10mins), thrombin (Thr, 0.1 IU/ml, 2mins) or both (Hyp & Thr). HCAEC were pretreated with (open bars) or without (filled bars) bromoenol lactone (BEL, 5  $\mu$ M, 10 mins) prior to hypoxia and/or thrombin experimental conditions. Data are means + SEM for 6 separate cell cultures. \*\*  $p < 0.01$  when compared with control values. ++ $p < 0.01$  when comparing hypoxia plus thrombin to either condition alone.

**FIGURE 7.** Arachidonic acid release from HCAEC incubated overnight with [ $^3$ H] arachidonic acid and incubated under hypoxia (10 mins) and/or thrombin stimulation (0.1 IU/ml, 2 mins). Data are means + SEM for 6 separate cell cultures. \*  $p < 0.05$ , \*\*  $p < 0.01$  when compared to control values. ++ $p < 0.01$  when comparing hypoxia plus thrombin to either condition alone.

**FIGURE 8.** Inhibition of endothelial cell cytosolic phosphatidate phosphohydrolase (PAPH) activity measured following 30 mins incubation with bromoenol lactone (BEL). Results represent data derived from 3 separate cell cultures. \*  $p < 0.05$ , \*\* $p < 0.01$  when compared to untreated activity.

**FIGURE 9.** Changes in HCAEC lysoplasmethylcholine (LPIsCho, left) and lysophosphatidylcholine (LPtdCho, right) content in response to thrombin stimulation (0.1IU/ml, 2mins) or hypoxia stimulation. Pretreatment with 5  $\mu$ M bromoenol lactone (BEL, open bars) completely inhibited choline lysophospholipid production. Values are mean + SEM for 6 different cell cultures. \*\*  $p < 0.01$  when compared to control values. ++ $p < 0.01$  when comparing hypoxia plus thrombin to either condition alone.

**FIGURE 10.** Long chain acylcarnitine accumulation in HCAEC exposed to hypoxia for increasing time intervals. Values are mean  $\pm$  SEM for 6 separate cell cultures. \*\* $p < 0.01$  when compared to cells under normoxic conditions.

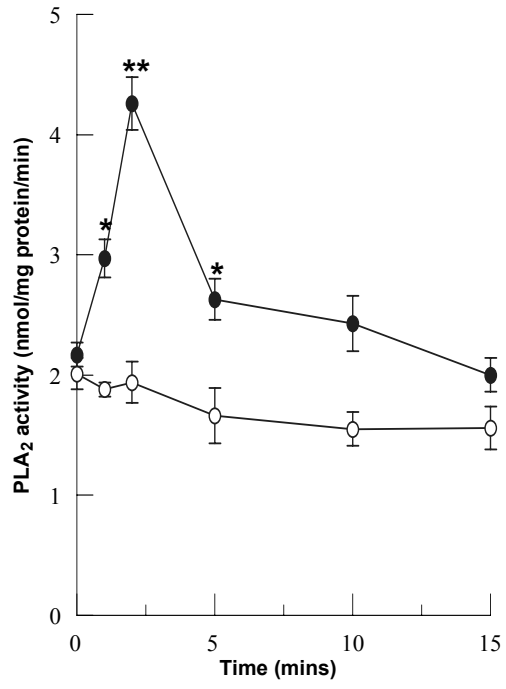


Figure 1A.

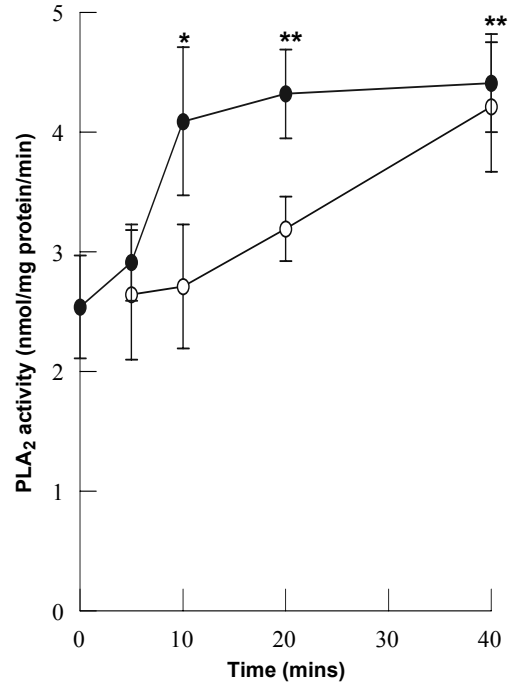


Figure 1B.

