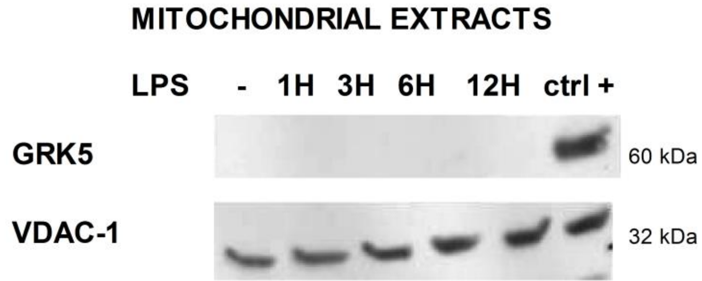
Supplementary Figure Legends

SF1: GRK5 does not localize in mitochondria. To evaluate the selective role of GRK2 in mitochondria, we tested the ability of GRK5 to localize in mitochondria. To this aim we performed a western blot in mitochondrial extracts using the specific antibody against GRK5 (Upstate). GRK5 does not localize in mitochondria neither in basal conditions nor after LPS stimulation. A whole lysates was used as positive control VDAC-1 was used as loading control. Images are representative of 3 independent experiments.

SF2: GRK2 kinase activity in mitochondria. To confirm the progressive accumulation of GRK2 in mitochondria after LPS stimulation, we performed a kinase assay in mitochondrial extracts using rhodopsin as substrate. After 6 hours of stimulation with LPS rhodopsin phosphorylation in mitochondrial extracts was increased respect to control. Images are representative of 3 independent experiments.

Supplementary Figure 1



Supplementary Figure 2

GRK2 kinase assay in mitochondrial extracts

Supplementary Methods

Reagents

Lipopolysaccharide (20 ng/ml, LPS) and 2′,7′-dichlorodihydrofluorescein (DCFH) were purchased from Sigma Aldrich; primary antibodies against GRK2, Actin, VDAC-1 and Gαi were from SantaCruz, NFκB and p-NFκB (Ser 536) were from Cell Signaling, Mn-SOD was from Upstate; all secondary antibodies were from SantCruz; primers for Real Time PCR were from PRIMM; reagents for Real Time PCR were from Applied Biosystem; the adenovirus encoding for the carboxy-terminal domain of GRK2 (AdβARK-ct) was a kind gift of Prof. WJ Koch (Temple University of Philadelphia) and Ad-Lac-Z was available in our lab and used as control.

Cell culture and transfection

Cultured macrophages RAW 264.7 were purchased from Lonza.. Cells were cultured in Dulbecco's MEM (DMEM) supplemented with 10% FBS at 37°C in 95% air and 5% CO2. The overexpression of IκBα in RAW264.7 was induced by transient transfection of IκBα plasmid. IκBα plasmid and the empty plasmid pcDNA3.1 (Invitrogen), used as control vector, were transfected in macrophages by means of X-treme GENE HP DNA transfection reagent (Roche) according to the manufacturer's instructions.

GRK2 gene deletion

The siRNA sequence against human GRK2, the siRNA scramble sequence used as control for gene silencing and the gene silencing procedure were described previously [26,27].

Membrane and mitochondrial extracts

Membrane and mitochondrial extracts were performed as previously described [25,26,28] and then analyzed by western blot. The procedures to obtain purified membranes and mitochondria are standardized in our laboratory as assessed by our previous paper [25,26,28]. To verify that our extracts are free from contaminants of cytosol, nuclei and reticulum, western blot analysis was performed using specific antibodies anti-actin, anti-H3 and anti-calnexin (SantaCruz), respectively.

Western blot

For total lysates, RAW 264.7 cells were lysed in RIPA/SDS, as previously described [29-31]. Protein concentration was determined by using BCA assay kit (Pierce). Lysates were electrophoresed by SDS/PAGE and transferred to nitrocellulose; reaction was visualized by using specific primary antibody, anti-rabbit and anti-mouse HRP-conjugated secondary antibody (Santa Cruz) and standard chemiluminescence (Pierce). Images were then digitalized and densitometric analysis was performed using dedicated software (ImageQuaNT). Data are presented as Arbitrary Densitometry Units (ADU) after normalization for internal control (VDAC-1 for mitochondria, Actin for cytosol and $G_{\alpha i}$ for membranes).

Real Time PCR

Macrophages were infected with AdβARK-ct or AdLac-Z, as control, and stimulated with LPS for the described time points. Total RNA was extracted using TRIzol reagent (Invitrogen) and then converted to cDNA by means of Thermo-Script RT-PCR System (Invitrogen), as described previously [33]. The reaction was visualized by SYBR Green Analysis (Applied Biosystem) on StepOne instrument (Applied Biosystem). Primer sequences for gene analysis are the following:

IL-1β Forward 5'-gccttgggcctcaaaggaaagaatc-3' and Reverse 5'-ggaagacacagattccatggtgaag-3';

IL-10 Forward 5'-gtgaagactttctttcaaacaaag-3' and Reverse 5'-ctgctccactgccttgctcttatt-3';

MCP1 Forward 5'-ggaaaaatggatccacaccttgc-3' and Reverse 5'-tctcttcctccaccaccatgcag-3';

GRK2 Forward 5'-ccctctcaccatctctgagc-3' and Reverse 5'-cggttggggaacaagtagaa-3'.

18S Forward 5'gtaacccgttgaaccccatt3' and Reverse 5'ccatccaatcggtagtagcg3'.

All values obtained were normalized to the values obtained with the 18S primers (endogenous control) and relative to the reference sample (basal control) using the formula $2^{-\Delta\Delta Ct}$. Obtained results are expressed as relative quantification (RQ).

Kinase assay and Mitochondrial biogenesis

Kinase assay and mitochondrial biogenesis were performed as previously described [25].

Measurement of ROS production

ROS generation in cells was assessed using the probe 2,7-dichlorofluorescein (DCF) (Sigma Aldrich). The membrane-permeable diacetate form of the dye (reduced DCF: DCFH) was added to cells at a final concentration of 5 μM for 15 min before stimulation with LPS. ROS in the cells oxidize DCFH, yielding the fluorescent product DCF. At different time points after stimulation with LPS, fluorescence was measured using an excitation wavelength of 485 ± 20 nm and an emitter bandpass of 535 ± 25 nm using a microplate reader, Infinite 200 Pro (Tecan group Ltd.).

Mitochondrial mass

MitoTracker probes are cell-permeant mitochondrion-selective dyes which passively diffuse across the plasma membrane and accumulate in active mitochondria. Thus, to label mitochondria, mitotracker (Invitrogen) was added to cells at the endpoint of stimulation for 30 minutes and fluorescence was measured using an excitation wavelength of 580 ± 20 nm and an emitter bandpass of 635 ± 35 nm in the microplate reader.

Statistical analysis

All values are presented as mean±SEM. Two-way ANOVA was performed to compare the different parameters between the different groups. A P value < 0.05 was considered to be significant. Statistics were computed with GraphPad Prism version 5.01 (GraphPad Software).