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Regulation of a truncated isoform of AMP-activated protein kinase α (AMPKα) in response to hypoxia in the muscle of Pacific oyster *Crassostrea gigas*

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Abstract:

AMP-activated protein kinase α (AMPKα) is a key regulator of energy balance in many model species during hypoxia. In a marine bivalve, the Pacific oyster *Crassostrea gigas,* we analyzed the protein content of adductor muscle in response to hypoxia during 6 h. In both smooth and striated muscles, the amount of full-length AMP-activated protein kinase α (AMPKα) remained unchanged during hypoxia. However, hypoxia induced a rapid and muscle-specific response concerning truncated isoforms of AMPKα. In the smooth muscle, a truncated isoform of AMPKα was increased from 1 to 6 h of hypoxia, and was linked with accumulation of AKT kinase, a key enzyme of the insulin signaling pathway which controls intracellular glucose metabolism. In this muscle, aerobic metabolism was maintained over the 6 h of hypoxia, as mitochondrial citrate synthase activity remained constant. In contrast, in striated muscle, hypoxia did not induce any significant modification of neither truncated AMPKα nor AKT protein content, and citrate synthase activity was altered after 6 h of hypoxia. Together, our results demonstrate that hypoxia response is specific to muscle type in Pacific oyster, and that truncated AMPKα and AKT proteins might be involved in maintaining aerobic metabolism in smooth muscle. Such regulation might occur in vivo during tidal intervals that cause up to 6 h of hypoxia.

Keywords: Marine bivalve ; *Crassostrea gigas* ; Hypoxia ; AMP-activated protein kinase ; Alternative splicing ; Adductor muscle

1. Introduction

Sessile benthic marine mollusks of the intertidal zone, such as the Pacific oyster *Crassostrea gigas*, are exposed to large changes in environmental conditions every day. Over the tidal cycle, temporal variation in environmental factors such as oxygen availability or food supply may be considerable (Newell 1979). Moreover, eutrophication may increase organic matter in the water column, reducing levels of available oxygen, which can lead to hypoxia and sometimes anoxia (Gray et al. 2002; Wu 2002). Sessile organisms cannot escape from unfavorable environmental conditions and must adapt to oxygen variation in their environment; therefore, they would be expected to be tolerant of oxygen deprivation. This context makes marine bivalves good model species to study mechanisms involved in oxygen deprivation response (Storey 1993).

Hypoxia is a key environmental condition that plays a role in changing energy management and resource utilization in bivalves. Bivalves can adapt filtration rate in response to hypoxia by changing either the ventilation rate over the gills or the duration of valve opening/closing (Tran et al. 2000). After prolonged hypoxia during 20 days, the oyster energetic metabolism is modified by a switch from the aerobic to the anaerobic ATP production pathway (Le Moullac et al. 2007a). The oxygen critical point threshold (PcO_2), at which oysters switch to anaerobic metabolism, was determined at approximately 3 mg L^{-1} of oxygen (close to 30 % oxygen saturation), depending on water temperature (Le Moullac et al. 2007b). To identify pathways that are activated or altered in response to hypoxia, a transcriptomic study was conducted in *C. gigas* during long-term hypoxia of 20 days at 30% oxygen saturation (Sussarellu et al. 2010). In the digestive gland, the principal biological processes that were up- or down-regulated were respiration, energetic metabolism assessed by the respiratory chain compartment and antioxidant defense in response to oxidative stress observed during hypoxia and/or normoxic recovery (Sussarellu et al. 2010). In *Crassostrea virginica*, a compensatory increase in activities of two mitochondrial enzymes, citrate synthase and cytochrome c oxidase, was reported after two weeks of hypoxia, suggesting that this species has both a better tissue aerobic capacity to compensate for reduced oxygen availability and a lower sensitivity to hypoxia than *C. gigas* (Ivanina et al. 2011).

In vertebrates, the AMP-activated protein kinase (AMPK) signaling pathway has been described as the key system regulating energy balance in cells in hypoxic conditions (Hardie 2007). AMPK is an heterotrimeric kinase composed of a catalytic α-subunit and two regulatory subunits, β and γ. Genes encoding the three subunits of AMPK are highly conserved among the eukaryotic species, including vertebrates, invertebrates, plants, fungi, and protozoa (Hardie 2003). In human, 2 or 3 genes encode each subunit, giving rise to 12 possible heterotrimeric combinations, and splice variants further increase the potential diversity of AMPK proteins (Towler and Hardie 2007).

In mammals, AMPK is activated by metabolic stresses such as glucose deprivation, oxidative phosphorylation, ischemia or lack of oxygen (Hardie 2008). A high cellular ratio of AMP/ATP triggers the phosphorylation and stimulation of AMPK kinase activity (Choi et al. 2001). AMPK activation is regulated at the post-translational level through phosphorylation and dephosphorylation. Many phosphorylation sites have been described in AMPK subunits α and β (Hawley et al. 1996; Woods et al. 2003; Mitchelhill et al. 1997). In rat liver, the AMPKα threonine 172 site has been identified as the determinant and required phosphorylation site for stimulation of AMPK kinase activity (Neumann 2003; Hawley et al. 1996; Suter et al. 2006; Stapleton et al. 1996; Carling et al. 2011)).

Once activated by low energy status, AMPK activates ATP-producing catabolic pathways such as lipolysis, glycolysis and glucose uptake, and deactivates ATP-consuming anabolic pathways such as glycogen synthesis and lipogenesis (Hardie 2004). Activated AMPK can

rapidly phosphorylate and activate a wide array of target key metabolic enzymes (Hardie 2004). Long-term effects of AMPK activity have also been demonstrated on the gene expression of several metabolic enzymes involved in glucose homeostasis (Viollet et al. 2003; Hardie 2003; Sato et al. 2011). Another signaling pathway linked to mammalian cell survival during energy deficiency in hypoxic conditions is the phosphatidyl inositol 3-kinases (PI3K)/protein kinase B (AKT) pathway, which regulates glucose metabolism and controls cell apoptosis (Kim et al. 2012; Parcellier et al. 2008; Alvarez-Tejado et al. 2001). The kinase AKT is activated by serine/threonine phosphorylation so as to increase glucose transport into cells to provide ATP to serve as fuel (Manning and Cantley 2007).

Few studies have investigated AMPK in mollusks or marine invertebrates, although AMPKα has been already described in a terrestrial pulmonate gastropod (Ramnanan et al. 2010), the brine shrimp, (Zhu et al. 2007), some crab species, lobster, one zooplankton species (Frederich et al. 2009; Jost et al. 2012) and a nemertean worm (Stricker 2011; Stricker et al. 2010). To date, only one study has characterized AMPKα threonine 172 phosphorylation in a marine invertebrate, the crab *Cancer irroratus*, subjected to oxygen depletion (Pinz et al. 2005). Up to date, no data are available on genes or AMPK protein subunits in oysters.

The main objectives of this study were to characterize the AMPKα isoforms in the Pacific oyster *C. gigas* and to define their potential regulation in the hypoxic response. The hypoxic response of oyster was explored in the adductor muscle, which is composed of smooth and striated muscle, by quantifying the protein content of AMPKα and AKT proteins using heterologous antibodies combined with citrate synthase activity measurement

2. Material and methods

2.1. Biological material

2.1.1. Hypoxia conditioning and muscle sampling

Oyster conditioning and sampling were performed as described in (Sussarellu et al. 2011). Briefly, one hundred and twenty wild Pacific oysters aged approximately 24 months were sampled during their sexual resting stage (January 2009) from an oyster farm in the Bay of Brest (Plougastel, France) and transported to the ORPHY laboratory facilities (UBO, Brest, France) where they were placed in eight 50 L tanks (n = 15 oysters per tank) and acclimated for 12 h in fully oxygenated through-flowing seawater.

