Online supplement

I_f blocking potency of ivabradine is preserved under elevated endotoxin levels in human atrial myocytes

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Supplemental Material and Methods

1. Isolation of human and guinea pig myocytes

Human right atrial appendages were obtained from 42 patients (27 men, 15 women) aged between 27 to 82 years (mean 65.83 ± 1.68). We used only appendages of patients in sinus rhythm before surgical intervention (aortic valve replacement, coronary bypass graft or aorta aneurysm repair) as well as showing normal cardiac parameters to avoid structural and/or electrophysiological alterations in the right atrial myocardium. Patients met following inclusion criteria: aortic valve stenosis with no patients in NYHA class III-IV or IV, ejection fraction more than 45%, no atrial rhythm disorders, normal left atrial diameter (patients with mitral and tricuspid insufficiency were excluded), coronary artery disease with no patients with unstable rest angina, no emergency cases, no hemodynamic instability during induction of anaesthesia, no evidence of elevated pulmonary pressure. All patients were under treatment with combinations of various drugs (see Supplemental Table 1). All experiments were carried out in accordance with the Declaration of Helsinki [1]. The ethics committee of the Medical University of Graz approved the use of human tissue and all patients gave informed consent. Atrial tissue samples were transported to the laboratory within 10 to 15 min in cold saline containing (in mmol/L): 90 NaCl, 30 KCl, 2 $NaHCO_3$, 2 HEPES/Na^+, 5.5 D(+)-glucose, 42 sucrose (pH 7.4, adjusted with NaOH). Tissues were cut into pieces of about 1 mm^3 and washed with saline to remove Ca^{2+}. Tissue pieces were transferred to a dissociation vessel and gently stirred at 37°C in saline containing 0.25% (w/v) trypsin (Sigma-Aldrich, St. Louis, MO, USA) for 2 min followed by saline containing 300 IU/mL collagenase (CLS-2, Cell Systems, Germany). The supernatant was replaced by collagenase solution every 15 min. After the third change, collagenase concentration was reduced to 150 IU/mL. Isolated myocytes were washed with saline followed by stepwise increase in Ca^{2+} to a final concentration of 500 µmol/L. Myocytes were then transferred to M199 cell culture medium (Sigma-Aldrich) containing penicillin 50 IU/mL, streptomycin 50 µg/mL (both Sigma-Aldrich) and kept at 37°C under 5% CO_2. Tissue transport and isolation procedure were carried out exactely as described [2]. Experiments were performed 6 h after isolation of myocytes within a 24 h time period.

Guinea pig (Dunkin Hartley, Charles River Laboratories, Germany) ventricular myocytes were isolated by Langendorff perfusion [3] and stored in M199 medium at 37°C. Experiments were performed 24 h after cell isolation.

2. Cell culture of the murine atrial cardiomyocyte cell line

HL-1 cells [4] were cultured in fibronectin (0.5%, w/v)/gelatin (0.02%, w/v) (Sigma-Aldrich) coated flasks and supplied with Claycomb medium (Sigma-Aldrich) containing 10% (v/v) fetal bovine serum (Sigma-Aldrich), 0.1 mmol/L norepinephrine (Sigma-Aldrich), 2 mmol/L L-glutamine (Sigma-Aldrich), 100 IU/mL penicillin and 100 µg/mL streptomycin at 37°C under 5% CO_2. Cells (10^4/well) were plated in 6-well plates.

3. Incubation protocol

In order to investigate acute effects of endotoxins on I_f, human myocytes were superfused with I_f-Tyrode containing 10 µg/mL S-form LPS (extracted from wild-type *Escherichia coli* serotype 0111:B4 [unless specified this S-form LPS was used] or *Salmonella enterica* serotype *Minnesota*, Sigma-Aldrich) or 10 µg/mL O-chain lacking R-form LPS (LPS Re of the deep rough mutant from *Salmonella enterica* serotype *Minnesota*strain R595 [kindly provided by Prof. Dr. Klaus Brandenburg, Research Center Borstel, Germany]) for 6 min. Chronic effects of endotoxins were evaluated after 6 to 10 h incubation of myocytes. In the present manuscript the S-form LPS is referred as S-LPS, while the R595-mutant LPS is referred as R595.

