EFFECTS OF ACUTE ADMINISTRATION OF CORTICOSTEROIDS DURING MECHANICAL VENTILATION ON RAT DIAPHRAGM

KAREN MAES, DRIES TESTELMANS, PASCAL CADOT, KEITH DE RUISSEAU, SCOTT POWERS, MARC DECRAMER and GHISLAINE GAYAN-RAMIREZ

ONLINE DATA SUPPLEMENT

Animal preparation and surgical procedure

The study was approved by the animal Experiments Committee of the Medical Faculty of the Katholieke Universiteit Leuven.

The study was performed on adult male Wistar rats (350-500g) randomly divided into four groups:

1) A control group consisting of acutely anesthetized animals free of intervention (C, n=8).
2) A group of rats breathing spontaneously for 24 hours and submitted to the same surgical procedure as the mechanically ventilated rats (SB, n=7).
3) A group of rats submitted to 24 hours of continuous controlled mechanical ventilation and receiving a single im injection of NaCl 0.9% at the beginning of the study (CMVS, n=10).
4) A group of rats submitted to 24 hours of controlled mechanical and receiving a single im injection of methylprednisolone 80mg/kg at the beginning of the study (CMVM, n=14).

In addition a spontaneous breathing group receiving a single intramuscular injection of 80 mg/kg of methylprednisolone was added to examine whether treatment with an acute high dose of corticosteroids in spontaneous breathing animals would affect diaphragm contractile properties and fiber type dimensions and proportions.

The surgery was performed under general anesthesia (sodium pentobarbital 60 mg/kg) and aseptic conditions. Body temperature was continuously maintained at 37°C using a heating blanket. The trachea was cannulated, as well as the right external jugular vein and carotid artery for infusion of anesthesia (sodium pentobarbital 2 mg/100g/ml/h) and heparin (2.8U:ml/h). Anesthetized animals were breathing humidified air enriched with O_2 and maintained at 37°C. For the mechanically ventilated rats, animals were ventilated with a volume-driven small-animal ventilator (Harvard Apparatus model 665A, Holliston, Ma) at a respiratory rate of 55-60 breath/min and a tidal volume of 0.5ml/100 g body weight. Arterial blood pressure was monitored during the experiment. Anesthesia depth was controlled throughout the experiment by evaluating foot reflex, corneal

reflex, and arterial blood pressure. During the duration of mechanical ventilation, continuous care to the animals was performed including expressing the bladder, lubricating the eyes, rotating the animal and passive movement of the limbs. Upon completion of the experiment, an arterial blood sample (90µL) was taken and blood gases analysis was performed (Synthesis 15u, Instrumentation Laboratory, Brussels, Belgium).

In vitro diaphragm contractile properties

After 24h, the diaphragm was quickly removed through a laparotomy, and immediately immersed in a cooled, curarized, oxygenated Krebs solution containing (in mMol/L) : NaCl 137, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, KH_2PO_4 1, $NaHCO_3$ 12, glucose 6.5. Two small rectangular bundles (width<2mm) from the middle part of the lateral costal region of each hemidiaphragm were obtained by careful dissection parallel to the long axis of the fibers. Both ends of each bundle were tied with silk sutures to serve as anchoring points. The bundles were suspended in a tissue bath containing Krebs solution and continuously aerated with 95% O_2 and 5% CO_2. Temperature was maintained at 37ºC using a thermostatically controlled water pump. The bundles were placed in between two large platinum stimulating electrodes, anchored at the bottom to a rigid support and at the top fastened to an isometric force transducer (Maywood Ltd., Hampshire, U.K.) connected to a micrometer. Signals were amplified and recorded on computer via analog to digital conversion (DT-2801A) using Labdat (Labdat/Anadat, RHT-Infodat, Montreal, Canada). Stimulations were delivered through a Harvard 50-5016 stimulator (Edenbridge, Kent, U.K.), connected to a power amplifier made from power one mode HS24-4.8, developed by computer technology resources centre, University of Virginia (R.J. Evans, 1983). Optimal muscle length (L_o) for peak twitch force was established for each bundle.

