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Metabolic profiling of heat or anoxic stress in mouse C2C12 myotubes using multinuclear magnetic resonance spectroscopy

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Abstract

In the present study, the metabolic effects of heat and anoxic stress in myotubes from the mouse cell line C2C12 were investigated by using a combination of ^{13}C, ^{1}H, and ^{31}P nuclear magnetic resonance (NMR) spectroscopy and enrichment with [^{13}C]-glucose. Both the ^{13}C and the ^{1}H NMR spectra showed reduced levels of the amino acids alanine, glutamate, and aspartate after heat or anoxic stress. The decreases were smallest at 42°C, larger at 45°C, and most pronounced after anoxic conditions. In addition, in both the ^{1}H and the ^{31}P NMR spectra, decreases in the high-energy phosphate compounds adenosine triphosphate and phosphocreatine with increasing severity of stress were identified. At anoxic conditions, an increase in ^{13}C-labeled lactate and appearance of glycerol-3-phosphate were observed. Accumulation of lactate and glycerol-3-phosphate is in agreement with a shift to anaerobic metabolism due to inhibition of the aerobic pathway in the mitochondria. Conversely, lower levels of unlabeled (^{12}C) lactate were apparent at increasing severity of stress, which indicate that lactate is released from the myotubes to the medium. In conclusion, the metabolites identified in the present study may be useful markers for identifying severity of stress in muscles.

1. Introduction

Increase in temperature or anoxic conditions in skeletal muscles disturb cellular homeostasis and trigger a cascade of responses. Understanding these cellular responses to stress is essential for developing protective strategies. Differentiated muscle cell cultures have been applied for studying basic cellular responses to stress because the use of cell cultures enables a strict and controlled environment [1-4].

Metabolites reflect the cellular activity; and as a consequence, changes in metabolite levels can provide a useful measure of the overall cellular response to stressors. Consequently, to obtain a better understanding of the effects of stress, it is essential to embrace an approach, such as a simultaneous measurement of a broad set of the metabolites present. High-resolution proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy is commonly applied to study metabolic perturbations in many different biological systems [5-8], and the success of the technique can be ascribed to the fact that ^{1}H NMR spectroscopy yields well-resolved signals from a large number of metabolites. However, combining ^{1}H with ^{13}C and ^{31}P NMR spectroscopy enables us to examine an even wider range of metabolic changes at the cellular level. After incubating cells with [^{13}C]-enriched glucose, the specific changes in glucose utilization can be monitored by using ^{13}C NMR spectroscopy [9-11]. In contrast, ^{31}P NMR allows an assessment of the energy-rich phosphate compounds such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), and phosphocreatine (PCr) that can be considered the essential energy fuel at the cellular level [11]. Accordingly, combining ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy on the same samples is expected to be a powerful technique for a simultaneous investigation of a broad set of metabolic

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changes related to stress exposure. Thus, the aim of the present study was to elucidate the integrated set of metabolic changes in myotubes derived from the mouse muscle cell line C2C12 exposed to temperature stress at 42°C or 45°C, or at anoxic conditions by using a combination of ^{13}C, ^{1}H, and ^{31}P NMR spectroscopy.

2. Materials and methods

2.1. Muscle cell cultures

The mouse myoblast cell line C2C12, originally derived from a mouse thigh muscle [12] (American Type Culture Collection, Manassas, VA), was grown to establish myotube cultures. A clone from this cell line, which previously has been isolated, where the myoblasts effectively fuse and form myotubes [13], was used between passages 8 and 10. The clone was grown in a 75-cm^2 culture flask in 10 mL of growth medium consisting of Dulbecco modified Eagle medium (DMEM) (Invitrogen, Paisley, UK, catalog no. 32430-027), 10% (vol/vol) fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 3 μg/mL amphotericin B, and 20 μg/mL gentamicin. Cells were maintained in an atmosphere of 95% air and 5% CO_2 at 37°C. Before confluence, cells were harvested in 0.25% trypsin and seeded in Petri dishes (140 mm in diameter) at a density of 10 000 cells per square centimeter. Cells were grown to confluence in growth medium and left to fuse in differentiation medium containing DMEM, 4% (vol/vol) fetal calf serum, and similar amounts of antibiotics as in the growth medium. After about 4 days, the cultures contained differentiated multinuclear myotubes and were ready for experimental use.

2.2. Incubation and extraction of cells

Differentiation medium made from glucose-free DMEM (Invitrogen, Paisley, UK, catalog no. 11966-025) was added with 5.5 mmol/L [$^{13}C_1$]glucose (Cambridge Isotope Laboratories, Andover, MA). The myotube cultures were incubated for 4 hours in the [$^{13}C_1$]glucose-containing medium (*a*) in an atmosphere of 95% air and 5% CO_2 at 37°C; (*b*) at 42°C for 1 hour, followed by 3 hours in an atmosphere of 95% air and 5% CO_2 at 37°C; (*c*) at 45°C for 1 hour, followed by 3 hours in an atmosphere of 95% air and 5% CO_2 at 37°C; (*d*) or in a chamber under low oxygen pressure (<1%) at 37°C for 4 hours (Fig. 1). To ensure anoxic conditions for the myotubes incubated under low oxygen pressure, the gasses were removed from the culture medium with a vacuum pump; and the degree of anoxia in the incubation chamber was verified with an O_2 electrode (CheckMate 9900; PBI Dansensor, Horsens, Denmark). After 4 hours of incubation, the [$^{13}C_1$]glucose-containing medium was discarded, the cells were washed 3 times with an ice-cold 0.9% NaCl solution to remove excess medium, and the cells were subsequently frozen in liquid nitrogen. For extraction of the cells, a methanol/chloroform extraction

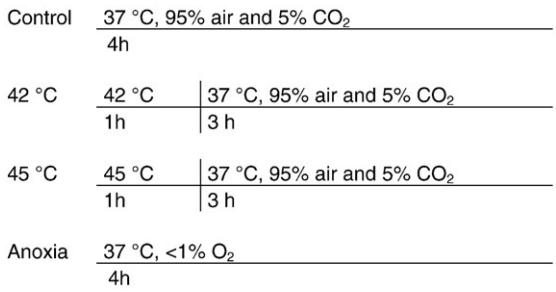

Fig. 1. The differentiated C2C12 mouse myotubes were incubated with [$^{13}C_1$]glucose for 4 hours and, during this incubation period, exposed to different treatments. See the text for further details.

method adopted from Serkova et al [14] was applied. Four milliliters of ice-cold methanol was added to the Petri dish with frozen myotubes, the cells were removed by scraping with a spatula, and the cell suspension was transferred to a glass vial. Four milliliters of ice-cold chloroform was added and, after vortex mixing, left on a crushed ice bath for 10 minutes. Subsequently, 4 mL of ice-cold water was added and, after vortex mixing, left overnight at 4°C for phase separation. The next day, the methanol/chloroform cell extraction was centrifuged at 1400*g* for 30 minutes at 4°C (Heraeus Multifuge 3 S-R, Kendro, Osterode, Germany). On a crushed ice bath, the methanol/water phase was transferred to a glass vial, taking care not to carry over the pellet debris. The samples were freeze-dried at −80°C and subsequently stored at −80 °C until NMR analysis.