The effect of ivabradine (kindly provided by Servier Laboratories, France, 1 µmol/L in I_f-Tyrode) on I_f was investigated after superfusion of myocytes for 7 min.

HL-1 cells were incubated with endotoxins for 6-24 h followed by cell lysis for RNA and protein isolation (see below).

4. Patch clamp solutions

(i) Superfusion (extracellular) solution: I_f-Tyrode solution contained (in mmol/L): 137 NaCl, 25 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 1 $BaCl_2$, 2 $MnCl_2$, 0.2 $CdCl_2$, 3 4-aminopyridine, 5 glucose, 5 HEPES (pH 7.35, adjusted with NaOH). Mn^{2+}, Ba^{2+}, Cd^{2+}

and 4-aminopyridine were added to avoid interference with potassium and calcium currents.
(ii) Pipette (intracellular) solution: Patch pipettes (2.5 to 4 MΩ) were filled with a solution containing (in mmol/L): 100 K^+-aspartate, 30 KCl, 5 Na^+-ATP, 4 $CaCl_2$, 11 EGTA, 10 HEPES (pH 7.2, adjusted with KOH).

5. Electrophysiological recordings and analysis

Electrophysiological recordings were performed by patch-clamp technique in the whole-cell mode at 36±1°C using the amplifiers List L/M-EPC 7 (List, Darmstadt, Germany) and Axopatch 200B (Molecular Devices, CA, USA) and the A/D - D/A converters Digidata 1322A and Digidata 1200 (Molecular Devices LLC). pCLAMP software (Molecular Devices LLC) was used for data acquisition and analysis. Only quiescent rod-shaped human atrial myocytes with clear cross-striation were used for patch-clamp experiments.
(i) Cell membrane capacitance was determined by numerical integration of the capacitive transient elicited by a 10 mV hyperpolarizing step from -50 mV.
(ii) Pacemaker current was measured by hyperpolarizing voltage steps (3 s duration) from -40 mV to -130 mV (10 mV increments, holding potential -40 mV, i.e. *protocol p1*).
(iii) As ivabradine is a use-dependent inhibitor its effect was measured employing the following protocol: Trains (total duration 9 min) of activating/deactivating voltage steps (-100 mV for 3 s; 0 mV for 0.4 s) from a holding potential of -40 mV at 1/6 Hz (i.e. *protocol p2*). Protocol *p1* preceded and followed protocol *p2* (see inset in Fig. 5a). After 2 min of protocol *p2* run ivabradine superfusion was started for 7 min.

In order to allow equilibration of the pipette solution with the cytosol current recordings were started 4 min after rupture of the membrane patch. I_f represents the difference between the instantaneous current immediately after the decline of the capacitive transient and the current at the end of the hyperpolarizing voltage steps. I_f was normalized to cell membrane capacitance and expressed as pA/pF in order to compensate for cell size variations. Mean cell capacitance of human atrial myocytes used in this study was 91.9±6.0 pF (n=87). Pacemaker current densities did not show any age dependency.

I_f conductance was calculated according to the following equation:

$$g_f = I_f/(V_m - V_{rev}) \qquad \textit{equation (1)}$$

g_f is the calculated conductance at a given membrane potential V_m, I_f is the measured current amplitude, and V_{rev} represents the reversal potential of I_f in human atrial myocytes amounting to -13 mV as described for an external potassium concentration of 25 mmol/L [5]. g_{fmax} was calculated by fitting the g_f values with the Boltzmann function according to

$$g_f = g_{fmax}/(1 + \exp[(V_{1/2} - V_m)/k]) \qquad \textit{equation (2)}$$

$V_{1/2}$ is the voltage at half-maximal activation and k is the slope factor. For calculation of steady-state activation curves conductances were normalized to maximal conductance and fitted by a Boltzmann equation. The liquid junction potential between the electrode tip and the external solution was calculated from Henderson equation and amounted to -11.7 mV [6]. Data were not corrected for the liquid junction potential.