The following measurements were performed at L_o, after a thermo-equilibration period of 15 min:

- Twitch characteristics: maximal twitch tension (P_t) was obtained from two successive twitch stimulations (1 Hz). The highest value with its corresponding time to peak tension (TPT) and half-relaxation time (1/2RT) were taken for further analysis.
- Maximal tetanic force and twitch-tetanus ratio: bundles were stimulated twice at 160 Hz, during 250 msec, with a two minute interval. Each pulse had a duration of 0.2 msec. Tetanic force (P_o) was taken as the maximal tension elicited at 160 Hz. Twitch-to-tetanus ratio (P_t/P_o) was calculated for each muscle bundle.
- The force-frequency relationship was measured, using the following order or frequencies with two minutes of interval in between the stimulations: 25, 50, 80, 120 Hz.
- Fatigue was induced by a 5 min stimulation run consisting of repeated 25 Hz stimulations of 330 ms, applied every three seconds.

At the end of the in vitro experiment, each muscle bundle was removed from the bath and its length, width and thickness were measured at L_o. They were blotted dry and weighed. All tensions were normalized for cross-sectional area (CSA).

Histological procedure and fiber type analysis

The right costal region of the diaphragm was folded, cut transversely, and placed at excised length on a cork holder, with the fibers oriented perpendicularly to the surface of the cork. The preparations were frozen in isopentane cooled with liquid N_2. Afterwards, serial sections parallel to the cork were cut at 10μm thickness with a cryostat kept at -20°C. Two sections of each muscle were stained for routine H&E, whereas the other serial sections were stained for adenosine triphosphatase (ATPase) after acid preincubation at pH 4.5 and 4.3. Based on their histochemical reactions, fibers were identified as slow-twitch type I, fast-twitch type IIa or fast-twitch type IIx/b

fibers. CSA's were determined from the number of pixels within the outlined borders using a Leitz Laborlux S. microscope (Wetzlar, Germany) at x20 magnification, connected to a computerized image system (Quantimet 500, Leica, Cambridge Ltd., U.K.). Around 150 fibers were used to calculate CSA and proportions of all fiber types.

Cytokine serum level determination

Serum levels of IL-1α, IL-1β, IL-2, IL-4, IL-10, TNF-α, and IFN-gamma were measured with a custom SearchLight rat cytokine proteome array (Perbio Science, Erembodegem, Belgium). Fifty microliters of serum were used in this assay according to the manufacturer's instructions. Samples were tested in duplicate. Sensitivity of this array was 1.6 pg/mL for IL-1α and IL-4, 6 pg/mL for IL-1β, IL-2, TNF-α, 0.8 pg/mL for IL-10 and 12 pg/mL for IFN-gamma. Plasma of LPS treated rats was used as positive control. All these measurements have been performed by the Searchlight multiplex sample testing services of the company.

Diaphragm lipid peroxidation measurement

To assess the magnitude of lipid peroxidation in the diaphragm we measured the hydroperoxide content of the muscle using a ferrous oxidation technique described by Hermes-Lima et al.(E1) Briefly, diaphragm muscle samples (~50 mg) were homogenized in ice-cold methanol (1:30, w:vol), centrifuged at 400 g for 10 min, and the supernatants (~200 μL) carefully removed for subsequent analysis. Fifty microliters of sample was combined with 950 μL of FOX reagent (in mM: $FeSO_4$, 0.25; H_2SO_4, 25.0; xylenol orange, 0.2) in 1.5 mL centrifuge tubes. Samples were incubated at room temperature in the dark for 24 h. All samples were assessed in triplicate. To calculate the concentration of peroxide in the samples, optical density values were obtained at 580 nM and plotted on a standard curve constructed from cumene hydroperoxide standards (12.5-100 μM concentrations). All samples were assayed on the same day.

Diaphragm muscle homogenates

Frozen diaphragm samples were homogenized in 10 volumes of cold KPO_4 buffer (100 mM) and centrifuged for 20 min at 17,000 g at 4°C. Protein content was determined in the supernatant with the method of Bradford (E2).

