METABOLIC CHANGES IN SKELETAL MUSCLE CELLS UNDER "CHEMICAL HYPOXIA"

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SUMMARY

The peri-mortem metabolism of muscle cells is of importance for meat quality, as it can influence the rate and extent of post-mortem pH fall. The mechanisms responsible for the variations of these parameters are not clear. The analysis of metabolism in a single muscle cell in hypoxic conditions can provide relevant information on the process that occur in muscle at slaughter.

We used muscle cell cultures for the study of both intracellular calcium and pH variations in cells under "chemical hypoxia". This hypoxia was induced by adding sodium cyanide (NaCN, inhibitor of the respiratory chain), Carbony cyanide m-chlorophenyl-hydrazone (CCCP, uncoupler of the oxidative phosphorylations) or iodoacetate (inhibitor of the glycolysis) to the medium. Muscle cells from the mouse cell line Sol 8 were grown until differentiation into myotubes. Prior to test, the cells were loaded with fluorescent probes Fura2-AM and BCECF-AM in order to estimate intracellular free calcium ($[Ca^{2+}]_i$) and pH (pH_i) respectively. Tests were carried out using different media containing one or a mixture of the hypoxic drugs, with or without Ca^{2+}. Fluorescence of the probes under dual wavelength excitation, was recorded every 2 min during 30 min at 37°C.

In control cells incubated during 30 min without any chemicals, $[Ca^{2+}]_i$ increases slightly when extracellular Ca^{2+} is present, pH_i is constant. With CCCP-NaCN-iodoacetate, the pH_i fell 0.5 units and $[Ca^{2+}]_i$ increased 3 times within 10 min after induction of hypoxia. In abscence of extracellular Ca^{2+}, pH_i fell to the same level, whereas $[Ca^{2+}]_i$ increased less. Impairment in mitochondrial function with CCCP or NaCN, in the absence or the presence of extracellular calcium, also induces a fall of pH_i and a slight increase of $[Ca^{2+}]_i$. When glycolysis is impaired, the fall of pHi is delayed and $[Ca^{2+}]_i$ is constant. These results show that the abrupt drop of pH_i induced by complete "chemical hypoxia", which is probably associated with depletion in high energy phosphates, does not seem to be related to glycolysis in our cellular model. The rapid increase in $[Ca^{2+}]_i$ evidenced in these experiments, was enhanced when extracellular calcium was present, suggesting early membrane damage and modification of metabolism in cell submitted to hypoxia.

INTRODUCTION

The anaerobic breakdown of ATP in post-mortem muscle induces acidification of the tissue. Variations in post-mortem pH fall are not well understood but lead to heterogeneity in meat quality. Many hypothesis have been put forward to explain cellular injury subsequent to ATP depletion. For example plasma membrane phospholipide degradation, activation of autolytic systems, cellular acidosis, superoxyde-induced membrane damage, and mitochondrial dysfunction have been suggested as playing a central role in ischemic injury (see for review Herman *et al.* 1990). A number of hypothesis involve Ca^{2+} ions as an accelerating factor in ATP turn-over and anaerobic breakdown of glycogen but there still lack proof for involving calcium in early post-mortem metabolism.

Zuurveld *et al.* (1985) demonstrated that skeletal muscle cells in culture are well comparable to mature muscle with respect to oxydative capacities and enzymes activities. Myotubes in absence of nerves present relatively high levels of glycolytic enzymes and are metabolically close to glycolytic fibers (Lawrence and Salsgiver 1983). These cells can therefore provide a model for study on muscle cell metabolism in conditions occuring in the muscle after slaughtering. In this study, muscle cells in culture have been used as a model for studying the effects of hypoxia on skeletal muscle metabolism. To mimic anoxia occuring in the muscle after slaughter, we substituted the lack of oxygen and substrat by metabolic inhibition with chemicals. The aim of this work was to investigate $[Ca^{2+}]i$ and pHi variations occuring during metabolic inhibition of muscle cells.