6. Immunoprecipitation of HCN channels

Male C57BL/6 mice (8-10 weeks, 20-30 g) were obtained from the Institute of Biomedical Research (Medical University of Vienna, Austria) and were kept on a 12 h light/dark cycle with free access to food and water. Mice were sacrificed by cervical dislocation, hearts were removed and homogenized in RIPA buffer (50 mmol/L Tris-HCl (pH 7.5), 300 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L NaF, 0.1% [v/v] Triton X-100, 0.02% [w/v] NaN_3, pH 7.4) containing a protease inhibitor cocktail tablet (Sigma-Aldrich). Tissue homogenates were centrifuged at 10,000 rpm (4°C, 10 min) to pellet debris. Heart protein lysates were equilibrated against immunoprecipitation buffer containing (in mmol/L): 50 Tris-HCl (pH 8.0), 10 $MgCl_2$ and 150 NaCl. Lysates containing equal protein amounts (1 mg) were mixed with 1 µg mouse monoclonal anti-HCN2 or rabbit polyclonal anti-HCN4 antibody (Abcam, Cambridge, UK) for 2 h at 4°C. Immune complexes were precipitated by mixing 20 µL of protein A/G plus Agarose (Santa Cruz Biotechnology, Heidelberg, Germany) overnight (4°C with shaking) [7]. Cell debris were pelleted by centrifugation at 10,000 rpm (4°C, 1 min). After washing 3 times with RIPA buffer, pellets were resuspended in 40 µL of 4 x NuPAGE LDS sample buffer and heated (70°C, 10 min). Western blot analyses were performed to identify immunoprecipitated HCN2 or HCN4 protein.

7. Western blot analysis

After treatment with endotoxins, 0.5-1 x 10^6 HL-1 cells were lysed in 100 µL lysis buffer (50 mmol/L Tris-HCl (pH 7.5), 300 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L NaF, 0.1% [v/v] Triton X-100, 0.02% [w/v] NaN_3, pH 7.4) containing a protease inhibitor cocktail tablet (Sigma-Aldrich) for 10 min on ice. Cells were scraped and centrifuged at 10,000 rpm (4°C, 10 min) to pellet debris. After protein estimation using the Lowry method, 50 µg of total protein were added to 10 µL of 4 x NuPAGE LDS sample buffer containing 2 µL sample reducing agent (Invitrogen, Austria) and heated (70°C, 10 min). Proteins and immunoprecipitates were separated by electrophoresis on NuPAGE 4-12% Bis-Tris gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) non-fat milk in TBST (Tris-buffered saline containing Tween 20) (25°C, 2 h) and incubated with anti-HCN2 or anti-HCN4 antibody (1:1000 in 5% [w/v] BSA) (4°C, overnight). Immunoreactive bands were visualized with HRP-conjugated goat anti-mouse/-rabbit IgG (1:100,000 in 5% [w/v] non-fat milk in TBST) (25°C, 2 h) followed by Super Signal West Pico Chemiluminescent substrate (Thermo Scientific, IL, USA) and developed by Bio-Rad ChemiDoc MP Imaging System. Membranes were stripped (58.4 g/L NaCl, 7.5 g/L glycine, pH 2.15) and incubated with monoclonal mouse anti-β-actin antibody (Santa Cruz Biotechnology) (1:1000 in 5% [w/v] BSA).

8. Immuno-dot-blot technique

Polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Vienna, Austria) were prewetted in absolute methanol. S-LPS and R595 (10 µL equivalent to 10 µg) were cross-linked (dotted) to PVDF membranes under UV conditions (10 min) and dried. Only methanol-prewetted (activated) but not dried (inactivated) PVDF membranes allow crosslinking of negatively charged molecules e.g. endotoxins or proteins. Dots were then incubated with 5 µL or 10 µL of immunoprecipitated HCN2/HCN4 protein or 10 µL (equivalent to 1 µg) solution containing high-density lipoprotein (HDL) (4°C, 2 h). HDL was isolated from human plasma as described [8]. Membranes were washed with TBST and blocked with 5% [w/v] non-fat milk in TBST (25°C, 5 h) followed by incubation with either anti-HCN2 or anti-HCN4 antibody (1:1000 in 5% [w/v] BSA) or rabbit anti-human apoA-I antiserum (1:3000 in 5% [w/v]

BSA, recognizing apoA-I (the major apolipoprotein of HDL) at 4°C overnight. To detect immunoreactive dots, membranes were incubated with HRP-conjugated goat anti-mouse/-rabbit IgG as secondary antibodies (1:100,000 in 5% [w/v] non-fat milk in TBST) (25°C, 2 h) followed by Super Signal West Pico Chemiluminescent substrate (Thermo Scientific) and developed by Bio-Rad ChemiDoc MP Imaging System.