The oysters were then kept out of the water for 3 h air drying to simulate a low tide, before being re-immersed in sea water in 8 tanks. In four tanks, oysters were maintained under normoxic conditions for 6 hours in fully oxygenated through-flowing seawater (oxygen at 8.8 mg L^{-1}, 100% oxygen saturation at 11°C, 34 salinity). In four other tanks, hypoxic conditions were maintained for 6 hours by reducing oxygen down to 2.6 mg L^{-1} (30% oxygen saturation at 11°C, 34 salinity), using an oxygen depletion system as described by (Pichavant et al. 2000). Hypoxic oxygen concentrations were reached by bubbling nitrogen though seawater in a reservoir tank supplying the 4 rearing tanks. Surface gas exchanges in the experimental tanks were limited by positioning the water outflow below the water surface. Oxygen concentration in the tank was monitored regularly and adjusted when necessary to keep hypoxia level constant throughout the experiment. Water flow rates were 0.7 and 3.6 L min^{-1} under hypoxic and normoxic conditions, respectively. Water was collected in a reservoir, re-oxygenated with electric pumps, filtered (0.22 µm) and UV-treated, before being re-circulated through the system. Samples of 5 oysters were taken from each tank after 1, 3, and 6 hours. These oysters were opened and dissected: the two parts composing the adductor muscle

(smooth and striated muscle) were separated, pooled (n = 5) and immediately frozen in liquid nitrogen. For each condition and sampling time (hypoxia and normoxia), 4 pools of 5 muscles (striated and smooth separately) were sampled, one for each tank. These pools were ground using a Dangoumeau homogenizer at -180°C and stored at -80°C for further analysis.

2.1.2. Wild oyster tissue sampling

To analyze a specific protein content in several oyster tissues, six wild Pacific oysters aged approximately thirty six months (live weight 59.5 ± 10.5 g) were collected during their sexual resting stage (January 2009) in the bay of Brest (Plougastel, France). The oysters were opened and their tissues rapidly dissected. The tissues sampled were mantle edge, digestive gland, mantle, palps, smooth muscle, striated muscle, heart, gills and visceral ganglia.

After dissection, specific tissues from several animals were pooled and immediately stored in liquid nitrogen for further utilization. For each tissues, 4 pools have been made of 4 oysters (n = 4). These pools were ground at -180°C using a Dangoumeau homogenizer and stored at -80°C for later analysis.

2.2. Methods

2.2.1. Cloning of AMPKα isoforms

Total RNA of striated muscle sampled from wild oyster was extracted using Extract-all (Eurobio), at a concentration of 50 mg frozen powder /1.5 ml buffer. RNA samples were then treated with DNAse I (1 U/µg total RNA, Sigma) and RNA quality was assessed using RNA nano chips and Agilent RNA 6000 nano reagents (Agilent Technologies) according to manufacturer's instructions. RNA concentrations were measured at 260 nm using an ND-1000 spectrophotometer (Nanodrop Technologies), with the conversion factor 1OD = 40µg/mL RNA. From 2 µg total RNA, RT-PCR amplifications were carried out as described in (Huvet et al. 2004).

Two primers were designed based on an *ampkα* sequence extracted from a draft genome assembly covering the start and stop codons: Cg-*ampkα*-Start (5'-CGGAGAAGTCCTCCTCCTCT-3') and Cg-*ampkα*-Stop (5'-CTATCTGGCCAGTGTGGTGA-3'). Putative *ampkα* cDNAs were amplified using the corresponding primers pair via PCR with an annealing temperature of 45°C (protocol in (Bacca et al. 2005). The PCR products were electrophoresed on 1% (w/v) agarose in 1X TAE buffer gels (TAE: Tris, acetic acid, 0.5M EDTA) and stained with ethidium bromide. Gel-extracted PCR products (QIAquick® Gel Extraction Kit, Qiagen) were cloned using the TOPO TA cloning kit vector (Invitrogen), plasmids were then purified (Nucleospin Plasmid DNA plasmid purification, Mascherey-Nagel), digested (restriction enzyme EcoR1) and sequenced (Beckman Coulter Genomics).

Homology searches were performed with the obtained DNA sequences using a BLASTX searches (Altschul et al. 1997). The DNA sequences were then converted into predicted amino acid sequences using the ExPASy translate tool (Gasteiger et al. 2003) and a multiple alignment was performed on selected sequences using CLUSTALW (Combet et al. 2000). Protein domain conservation was performed using ExPASy prosite (de Castro et al. 2006) and SMART (Schultz et al. 1998; Letunic et al. 2011).

2.2.2. Total protein extraction

Total protein extracts were obtained from the tissues sampled in wild *C. gigas* oysters and from pools of striated and smooth muscle from the hypoxia and normoxia conditioned oysters, according to (Corporeau and Auffret 2003). To solubilize proteins, powdered tissues were homogenized in a lysis buffer containing phosphatase and protease inhibitors (150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% Igepal, 5 ml phosphatase inhibitor cocktail II, 2 tablets Complete EDTA free protease inhibitor cocktail; pH 8.8 at 4°C). Total protein extracts were then quantified using a DC protein assay (Biorad) and concentration was determined quantitatively using 96-well micro-plates (Nunc) and a micro-plate reader connected to KC4 v3 software (Bio-Tek Instruments, Inc).

2.2.3. Citrate synthase activity in muscle

Prior to quantification of citrate synthase activity in striated and smooth muscle, each protein lysate was adjusted to a final concentration of 3.5 mg/ml by adding lysis buffer. CS activity was measured in a medium containing 100 mM Tris-HCl pH8, 0.2 mM acetyl coA, 0.1 mM 5.5'-Dithio-bis(2-nitrobenzoic acid) (DTNB). The reaction was initiated by addition of 0.5 mM oxaloacaetate (final concentration), in a total volume of 200 µl and activity was measured for 10 minutes at room temperature at 412 nm (ε = 13600 L mol^{-1} cm^{-1}).

2.2.4. Western-blot analysis

Prior to western-blot analysis, each protein lysate was adjusted to a final concentration of 5 mg/ml by adding lysis buffer. Protein samples were then denatured by Laemmli solution (0.6 M Tris-HCl, 1 ml glycerol, 10% w/v SDS, 0.5 ml 0.1% w/v bromophenol blue, 0.5 ml β-mercaptoethanol) and 90 µg of each protein extract was loaded onto a 10% SDS polyacrylamide gel. Proteins were then transferred to a PVDF membrane (Biorad) for immunodetection on western blot. Immunodetection was performed with several heterologous antibodies directed against AMPKα that were validated for their cross-reactivity in C. gigas: Cell Signaling Technology (CST) technical services blasted the antigenic epitope sequence of heterologous anti-AMPKa antibodies against the genome of C. gigas, very recently available (Zhang et al., Nature, 2012 (doi:10.1038/nature11413)), and demonstrated that only AMPKα protein of C. gigas could be recognized by these antibodies (Cell Signaling Technology; personal communication). Thus, immunodetection was performed with several anti- AMPKα antibodies (Ozyme, Cell Signaling Technology): a rabbit monoclonal anti-AMPKα antibody directed against amino-terminal sequence of human AMPKα (dilution 1:1000; CST # 2603), a rabbit monoclonal anti-AMPKα antibody directed against residues surrounding Lys40 of human AMPKα (dilution 1:1000; CST # 5832), and a mouse monoclonal antibody directed against purified recombinant human AMPKα1 protein (dilution 1:1000; CST # 2793). We also used a polyclonal anti-AKT antibody directed against carboxy-terminal sequence of mouse Akt (dilution 1:1000; CST # 9272). Blots were revealed using an HRP-linked secondary goat anti-rabbit or anti-mouse antibody (dilution 1:5000) and a HRP detection kit (Biorad). The amount of protein detected was quantified using MULTI-ANALYST software (Biorad), with the background signal removed. The value obtained is expressed in OD/mm^2 and represents the band intensity expressed as mean count per pixel multiplied by the band surface.