2.3. NMR spectroscopy

The freeze-dried methanol/water phase from the cell extracts from 2 Petri dishes was pooled and dissolved in 600 μL D_2O containing 0.025% (wt/vol) sodium trimethylsilyl-[2,2,3,3-D_4]-1-propionate and 0.01% (vol/vol) dioxane. The ^{13}C and ^{1}H NMR spectra of the cell extracts were recorded at 25°C on a Bruker Avance 800 spectrometer, operating at a ^{13}C frequency of 201.01 MHz and a ^{1}H frequency of 799.40 MHz, equipped with a 5-mm ^{1}H observe cryoprobe (Bruker BioSpin, Rheinstetten, Germany). Proton-decoupled ^{13}C NMR spectra were recorded using Bruker standard homonuclear pulse programs, and each spectrum was the sum of 1024 free induction decays (FIDs). A 90° pulse was applied with a repetition time of 3 seconds and an acquisition time of 0.682 second. Standard 1-dimensional ^{1}H NMR spectra were acquired using single 90° pulse experiment, and each spectrum was the sum of 16 FIDs. Water suppression was achieved by irradiating the water peak during the relaxation delay of 5 seconds, and 32 K data points spanning a spectral width of 13.03 ppm were collected. In addition, ^{13}C-decoupled ^{1}H NMR spectra were recorded to verify the spectral assignment of signals from ^{13}C-labeled metabolites in the ^{1}H NMR spectra. All spectra were referenced to sodium trimethylsilyl-[2,2,3,3-D_4]-1-propionate at 0 ppm. Dioxane (δ 69.44 ppm) was used as a secondary reference in

the ^{13}C NMR spectra. In addition, to aid spectral assignment, 2-dimensional ^{1}H-^{1}H COSY and 2-dimensional ^{1}H-^{13}C HSQC spectra were recorded on selected cell samples using water suppression. ^{13}C and ^{1}H NMR spectra were obtained on 3 replicates of each treatment group (*a*-*d*) described above and on an additional sample for the control group (*a*). Each replicate represents the pooling of the freeze-dried methanol/water phase from the cell extracts from 2 Petri dishes.

^{31}P NMR spectra were recorded at 25°C on a Bruker Avance III 600 spectrometer (Bruker BioSpin) operating at a ^{31}P frequency of 242.94 MHz. The ^{31}P NMR spectra were obtained as the sum of 8192 FIDs acquired with a repetition time of 5 seconds and an acquisition time of 1.346 seconds. The spectral width was 12 kHz, and the chemical shift was referenced to the resonance of PCr at 0 ppm. ^{31}P NMR spectra were obtained on samples of the treatment groups *a*, *c*, and *d* described above.

2.4. Data analysis

Quantification of the various metabolites was carried out by integration of peak areas using the Topspin software (Bruker BioSpin). The ^{13}C NMR spectra were integrated in the spectral range 19.00 to 100.00 ppm, excluding the dioxane signal at 69.00 to 70.00 ppm. The ^{1}H NMR spectra were integrated in the spectral range 1.30 to 9.50 ppm, excluding the interval 4.70 to 4.90 ppm containing the residual water signal and the interval 3.74 to 3.79 ppm containing the dioxane signal. All the data were analyzed by using the mixed procedure of SAS (SAS Institute, Cary,