9. RNA isolation and real time RT-PCR

Total RNA was isolated from HL-1 cells after S-LPS or R595 (10 µg/mL each) treatment by using QIAshredder and RNeasy Mini Kit (Qiagen, UK). One µg of RNA was subjected to reverse transcription [9]. Six ng cDNA per template were used for gene quantification by using SYBR Green PCR Kit (Qiagen) and gene specific primers. Real time RT-PCR protocol was performed using LightCycler 480 system (Roche Diagnostics, Vienna, Austria). Following primers were used:
GAPDH (Mm_Gapdh_3_SG, Qiagen); HCN2 (NM_008226) F: 5'-CTGCGGCTATCACGGCTCAT-3', R: 5'-CAACCGTCCCAGTGGCAGA-3'; HCN4 (NM_001081192) F: 5'-GTCCATGCGCAAGCGGCTCTA-3', R: 5'-TGCTCCTTCGGCTGGGGTCC-3'. Relative gene expression levels compared to GAPDH were calculated by using ΔΔCT method.

10. Analysis of ivabradine concentrations in guinea pig ventricular myocytes

Ivabradine was quantified using a fluorometric HPLC technique [10]. Briefly, untreated (controls) and endotoxin-treated (S-LPS or R595, 10 µg/mL, 6 h) cardiomyocytes were incubated with ivabradine (1 µmol/L, 15 min) followed by four washing steps with 1.5 mL Tyrode solution (in mmol/L: 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.1 $MgCl_2$ x $6H_2O$, 2.2 $NaHCO_3$, 0.4 NaH_2PO_4 x H_2O, 5.6 D-glucose, 10 HEPES, pH 7.4). Finally, cells were deproteinized with 200 µL $HClO_4$ (0.4 mol/L). After centrifugation (1500 rpm, 5 min) the supernatant was used for ivabradine determination and cell debris were lysed in 1 mL NaOH (0.1 mol/L) for protein determination (BCA Protein Assay, PIERCE, Rockford, IL, USA). Additionally, the final washing solution (Tyrode – 4^{th} step) was also analyzed for ivabradine to ensure the efficacy of the washing procedure. Ivabradine was isocratically separated on a 5-µm ODS Hypersil column (250 x 4.6 mm) guarded by a 5-µm ODS Hypersil column

(10 x 4.6 mm; Uniguard holder) with a mixture of acetonitrile and 0.025 mmol/L KH_2PO_4 buffer (40:60, v/v, pH 1.7) the latter containing 0.3% (v/v) of 1 mol/L HCl. The flow rate was 0.8 mL/min (25°C); the injection volume was 40 µL. For calibration, ivabradine dilutions (ranging from 0.01 to 1 µmol/L) were prepared freshly in Tyrode solution. Fluorescence was monitored at excitation/emission wavelengths of 283 and 328 nm. The HPLC apparatus consisted of an L-2200 autosampler, L-2130 HTA pump and L-2480 fluorescence detector (VWR Hitachi, Tokyo, Japan). Detector signals were recorded with a personal computer. The program EZchrom Elite (Scientific Software Inc., San Ramon, CA, USA) was used for data acquisition and analysis.