To ensure that identical amounts of total protein samples were loaded into the gels, membranes were dehybridized by incubation for 1 h at room temperature in dehybridizing buffer (100 mM glycine, 100 mM NaCl pH 3.2), and rehybridization was done with an anti-histone H3 antibody directed against the carboxy-terminal sequence of human histone H3 (dilution 1:5000;CST # 9715;), as described in (Fabioux et al. 2009).

2.2.5. Statistical analysis

Statgraphics software (StatPoint Technologies, Inc.) was used for all statistical analyses. For multiple comparisons within the same factor, we used the Fisher's least significant difference (LSD) procedure at the 95% confidence interval. Results are expressed as mean ± standard deviation (SD).

3. Results

3.1. Cloning and characterization of Crassostrea gigas ampkα

Using specific primers, we identified in the muscle of *C. gigas* three *ampkα* clones of 1,212, 1,284 and 1,647 base pairs respectively. These sequences encoded three isoforms of AMPKα protein, named *C. gigas*_alpha_truncated 1 (GenBank accession number JX104212), *C. gigas*_alpha_truncated 2 (GenBank accession number JX104213) and *C. gigas*_alpha_like (GenBank accession number JX104214). As shown in figure 1, both truncated isoforms of AMPKα corresponded to alternative spliced variants of the *ampkα* gene (GenBank accession number JX402632, Fig. 1). The 1,647 base pairs cDNA encoded an entire AMPKα protein of 62 kDa, and the 1,212 and 1,284 base pairs cDNAs encoded two truncated isoforms of 46 and 48 kDa, respectively (Fig. 2).

The three AMPKα sequences showed more than 70% conservation with AMPKα subunits in other species. The entire AMPKα protein of 62 kDa contains the kinase domain, the binding domain for β and γ subunits, and the conservation of the threonine 172 active site and of two motifs surrounding threonine 172, (Asp-Phe-Gly and Ala-Pro-Glu) which is a characteristic phosphorylation site in kinase (Hardie et al. 1998) (Fig. 2). Blast results didn't reveal closer relationship to AMPKα isoforms characterized in vertebrate as α1, α2. Both truncated AMPKα isoforms of 46 and 48 kDa were deleted in the kinase domain and lacked the threonine 172 phosphorylation site, although they conserved the C- terminal binding domain for β and γ subunits to form an heterotrimer (Towler and Hardie 2007) (Fig. 2). *C. gigas*_alpha_truncated 2 contained an additional group of 24 amino acids that was not characterized in the *C. gigas*_alpha_truncated 1 in the C-terminal region. All three *C. gigas* AMPK conserved the threonine 258 but missed the serine 485 phosphorylation sites (Fig. 2).

3.2. Characterization of *Crassostrea gigas akt*.

Among the *C. gigas* EST sequences in GigasDatabase (Fleury et al. 2009), we identified a sequence corresponding to the *C. gigas akt* gene (GenBank accession number FP004887). The corresponding partial amino acid sequence showed more than 60% conservation with *akt2* of Atlantic salmon *Salmo salar* (FJ969488), *akt* of blackback land crab *Gecarcinus lateralis* (ADM87425) and *akt2* of ferret *Mustela putorius furo* (AER94007). We also demonstrated the conservation of the pleckstrin homology (PH) domain needed for anchorage of AKT to the cell membrane (Alessi et al. 1996) (Fig. 3).

3.3. Tissue-specific detection of AMPKα isoforms protein

All the anti- AMPKα antibodies we used could recognize two bands in western-blot, one at 62 kDa and one other at 48 kDa, but not at the same level of specificity, depending on the antibody we used. The band at 62 kDa corresponded to the predicted size of the full-length AMPKα protein while the band at 48 kDa corresponded to truncated isoforms of AMPKα.

The anti-AMPKα antibody CST#2793 (produced against a human recombinant AMPKα1 protein) recognized mainly the full-length AMPKα protein of 62 kDa and very lightly recognized the truncated isoforms at 48 kDa (very poor signal obtained after long exposure to ECL). This antibody allowed us to show that the entire AMPKα protein was expressed in all oyster tissues (data not shown).

In contrast, the anti-AMPKα antibody CST#2603 (produced against a synthetic peptide derived from the amino-terminal sequence of human AMPK alpha) and the anti- AMPKα antibody CST#5832 (produced against a synthetic peptide corresponding to residues surrounding lys40 of human AMPKα) recognized very rapidly the truncated isoforms at 48 kda and very poorly the full-length AMPKα protein at 62 kDa after a long ECL exposure. Using these antibodies, we were able to show that truncated AMPKα isoforms at 48 kDa were specific for several tissues, mainly expressed in smooth and striated muscles (Fig. 4). Based on the calculated molecular weight of truncated AMPKα isoforms identified in *C. gigas* muscle, this band could be assigned to specific antibody recognition of either one variant or both variants of truncated AMPKα isoforms in *C. gigas*. We could not determine whether this band corresponded to a specific truncated isoform of 46 or 48 kDa AMPKα, or both, since the band was large with rapid and high density and obtained in low resolution SDS-Page gels (10% acrylamide). Figure 4 illustrates how *C. gigas* truncated AMPKα isoform was specifically expressed in five different tissues of oysters sampled at the sexual resting stage: the striated muscle had the highest density with 73.46 OD/mm², followed by the smooth muscle with 42.20 OD/mm². Much lower densities were observed for the mantle edge (2.43 OD/mm²), heart (0.34 OD/mm²) and visceral ganglia (0.07 OD/mm²). Bands of even lower density were detected in the other tissues.

3.4. AMPKα protein level during hypoxia

Figure 5 A shows western-blot representative images obtained for the detection of the entire AMPKα protein at 62 kDa. Its quantification demonstrated that the entire AMPKα amount did not vary statistically during normoxia and hypoxia in both muscles (Two-Way ANOVA: $F = 1.55$; $p = 0.2253$; data not shown).

In smooth muscle, the protein content of truncated AMPKα isoform at 48 kDa significantly increased as early as 1 h after the hypoxia treatment started, compared with normoxia, and this increase was observed though the 6 h of hypoxia in the experiment (Two-Way ANOVA: $F = 8.92$; $p = 0.0002$; Fig. 5 B). In striated muscle, no significant difference was observed in the protein content of truncated AMPKα isoform between hypoxic and normoxic conditions (Two-Way ANOVA: $F = 1.62$; $p = 0.2058$; Fig. 5 C).

3.5. Citrate synthase activity during hypoxia

In striated muscle, the activity of the citrate synthase was significantly modified (Two-Way ANOVA: $F = 3.75$; $p = 0.00167$; Fig. 6 B). This activity increased slightly after 3 h of hypoxia (+ 12.3 %) before significantly decreasing at 6 h (- 22.5 %) in comparison with normoxia, where it remained stable. In smooth muscle, no significant difference was measured in the activity of the citrate synthase either between treatments or between points in time within treatments (Two-Way ANOVA: $F = 0.57$; $p = 0.7206$; Fig. 6 A).

