Supplemental Information for:

Natriuretic peptides enhance skeletal muscle oxidative capacity in humans

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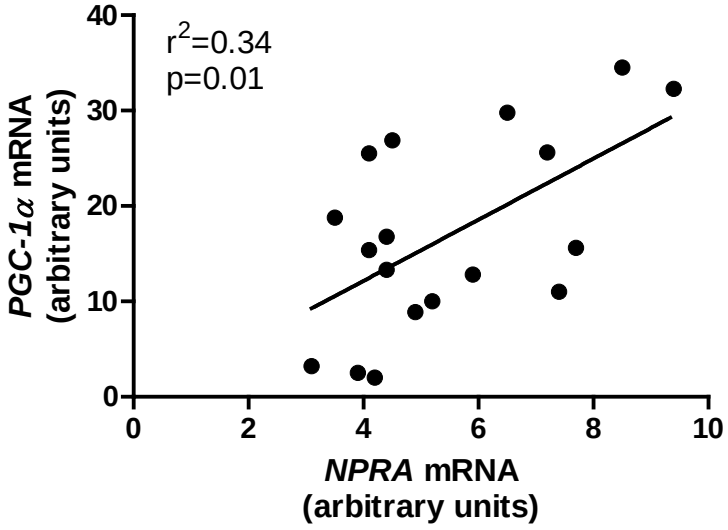
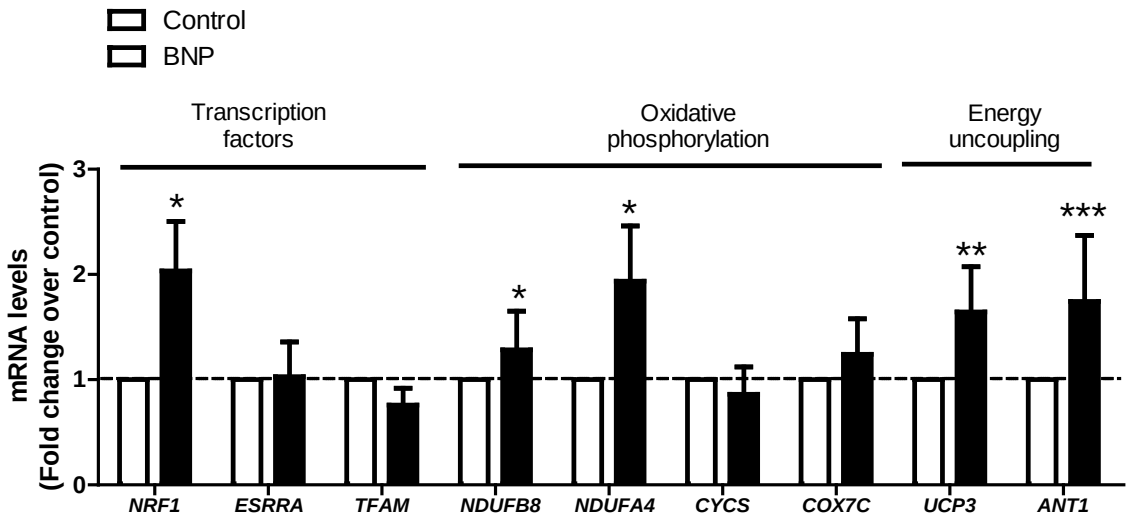
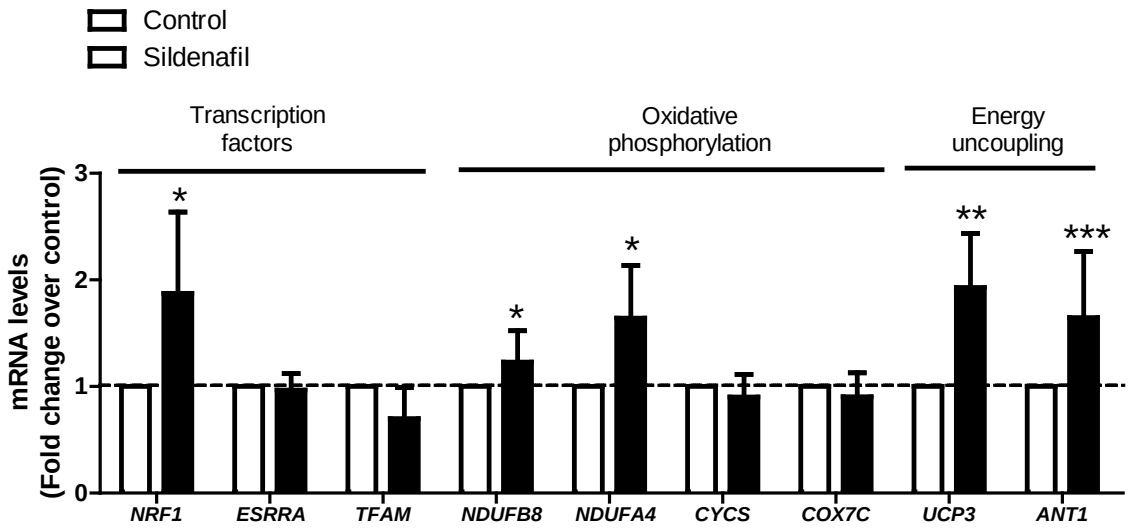
Inventory of Supplemental Information:



