Supplemental Online Information

Blockade of ActRIIB signaling triggers muscle fatigability and metabolic myopathy

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SUPPLEMENTAL METHODS

Measurement of contractile properties

The contractile properties of *extensor digitorum longus* (*EDL*) and *soleus* muscles were studied *in vitro* according to previously published protocols.[1] Muscles were soaked in an oxygenated Tyrode solution (95% O_2 and 5% CO_2) containing 58.5 mM NaCl, 24 mM $NaHCO_3$, 5.4 mM KCl, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM glucose (pH7.4) and maintained at a temperature of 22°C. One muscle tendon was attached to a lever arm of a servomotor system (300B, Dual-Mode Lever, Aurora, Dublin; Ireland). After equilibration (30 min), field electrical stimulation was delivered through electrodes running parallel to the muscle. Pulses of 1 ms were generated by a high power stimulator (701B, Aurora). Absolute maximal isometric tetanic force (P0) was measured during tetanic contractions (frequency of 50-100 Hz, train of stimulation of 1, 500 ms for *soleus* and 750 ms for *EDL*). The muscle length was adjusted to an optimum (L0) that produced P0. Specific maximal isometric force (sP0) was calculated by dividing the force by the weight of the muscle. Fatigue resistance was then determined after a 5 min rest period. The muscles were stimulated at 75 Hz during 500 ms, every 2 s, for 3 min. The time taken for initial force to fall by 50% (*EDL*) or 30% (*soleus*) was then measured. All data were recorded and analyzed on a microcomputer, using the PowerLab system (4SP, ADInstruments, Oxford, UK) and software (Chart4, ADInstruments).

Histology

For CD31 expression, frozen unfixed 12 µm sections of *EDL* and *soleus* muscles were blocked 1 h in PBS plus 2% BSA and 2% SVF. Sections were then incubated overnight with primary antibodies: anti-CD31 (Pharmigen, Ranges, France) and anti-laminin (Dako, Les Ulis, France). After washes in PBS, sections were incubated 1 h at room temperature with secondary antibodies with various fluorophores (Alexa Fluor®, Invitrogen, Saint Aubain, France). After washes in PBS, slides were mounted in Fluoromount-G (Southern Biotech, Birmingham, Alabama, USA).

For expression analysis of myosin heavy chain (MHC) isoforms, primary antibodies were: anti-MHCI (hybridoma#BA-D5, Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ, Braunschweig, Germany) and anti-MHCIIa (hybridoma#SC-71, DSMZ). For MHC-immunohistochemistry, frozen unfixed 12 µm sections were blocked 1 h in PBS plus 2% BSA and 2% SVF. Sections were then incubated overnight with primary antibodies against laminin (Dako) and MHCI and MHCIIa isoforms. After washes in PBS, sections were incubated 1 h with secondary antibodies with various fluorophores (Alexa Fluor®, Invitrogen). After washes in PBS, slides were finally mounted in Fluoromont-G. Morphometric analyses were made on whole sections of *EDL* and *soleus* muscles. Images were captured using a digital camera (Hamamatsu ORCA-AG, Massy, France) attached to a motorized fluorescence microscope (Zeiss AxioImager Z1, Jena, Germany), and morphometric analyses were made using the MetaMorph v7.5 software (Molecular Devices, Sunnyvale, California, USA).

For Nos1 expression, frozen unfixed 12 µm sections of EDL muscles were rehydrated in PBS, fixed with 4% PFA for 10 min, blocked in 4% BSA for 1 h. Sections were then incubated overnight at room temperature with primary antibodies: anti-Nos1 (Abcam, Paris France). After washes in PBS, sections were incubated 1 h at room temperature with secondary Alexa594-conjugated antibodies (Alexa Fluor®, Invitrogen). After washes in PBS, slides were mounted in Fluoromount-G. Images were captured by confocal laser scanning microscope (Leica SPE DM2500, Wetzlar, Germany).