3.6. AKT protein level during hypoxia

To quantify AKT protein content in muscles, we used a heterologous monoclonal anti-AKT antibody. This antibody allowed us to detect a single band in SDS-PAGE at approximately 38 kDa (Fig. 7). In smooth muscle (Fig. 7 A), this antibody showed that the AKT protein content was significantly (by 6.4 times) higher in the hypoxic condition than in the normoxic condition

at 1 h (Two-Way ANOVA: F = 3.00; p = 0.0382). A higher AKT protein content was maintained in hypoxia compared with normoxia after 3 and 6 h of the treatment (1.5 and 4.6 relative OD/mm^2, respectively), but these differences were not significant (Fig. 7 A). In striated muscle (Fig. 7 B), no significant difference was observed in AKT protein content between hypoxic and normoxic conditions (Two-Way ANOVA: F = 0.82; p = 0.5543).

4. Discussion

4.1. Crassostrea gigas AMPKα isoforms

An important finding of the present study is that the entire AMPKα of 62 kDa was ubiquitously expressed and that we identified two truncated AMPKα protein isoforms of 46 and 48 kDa that were tissue-specifically expressed in *C. gigas,* mainly in smooth and striated muscles. Both truncated isoforms resulted from alternative splicing of the full-length *ampkα* gene. Alternative splicing was already reported for some genes in *C. gigas* and the spliced variants may present a tissue-specific expression and a specific function (Rodet et al. 2008).

As demonstrated for genes involved in stress response in *C. gigas* (Kawabe and Yokoyama 2011a), we can hypothesize that hypoxia could have increase the alternative splicing leading to a rapid accumulation, as soon as 1 h after hypoxia, of truncated AMPKα in smooth muscle.

In all species, the size of the full-length AMPKα protein is around 62 kDa (Apfeld et al. 2004; Beri et al. 1994; Carling et al. 1989; Yoshida et al. 1999; Zhu et al. 2007) as in oyster, where we demonstrated that the entire AMPKα had a molecular weight of 62 kDa. However, both truncated AMPKα were 33% shortened. They contained a truncated kinase domain and missed the threonine 172 phosphorylation site, important for AMPK activation in all species (Stapleton et al. 1996; Carling et al. 2011). Here we described the *in vivo* expression of these truncated isoforms of AMPKα in smooth and striated muscle as compared with other tissues.

In *Artemia franciscana,* one truncated isoform of AMPKα was found in addition to the entire AMPKα. In fact, the entire AMPKα2 is a 515-amino acid protein with a calculated molecular mass of approximately 58 kDa, and the truncated isoform results from a 244 base pairs deletion in its encoding mRNA that caused a frameshift, introducing a new translation stop codon that shortened the C-terminal of AMPKα2. This truncated protein had 313-amino acids with a calculated molecular mass of 36 kDa (Zhu et al. 2007). Nevertheless, this truncated isoform of AMPKα kept its entire kinase domain and its activation through phosphorylation (Carling et al. 1989; Zhu et al. 2007). As reported in wormbase.org, in *Caenorhabditis elegans*, an AMPKα isoform named AAK-1b is truncated in its kinase domain and conserved its threonine 172 phosphorylation site but no functional data were obtained on this truncated kinase isoform (Yook et al. 2012).

4.2. Muscle specific regulation of truncated AMPKα and AKT

Crassostrea gigas inhabits the intertidal zone and shows high tolerance to stress conditions such as hypoxia and heat shock (Kawabe and Yokoyama 2011a, b). In *C. gigas*, the adductor muscle is one of the primary organs involved in responses to biotic or abiotic environmental variations, such as hypoxia (Le Moullac et al. 2007a; Samain and McCombie 2008). Valve closure is usually considered as a general stress response of marine bivalves to a wide variety of environmental stressors (Manley 1983; Akberali and Trueman 1985). Valve closure isolates the soft tissues from the external environment, which leads to a gradual decline of the oxygen in the mantle cavity water, and ultimately anoxia (Heinonen et

al. 1997). In *C. gigas*, a prolonged 24 h period of hypoxia at 2 mg L^{-1} of oxygen was already reported to significantly reduce valve opening (Le Moullac 2008). At the cellular level, hypoxia was shown to slow down glycolysis (Le Moullac et al. 2007a) and to induce increased activity of the electron transfer system within the respiratory chain (Le Moullac et al. 2007b). In the context of the frequent environmental changes to which the species is exposed, energy supply to the adductor muscle could well be a priority at the organism level to ensure survival of the whole animal by keeping the valves closed more often/longer to protect the oyster against external stresses.

Indeed, in many species, the AMPK signaling pathway is involved in the coordination of anabolic and catabolic metabolic processes in various tissues, including cardiac and skeletal muscle, adipose tissue, pancreas and liver (Khan et al. 2006). In vertebrates, AMPK subunits have been reported in both striated muscle (Saha et al. 2000; Musi and Goodyear 2003) and smooth muscle, mainly cardiovascular smooth muscle (Ferri 2011; Sung et al. 2011), which led to the identification of AMPK as an important intracellular signaling pathway in these muscles.

In our study, we demonstrated a rapid and muscle-specific regulation of truncated AMPKα protein content by hypoxia in oysters while the amount of the entire AMPKα remained constant. Truncated AMPKα protein content was accumulated in smooth muscle after as little as 1 h of hypoxia and this continued throughout the 6 h of the treatment. We demonstrated that citrate synthase activity was maintained during 6 h of hypoxia in smooth muscle, while it slightly decreased in striated muscle. Mitochondrial citrate synthase is a key enzyme in aerobic energy production and is a useful indicator of general physiological status of *C. gigas* (Garcia-Esquivel et al. 2001; Garcia-Esquivel et al. 2002; Pernet et al. 2012). Citrate synthase activity reflects the mitochondrial oxidation capacity resulting from changes in mitochondrial number and activity (Tripathi and Verma 2004). In our experiment, as citrate synthase activity in smooth muscle did not show any difference between normoxic and hypoxic conditions, we suggest that its aerobic capacity was maintained during the 6 h of hypoxia, resulting in a higher metabolic performance than in the striated muscle. Since adductor muscle is not a storage tissue for glycogen in *C. gigas* (Berthelin et al. 2000), it needs to be supplied with glucose at a high rate to maintain an aerobic pathway of energy production. In the two oyster species *C. gigas* and *C. virginica*, the adductor muscle has been seen to use the PEPCK or PK pathway to allow high glycolysis in order to provide ATP for contractile activity (Greenway and Storey 1999; Le Moullac et al. 2007a)**.** In adductor muscle of *C. virginica*, hypoxic conditions up-regulate mitochondrial enzymes, such as citrate synthase, in order to avoid metabolic depression and to provide better survival by minimizing physiological stress of oysters (Ivanina et al. 2011). Citrate synthase activity is an excellent marker of aerobic metabolism in vertebrates (Hanke et al. 2008) and invertebrates such as oysters (Ivanina et al. 2011; Pernet et al. 2012).