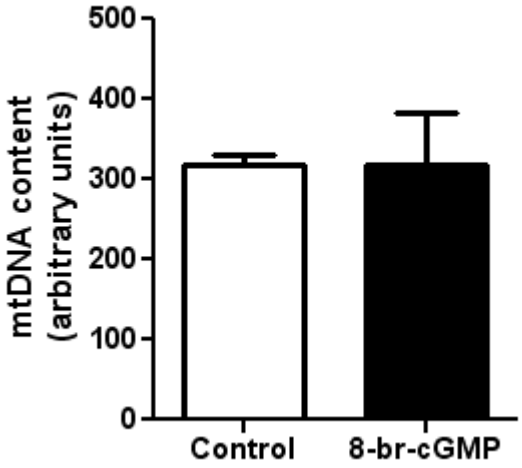
Supplemental Figure 1: Link between NPRA and PGC-1α in human skeletal muscle. Linear regression between *NPRA* and *PGC-1α* mRNA levels in human skeletal muscle of healthy obese subjects at baseline (n=18).



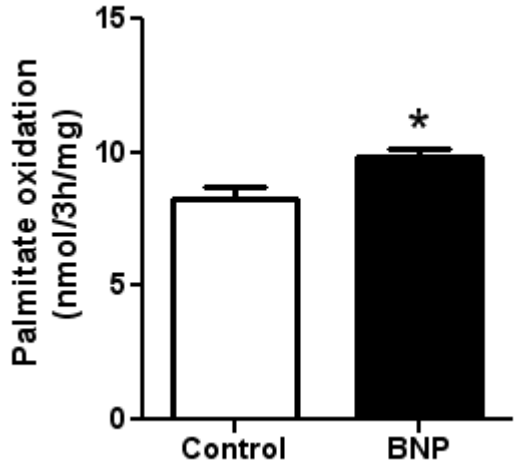
Supplemental Figure 2: BNP-induced mitochondrial genes in human myotubes. Changes in transcription factors, oxidative phosphorylation, and energy uncoupling genes in response to 48h treatment with BNP 10 nM (n=6). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control.



