ACTIVATION OF UBIQUITIN-PROTEASOME PATHWAY IN THE DIAPHRAGM IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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Online data supplement

METHODS

Isolation of 20S proteasomes and measurement of proteolytic activity

The 20S proteasome isolation and proteolytic activity measurements were determined according to Hobler et al. [1], with minor modifications. To isolate 20S proteasomes, diaphragm samples were homogenized in ice-cold buffer (pH 7.5) containing (in mM) 50 Tris-HCl, 5 $MgCl_2$, 250 sucrose, 1 dithiothreitol, 0.2 phenylmethylsulphonylfluoride, and protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, the Netherlands) by means of a Dounce homogenizer. Subsequently, the proteasomes were isolated from the homogenates by three sequential centrifugation steps; the first centrifugation was at 10,000 *g* for 20 min. The supernatant was centrifuged at 100,000 *g* for 1h. The obtained supernatant was then centrifuged at 100,000 *g* for 5h. The final pellet, containing the 20S proteasomes was resuspended in buffer (pH 7.5) containing 50 mM Tris-HCl, 5 mM $MgCl_2$, and 20% glycerol. The protein content of the proteasome preparation was determined based on Bio-Rad Protein Assay (Bio-Rad, Veenendaal, the Netherlands). The proteolytic activity of the 20S proteasomes was determined by measuring the activity against the fluorogenic substrates succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin (LLVY) and N-carbenzoxy-leu-leu-glu-7-amido-4-methylcoumarin (LLE) (Sigma-Aldrich, Zwijndrecht, the Netherlands). These substrates are preferentially hydrolyzed by the chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing peptidase activities of the 20S proteasome, respectively [2]. To measure proteolytic activity, 15 μg proteasome extract was added to 60 μL of medium containing 62.5 mM tris-HCl, 12.5 mM $MgCl_2$, 1.25 mM 1.4-dithiothreitol, 0.01 U apyrase, and 100 μM of LLVY or 375 μM of LLE. The reaction took place at 37°C for 45 min. The peptidase activity was determined by measuring the generation of the fluorogenic cleavage product (methylcoumarylamide) at 380 nM excitation wavelength and 440 nM emission wavelength

continuously with a spectrophotometer. Standard curves were established for the fluorogenic product, and peptidase activity was expressed as picomoles per microgram protein per minute. Addition of the proteasome inhibitor MG132 to the reaction resulted in complete inhibition of methylcoumarylamide production, indicating successful isolation of proteasomes without the presence of significant amounts of other proteases. For a representative activity vs time plot, see figure E1.

MAFbx and MURF-1 mRNA determination with real-time quantitative PCR

Total RNA was extracted from diaphram samples using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The extracted RNA was dissolved in diethylpyro-carbonate (DEPC)-treated water and the concentration was determined by spectroscopy at 260 nm using the Ultrospec 1000 UV/Visible Spectrophotometer (Pharmacia Biotech, Foster City, CA). Total RNA was then reverse transcribed into cDNA using 50 ng of total RNA in a 20µl reaction volume by using SuperScript^{TM} Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was performed in a total reaction volume of 25 µl per reaction. The 25 µl reaction mixture contained 12.5 µl of a SYBR green mix (Bio-Rad, Salt Lake City, UT), 10 pmol of each forward and reverse primer, 1µl cDNA and nuclease-free water to make up the reaction volume. Specific primers were selected using express software (Applied Biosystems, Foster City, CA). Forward and reverse oligonucleotides used were as following: MAFbx, 5'-CATCCTTATGTACACTGGTCCA-AAGA-3' and 5'-ATCCGATACACCCACATGTTAATG-3', MuRF-1, 5'-AACTTGGAGAAGCAGCTGATCTG-3' and 5'-TAGGGATTTGCAG-CCTGGAA-3'; Glyseraldehyde-3-phosphate dehydrogenase **(**GAPDH), 5'-ATTCCACCCATGGCAAATTC -3' and 5'- AT-TCCACCCATGGCAAATTC -3'. These primers were synthesized by Sigma Genosys. PCR runs were performed in triplicate using MyiQ real time PCR detection system

(Bio-Rad, Salt Lake City, UT). Levels of MAFbx and MuRF-1 mRNA were normalized to that of GAPDH in arbitrary unit.

Western blot analysis

Diaphragm samples were homogenized in ~200 µl ice-cold buffer (pH 7.5), containing 50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride (PMSF), and protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, the Netherlands), by means of a Dounce homogenizer (Polytron, Rekken, the Netherlands). Homogenates were centrifuged at 10,000 x *g*, 4 °C for 10 min and the protein concentration of the resulting supernatants was determined using the Bio-Rad protein assay (Bio-Rad, Veenendaal, the Netherlands). Soluble proteins (10 µg) were subjected to routine Western blotting using 10% polyacrylamide sodium dodecyl sulfate (SDS)-gels and antibodies against the 20S proteasome subunit C8 (MCP72; mouse monoclonal; 1:10,000; Affiniti, Gorinchem, the Netherlands), and against myosin heavy chain (A4.1025; mouse ascites; 1:2500; Upstate, Lelystad, the Netherlands). After washing, blots were incubated with a horseradish peroxidase conjugated goat anti-mouse Ig (1:10,000, Pierce, Etten-Leur, the Netherlands). Chemiluminescence was performed using a ECL™ Western blotting analysis system (Amersham Biosciences, Roosendaal, Belgium). Protein bands were quantified using optical densitometry. Coomassie Blue-stained protein gels of the samples used for myosin heavy chain and C8 immunoblotting demonstrated equivalent amounts of protein loaded per lane.