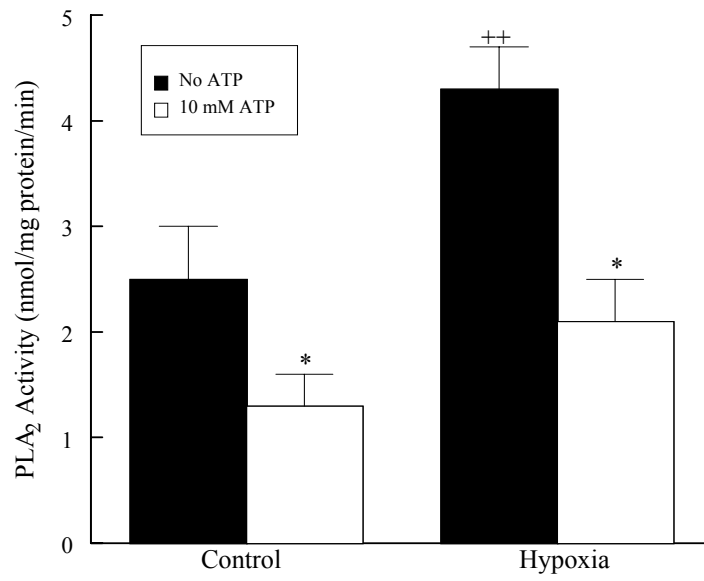


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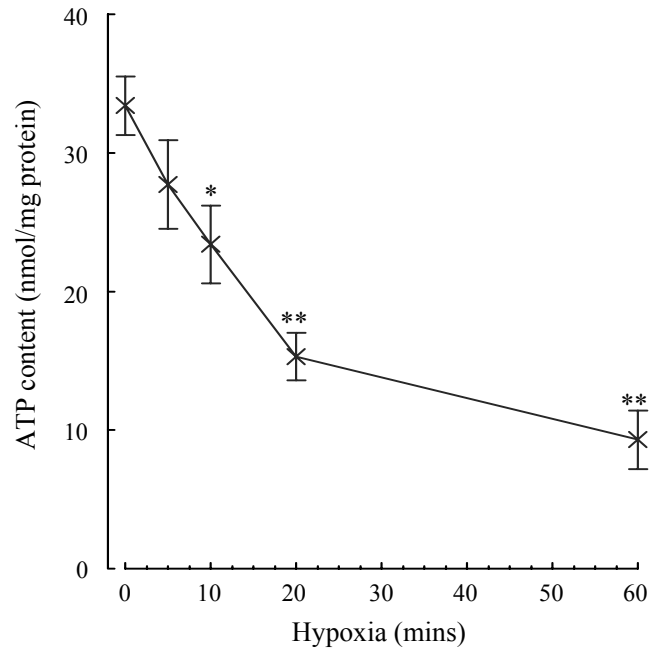


Figure 3.

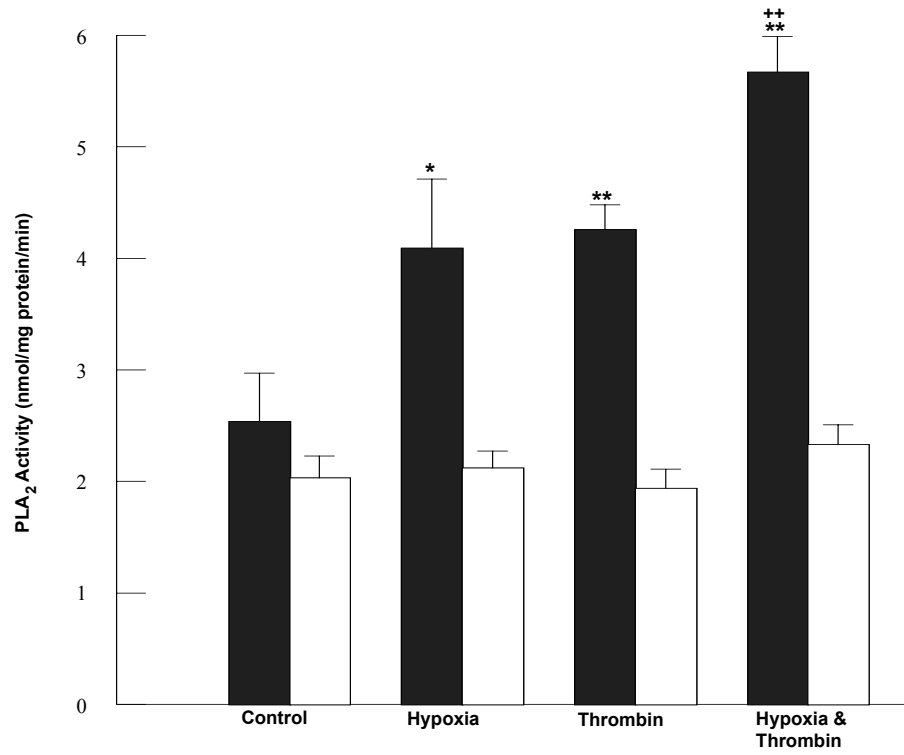


Figure 4.

