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Differential gene expression upon depolarization of skeletal muscle cells

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It has been extensively reported that adaptive response of skeletal muscle to exercise or to electrical stimuli involves changes in specific gene expression, nevertheless the cellular mechanisms underlying muscle adaptation remain unknown (Pette, 1998). The slow calcium transient that follows membrane depolarization in skeletal muscle cells (Jaimovich et al. 2000; Araya et al. 2003) is involved in initial steps of signaling pathways leading to transcriptional activation of some early genes (Carrasco et al. 2003). To further explore on the subject we analyzed Interleukin-6 (IL-6) expression in K+ depolarized myotubes, since IL-6 has been reported as one of the genes differentially expressed when skeletal muscle is submitted to work overload (Carson et al. 2002). Myotubes in primary culture were obtained from neonatal rats (humanely killed; Jaimovich et al. 2000). Using semi quantitative RT-PCR, we have shown that K+-evoked depolarization induces a transient increase (357±19%, mean±SEM, n=3) in IL-6 mRNA levels, with a maximum at 3 h. Depolarization performed in the presence of slow calcium signals suppressors like U73122 (138±17%, n=3) and 2-aminoethoxydiphenyl borate (94±6%, n=3), resulted in decreased activation of IL-6 gene expression. These results suggest a regulatory role for the slow calcium signal on IL-6 gene transcription.

To identify other genes that could be regulated by the same depolarizing stimuli we made use of oligonucleotide microarrays analysis (Compugen 22K oligomouse, 21.920 genes). Total RNA from C2C12 myotubes was obtained at different times after 5 min of high K+ stimulation. cDNA prepared from control and depolarized samples was labelled with cyanine 3 or 5 prior to microarray hybridization. Analysis (6 arrays for each time) was performed using NCI— Center for Information Technology of NIH Microarray Database tools. We focused our interest on 58 genes whose expression changed at least 2-fold up or down related to non-depolarized conditions. The main differences in the transcriptional response were observed for genes involved in metabolism, signal transduction and stress response. Evidence involving slow calcium signals in the expression of some of these selected genes could contribute to clarify the mechanisms that link changes in membrane potential and the adaptive response in skeletal muscle cells.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Transcriptional modulation of the human equilibrative nucleoside transporter 1 in fetal endothelium from gestational diabetes mellitus

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Gestational diabetes mellitus (GDM) is associated with foetal endothelial dysfunction, and human umbilical vein endothelial cells (HUVEC) isolated from pregnancies with GDM show increased nitric oxide (NO) synthesis and L-arginine transport. These effects of GDM involve MAP kinases, protein kinase C (PKC) and NO cell signaling cascades and are blocked by the A_{2a} purinoceptor antagonist ZM-241385 (Vázquez *et al.* 2004). GDM is also associated with reduced adenosine transport in HUVEC (Sobrevia *et al.* 1994) and increased extracellular adenosine (Vázquez *et al.* 2004). We studied the transcriptional regulation of the human equilibrative nucleoside transporter 1 (hENT1) by GDM and the involvement of A_{2a} purinoceptors.

HUVEC from normal or GDM pregnancies (Ethic committee approval and informed patient consent were obtained) were cultured in medium 199 (M199, containing 20% bovine sera, 3.2 mM L-glutamine) up to passage 2. hENT1 mRNA levels was quantified with real-time PCR using SYBR green in cells cultured in M199 containing nitrobenzil-tyoinosine (NBMPR, 1 mM) or ZM-241385 (100 nM). Genomic DNA was extracted and several fragments of the promoter region of the *hENT1* gene were amplified by PCR (−3100, −2056, −1016 and −697 bp). The PCR products were cloned into pGL3 upstream luciferase reporter gene. These constructs were used to transfect HUVEC by electroporation (320 Volt, 30 msec) in M199 (8-10% efficiency) using GFP as transfection control.

GDM reduced hENT1 mRNA expression (32 ± 3%, unpaired Student's t test, means ± S.E.M., n=4). The inhibitory effect of GDM on the hENT1 mRNA level was blocked by ZM-241385. To determine elements implicated in the pathological down-regulation of hENT1 transcription, HUVEC were transfected with plasmid constructs containing the different fragment of the promoter region. Endothelial cells from normal pregnancies show repressor elements from −2056 to −3100 bp. In addition, preliminary assays show that in GDM HUVEC the inhibitory effect of these elements are blocked by ZM-241385. These results support the possibility that the reported reduced adenosine transport in HUVEC from GDM could be associated with reduced hENT1 transcription modulated by distal repressor elements in the gene promoter region of *hENT1*, involving the activation of A_{2a} purinoceptors.

Sobrevia *et al.* (1994). *Am J Physiol* **267**, C39-C47.