Diaphragm protein oxidation measurements

Protein carbonylation in diaphragm homogenates were measured with a commercial kit (Oxyblot, Chemicon, Heule, belgium). The assay is based on derivatization of carbonyl groups in the presence of 2,4-dinitrophenylhydrazine (DNPH). In brief, 15 µg of protein was used per derivatization reaction. Proteins were then denatured by addition of 12% SDS. The samples were subsequently derivatized by adding 10 µl of 1x DNPH solution and incubated for 15 min at room temperature. Finally, 7.5 µl of neutralization solution was added to the sample mixture. To evaluate the selectivity of carbonyl measurements, muscle protein samples also underwent a protein carbonyl detection procedure without the derivatization step (negative controls). DNP-derivatized proteins were separated on a 12% polyacrylamide gel. Proteins were transferred to polyvinyldifluoride (PVDF) membranes using a semi-dry blotting system. Membranes were stained with PonceauS and were subsequently incubated with a polyclonal anti-DNP moiety antibody, followed by incubation with horseradish peroxidase (HRP)–conjugated secondary antibody. Specific proteins were detected with a chemiluminescence kit (GE Healthcare, Diegem, Belgium). To quantify the amount of oxidation and allow the comparison between the various samples the oxidative index was used (E3,4). This index is the ratio between densitometric values of the oxidized proteins and the total proteins. Quantification was determined with the software package (Bio 1D) of the imaging system (Photo Print, vilber, France).

Western blot analysis of different nitric oxide synthase (NOS) isoforms

Proteins were separated on a 7.5% polyacrylamide gel and transferred onto PVDF membrane using a semi-dry blotting system. Blots were incubated overnight at 4°C with monoclonal antibodies against eNOS, nNOS and iNOS (BD Biosciences, Erembodegem, Belgium). Blots were washed and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibody. Positive controls were included on each gel, including human endothelial cell lysate, rat cerebrum lysate, and mouse macrophage stimulated with IFN-γ/LPS for eNOS, nNOS, and iNOS, respectively. Furthermore, equal laoding was ensured by staining the membranes with PonceauS.

Western blot analysis of MyoD and myogenin

Frozen diaphragm samples were homogenized in 10 volumes of 50 mM TRIS-HCl (pH 7.4), 5 mM EDTA, 10 μg/ml (4-amidinophenyl)-methanesulfonyl fluoride, and 0.2% NP-40 with a teflon Potter homogeniser and centrifugated at 1340 g for 25 min. Protein content in the supernatant was measured with the Bradford assay (E2). Proteins (20 and 60 μg for MyoD and myogenin experiment, respectively) were separated by SDS-PAGE in a 4% stacking gel. Proteins were then transferred onto polyvinyldifluoride membrane (PVDF) using a semi-dry blotting system. The blots were incubated overnight at 4°C before with the primary antibodies. The primary antibodies were polyclonal rabbit anti-MyoD (1:400 dilution, sc-760, Santa Cruz Biotechnology, CA) and anti-myogenin (1:1000 dilution, sc-576, Santa Cruz Biotechnology, CA). The blots were then washed and incubated with horseradish peroxidase-conjugated rat anti-rabbit IgG (1:1000, Dako, Heverlee, Belgium) for 1 hour. Proteins were visualized with a tetramethylbenzidine membrane peroxidase system and analyzed semiquantitatively with the software package (Bio 1D) of the imaging system

(Photo Print, vilber, France). Each experiment was performed in triplicate. Quantification was normalized to protein levels determined by PonceauS staining.

mRNA expression of MyoD and myogenin

Total RNA isolation.

A portion of the costal diaphragm was homogenized in 0.5 ml of Trizol (Invitrogen, Belgium) and processed according to the manufacture's instructions. The sample was centrifuged at 12,000 g for 10 minutes to remove insoluble material. 0.2 ml chloroform was added to the tube, incubated for 3 min at room temperature and centrifuged for 15 min at 12,000 g. The RNA portion, the upper aqueous phase, was transferred and incubated at room temperature for 5 minutes. The RNA was precipitated with isopropanol and washed with 75% ethanol. The pellet was resuspended in RNAse free water. The concentration and purity of the total RNA extracted was measured.

Reverse transcription

Briefly, reverse transcription was performed using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Belgium) according to the manufacturer's instructions. First strand cDNA was subsequently treated with 2 units of RNase H.