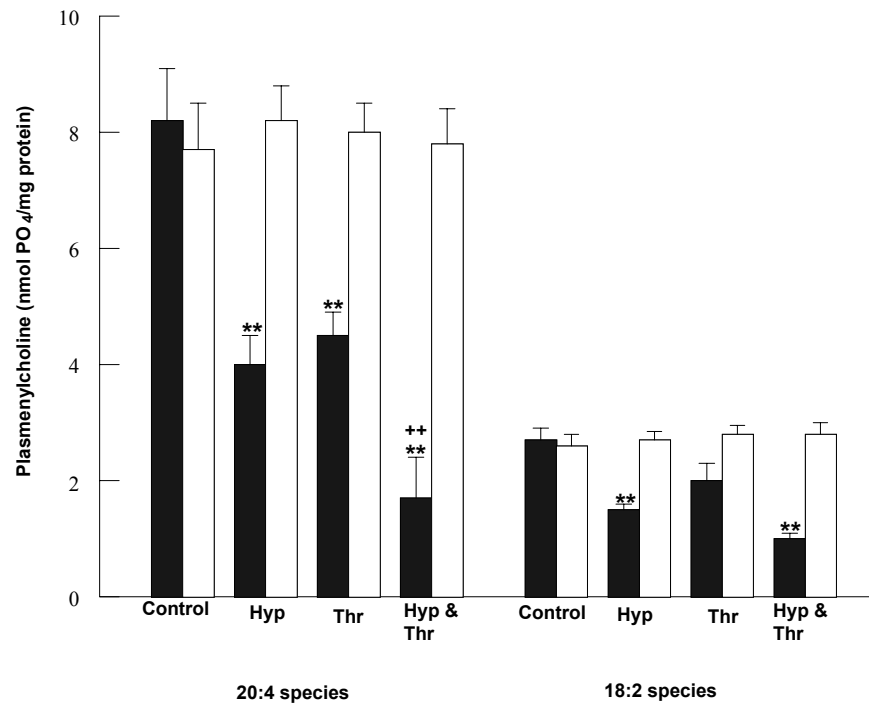


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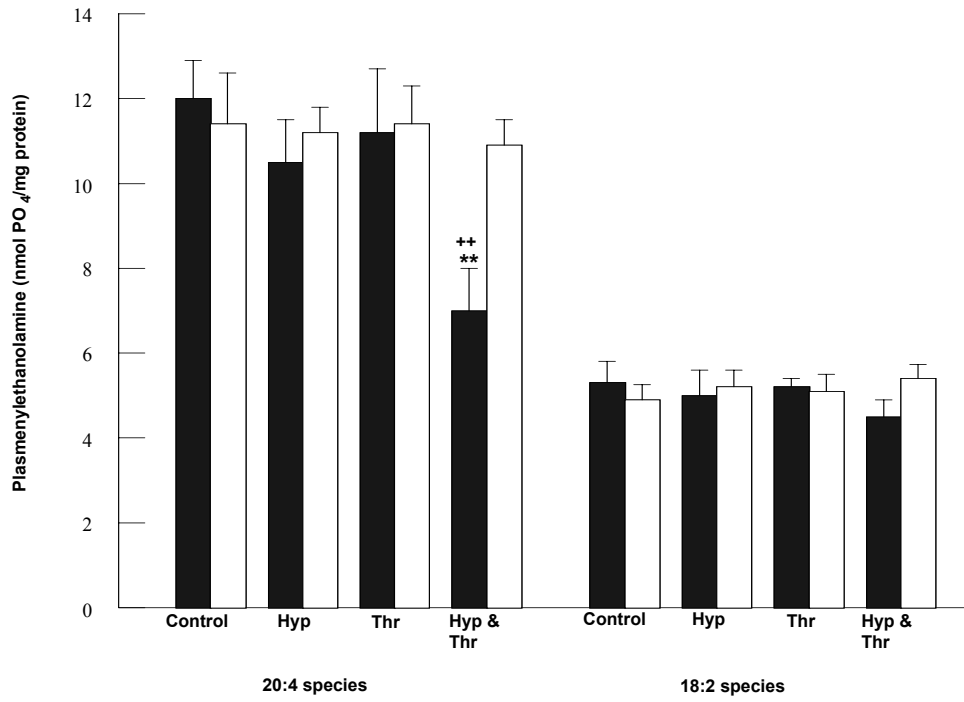


Figure 6.