MATERIALS AND METHODS

Cell culture : Mouse skeletal muscle cell line Sol 8 (kindely provided by C. Pinset, Inst Pasteur Paris) were grown in monolayer culture in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% fetal calf serum, L-glutamine (4 mM), penicilline (50 unit/ml) and streptomycine (50 ml/ml) in a water saturated atmosphere of 5% CO2 and 95% air at 37°C. For the experiments, cells were cultured in silicone wells (Flexiperm Heraeus), on a glass coverslip coated with 2% gelatine. At confluence, cells were differentiated in DMEM containing 2% horse serum, L-glutamine and antibiotics. The media were renewed every 2 days. Experiments were performed on myotubes after 2-3 days of differentiation.

Simultaneous measurement of $[Ca^{2+}]i$ and pHi :
pHi and $[Ca^{2+}]i$ were determinated by using the fluorescent indicator 2',7'-bis-(carboxyethyl)5-(6')carboxyfluorescein acetoxymethyl ester (BCECF-AM, SIGMA) and fura2-AM (SIGMA) respectively. Dual loading of myotubes was performed by incubation in Minimum Essential Medium Eagle (MEM) containing 2 μM BCECF-AM, 10 μM fura2-AM and 0.02% pluronic F127 during 45 min at room temperature, in the dark. The culture were rinsed for 30 min before measurements.

The fluorescence was recorded using an epifluorescence microscope (Olympus IMT2) connected to a DMX 1000 spectrofluorimeter (SLM AMINCO) by an optic fiber, for dual excitation fluorescent measurement. Loaded myotubes were alternatively excited at l=350 and 380 nm, then sequentially at 440 and 495 nm by means of a xenon lamp and a computer-controled rotating mirror wheel. The ratio 350/380 for $[Ca^{2+}]i$ determination and 495/440 for pHi determination were calculated from emitted fluorescence respectively through a 480 and 530 nm filter. Endogenous fluorescence and background recorded from non-loaded cells were substracted from each measurements. Fluorescence was measured every 2 min during 30 min.

Calibration of pH measurement (Rink *et al.* 1982) was performed with buffers (130 mM KCl, 20 mM NaCl and 10mM Hepes) adjusted to various pH values with KOH. Equilibrium of pHi with external pH in these buffers was obtained by extracellular application of the K^+/H^+ exchanger nigericine (5 μM). The calibration curves were linear over the pH range between 6.4 - 7.2.

Calibration and calculation of free cytosolic Ca^{2+} were performed by the procedure described by Grynckiewicz *et al.*(1985). Rmax (maximal fluorescence ratio) was obtained by adding ionomycin at final concentration of 2 μM in buffer containing 5 mM Ca^{2+}. Rmin (minimal fluorescence ratio) was obtained by adding ionomycin and EGTA at a final concentration of 10 mM in Ca^{2+}-free buffer.

Model of hypoxia :
At the beginning of each experiment, myotubes were perfused with an Hepes buffer (130 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose ±1.8 mM $CaCl_2$) at pH=7.2. Chemical hypoxia was obtained by adding 2 mM sodium cyanide (NaCN, inhibitor of the respiratory chain) or 2 mM Carbony cyanide m-chlorophenyl-hydrazone (CCCP, uncoupler of the oxidative phosphorylations) or 0.5 mM iodoacetate (inhibitor of the glycolysis) or a mixture of this tree chemicals in the incubating solution. Experiment took place on a thermostatically regulated table of the inverted microscope at 37°C. Incubation in Ca^{2+}-free buffer were carried out in the presence of 0.1mM EGTA.

Cell viability :
Cell viability was monitored by incubating the myotubes at the end of the experiment with Trypan Blue.