PCR

PCR experiments were performed by using the Accuprime Taq DNA polymerase system (Invirogen, Belgium). Primers for MyoD and myogenin were obtained from Invitrogen. Primer sequences for MyoD are as follows: Forward,TGGCGCCGCTGCCTTCTACG ;Reverse, ACACGGCCGCACTCTTCCCTG. Primer sequences for myogenin are as follows: Forward, GACCTGATGGAGCTGTAT; Reverse, AGACAATCTCAGTTGGGC. QuantumRNA 18S (Ambion, Lennik, Belgium) was used as an internal standard. An optimized ratio 1:9 of 18S primer: competimer was mixed with myogenin primers and a ratio 3:7 of 18S primer:competimer with MyoD primers. Amplification products were analysed by electrophoresis and stained with Vistra

Green (GE Healthcare, Diegem, Belgium). Qunatificantion was determined with the software package (Bio 1D) of the imaging system (Photo Print, vilber, France). Expression of MyoD and myogenin was normalized to its 18S internal standard.

Calpain activity

Indirect measurement of calpain activity was assessed by analyzing the 145/150 kDa cleavage products of the protein αII-spectrin by western blotting. However, because αII-spectrin is a substrate for both calpain and caspase-3 and since the cleavage products of intact spectrin by calpain (150 and 145 kDa fragments) and caspase (150 and 120 kDa fragments) were not easy to dissociate on Western blotting, the 190 kDa calpain specific degradation product of talin was also measured by Western blotting since talin is specifically degraded by calpain. Frozen samples were homogenized in 10 volumes of cold KPO_4 buffer (100 mM) and centrifuged for 20 min at 17,000 g at 4°C. Protein content was determined in the supernatant with the method of Bradford (E2). Proteins were separated with SDS-PAGE on an 8% polyacrylamide gel for αII-spectrin and a 6% polyacrylamide gel for talin and than transferred on polyvinyldifluoride (PVDF) membrane via a semi-dry blotting system. A mouse monoclonal primary antibody against αII-spectrin (Biomol, Plymouth Meeting, PA) or against talin (Sigma-Aldrich, Bornhem, Belgium) was used overnight at 4°C. After washing, membranes were incubated for 60 minutes at room temperature with an anti-mouse IgG-horseradish peroxidase (HRP)-conjugated secondary antibody (DAKO, Glostrup, Denmark). Proteins were revealed with a tetramethylbenzidine membrane peroxidase system or with chemiluminescence detection (GE Healthcare, Diegem, Belgium) and analyzed semiquantitatively with the software package (Bio 1D) of the imaging system (Photo Print, vilber, France). Bands at 260 kDa represent intact αII-spectrin and bands at 145/150 kDa are the cleavage products of αII-spectrin. Bands at 230 kDa represent intact talin and bands at 190 kDa

are the calpain specific degradation products. The intensity of the cleaved bands was expressed as the intensity of the intact bands and finally expressed as a percentage of the control group. Each experiment was performed in triplicate.

REFERENCES

E1. Hermes-Lima M, Willmore WG, Storey KB. Quantification of lipid peroxidation in tissue extracts based on Fe(III)xylenol orange complex formation. Free Radic.Biol.Med 1995:19:271-280.

E2. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem. 1976:72:248-254.

E3. Canton M, Neverova I, Menabo R, Van EJ, Di LF. Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts. Am.J.Physiol Heart Circ.Physiol 2004:286:H870-H877.

E4. Vescovo G, Ravara B, Dalla LL. Skeletal muscle myofibrillar protein oxidation and exercise capacity in heart failure. Basic Res.Cardiol. 2008:103:285-290.

FIGURE LEGENDS

Figure E1: Diaphragm force generated during the force-frequency relationship in spontaneous breathing animals treated with saline (SB, closed circles) or 80 mg/kg methylprednisolone (SB-methylprednisolone, open circles). Values are means ± SD.

Figure E2: Diaphragm cross-sectional area (CSA) of the type I, type IIa and type IIx/b fibers in the spontaneous breathing animals treated with saline (SB, solid bars) or 80 mg/kg methylprednisolone (SB-methylprednisolone, open bars). Values are means ± SD.

Figure E3: Western-blotting data of eNOS in the diaphragm of the controls (C,) spontaneous breathing animals (SB,), controlled mechanical ventilation animals treated either with saline (CMV-Saline) or with methylprednisolone (CMV-Methylprednisolone). Values are means ± SD. Values were normalized to total protein levels determined by PonceauS staining (showed on the left).