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Cellular Distribution of Reduced Glutathione During Cell Cycle in 3T3 Fibroblasts

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Reduced glutathione (GSH) is a low molecular weight thiol and the most abundant no enzymatic antioxidant in the cell. Its property to maintain cellular thiol/disulfide redox state gives it a central place in the control of vital cell processes of great diversity including cell proliferation (Jones et al. 2002).
Our group has recently demonstrated that the peak of total GSH in 3T3 fibroblasts coincides with the peak of telomerase activity at 24h in culture and precedes the exponential phase of cell growth (Borras et al. 2004). Considering the growing evidence that shows the importance of GSH compartimentation (Voehringer et al. 1998), and its role in numerous processes that occur in the nucleus, we have focused our attention in following the changes in the GSH distribution throughout the cell cycle.
To visualize GSH localization, cells were observed with a Leica confocal microscope connected to a PC with software for microscope control and image analysis. 3T3 fibroblasts were maintained in culture as described previously (Borras et al. 2004) and plated in 2cm2 chamber slides 5 days, 72h, 48h, 24h, 12h and 6h before the experiment, so cells in all phases of the cell cycle were dyed and analyzed on the same day. Triple staining was applied: propidium iodide 2µg/ml (PI) to identify dead cells, Hoechst 2µg/ml to localize nucleus and CellTracker green 5-chloromethylfluorescein diacetate 5 µM (CMFDA), which marks GSH (specificity 95%). We captured the emission of red (excitation 543nm), blue (excitation 364nm) and green (excitation 488nm) fluorescence to identify IP, Hoechst and CMFDA staining, respectively. Z-series obtained were converted to 2-D images of maximal fluorescence and analyzed by drawing regions around the cell and its nucleus. We calculated the nucleus/cell ratio (n/c) of the mean of CMFDA fluorescence in 3 separate experiments (100 cells per condition).
Our results show that in 3T3 fibroblasts GSH concentrates in the nucleus during the first 24h (n/c=2.40±0.46) of culture (Borras et al. 2004). This phenomenon, to a lesser extent, is maintained until 48h (n/c=1.94±0.09), which coincides with exponential phase of cell growth (Borras et al. 2004). At 72h the majority of cells are characterized by evenly distributed GSH (n/c=1.33±0.10, p≤0.05 vs. 24h). At 5 days, as cells reach confluence and enter quiescence, the intensity of green fluorescence is very low and distribution is homogeneous (n/c=1.17±0.09, p≤0.05 vs. 24h).
We believe that the start of active proliferation of 3T3 fibroblasts requires a reduced environment in the nucleus and that the level of GSH compartimentation regulates the rhythm of the cell cycle.

Jones DP et. al. (2002) Methods Enzymol 348: 93-112

Borras C et. al. (2004) J Biol. Chem 279: 34332-34335

Voehringer DW et. al. (1998) Proc. Natl. Acad. Sci. USA Cell biology 95, 2956-2960

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On the mechanism that opens Connexin 43 hemichannels in metabolically inhibited rat astrocytes.

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Gap junction hemichannels (HCs) are hexamers of protein subunits named connexins (Cxs). Unapposed HCs can open to connect intra- and extra-cellular space and allow permeation by ions and small molecules. In astrocytes subject to metabolic inhibition (MI) increased opening of connexin43 HCs (Cx43-HCs) contributes to cell death (Contreras et al, 2002). Desphosphorylation and/or oxidation of Cx43-HCs have been proposed as gating mechanisms that open Cx43-HCs during MI or ischemia. Our objectives were to determine the effects of MI on the phosphorylation state and function of Cx43-HCs and to study the effect of reducing agents on Cx43-HCs during MI. Primary cultures of cortical astrocytes obtained from humanely killed neonatal rats were MI with 100 µM iodoacetic acid and 5 ng/ml antimycin A for different periods up to 75 min at 37°C. Membrane proteins of the extracellular surface were labeled with sulfo-NHS-SS-biotin and isolated with neutravidin beads. The phosphorylation state of Cx43 was analyzed by immunoblot determination of mobility using an antibody to the C-terminal. Cx43 at the cell surface of unpermeabilized cells were visualized by immunofluorescence using an antibody to the first extracellular loop. Opening of Cx43-HCs was evaluated through uptake of ethidium bromide (100 µM) in cells exposed to the dye for 5 min. Under basal conditions, 8.8±2.6‰ (mean±SD, n=4) of total Cx43 was at the cell surface and was mainly phosphorylated. After 15 min MI, surface Cx43 was 1.8±0.5 (mean±SD, n=4) times that in control cells and remained high for at least 60 min. In agreement, surface Cx43 immunoreactivity was greatly increased by MI. After 15-30 min MI levels of unphosphorylated Cx43 increased at least up to 75 min. Dye uptake after 50 min MI was decreased by 10 mM DTT or 100 µM trolox added 30 min before the uptake assay. DTT did not prevent the increase in unphosphorylated Cx43 levels after MI. We propose that the massive Cx43-HC opening observed in metabolic inhibited astrocytes results from a combination of HC recruitment to the cell surface and oxidation, presumably of Cx43.

Contreras J.E. et al., Proc. Natl. Acad. Sci. USA 99: 495-500, 2002

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.