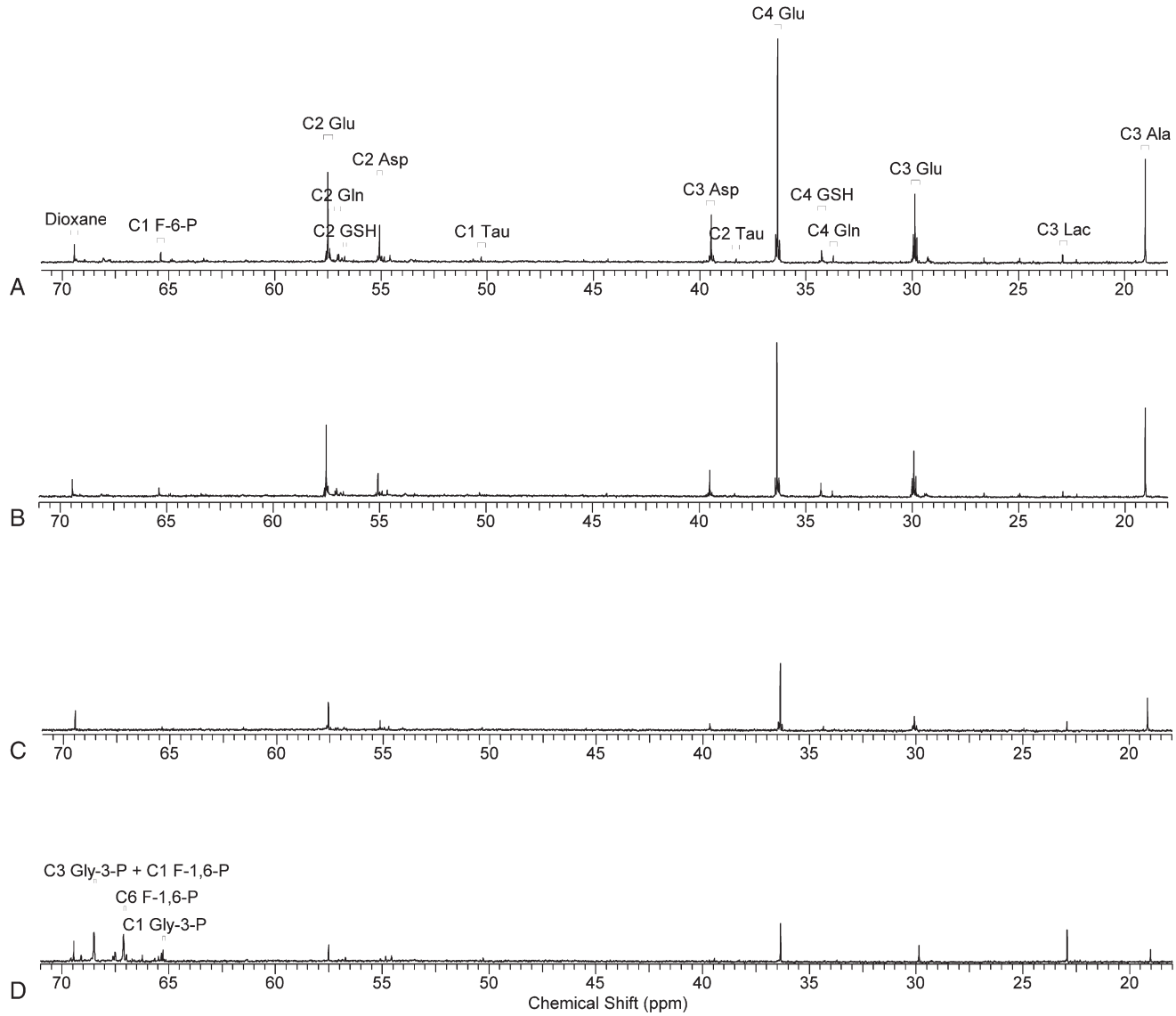

Fig. 2. ^{13}C NMR spectra of C2C12 mouse myotubes after incubation with [$^{13}C_1$]glucose for 4 hours. The spectra were obtained on the freeze-dried methanol/water extractions of the cells. (A) Control. (B) 42 °C for 1 hour. (C) 45°C for 1 hour. (D) Anoxia for 4 hours. Dioxane is an internal reference. F-6-P indicates fructose-6-phosphate; Glu, glutamate; Gln, glutamine; GSH, γ-glutamyl-glutathione; Asp, aspartate; Tau, taurine; Lac, lactate; Ala, alanine.

NC). The model included treatment as a fixed effect and replicates as a random effect. Absolute integral intensities are given as least squares means (LSMeans) ± standard errors of LSMeans (SEM).

3. Results

3.1. ^{13}C, ^{1}H, and ^{31}P NMR spectra

After incubating the C2C12 mouse myotubes with [$^{13}C_1$] glucose for 4 hours at standard conditions, the ^{13}C label was found mainly in [$^{13}C_3$]alanine; in [$^{13}C_2$], [$^{13}C_3$], and [$^{13}C_4$] glutamate; as well as in [$^{13}C_2$] and [$^{13}C_3$]aspartate (Fig. 2, Table 1). Labeling was also found and identified for [$^{13}C_3$] lactate, [$^{13}C_2$] and [$^{13}C_4$]glutamine, [$^{13}C_2$] and [$^{13}C_4$]γ-glutamyl-glutathione, [$^{13}C_1$]fructose-6-phosphate, and [$^{13}C_1$]α- and β-glucose (not shown in Fig. 2). The chemical shifts for C_3 glutamine and γ-glutamyl-glutathione are indistinguishable, and other minor signals remain unidentified. Taurine signals from natural abundance ^{13}C were also detected. Under standard incubation conditions, several of the metabolites identified in the ^{13}C NMR spectra and additional metabolites were identified in the ^{1}H NMR spectra (Figs. 3 and 4, Table 2) and in the ^{31}P NMR spectra (Fig. 5), respectively.

3.2. Effects of heat and anoxia

Heat or anoxic stress resulted in lower levels of several metabolites including γ-glutamyl-glutathione (Fig. 2, Table 1), alanine, glutamate, aspartate, glutamine, and taurine (Figs. 2-4, Tables 1 and 2), fructose-6-phosphate (Figs. 2 and 5, Table 1), creatine, tyrosine, and phenylalanine (Table 2), PCr, ATP, ADP, and NAD^+ (Table 2, Fig. 5), glycerophosphocholine, glycerophosphorylethanolamine, and uridine 5'diphosphoglucose (Fig. 5). The decreases in the metabolites were generally small at 42°C, more apparent at 45°C, and most pronounced after anoxic stress.

Conversely, higher levels of Pi were apparent in the ^{31}P NMR spectra with increasing severity of the stress exposure (Fig. 5). In the ^{13}C NMR spectra, higher levels of [$^{13}C_3$]lactate were observed after stress exposure, which was significant for anoxic stress when compared with control (Fig. 2, Table 1). In the ^{1}H NMR spectra, higher levels of the ^{13}C-labeled lactate were also observed at stress; however, these were not significant (Fig. 3, Table 2). Conversely, lower levels of the unlabeled (^{12}C) lactate were identified after stress exposure, significantly lower at 45°C and anoxic stress, when compared with control (Fig. 3, Table 2). Under anoxic conditions, ^{13}C was found in [$^{13}C_1$] and [$^{13}C_3$]glycerol-3-phosphate and in [$^{13}C_1$] and [$^{13}C_6$] fructose-1,6-bisphosphate (F-1,6-P) in the ^{13}C NMR spectra (Fig. 2, Table 1). Glycerol-3-phosphate (Gly-3-P) and fructose-1,6-bisphosphate were also identified in the ^{31}P NMR spectra and, additionally, glucose-6-phosphate (G-6-P) at anoxia (Fig. 5). Finally, at anoxia, an increase in unlabeled (^{12}C) glutamate was observed in the ^{1}H NMR spectra (Fig. 4, Table 2).