Measurement of caspase-3 activity

Caspase-3 activity was determined as described by Du et al. [3], with minor modifications. Frozen diaphragm samples were pulverized and homogenized on ice in a buffer containing 100 mM HEPES (pH 7.5), 10% sucrose, 0.1 % Nonidet P-40, 10 mM dithiothreitol, and

protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, the Netherlands). Homogenates were subjected to three cycles of freeze-thaw before centrifugation at 18,000 g for 30 min. The supernatant (85 ug protein) was added to reaction buffer consisting of 100 mM HEPES (pH 7.5), 10% sucrose, and 10 mM dithiothreitol. The fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was then added and the reaction was performed at 30°C for 60 min. The caspase-3 activity was determined by measuring the generation of the fluorogenic cleavage product (methylcoumarylamide) at 360 nM excitation wavelength and 460 nM emission wavelength with a spectrophotometer. Results were expressed as fluorogenic units per minute. Addition of N-Acetyl-Asp-Glu-Val-Asp-CHO (a caspase-3 inhibitor) to the reaction resulted in complete inhibition of methylcoumarylamide production. For a representative activity vs time plot, see figure E2.

Reference List

E1. Hobler, S. C., A. Williams, D. Fischer, J. J. Wang, X. Sun, J. E. Fischer, J. J. Monaco, and P. O. Hasselgren. 1999. Activity and expression of the 20S proteasome are increased in skeletal muscle during sepsis. *Am.J.Physiol* 277:R434-R440.

E2. Craiu, A., M. Gaczynska, T. Akopian, C. F. Gramm, G. Fenteany, A. L. Goldberg, and K. L. Rock. 1997. Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. *J.Biol.Chem.* 272:13437-13445.

E3. Du, J., X. Wang, C. Miereles, J. L. Bailey, R. Debigare, B. Zheng, S. R. Price, and W. E. Mitch. 2004. Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J.Clin.Invest* 113:115-123.

LEGENDS TO FIGURES

Figure E1. Representative plot of the proteolytic activity of isolated 20S proteasomes against the fluorogenic substrate LLVY vs time from a COPD and non-COPD patient. Addition of the proteasome-inhibitor MG132 almost completely abolished substrate cleavage.

Figure E2. Representative plot of the activity of diaphragm homogenates from a COPD and non-COPD patient against the caspase-3-specific fluorogenic substrate Ac-DEVD-AMC vs time. Addition of the caspase-3-inhibitor DEVD-CHO almost completely abolished substrate cleavage.

Figure E1.

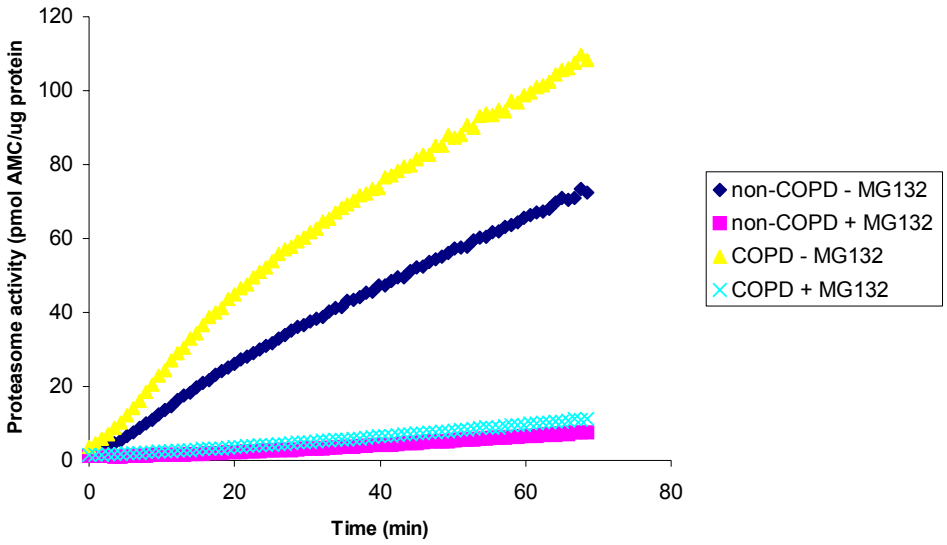
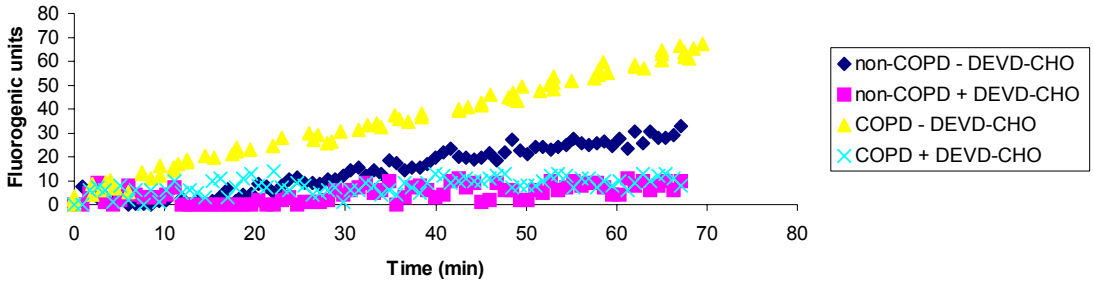

120
100
80
60
40
20
0
Proteasome activity (pmol AMC/ug protein
0
20
40
60
80
Time (min)
non-COPD - MG132
non-COPD + MG132
COPD - MG132
COPD + MG132

Figure E2.


Fluorogenic units
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
Time (min)
non-COPD - DEVD-CHO
non-COPD + DEVD-CHO
COPD - DEVD-CHO
COPD + DEVD-CHO