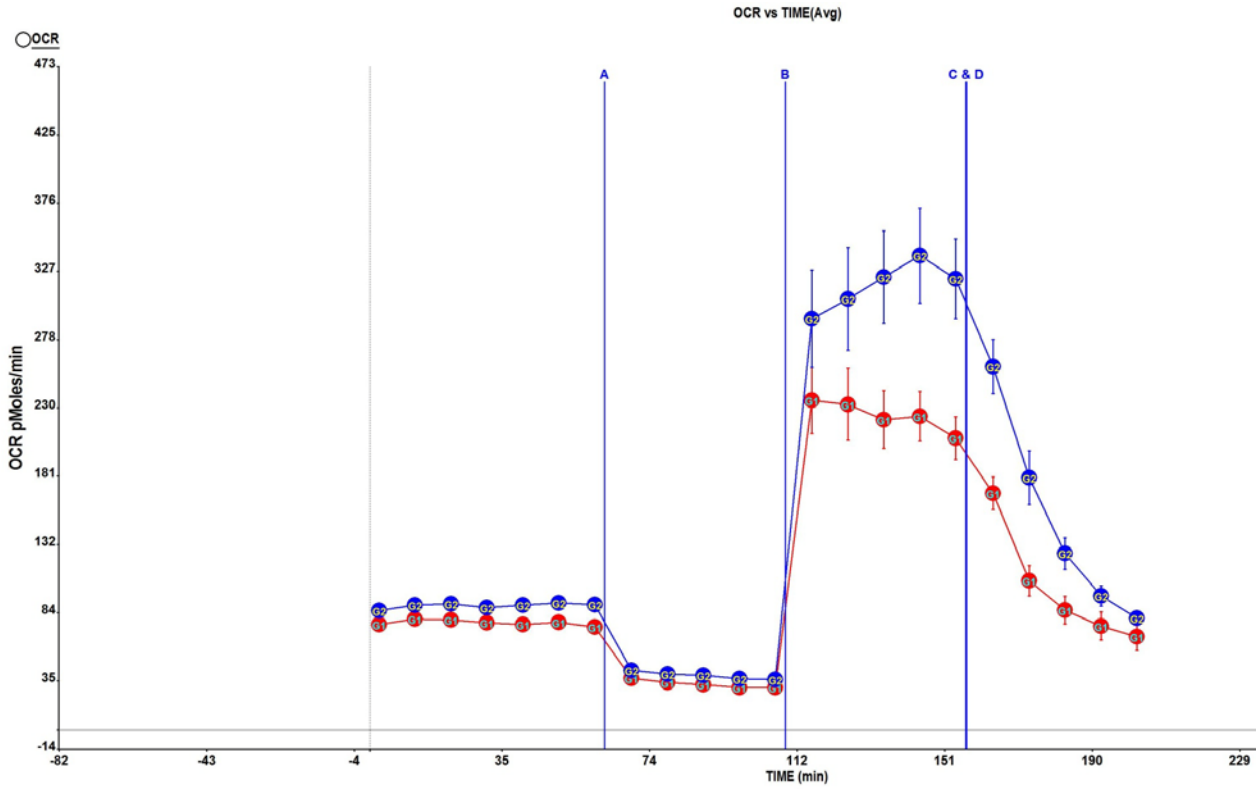
Supplemental Figure 3: Sildenafil-induced mitochondrial genes in human myotubes. Changes in transcription factors, oxidative phosphorylation, and energy uncoupling genes in response to 48h treatment with the selective phosphodiesterase-5 inhibitor, sildenafil 1 µM (n=6). * p<0.05, ** p<0.01, *** p<0.001 versus control.



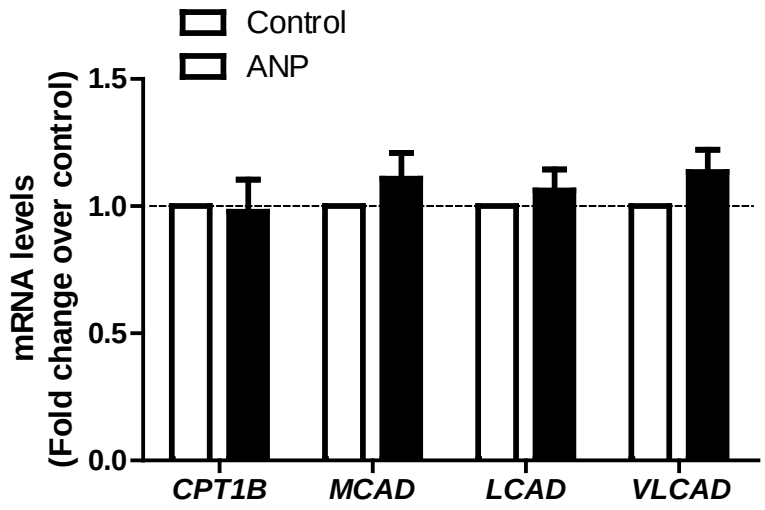
Supplemental Figure 4: cGMP does not increase mtDNA in human myotubes. Mitochondrial DNA content measured after 72h treatment with 8-bromo-cGMP 100 µM (n=3-6).



Supplemental Figure 5: BNP-induced fat oxidation in human myotubes. Palmitate oxidation measured after 72h treatment with BNP 10 nM (n=6). * $p<0.05$ versus control.