4. Discussion

In the present study, the metabolic effects of heat or anoxic stress in mouse myotubes were quantitatively

Table 1
^{13}C NMR chemical shift ranges and LSMeans of the absolute integral intensities, with calculated SEM

Metabolite	Shift range (ppm)	Control (n = 4)	42°C (n = 3)	45°C (n = 3)	Anoxia (n = 3)	SEM
C_3 alanine	19.00-19.14	3.71	3.62	1.69*	0.49*	0.52
C_3 lactate	22.92-22.97	0.25	1.20	0.67	2.03*	0.60
C_3 glutamate	29.81-30.06	7.10	5.24†	2.15†	0.57†	0.20
C_4 glutamine	33.70-33.83	0.26	0.19	0.07*	0.03*	0.03
C_4 γ-glutamyl-glutathione	34.22-34.36	1.01	0.86	0.41*	0.02†	0.12
C_4 glutamate	36.32-36.37	13.75	9.47*	4.05†	1.60†	0.70
C_2 taurine	38.22-38.40	0.18	0.17	0.05*	0.06*	0.02
C_3 aspartate	39.40-39.67	3.43	2.12*	0.74†	0.09†	0.18
C_1 taurine	50.22-50.35	0.31	0.18	0.05*	0.13*	0.08
C_2 aspartate	54.93-55.14	2.89	1.59*	0.66*	0.06†	0.30
C_2 γ-glutamyl-glutathione	56.82-56.89	0.39	0.29	0.21	0.10	0.15
C_2 glutamine	57.00-57.33	1.01	0.63	0.27*	0.05*	0.20
C_2 glutamate	57.49-57.56	6.93	4.70*	2.05†	0.53†	0.44
C_1 glycerol-3-phosphate	65.23-65.31	ND	ND	ND	0.64*	0.09
C_1 fructose-6-phosphate	65.35-65.40	0.46	0.40	0.14*	0.10*	0.11
C_6 F-1,6-P	67.07-67.16	ND	ND	ND	2.14*	0.45
C_3 glycerol-3-phosphate/C_1 F-1,6-P	68.45-68.57	ND	ND	ND	2.53*	0.45
C_1 α-glucose	94.89-94.95	0.07	0.30	0.12	0.38	0.17
C_1 β-glucose	98.72-98.80	0.09	0.41	0.21	0.47	0.24

ND indicates not detectable.

* LSMeans differ significantly ($P < .05$) when compared with the control cells.

† LSMeans differ significantly ($P \leq .0001$) when compared with the control cells.

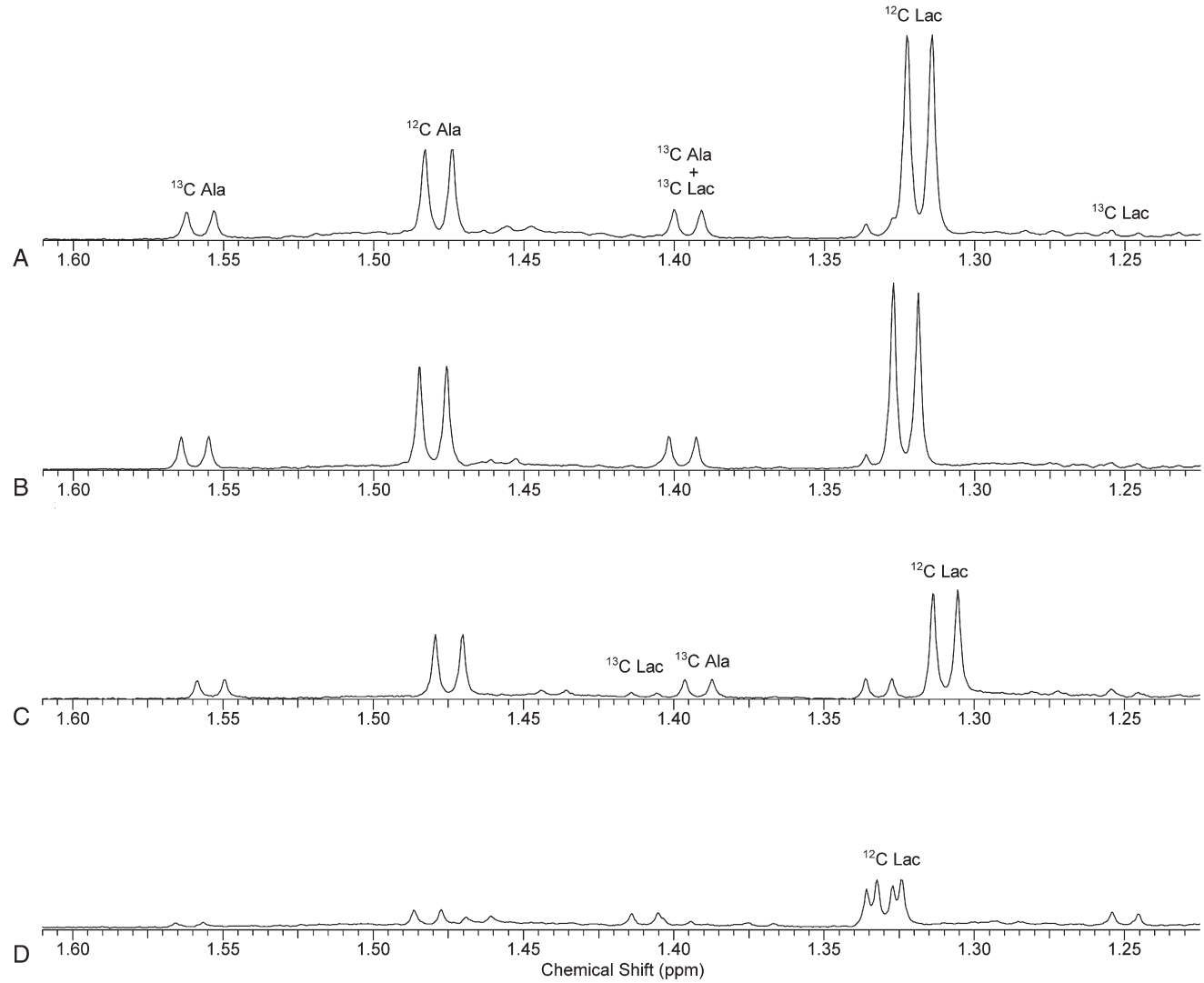

Fig. 3. Expanded region showing lactate and alanine signals in the ^{1}H NMR spectra of C2C12 mouse myotubes after incubation with [$^{13}C_1$]glucose for 4 hours. The spectra were obtained on the freeze-dried methanol/water extractions of the cells. (A) Control. (B) 42°C for 1 hour. (C) 45°C for 1 hour. (D) Anoxia for 4 hours. The duplet for ^{13}C-labeled lactate at approximately 1.40 ppm is overlapping with the duplet for ^{13}C-labeled alanine; and hence, the integral values for these duplets are not given. The integral values for the duplet for the ^{13}C-labeled lactate at approximately 1.25 ppm and the ^{13}C-labeled alanine duplet at approximately 1.56 ppm, respectively, are given in Table 2.

investigated using a combination of high-resolution ^{13}C, ^{1}H, and ^{31}P NMR spectroscopy. The metabolites identified in the ^{13}C, ^{1}H, and ^{31}P NMR spectra were primarily metabolites derived from the glycolysis and the Krebs cycle (Fig. 6). ^{13}C was mainly incorporated in the cytosolic-derived metabolite alanine and the mitochondrial-derived metabolites glutamate and aspartate; and heat or anoxic stress resulted in lower levels of these metabolites, indicating impaired glucose metabolism. The decreases were smallest at 42°C heat stress exposure. A more significant reduction in the metabolites was apparent at 45°C heat stress exposure, whereas anoxia resulted in an even more profound reduction. These results are consistent with effects of anoxia observed in glioma and hepatoma cells [10].