Figure E4: Representative Western blots illustrating the diaphragmatic protein levels of nitric oxide synthase (NOS). A: neuronal NOS (nNOS); B: inducible NOS (iNOS) in controls (C,) spontaneous breathing animals (SB,), controlled mechanical ventilation animals treated either with saline (CMV-Saline) or with methylprednisolone (CMV-Methylprednisolone). Positive control for iNOS: mouse macrophage exposed to IFN-γ and LPS, positive control for nNOS: rat cerebellum lysate. . PonceauS staining (showed on the left) was used as loading control.

Figure E5: Representative blots stained with PonceauS, illustrating equal loading for MyoD protein (A) and myogenin protein (B).

Table E1. Diaphragm fiber type proportions

	SB-Methylprednisolone	SB
Type I	44.1 %	40.9 %
Type IIa	29.7 %	33.7 %
Type IIx/b	26.2 %	25.4 %

SB-Methylprednisolone: rats breathing spontaneously and treated with methylprednisolone;

SB: rats breathing spontaneously and treated with saline

Figure E1

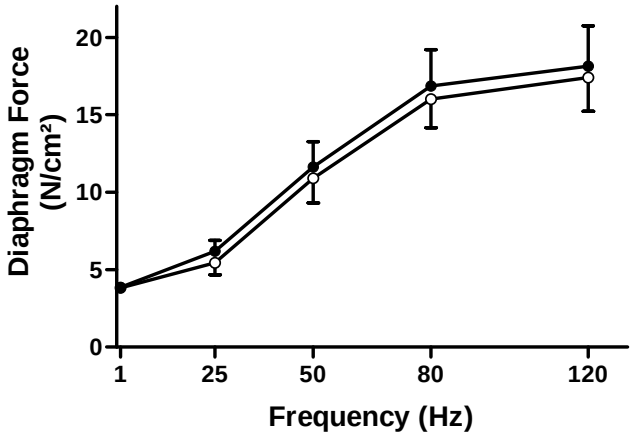


Figure E2

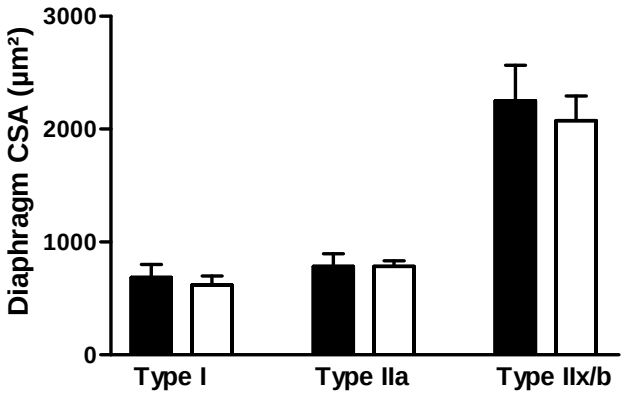
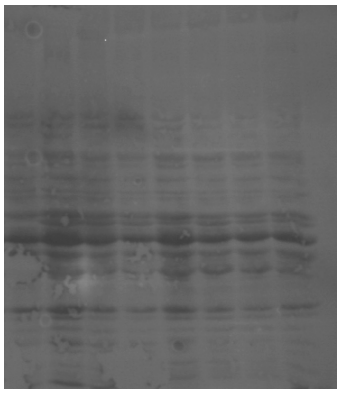
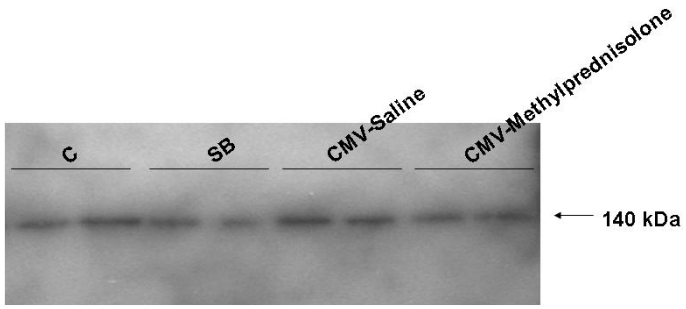
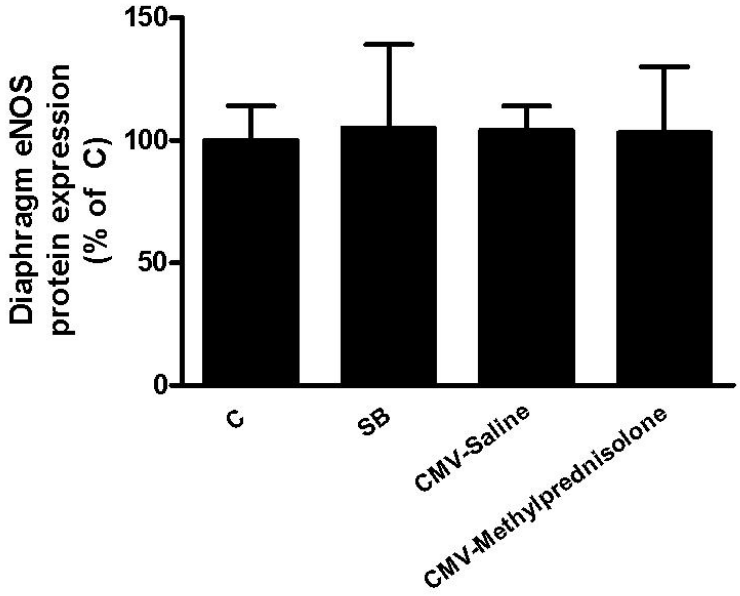