Supplemental Figure 6: ANP enhances oxygen consumption in human myotubes. Representative run of one XF24 analyzer experiment in control myotubes (red symbols) and in myotubes treated for 72h with ANP 10 nM (blue symbols) (n=9 replicates). A: injection of oligomycin (ATP synthase inhibitor); B: injection of FCCP (uncoupling agent); C & D: injection of rotenone (complex I inhibitor) and myxothiazol (complex III inhibitor). OCR: oxygen consumption rate.



Supplemental Figure 7: ANP does not significantly change fat oxidation gene expression in human myotubes. Changes in fatty acid oxidation genes in response to 72h treatment with ANP 10 nM (n=6). * $p<0.05$, ** $p<0.01$ versus control.

Supplemental Table 1. Relationship between *NPRA* and OXPHOS mRNA levels in human skeletal muscle.

	NPRA		
Genes	r	p value	p_{adj} value
Oxidative phosphorylation			
NDUFC2	0.56	0.03	0.81
SDHA	0.69	0.005	0.15
COX15	0.81	0.0002	0.0064
ATP6AP1	0.68	0.005	0.145
ATP6V1F	0.59	0.02	0.56
Substrate oxidation			
CPT1B	0.52	0.04	1.04
PDHA1	-0.72	0.003	0.093

r: Pearson correlation coefficients from microarray data at baseline; non adjusted p value; p_{adj} value: Benjamini-Hochberg false discovery rate considered statistically significant if ≤ 15%.

Supplemental Table 2. Primers sequences used for gene expression analyses.

Gene symbol	Primers sequence
LCAD	Fwd: 5‘-AACCATGGCTCAGAAGAACAGATT-3‘ Rev: 5‘-TGTCATTGCTATTGCACCAATACA-3‘
VLCAD	Fwd: 5‘-TGGCACGGATGGTTATGCT-3‘ Rev: 5‘-CGAGCCAAAGATTTTGCTGAT-3‘
TFAM	Fwd: 5‘-GATTCCAAGAAGCTAAGGGTGATTC-3‘ Rev: 5‘-TCAGAGTCAGACAGATTTTTCCAGTT -3‘
UCP3	Fwd: 5‘-TCACCTCCAGGCCAGTACTT-3‘ Rev: 5‘-CGTTAGCTACCAGTGGCCTT-3‘
SLC25A4 (ANT1)	Fwd: 5‘-GCTGCCTGACCCCAAGAAC-3‘ Rev: 5‘-CTGCGACTGCCGTCACACT-3‘
MCAD	Fwd: 5‘-AGCTCCTGCTAATAAAGCCTTTACTG-3‘ Rev: 5‘-CATGTTTAATTCCTTTCTCCCAATC-3‘
CPT1B	Fwd: 5‘-TACAACAGGTGGTTTGACA-3‘ Rev: 5‘-CAGAGGTGCCCAATGATG-3‘

Supplemental Methods

In vivo experiments

Design of the study. Before the investigation, body composition (considering a 3-compartments model) was determined at baseline using a total body Dual-Energy X-ray Absorptiometer (Lunar-DPX). One week before the investigation, maximal oxygen uptake (VO_{2max}) was assessed using a graded exercise test conducted on an electromagnetically braked bicycle ergometer (Ergometrics 800, Ergoline, Jaeger, Germany) as previously described (1, 2). The initial workload was 50 watts and it was increased by 30 watts every 3 min until exhaustion. Heart rate was continuously monitored by telemetry using a heart rate monitor (Ergocard, Jaeger, Germany) and blood pressure was measured with an exercise-adapted monitor (Tango Stress Test BP Monitor; Suntech Medical Instruments Inc., Raleigh, North Carolina, USA). We considered that the subjects achieved their VO_2max when all the following usual and accepted criteria were achieved: maximal heart rate measured at exhaustion was higher than 90% of the age-predicted maximal heart rate, Respiratory Quotient (RQ, i.e. VCO_2/VO_2) measured at exhaustion was higher than 1.1, the subjects could not sustain a sufficient rate of cycling. On the experimental day, subjects were investigated after a 10h overnight fast. They ate standardized meals composed mainly of carbohydrate (49%), protein (16%) and fat (35%) (corresponding to their daily metabolic requirements) 48h prior to the experimental day.