Likewise, stressful conditions caused lower levels of ATP, ADP, and PCr in the ^{31}P NMR spectra both at 45°C heat stress and at anoxia compared with the control cells; and the reductions in both ATP/ADP and PCr were confirmed in the ^{1}H NMR spectra. The reductions were most severe at anoxic stress, where a depletion of ATP and ADP was apparent in the ^{31}P NMR spectra. Concomitantly with decreases in ATP, ADP, and PCr, higher levels of Pi were found in the ^{31}P NMR spectra at increasing stress conditions, which are in agreement with previous observation in rat [15-17], guinea pig [8], and rabbit [18] hearts at anoxic conditions and in microglial cells exposed to 45°C heat stress [19]. Hence, the high-energy phosphate in the myotubes decreased at both heat and anoxic stress exposure, most severely at anoxic stress with an almost complete depletion of the ATP reserve, thus pointing to a very low rate or termination of anaerobic ATP production.

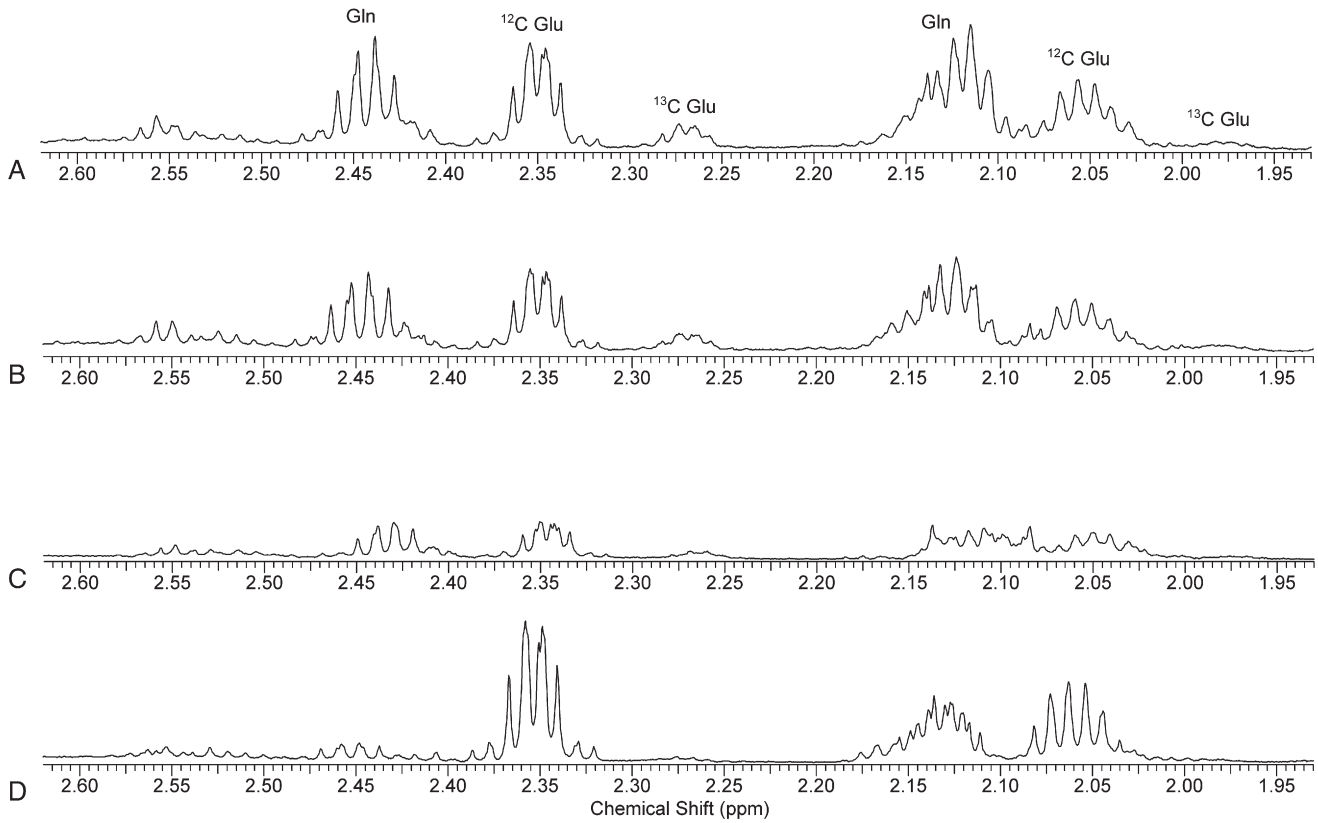

Fig. 4. Expanded region showing glutamate and glutamine signals in the ^{1}H NMR spectra of C2C12 mouse myotubes after incubation with [$^{13}C_1$]glucose for 4 hours. The spectra were obtained on the freeze-dried methanol/water extractions of the cells. (A) Control. (B) 42°C for 1 (C) 45°C for 1 hour. (D) Anoxia for 4 hours. The integrals for the ^{13}C-labeled glutamate multiplet signals at approximately 2.27 ppm are given in Table 2. The multiplet signals for the ^{13}C-labeled glutamate at approximately 1.98 ppm are weak, and the 2 other satellites ^{13}C-labeled glutamate multiplet signals overlap with glutamine. Hence, the integrals for these signals are not given. Approximate integral values for glutamine are given (Table 2).

At anoxic conditions, the phosphomonoesters Gly-3-P; F-1,6-P; and G-6-P were observed in the ^{1}C and/or ^{31}P NMR spectra (Table 1, Figs. 2 and 5). Appearance of phosphomonoesters at anoxic stress is in agreement with Jeffrey et al [20], who found increase in phosphomonoesters in rat hearts with increasing exposure time to anoxic conditions. Most marked is the accumulation of Gly-3-P and G-6-P (Table 1, Fig. 2 and 5), which also were the phosphomonoesters that were found to increase the most in rat hearts when exposed to anoxic conditions [20].