Our hypothesis is that in the smooth muscle of *C. gigas*, the increase of truncated AMPKα and AKT could be related to the maintaining of mitochondrial citrate synthase activity under short-term hypoxia to avoid metabolic depression. In many species, the role of oxygen stress on AMPK has been largely studied in relation to the fact that AMPK is viewed as a master regulator of energy (Hardie 2003). In general, AMPK switches on catabolic processes that provide alternative routes for generating ATP, such as glucose uptake, glycolysis, fatty acid oxidation or mitochondrial biogenesis (Hardie 2008). In rat skeletal muscle, conditions that cause reduction in cellular fuel status, including contraction, hypoxia, inhibition of oxidative phosphorylation, and hyperosmolar stress, have been demonstrated to increase AMPK activity and glucose transport (Hayashi et al. 2000). In cultures of rat skeletal muscle cells, AMPK can regulate glucose consumption (Hong et al. 2012). In skeletal muscle of mice, AMPK appears to be an obligate intermediate in the pathway to accelerate glucose uptake during hypoxia, to sustain ATP availability and cell survival in this tissue (Mu et al. 2001). Our experiment showed a rapid (from 1 h of hypoxia) and persistent (up to 6 h) increase in AKT

protein content in the smooth muscle concomitant with the increase in truncated AMPKα protein content, supporting the hypothesis of an increased glucose uptake in this muscle under hypoxic conditions. AKT is the key kinase, regulated by phosphorylation at threonine 308 and serine 473, involved in the insulin signaling pathway, which regulates glucose metabolism, glucose entry into the cell and controls cell survival, especially under stressful conditions (Manning and Cantley 2007). Indeed, in rat skeletal muscle, AKT is activated in response to a high energy demand during prolonged exercise, and leads to enhance glucose transport and survival by increasing insulin-sensitivity (Funai et al. 2010).

Some elements of the insulin pathway have already been characterized in *C. gigas*: an insulin-related peptide cDNA (Hamano et al. 2005), an insulin-like receptor CIR (Gricourt et al. 2003) and Ras, Pten and p70S6K, three potential elements of the oyster insulin pathway (Jouaux et al. 2011). In vertebrates, hypoxia is known to activate the AKT cell survival pathway to maintain protection from apoptosis (Alvarez-Tejado et al. 2001; Kim et al. 2012). In vertebrate cell culture, the hypoxia-induced activation of AKT is observed even after 3-4 h of hypoxia but can be prevented by treatment with cycloheximide, suggesting that *de novo* protein synthesis is required (Alvarez-Tejado et al. 2001). AKT is also important for ischemia-protection in human heart (Mullonkal and Toledo-Pereyra 2007). In mouse soleus muscle, glycogen content, AMPK activity and AKT activity did not differ significantly between hypoxia and control treatments, but insulin stimulation led AKT phosphorylation to be significantly higher in hypoxia than the control. This experiment emphasized the key role of insulin in AKT pathway under hypoxic conditions (Gamboa et al. 2011). In mouse cardiomyocytes, activation of both AKT and AMPK are important for maintaining glucose metabolism during oxidative stress (Horie et al. 2008), and a direct link was recently demonstrated between AMPK and AKT signaling pathways for promoting energy conservation and survival of skeletal muscle exposed to ischaemia (Chopra et al. 2011). In fact, evidence has been found for a direct cross-talk between AKT and AMPK that promotes cell survival in low energy status conditions under hypoxia in brain and heart in vertebrates (Kovacic et al. 2003; Bertrand et al. 2006). Such a relationship was also recently demonstrated in crucian carp heart (Stenslokken et al. 2008).

4.3. Putative in vivo function for truncated AMPKα during hypoxia

Many studies have shown that phosphorylation of threonine 172 is required for AMPK kinase activity to produce the downstream cellular effects of activated AMPK signaling (Neumann 2003; Hawley et al. 1996; Hardie et al. 1998). Indeed, under hypoxic conditions, AMPKα is regulated in the muscle at the post-translational level through threonine 172 phosphorylation, leading to AMPK kinase activation and downstream events in vertebrate models (Wadley et al. 2006). Due to its high conservation in *C. gigas*, we can assume that the kinase activity of the entire AMPKα protein of 62 kDa could also be regulated through threonine 172 phosphorylation to act on downstream targets in both striated and smooth muscles. Indeed, in the liver of anoxia-tolerant goldfish (*Carassius auratus*) after 30 min of hypoxia (oxygen at 0.3 mg L^{-1}), no changes in the quantity of total protein were observed, despite an increase in AMPK activity of nearly 5.5 fold (Jibb and Richards 2008). In contrast, in heart of crucian carp *Carassius carassius* after 10 days of hypoxia (oxygen at 0.3 mg L^{-1}), no significantly changes in total protein and activity of AMPKα were observed compared with control conditions (Stenslokken et al. 2008).

As far as we know, our stduy is the first description of the accumulation of truncated AMPKα isoforms in response to hypoxia. As demonstrated, the truncated AMPKα did not contain neither the entire kinase domain nor the threonine 172 phosphorylation site. We thus can suppose that it might have another function than the entire AMPKα in response to hypoxia in the smooth muscle in *C. gigas*. In fact, (Noy et al. 2012) demonstrated that an *in vivo* truncated kinase, named Casein kinase II (CK2), that was deleted in its kinase domain (i.e. kinase-dead), can act as a regulator of the corresponding full-length CK2 function, by

sequestration of the partners of full-length CK2. We can hypothesize that in oyster, the truncated AMPKα isoforms could serve as an *in vivo* modulator of the dowsntream AMPKα signaling during hypoxia in the smooth muscle. Indeed, since the truncated isoforms conserved the ligand binding domain for β and γ subunits, these truncated AMPKα could have kept the ability to bind AMPKα ligands such as β and γ subunits and/or downstream binding partners. Indeed, using a constructed mutant, (Ramanathan et al. 2010) demonstrated that a truncated human AMPKα in its kinase domain can conserve its binding capacity with β and γ subunits. Moreover, the truncation of the kinase domain in human AMPKα rendered the heterotrimer less soluble, indicating that truncated AMPKα could exert its regulatory function through modulation of biochemical properties of AMPK complex and/or partners. Here we can conclude that the rapid increase in the protein amount of truncated AMPKα in smooth muscle could modulate the signaling pathway of the full-length AMPKα, and could have led to the protection of the citrate synthase activity during 6 h of hypoxia. Further investigations are needed to quantify the activity of the entire AMPKα response in presence of the truncated isoforms in *C. gigas*.

Response to hypoxia through the expression of truncated AMPKα was smooth muscle-specific. Although both form part of the adductor muscle, smooth and striated muscles do not have the same metabolism and function. Indeed, in molluscs, the slow-acting smooth muscle can go into a catch state after contraction, and tension can be maintained for many hours with a small ATP turnover. In contrast, the quicker-acting muscle, usually striated to some extent, has phasic activity responsible for the rapid closure of the shell (Elliott and Bennett 1982). Here we showed that in oyster, the aerobic metabolism in the smooth muscle might be more protected against an environmental stress such as hypoxia and that the hypoxic response of smooth muscle seems to be mediated by the regulation of AMPKα isoforms.

5. Conclusion

In this manuscript, we showed that two truncated AMPKα isoforms, that lost the kinase domain and the determinant threonine 172 phosphorylation site, might play a role in the metabolic response during hypoxia in the smooth muscle of *C. gigas*. Our experiments suggested that the *in vivo* accumulation of truncated AMPKα, through alternative splicing, could serve as a modulator of the entire AMPKα response in order to maintain aerobic capacity and glucose homeostasis via AKT. Pacific oysters thus appeared as a good model to understand the environmental regulation of AMPKα signaling.

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The authors declare that they have no conflict of interest.