Figure E3

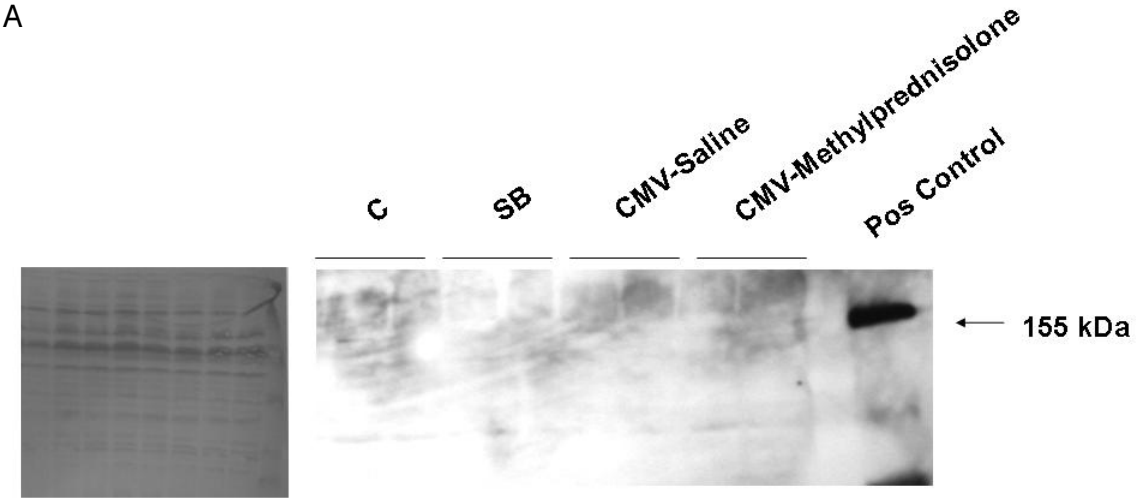



C
SB
CMV-Saline
CMV-Methylprednisolone
140 kDa


Diaphragm eNOS protein expression (% of C)
150
100
50
0
C
SB
CMV-Saline
CMV-Methylprednisolone