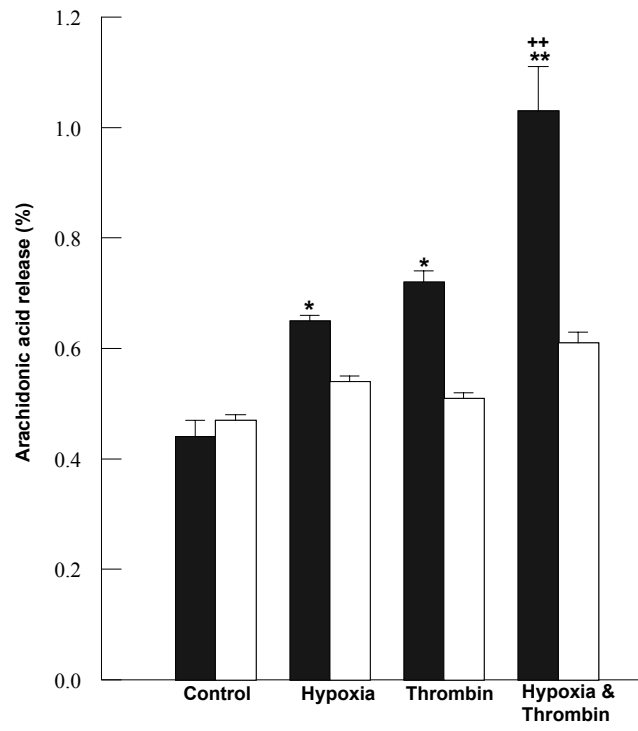


Figure 7.

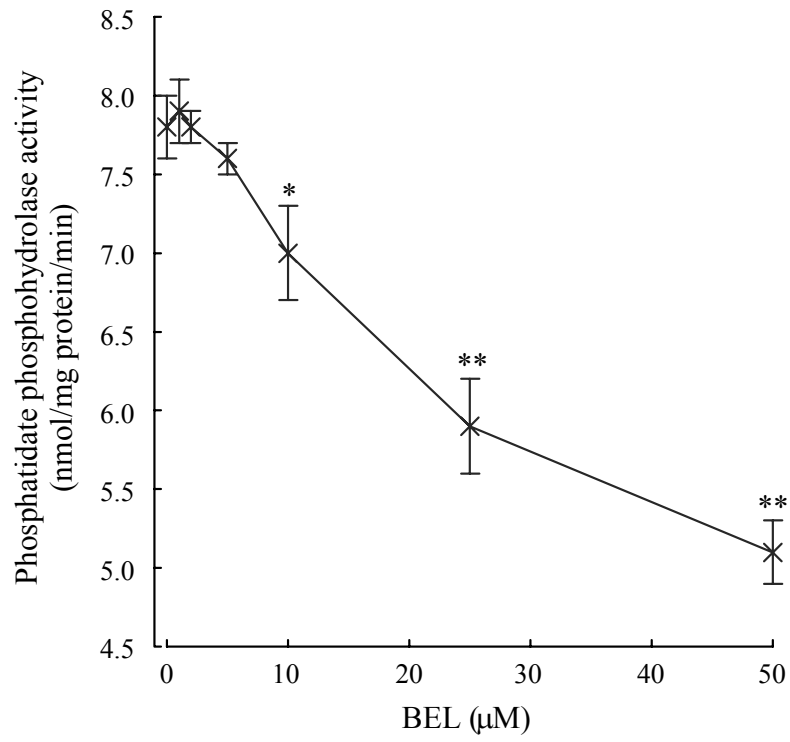


Figure 8.