The Gly-3-P functions together with dihydroxyacetone phosphate as a shuttle to transport electrons from the cytosol to the mitochondria. Accordingly, the appearance of Gly-3-P in the ^{13}C and the ^{31}P NMR spectra at anoxic stress is consistent with a blockade of this shuttle and a shift to anaerobic metabolism due to inhibition of the aerobic pathway in the mitochondria. The shift to anaerobic metabolism was confirmed by increased levels of ^{13}C-labeled lactate at anoxic conditions. ^{13}C-labeled lactate also increased after heat stress, suggesting that also heat stress causes a shift to the anaerobic glycolytic pathway. The appearance or increase in the Gly-3-P level, concomitantly with release or accumulation of lactate, has also been observed in perfused rat and guinea pig hearts at anoxic conditions [8,21].

An increased lactate production at an increasing stress level and an increase in Gly-3-P at anoxic stress will result in a higher yield of NAD^+ (Fig. 6); however, this NAD^+ will be rapidly converted to NADH because of the high glycolytic rate, converting glyceraldehyde-3-phosphate to phosphoenolpyruvate. Hence, this is consistent with the decreases in NAD^+ observed in the ^{1}H and the ^{31}P NMR spectra at increasing stress levels (Table 2, Fig. 5) and is in agreement with the findings on rat hearts exposed to anoxia [22].

With increasing severity of stress, lower levels of almost all detected metabolites, which are mainly amino acids, were observed in the ^{13}C and the ^{1}H spectra. This indicates that both unlabeled (^{12}C) amino acids (as shown in Fig. 6) and possibly ^{13}C labeled amino acids are released from the myotubes to the medium, with increasing release at increasing severity of stress. Other studies have shown release of amino acids (alanine, glutamine, and total amino acid nitrogen) from human leg muscles into the bloodstream after stress exposure induced by infusion of hormones [23], and release of amino acids (phenylalanine, alanine, and glutamine) has also been observed in human skeletal muscles

Table 2
^{1}H NMR chemical shift ranges and LSMeans of the absolute integral intensities, with calculated SEM

Metabolite	Shift range (ppm)	Control (n = 4)	42°C (n = 3)	45°C (n = 3)	Anoxia (n = 3)	SEM
Lactate (CH_3) (^{13}C labeled)	1.24-1.25	0.10	0.21	0.13	0.18	0.06
Lactate (CH_3) (unlabeled)	1.32-1.33	2.41	2.06	1.09*	0.78*	0.22
Alanine (CH_3) (unlabeled)	1.47-1.48	0.97	1.01	0.49*	0.14†	0.07
Alanine (CH_3) (^{13}C labeled)	1.55-1.56	0.41	0.37	0.21*	0.11†	0.04
Glutamate (β-CH_2) (unlabeled)	2.05-2.06	3.08	2.51	1.47*	2.89	0.36
Glutamine (β-CH_2)	2.11-2.14	4.52	3.41*	1.53†	2.41†	0.24
Glutamate (γ-CH_2) (^{13}C labeled)	2.27-2.28	1.11	0.88	0.47*	0.26†	0.10
Glutamate (γ-CH_2) (unlabeled)	2.35-2.36	3.22	2.62	1.24*	3.56	0.34
Glutamine (γ-CH_2)	2.43-2.46	2.15	1.47*	0.77†	0.37†	0.15
Aspartate (β-CH_2)	2.81-2.82	1.89	1.39	0.94	0.98	0.51
Taurine (NCH_2)	3.38-3.42	1.81	1.70	0.43*	0.80*	0.25
Creatine (CH_2)	3.93-3.94	0.95	1.07	0.56	0.99	0.18
PCr (CH_2)	3.95-3.96	1.82	1.27*	0.67†	0.41†	0.13
ATP/ADP (ribose H1)	6.15-6.16	0.70	0.52*	0.30†	0.09†	0.04
Tyrosine (H3/H5)	6.88-7.20	0.49	0.41	0.25*	0.16*	0.04
Phenylalanine (H2/H6/H4/H/H5)	7.38-7.39	0.61	0.55	0.35*	0.26*	0.06
ATP/ADP (ring H2)	8.27-8.28	0.65	0.46*	0.29†	0.09†	0.03
ATP/ADP (ring H8)	8.53-8.54	0.67	0.48*	0.27†	0.07†	0.03
NAD^+	9.33-9.34	0.07	0.06*	0.03†	0.02†	–

* LSMeans differ significantly ($P < .05$) when compared with the control cells.
† LSMeans differ significantly ($P \leq .0001$) when compared with the control cells.

when exposing patients with chronic heart failure to light exercise [24].

Surprisingly, very small increases in lactate were observed in the ^{13}C spectra at stress conditions; and the increase was only significant in the ^{13}C spectra at anoxic conditions. In the ^{1}H NMR spectra, contrary to the ^{13}C NMR spectra, even lower levels of lactate designated by the peaks at a chemical shift of 1.32 to 1.33 ppm were observed at both heat and anoxic stress (Fig. 3, Table 2). These peaks represent unlabeled (^{12}C) lactate; and the levels of lactate were, when compared with control, significantly lower at 45°C stress and at anoxic stress exposure. At 1.24 to 1.25 ppm and approximately 1.40 ppm, the peaks for the ^{13}C-labeled lactate are apparent, which increase at stress (Fig. 3, Table 2), as also seen in the ^{13}C NMR spectra (Fig. 2, Table 1). From these results, it is likely that lactate is released from the myotubes as illustrated in Fig. 6. This is in agreement with previous findings in C2C12 myoblasts and myotubes at anoxic conditions [2,25]. Furthermore, the experiments by Brown et al [23] showed release of lactate from the skeletal muscles concomitantly with the release of amino acids.