RESULTS AND DISCUSSION

Basal level of $[Ca^{2+}]i$ in myotubes is 100±6 nM in Ca^{2+} -buffer (fig 1) and 60±5 nM in Ca^{2+} free buffer (fig 3). Similar $[Ca^{2+}]i$ were measured in myotubes from mouse and rat (Giovanelli *et al.* 1991 ; Muller et *al.* 1992) and also in intact isolated fiber of muscle from pigs (Iaizzo *et al* 1988). When myotubes were incubated in Ca^{2+}-medium during 30 min, a slow and slight increase of $[Ca^{2+}]i$ occurs likely due to a low entrance of extracellular calcium. Basal pHi value is 7.12± 0.02. This value is not modified significantly over 30 min of incubation in normal medium (fig 2 and 4).

-Mitochondrial inhibition:
In the presence of NaCN or CCCP, a transient increase of cytosolic Ca^{2+} to 190 nM is observed about 4 min after drug application, independently of the presence of calcium in the incubating medium (fig 1 and 3). This result suggests that this early peak correspond to release of Ca^{2+} from intracellular stores. pHi decreases markedly within 10 min to 6.72±0.05 for CCCP and to 6.81±0.04 for NaCN (fig 2). Thenafter pHi values stabilize around 6.60 and

6.75 for CCCP and NaCN respectively. Fall in pHi is more dramatic for CCCP than NaCN. NaCN inhibits respiratory chain and CCCP is an uncoupler of oxidative phosphorylations. When mitochondrial oxidative phosphorylation is impaired, glycolytic energy production is enhanced. Gulden (1993) showed that inhibition and uncoupling of mitochondrial oxydative phosphorylation induce an increase in glucose consumption of skeletal muscle cell in culture to a maximum of 170-210% of the controls. The enhanced glucose consumption is considered to reflect an increased rate of glycolysis as a consequence of reduced supply of ATP from mitochondrial oxidative phosphorylation (Pasteur effect). The dramatic fall of pHi observed in our experiments may correspond to the production of lactate and H^+ issued from the exacerbated glycolysis and the ATP hydrolysis for maintaining vital cellular functions.

-Glycolysis inhibition:
When glycolysis is impaired by iodoacetate, myotubes present a slight and progressive increase of $[Ca^{2+}]i$ as observed in the control, with a slight entrance of extracellular Ca^{2+} (fig 1). Compared to mitochondrial inhibition, the beginning of pHi fall is delayed, in Ca^{2+} buffer as in Ca^{2+}-free buffer (fig 2 and 4). A significant decrease occurs after the first 4 min, reaches the value of 6.66 after 10 min and no more significantly vary. When glycolysis is impaired, energy supply comes from oxydative metabolism. Buffering capacities of cytoplasm and H^+ transport system may account for the delayed pHi fall. Thenafter, imbalance between energy demand and supply may lead to H^+ accumulation and fall of pHi.

-Chemical hypoxia:
Exposure of myotubes to NaCN+CCCP+Iodoacetate for 30 min resulted in a strong increase of $[Ca^{2+}]i$ (1757±275nM after 8 min), the maximum value of 2722±1647nM was reached after 30 min (fig 1). Regarding the pHi, a rapid acidification was observed (6.62±0.01 after 4 min), then pHi slowly decreased until stabilization around 6.5 (fig 2). In the absence of extracellular Ca^{2+}, the $[Ca^{2+}]i$ increase was limited to 366±44 nM in 30 min (fig 3) whereas pHi decreased in the same order of rate and magnitude than in the presence of extracellular Ca^{2+} (fig 4). After the 30 min of incubation in these conditions, some myotubes exhibited blebs which are generally associated with cellular damages (Duncan and Shamsadeen 1989) or were found dead. These results suggest an early membrane damage in cells under hypoxia.