Figures E4

A



B

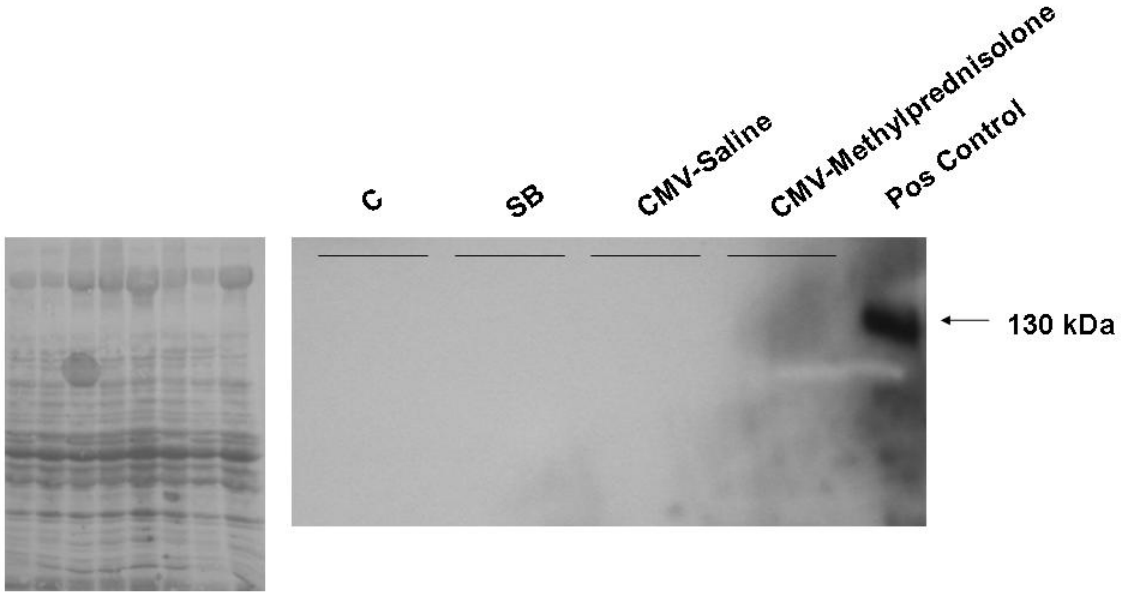
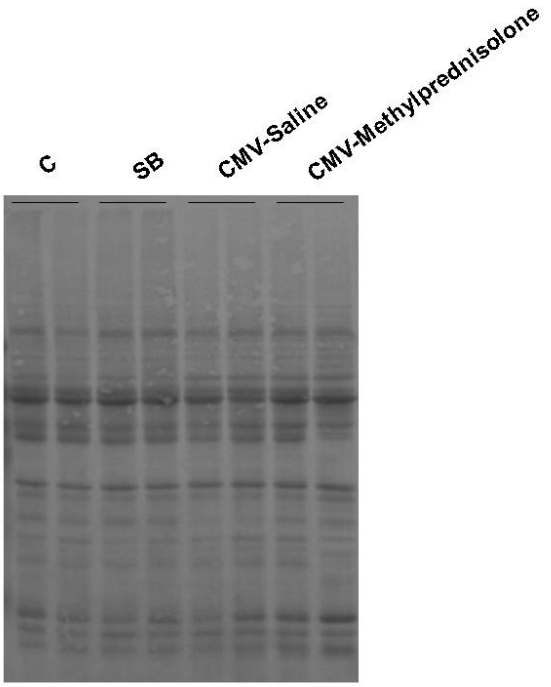
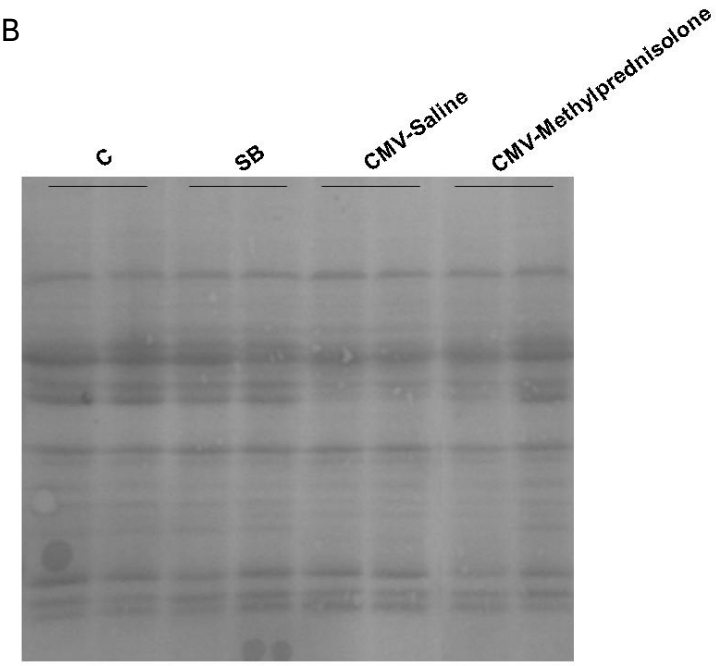


Figure E5

A


C
SB
CMV-Saline
CMV-Methylprednisolone

B


C
SB
CMV-Saline
CMV-Methylprednisolone