Only at anoxic stress was an increase in an amino acid (glutamate) apparent, and this was only as an increase in unlabeled (^{12}C) glutamate (Fig. 4). As described above, de novo synthesis of ^{13}C-labeled glutamate is decreasing; or de novo synthesized ^{13}C glutamate is released from the myotubes at anoxia. On the contrary, unlabeled (^{12}C) glutamine is available in the medium and may be taken up by the myotubes and converted to glutamate, which would explain the accumulation of unlabeled (^{12}C) glutamate at anoxic conditions (Fig. 6). Unlabeled (^{12}C) glutamate may be converted to unlabeled (^{12}C) α-ketoglutarate and enter the Krebs cycle. This anaplerotic replenishment of intermediates for the Krebs cycle by uptake of glutamine and its conversion to glutamate has been demonstrated in a variety of proliferating cell types [26], and glutamine uptake has also been observed in rat myotubes [27]. Amino acids crossing the cell membrane do not consume energy [28], and glutamate may through conversion in the Krebs cycle contribute to anaerobic energy production (Fig. 6). Also other unlabeled (^{12}C) amino acids may be fueled through other entries into the Krebs cycle, concomitantly with energy production, as indicated in Fig. 6. Anaplerotic replenishment of intermediates for the Krebs cycle by uptake of glutamate and other amino acids from perfusion solutions or the bloodstream, accompanied with GTP and ATP energy formation in the Krebs cycle, has been observed in hearts at anoxic conditions [29-31]. Supply of glutamate and other amino acids in the perfusion solution has been shown to have a protective effect in anoxic hearts by preserving the energy level of ATP, GTP, and PCr [30,31]. Amino acids have also been shown to have protective effect against ischemia/reperfusion injury [30,31]. Furthermore, Wiesner et al [29] have shown that anoxic conditions in dog hearts increase uptake of glutamate from the bloodstream, thereby replenishing the pool of intracellular glutamate. Hence, the accumulation of unlabeled (^{12}C) glutamate at anoxic conditions in the present experiments does not indicate anaerobic energy metabolism as a prominent mechanism; but the accumulated glutamate may facilitate the recovery of the myotubes at reoxygenation.

Besides changes in metabolites related to the glycolysis and the Krebs cycle, changes in taurine levels in the ^{1}H NMR spectra and the natural ^{13}C-containing taurine in the ^{13}C NMR spectra were also observed at stress conditions. Lower levels of taurine were apparent at stressful conditions, and

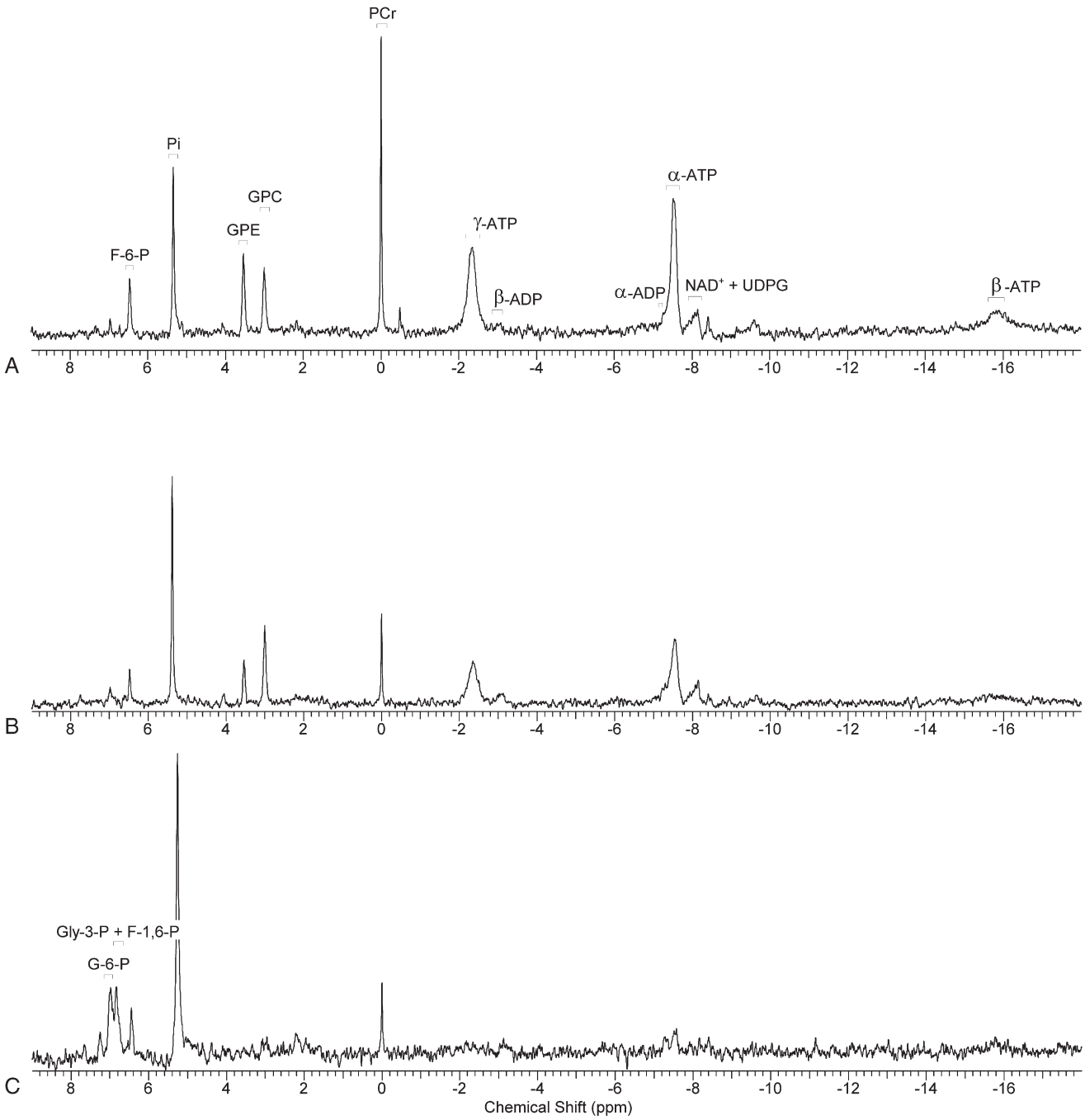

Fig. 5. ^{31}P NMR spectra of C2C12 mouse myotubes obtained on the freeze-dried methanol/water phase of the cell extractions. (A) Control. (B) 45°C for 1 hour. (C) Anoxia for 4 hours. Pi indicates inorganic phosphate; GPE, glycerophosphorylethanolamine; GPC, glycerophosphocholine; UDPG, uridine 5'diphosphoglucose.

the lowest levels appeared after exposure to 45°C. Reduced levels of taurine at anoxic conditions in muscle myotubes are thought to be caused by release of taurine from the cells, mediated by reactive oxygen species [4].