RT-qPCR

Total RNA was isolated from frozen muscle after pulverization in liquid nitrogen from C_2C_{12} cell pellets and from endothelial human cells with the Trizol® (Invitrogen) extraction protocol. Isolated RNA was quantified using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Schwerte, Germany) and cDNA was synthesized using the Thermoscript® RT PCR System (Invitrogen). After cDNA synthesis, Real Time PCR was performed by using the SYBR Green® PCR Master Mix Protocol (Applied Biosystems, Darmstadt, Germany) in triplicate on the ECO Real-Time PCR System (Illumina, Eindhoven, The Netherlands) with a hotstart-Taq polymerase. A 10 min denaturation step at 94°C was followed by 40 cycles of denaturation at 94°C for 10 s and annealing/extension at 60°C for 30 s. Before sample analysis we had determined for each gene the PCR efficiencies with a standard dilution series (10^0-10^7 copies/µl),f which subsequently enabled us to calculate the copy numbers from the C_t values.[2] mRNA levels were normalized to 18S rRNA. The sequences for the primers used are listed below:

Table of oligonucleotides used for RT-qPCR of mouse and human tissues

Gene	Primer sequence (5'–3')	Direction
Oligonucleotide primers used for mice		
Pparβ	AGCCACAACGCACCCTTT	forward
	CGGTAGAACACGTGCACACT	reverse
Pgc1α	GAAAGGGCCAAACAGAGAGA	forward
	GTAAATCACACGGCGCTCTT	reverse
Vegf-A	AAGCCAGCACATAGGAGAGATGA	forward
	TCTTTCTTTGGTCTGCATTCACA	reverse
Cpt1b	TCGCAGGAGAAAACACCATGT	forward
	AACAGTGCTTGGCGGATGTG	reverse
Pdk4	AGGTCGAGCTGTTCTCCCGCT	forward
	GCGGTCAGGCAGGATGTCAAT	reverse
Nos1	AAGGAGCAAGGAGGCCATAT	forward
	ATATGTTCTGAGGGTGACCCC	reverse
Vdac1 (Porin)	ACTGTGGAAGACCAGCTTGC	forward
	TGCTCCCTCTTGTACCCTGT	reverse
MTCO2	GCCGACTAAATCAAGCAACA	forward
	CAATGGGCATAAAGCTATGG	reverse
18S rRNA	CATTCGAACGTCTGCCCTATC	forward
	CTCCCTCTCCGGAATCGAAC	reverse
Oligonucleotide primers used for humans		
ACVR2B	AGCCGTCTATTGCCCACA	forward
	CATGTACCGTCTCGTGCCTA	reverse
ACVR2A	AGGTTGTTGGCTGGATGAT	forward
	GCCCTCACAGCAACAAAAAT	reverse
ALK4	GTCTTGGTTCAGGGAAGCAG	forward
	GGACCCGTGCTCATGATAGT	reverse
ALK5	TTGCTCCAAACCACAGAGTG	forward
	TGAATTCCACCAATGGAACA	reverse
18S rRNA	CATTCGAACGTCTGCCCTATC	forward
	CTCCCTCTCCGGAATCGAAC	reverse

Measurement of enzyme activities

[1] Enolase: Enolase-catalyzed conversion of 2-phospho-d-glycerate to phosphoenolpyruvate at 25°C was monitored spectrophotometrically at 340 nm. Activity was expressed in international units (IU) per mg of protein.

[2] Citrate synthase: Citrate synthase-catalyzed conversion of oxaloacetate and acetyl-CoA to citrate at 30°C was monitored spectrophotometrically at 412 nm. Activity was expressed in international units (IU) per mg of protein.

[3] Cytochrome C oxidase (COX): Cytochrome C oxidase-catalyzed oxidation of cytochrome C at 30°C was monitored spectrophotometrically at 550 nm. Activity was expressed in international units (IU) per mg of protein.

[4] Hydroxyacyl CoA dehydrogenase (HADHA): HADHA-catalyzed conversion of acetoacetyl-CoA to hydroxybutyryl-CoA at 30°C was monitored spectrophotometrically at 340 nm. Activity was expressed in international units (IU) per mg of protein.

SUPPLEMENTAL REFERENCES

1. Agbulut, O, Vignaud, A, Hourde, C, Mouisel, E, Fougerousse, F, Butler-Browne, GS *et al.* (2009) Slow myosin heavy chain expression in the absence of muscle activity. *Am J Physiol Cell Physiol* **296**:C205-C214.

2. Pfaffl, MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**:e45.