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Figures

Figure 1: Diagram representing the intron-exon structure of the *C. gigas* a*mpk alpha* gene (GenBank accession number JX402632) and the three AMPK mRNAs obtained by alternative splicing of exons. AMPK alpha_like: GenBank accession number JX104214. AMPK alpha_truncated 1: GenBank accession number JX104212. AMPK alpha_truncated 2: GenBank accession number JX104213.

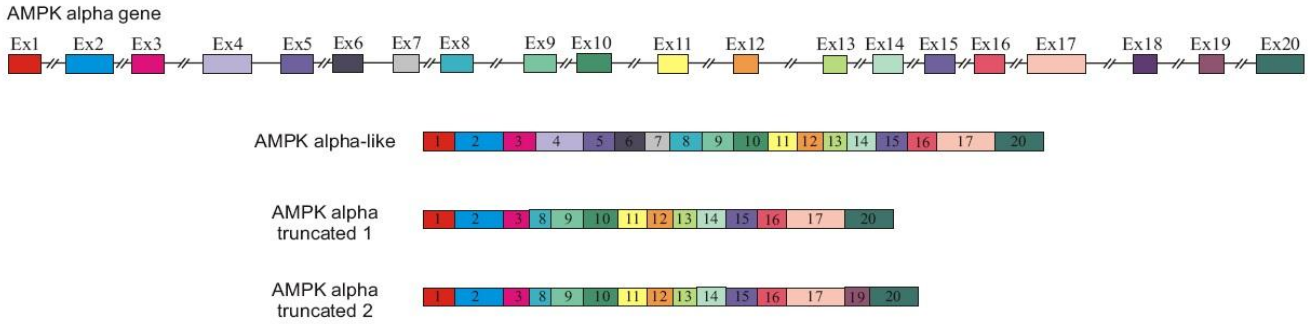

Figure 2: Amino acid alignment of *Crassostrea gigas* AMPK alpha_Truncated 1 (GenBank accession number JX104212), AMPK alpha_Truncated 2 (GenBank accession number JX104213), with the full-length AMPK_alpha_like (GenBank accession number JX104214), *Homo sapiens* AMPK α1 and α2 (NM006251 and AAH69823). Sequence conservation is indicated in red for complete conservation among the compared species, in green for strong similarity, in blue for weak similarity and black where there is no conservation. Red, blue and orange arrows: Threonine 172,Threonine 258 and Serine 485 phosphorylation sites, respectively. Green squares: characteristic motifs surrounding the threonine 172 site in kinase (Asp-Phe-Gly and Ala-Pro-Glu). Orange square: binding domain for β and γ subunits. "Prim.cons" line contains the consensus sequence (the most common amino acid at position). In case of a tie between two amino acid frequencies, a number is reported in this line.

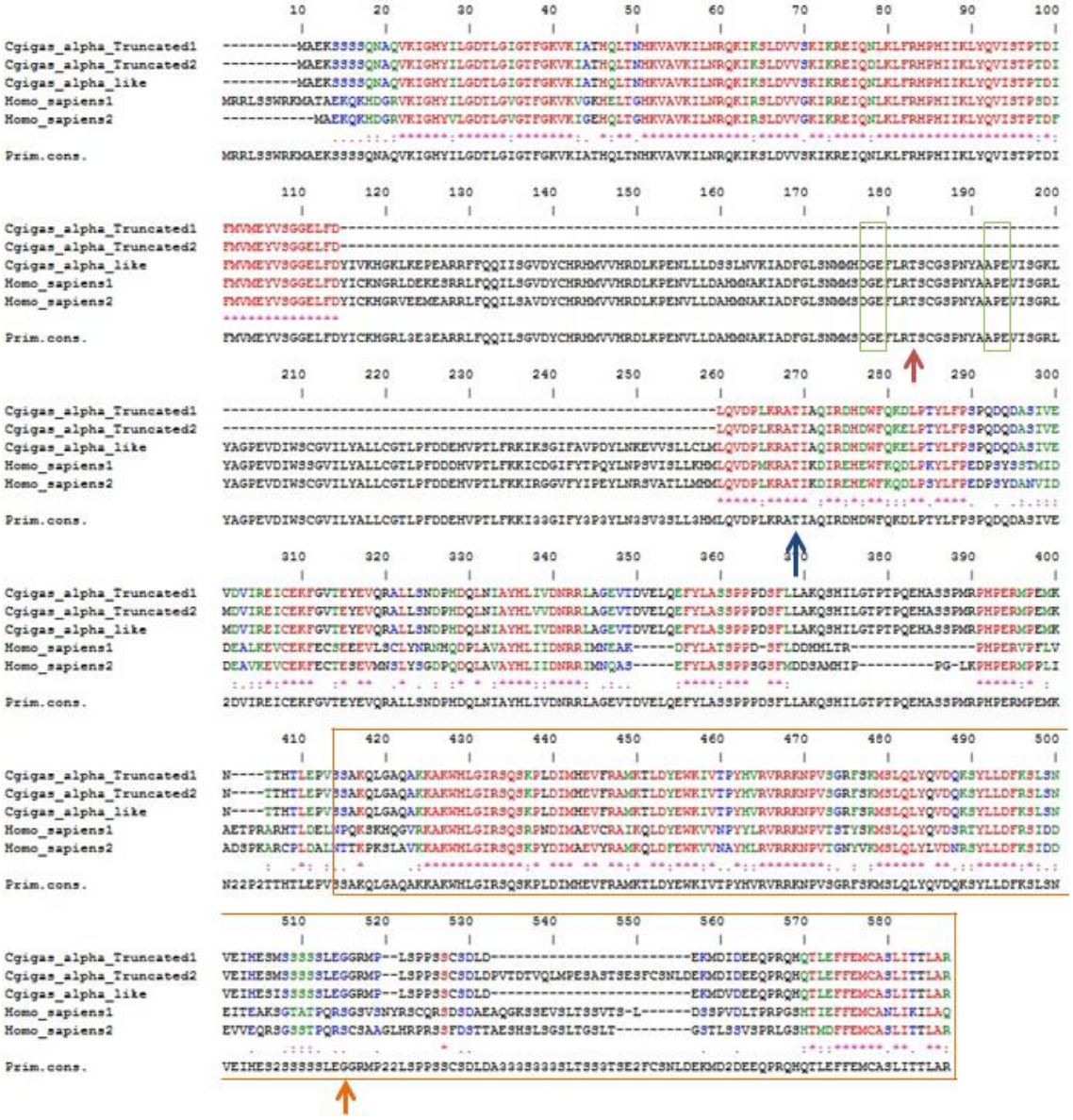

Figure 3: Amino acid alignment of *Crassostrea gigas* AKT *C. gigas* (GenBank accession number FP004887), AKT2 of Atlantic salmon *Salmo salar* Ssalar2 (GenBank accession number FJ969488), AKT of blackback land crab *Gecarcinus lateralis* Glateralis (GenBank accession number ADM87425), AKT3 of *Homo sapiens* Hsapiens3 (GenBank accession number BC121154) and AKT2 of Ferret *Mustela putorius furo* Mputorius3 (GenBank accession number AER94007). Sequence conservation is indicated in red for complete conservation among the compared species, in green for strong similarity, in blue for weak similarity, and in black where there is no conservation. Red square: pleckstrin homology (PH) domain. “Prim.cons” line contains the consensus sequence (the most common amino acid at position). In case of a tie between two amino acid frequencies, a number is reported in this line.