Reduced levels of taurine together with the metabolites alanine, glutamate, and glutamine have been identified with ^{1}H NMR as useful markers for pig hearts exposed to anoxia compared with control hearts by use of multivariate modeling [32]. In the present study, these and additional metabolites were also indicators of stress, with generally lower levels of the metabolites with increasing severity of stress. However, from these results, it is also apparent that one needs to be careful if glutamate is used as a marker for stress. Decreases in glutamate at increasing severity of stress were apparent from the ^{13}C labeling of glutamate; however, in the ^{1}H spectra at anoxic stress, increase in unlabeled (^{12}C) glutamate was observed. Likewise, increases in lactate at stress were apparent from the de novo synthesized ^{13}C-

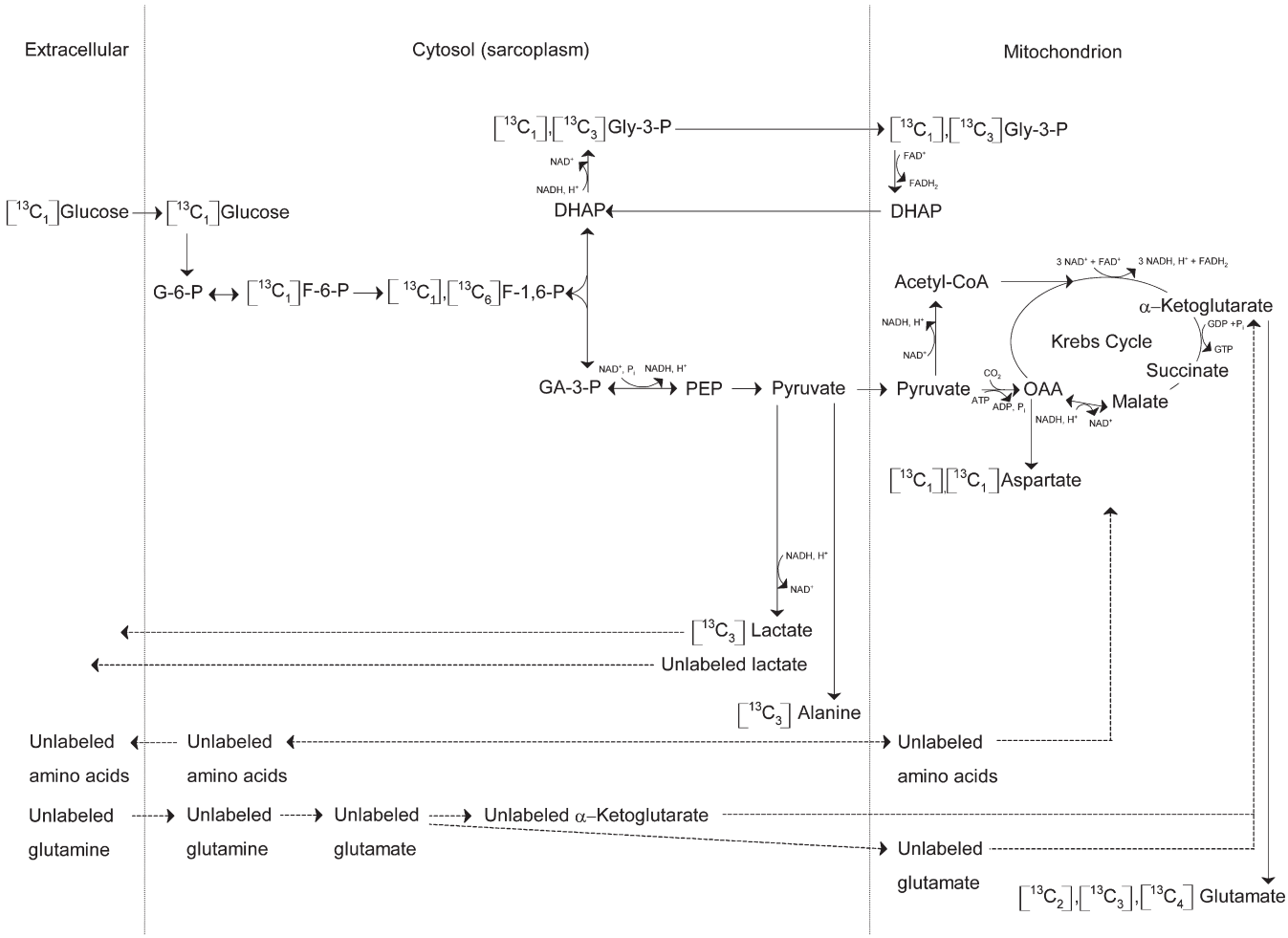

Fig. 6. Schematic representation of the main reactions involved in [$^{13}C_1$]glucose metabolism in C2C12 mouse myotubes. The aerobic pathway occurs when pyruvate is oxidized in the Krebs cycle at standard incubation conditions. At heat stress and anoxia, [$^{13}C_1$]lactate is produced through the anaerobic glycolytic pathway; and anoxia also leads to [$^{13}C_1$],[$^{13}C_3$]Gly-3-P accumulation. Under stressful conditions, lactate is released from the myotubes to the medium. Solid arrows indicate metabolism of [$^{13}C_1$]glucose, whereas dashed arrows indicate release or uptake of metabolites. DHAP indicates dihydroxyacetone phosphate; GA-3-P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; OAA, oxaloacetate.

labeled lactate in the ^{13}C and the ^{1}H spectra, whereas decreases in unlabeled (^{12}C) lactate were apparent from the ^{1}H spectra.

Hence, in the present experiments, the metabolic markers previously identified as indicators of anoxic stress are confirmed, that is, lower levels of taurine, alanine, glutamine, and newly synthesized glutamate were identified in both ^{13}C and ^{1}H spectra as markers of anoxia. These metabolites were also markers for 42°C and 45°C heat stress; however, only at 45°C were the lower levels of metabolites found to be significant for all the 4 metabolites in both ^{13}C and ^{1}H spectra. Furthermore, changes in the levels of additional metabolites were identified at heat and/or anoxic stress in the present study. Most marked was the occurrence of Gly-3-P and G-6-P only apparent at anoxic stress, with Gly-3-P evident in ^{13}C and ^{31}P spectra and G-6-P in ^{31}P spectra. All these metabolites identified in myotubes from mouse muscle cells may serve as stress markers for heat stress and/or anoxia, and may furthermore be useful markers for identifying severity of stress in general in muscles.

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