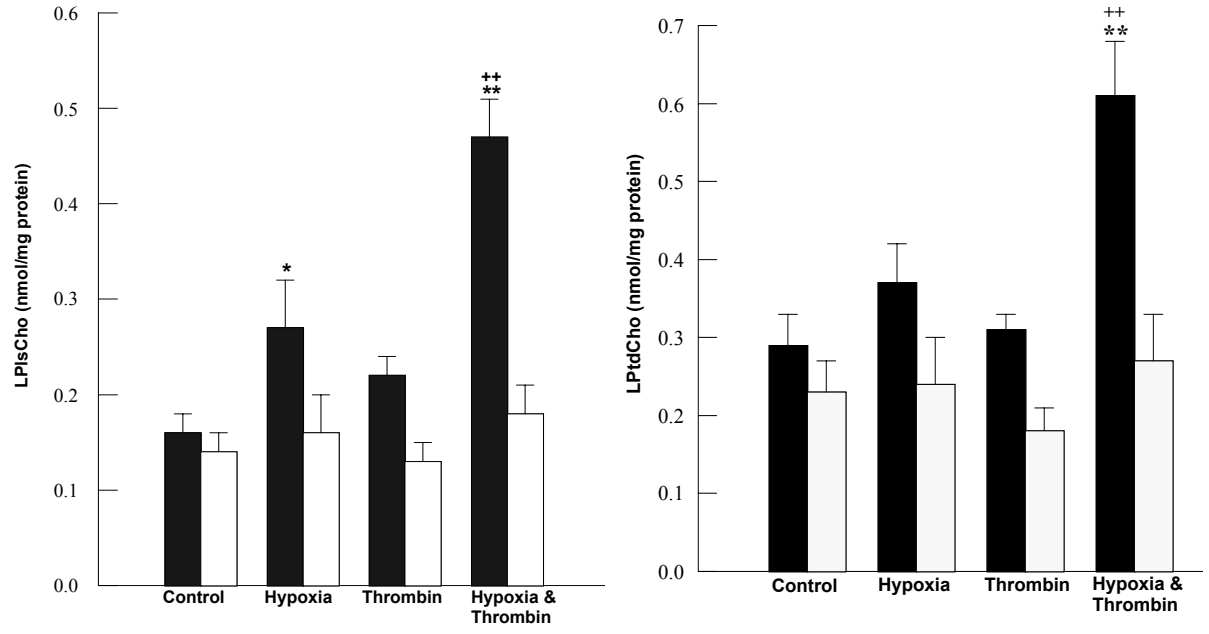


Figure 9.

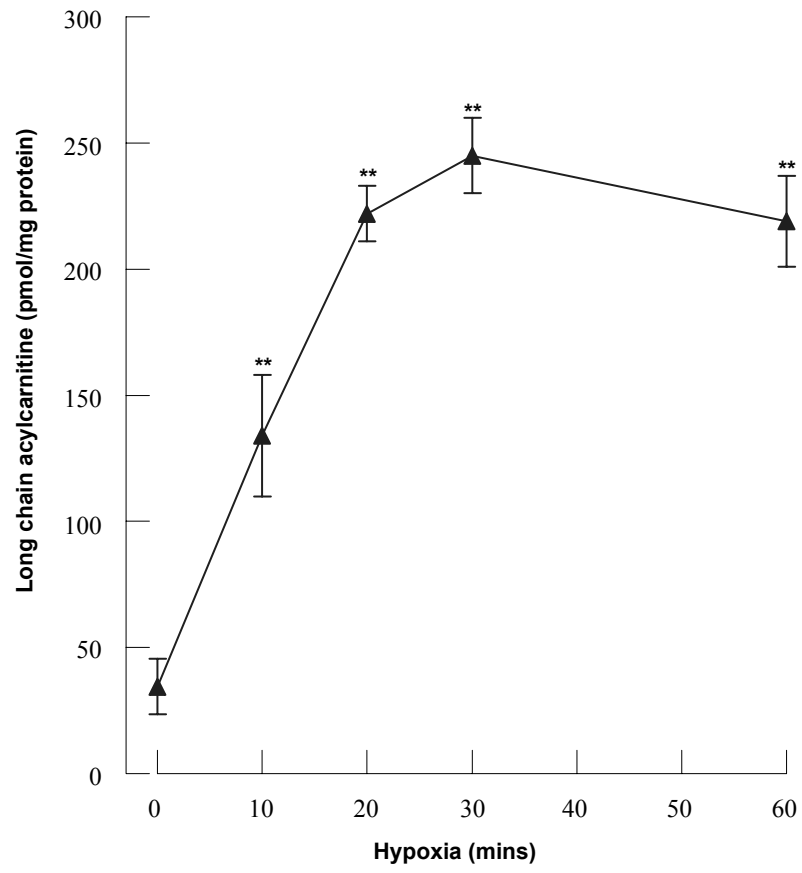


Figure 10.