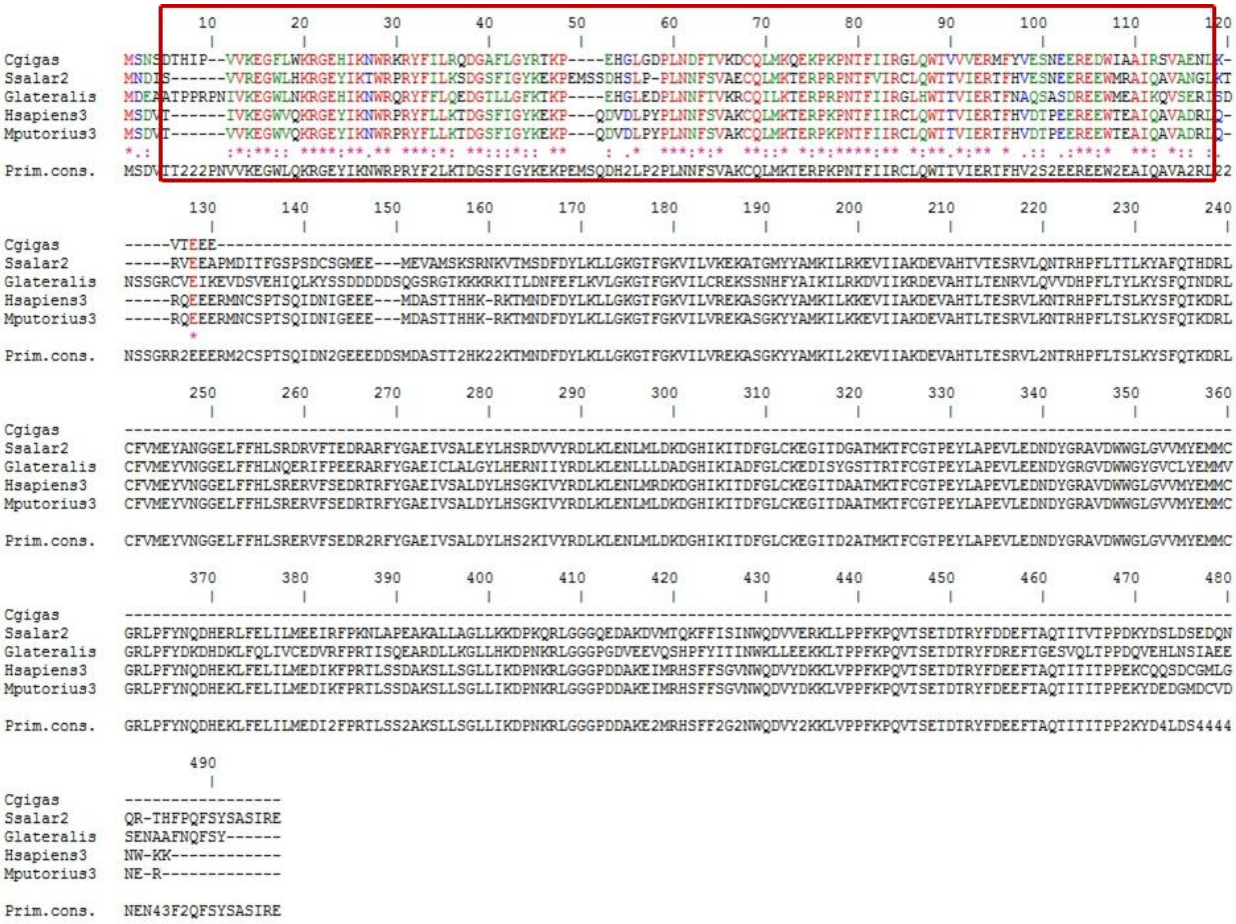

Figure 4: Levels of truncated AMPKα protein quantified on western blot (expressed in relative OD/mm^2); for different oyster tissues sampled in wild oysters at the sea shore : mantle edge, digestive gland, mantle, labial palps, smooth muscle, striated muscle, heart, gills and visceral ganglia during sexual resting stage (n = 4). The protein values presented on the graph were calculated from 4 blots and a representative western blot of truncated AMPKα is shown under the graph. Columns and bars show mean ± SD. Different letters indicate a significant difference between tissues (P < 0.05).

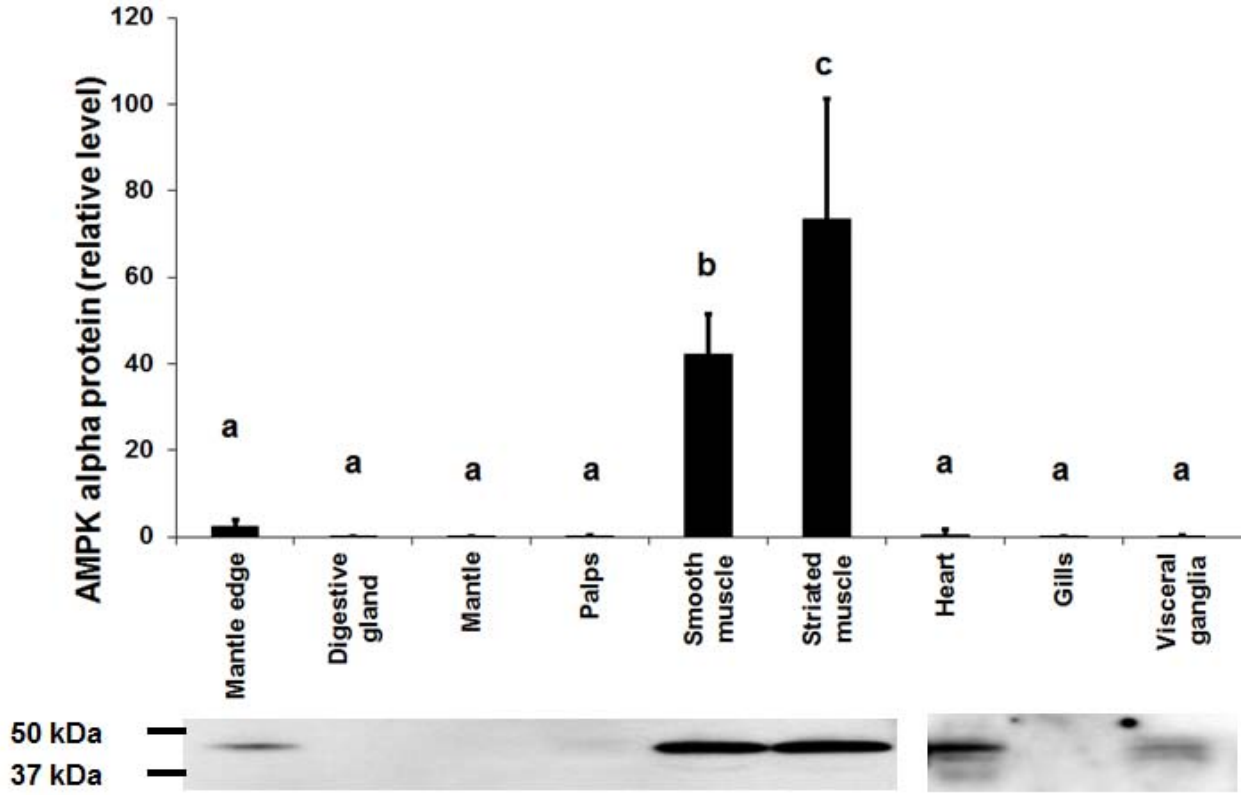

Figure 5: Western-blot of entire AMPKα and truncated AMPKα protein in smooth and striated muscle of oysters exposed to normoxia (n = 4) or hypoxia (n = 4) measured after 1, 3 and 6 h of challenge. (A) Representative images of entire AMPKα detection at 62 kDa and truncated AMPKα at 46-48 kDa. (B) Quantification of truncated AMPKα protein during normoxia (white bars; n = 4) or hypoxia (black bars; n = 4) in smooth muscle. (C) Quantification of truncated AMPKα protein during normoxia (white bars; n = 4) or hypoxia (black bars; n = 4) in striated muscle. The protein values presented on the graph were quantified on 4 western blots and expressed in relative level of OD/mm2. Columns and bars show mean ± SD. Asterisks indicate a significant difference between conditions ($P < 0.05$).