Exercise training. Aerobic exercise was performed at the Centre de Ressources, d'Expertise et de Performance Sportives (CREPS) of Toulouse. Exercise sessions consisted mainly of cycling and running, 5 times per week for 8 weeks. Subjects exercised 3 times per week under supervision during the first 4 weeks and 2 times per week during the last 4 weeks. They exercised on their own during other sessions. All daily sessions consisted of at least 20 min warm-up at 35% VO_{2max} followed by progressively increasing exercise intensity (up to 85% VO_{2max}) and duration (up to 1h) throughout the training program. The subjects exercised at a target heart rate corresponding to 35–85% of their maximal oxygen uptake (VO_{2max}). Heart rate was monitored with a Suunto T3 Cardiometer (MSE, Strasbourg, France). Compliance with training was good, as checked by a training diary including day-to-day activities. The percentage of sessions completed was greater than 85%. Food and calorie intake were not modified during the training protocol.

Indirect calorimetry. At rest, gas exchanges were measured for 20 min, and values were averaged on the last 15 min. VO_2 and VCO_2 were measured and the RQ calculated as previously (2). Breath-by-breath measurements were taken at rest and throughout exercise to assess air flow and O_2 and CO_2 concentrations in expired gases by using a computerized ergospirometer (Ultima PFX, Medical Graphics, USA). Oxygen concentration was analysed by a zirconium cell and CO_2 concentration by an infrared analyser. Certified calibration gases were used to calibrate the analysers every day before the beginning of the assay. The VO_{2max} exercise trial occurred in a ventilated room to ensure a constant room temperature and hygrometry from the calibration just before the trial.

In vitro experiments

Microarrays. Briefly, target RNA was generated from 500 ng of total RNA with the Agilent low RNA input amplification kit (Agilent Technologies). Microarray experiments were performed using a reference design and whole genome 4x44k oligonucleotide arrays (Agilent Technologies). Data acquisition was performed with an InnoScan 710A scanner (Innopsys, Carbonne, France) and images were processed with Mapix software version 5.5.0 (Innopsys). Hybridization was quality checked using control spikes resulting in the analysis of microarray data from 8 subjects. Outlier replicates and spots with a signal to noise ratio less than 2 on both red and green channels were eliminated from the analyses. Mean log ratios were calculated before normalization. Raw data were normalized with a global Loess procedure and filtered with the R package LIMMA (Bioconductor). Mean spot calculation according to Gene Symbol resulted in 7859 exploitable gene IDs. Principal component analysis was used to check that all microarray experiments provided consistent data. Microarray data were analyzed using repeated measures ANOVA using Partek Genomics Suite version 6.5 (Partek Inc., St. Louis, MO, USA) resulting in 608 gene symbols. Functional analysis was done using Ingenuity Pathway Analysis (IPA) version 7.5 (3). Ingenuity's Pathways Knowledge Base was seeded with the 608 gene symbols together with the array mean fold change of the differentially expressed genes. A Benjamini-Hochberg multiple testing correction p-value was applied to the canonical pathways analysis of the 489 eligible gene symbols of the dataset. Data were considered significant for p value < 0.05. The microarray database has been deposited in Gene Expression Omnibus (GSE40551).

Real-time qPCR. Total RNA from muscle tissue and cultured myotubes was isolated in RNeasy Lysis Buffer + β-mercaptoethanol reagent (Qiagen GmbH, Hilden, Germany) as previosuly (4, 5). The quantity of the RNA was determined on a Nanodrop ND-1000 (Thermo Scientific, Rockford, IL, USA). Reverse-transcriptase PCR was performed on a GeneAmp PCR System 9700 using the Multiscribe Reverse Transcriptase method (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR (qPCR) was performed to determine cDNA content. Sequences of SYBR primers are given in Supplemental Table 2. All other primers were bought from Applied Biosystems (Foster City, CA, USA). Primers used were: *18S* (Taqman assay ID: Hs99999901_s1), *CD36* (Hs00169627_m1), *PPARD* (Hs00602622_m1), total *PPARGC1A* transcripts (Hs00173304_m1), *FABP3* (Hs00269758_m1), *SLC27A1* (FATP1) (Hs01587917_m1), *NPR1* (Hs00181445_m1), *PRKG1* (Hs00183512_m1), *PDE5A* (Hs00153649_m1), *NRF1* (Hs00602161_m1), *ESRRA* (Hs00607062_m1), *NDUFB8* (Hs00428204_m1), *NDUFA4* (Hs00800172_s1), *CYCS* (Hs01588974_g1), *COX7C* (Hs01595220_g1), and *NPR3* (Hs00168558_m1). Quantitative PCR was then performed on a StepOnePLus real-time PCR system (Applied Biosystems, Foster City, CA, USA). For each primer, a standard curve was made prior to mRNA quantification to assess the optimal total cDNA quantity. All expression data were normalized by the $2^{(\Delta\Delta Ct)}$ method using 18S rRNA as internal control.