The association of a transient or persistent increase in $[Ca^{2+}]i$ with a decrease in pHi is dependent of the cell type. Herman *et al.* (1990) noted little or no change in free Ca^{2+} and acidosis in hepatocytes, whereas Bond *et al.* (1993) observed a progressive increase of $[Ca^{2+}]i$ accompaniing acidosis in cardiomyocytes. Bowers *et al.* (1992) by exposing cardiomyocytes and hepatocytes to chemical hypoxia (CN^- and 2-Deoxyglucose), provoked a fall of ATP, instantaneous in hepatocytes and delayed by few minutes in cardiomyocytes because of the presence of creatine phosphate in this latter. In Sol 8 skeletal muscle cells it occurs an immediate response in $[Ca^{2+}]i$ and pHi, as a result of energy depletion induced by chemical hypoxia. This is known to be associated with ultrastructural and membranar damages in muscle cells (Duncan and Jackson 1987). However, such extrem hypoxic condition does not likely represent those of muscle cell in slaughtered animal since glycogen is generally present ar early post-mortem stage.

Mitochondrial impairment may correspond to the anoxia occuring in muscle tissue at slaughter. In the muscle, the oxygen deprivation leads to a virtually absolute anoxia within minutes (Bendall and Taylor 1972). Such anoxia inhibits mitochondrial phosphorylation completely and the resultant decrease of ATP and increase of ADP and AMP stimulates glycolysis. However pH decreases by about 2 units in post-mortem muscle whereas it only felt of 0.6 units in Sol 8 myotubes, even for incubation as long as three hours in hypoxic conditions (data not shwon). This can be explained by an easier diffusion of intracellular lactate and H^+ in myotubes in culture compared with muscle tissue. Moreover, the mouse myogenic cell line Sol 8 cultured in our conditions could probably not differentiate enough to get, at least partly, the characteristic of adult myofibres (Pinset *et al*, 1991). This can also minimize the effect of hypoxia on intracellular pH and Ca^{2+}.

CONCLUSION

In summary, the extent of acidosis occuring in skeletal muscle cell in culture under hypoxic condition is not governed by $[Ca^{2+}]i$. On the other hand, a high rate of pH fall induced by total inhibition of energy metabolism is associated with a strong increase of $[Ca^{2+}]i$, these ions being provided both by intracellular stores and extracellular medium when available. This, in turn, can activate enzymatic systems such as Ca-ATPase with consequent depression of cellular energy stores and decrease in pHi in tissues at post-mortem stages.

It is now interesting and necessary to investigate relationship between pHi and $[Ca^{2+}]i$ and to define their

role on energetic metabolism, on membranar and structural states in injuries leading to cell death.

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Figure 1: Effect of chemical hypoxia on $[Ca^{2+}]i$ in myotubes incubated in Ca^{2+}-buffer containing 2 mM NaCN (n=5) or 2 mM CCCP (n=3) or 0.5 mM iodoacetate (n=4) or a mixture of the three chemicals (n=3). Control (n=4) correspond to myotubes in buffer without chemical. Values are means ± SEM.

Figure 2 : Effect of chemical hypoxia on pHi in myotubes incubated in Ca^{2+}-buffer containing 2 mM NaCN (n=5) or 2 mM CCCP (n=3) or 0.5 mM iodoacetate (n=4) or a mixture of the three chemicals (n=3). Control (n=4) correspond to myotubes in buffer without chemical. Values are means ± SEM.

Figure 3 : Effect of chemical hypoxia on $[Ca^{2+}]i$ in myotubes incubated in Ca^{2+}free-buffer containing 2 mM NaCN (n=5) or 2 mM CCCP (n=4) or 0.5 mM iodoacetate (n=4) or a mixture of the three chemicals (n=5). Control (n=4) correspond to myotubes in buffer without chemical. Values are means ± SEM.

Figure 4 : Effect of chemical hypoxia on pHi of myotubes incubated in Ca^{2+}free-buffer.containing 2 mM NaCN (n=5) or 2 mM CCCP (n=4) or 0.5 mM iodoacetate (n=4) or a mixture of the three chemicals (n=5). Control (n=4) correspond to myotubes in buffer without chemical. Values are means ± SEM.