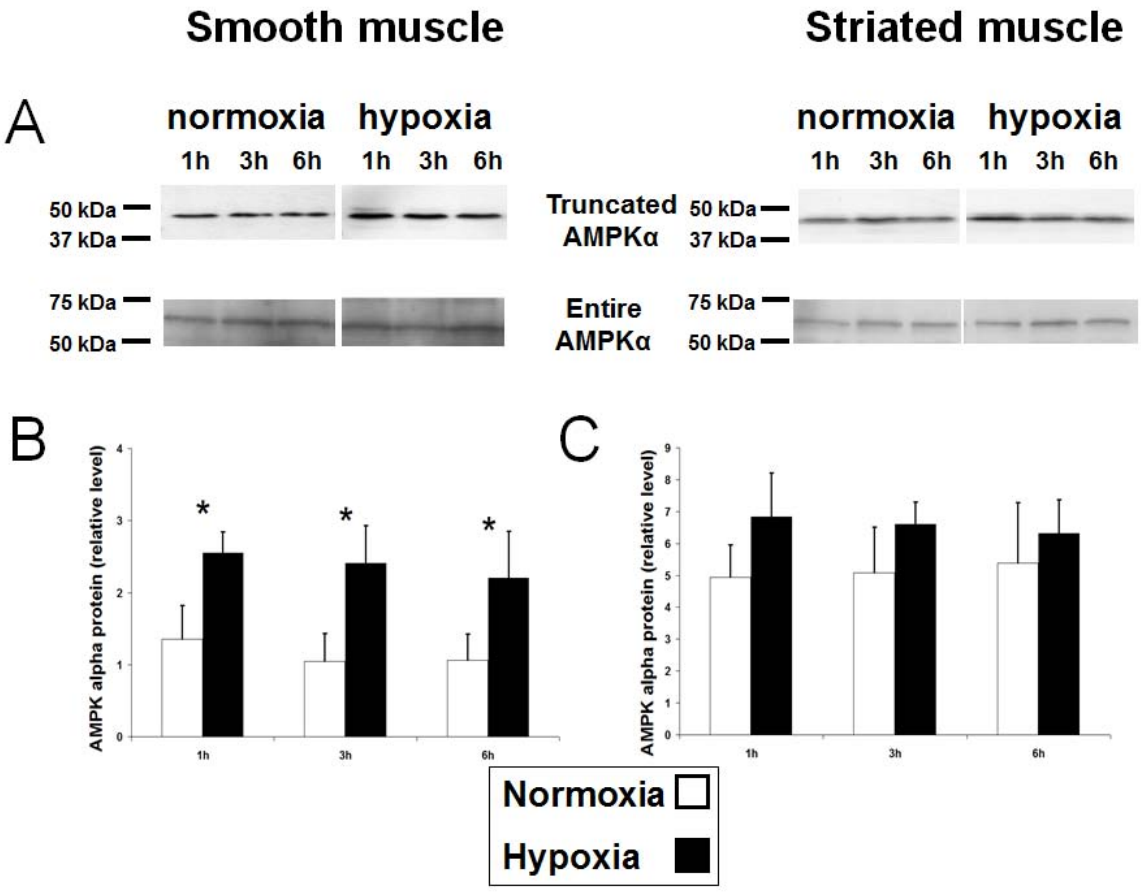

Figure 6: Enzymatic assay of citrate synthase in smooth and striated muscle of oysters exposed to normoxia (n = 4) or hypoxia (n = 4) measured after 1, 3 and 6 h of challenge. (A) Citrate synthase activity during normoxia (white bars; n = 4) or hypoxia (black bars; n = 4) in smooth muscle; (B) Citrate synthase activity during normoxia (white bars; n = 4) or hypoxia (black bars; n = 4) in striated muscle. Different letters indicate significant differences along the temporal sampling ($P < 0.05$). Asterisks indicate significant differences between conditions ($P < 0.05$).

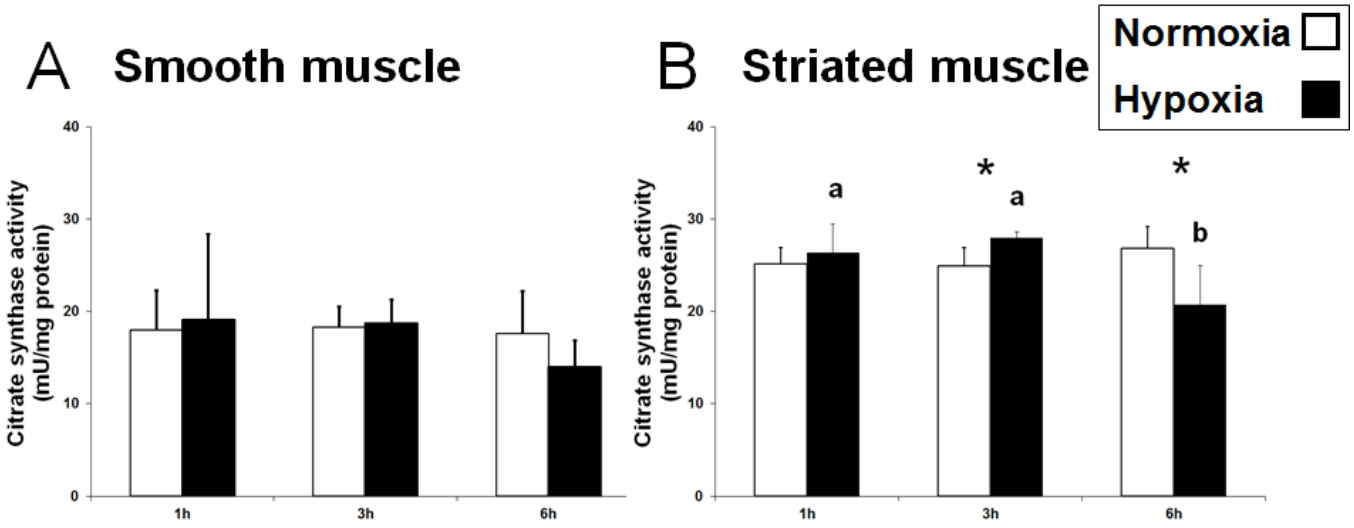

Figure 7: Western-blot of AKT protein in smooth and striated muscle of oysters exposed to normoxia (n = 4) or hypoxia (n = 4) measured after 1, 3 and 6 h of challenge. (A) Quantification of AKT protein during normoxia (white bars; n = 4) or hypoxia (black bars; n = 4) in smooth muscle. (B) (A) Quantification of AKT protein during normoxia (white bars; n = 4) or hypoxia (black bars; n = 4) in striated muscle. The protein values presented on the graph were calculated from 4 blots and a representative western blot of truncated AMPKα is shown under the graph. Columns and bars show mean ± SD. Asterisks indicate significant differences between conditions (P < 0.05).