Western blotting. Muscle tissues and cell extracts were homogenized in a buffer containing 50 mM HEPES, pH 7.4, 2 mM EDTA, 150 mM NaCl, 30 mM NaPPO4, 10 mM NaF, 1% Triton X-100, 10 μl/ml protease inhibitor (Sigma-Aldrich), 10 μl/ml phosphatase I inhibitor (Sigma-Aldrich), 10 μl/ml phosphatase II inhibitor (Sigma-Aldrich), and 1.5 mg/ml benzamidine HCl (6). Tissue homogenates were centrifuged for 25 min at 15,000*g*, and supernatants were stored at -80°C. Solubilized proteins from muscle tissue and myotubes were run on a 4-12% SDS-PAGE (Biorad), transferred onto nitrocellulose membrane (Hybond ECL, Amersham Biosciences), and incubated with the primary antibody OXPHOS (MitoSciences) and PGC1α (Santa Cruz). Subsequently, immunoreactive proteins were determined by enhanced chemiluminescence reagent (GE Healthcare) and visualized by exposure to Hyperfilm ECL (GE Healthcare). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology) served as an internal control.

cGMP measurements. Human primary myotubes were pretreated for 15 min with 0.5 µM IBMX and treated for 20 min with ANP or BNP (0.01-1.0 µM). Myotubes were then homogenized in ice cold methanol/acetonetrile. cGMP concentration was measured by ELISA (ENZO Life Sciences, cGMP complete EIA kit, Lörrach, German), and normalized by total protein content.

Determination of mitochondrial content. For quantification of mitochondrial content, we measured the mitochondrial (mt) to nuclear DNA ratio as previously described (7). The sequences for the primer sets used for determination of mtDNA for NADH dehydrogenase subunit 1 (*ND1*) were forward primer CCCTAAAACCCGCCACATCT, reverse primer GAGCGATGGTGAGAGCTAAGGT, and of nuclear DNA for lipoprotein lipase (*LPL*) were forward primer CGAGTCGTCTTTCTCCTGATGAT, reverse primer TTCTGGATTCCAATGCTTCGA. We also determined mitochondrial mass in myotubes using Mitotracker Green FM (Invitrogen, Carlsbad, CA) which stains mitochondrial matrix protein irrespective of the membrane potential and thus provides an accurate assessment of mitochondrial mass. Briefly, cells were washed with 1X PBS and incubated at 37°C for 30 minutes with 100nM of each Mitotracker. Cells were then harvested using trypsin/EDTA and re-suspended in 1X PBS. Fluorescence intensity was measured on a fluorometer and values corrected for total protein.

Palmitate oxidation assay. Myotubes were preincubated for 3-hour with [1-^{14}C]palmitate (1 µCi/ml; Perkinelmer, Boston, MA) and nonlabeled (cold) palmitate. Palmitate was coupled to a fatty acid–free BSA in a molar ratio of 5:1. Following incubation, $^{14}CO_2$ and ^{14}C-ASM was measured as previously described (4). Briefly, assayed medium is transferred into a custom–made Teflon 48-well trapping plate. The plate is clamped and sealed, and perchloric acid is injected through the perforations in the lid into the medium, which drives CO_2 through the tunnel into an adjacent well, where it is trapped in 1N NaOH. Following trapping, the media is spun twice and ^{14}C-ASM measured by scintillation counting. Aliquots of NaOH and medium are transferred into scintillation vials, and radioactivity is measured on a multipurpose scintillation counter (Tricarb 2100 TR, Packard). All assays are performed in triplicates, and data are normalized to protein content.

Supplemental References

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