11. Computer simulation

A modeling approach was used to get first indications of the I_f-mediated effect of ivabradine on sinoatrial beating rate under elevated S-LPS levels. Due to unavailability of an appropriate human computational model a recently published rabbit sinoatrial pacemaker cell model was used [11]. This model reproduces the effect of I_f inhibition on beating rate in good agreement to experimental findings (e.g. ~ 20% rate reduction by 3 µmol/L ivabradine). The dynamics of the rabbit sinus node model were computed for I_f conductance values scaled between 0.0 and 1.0 (5% increment) and for I_f $V_{1/2}$ steady-state activation values between -52.5 mV (i.e. the default value in the original model code [11]) and -66.5 mV (1 mV step size). To achieve a stable cycle length of spontaneous action potentials the model was simulated 70 s for each parameter setting. Cycle length (CL) was calculated within the last 10 s. The differential equations system of the model was implemented in Matlab and a variable step solver (ode15s) was used for numerical integration.

12. Statistics

Results are presented as mean ± standard error of mean (SEM), n represents number of experiments or number of cells/number of patients. Statistical significance was tested by Student´s t-test or one-way ANOVA with adequate post hoc tests (Tukey, Dunett), using IBM SPSS software. P-values ≤ 0.05 were considered statistically significant. All tests were 2-sided.

Supplemental References

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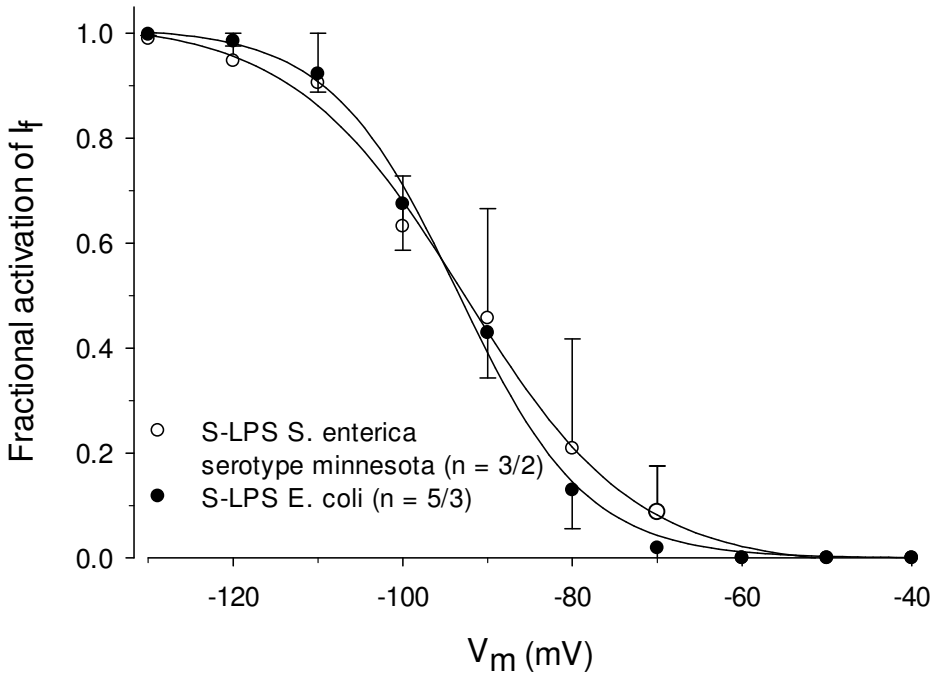
Patients	ACE inhibitor	Angiotensin receptor antagonist	Antiarrhythmics	Anticoagulants	β-blocker	Calcium channel blocker	Digitalis	Diuretics	Nitrates	Statins
1		+								
2		+		+	+	+				
3	+			+	+			+	+	
4	+					+		+		
5		+								
6				+	+				+	+
7										
8					+					+
9									+	
10	+				+					
11				+	+					
12					+			+		+
13						+		+		
14	+			+	+					+
15	+				+					+
16	+			+	+			+		
17					+	+		+		
18					+					
19	+				+					
20	+									+
21					+		+	+		
22				+	+	+				+
23					+					+
24					+			+		+
25				+		+			+	+
26				+					+	
27	+			+	+					+
28	+			+				+		+
29	+			+		+				+
30	+				+	+		+		
31	+			+		+				+
32	+				+					+
33		+		+	+			+		
34	+			+	+					+
35	+			+	+			+		
36		+		+				+		
37					+					
38			+		+					
39					+			+		+
40	+				+	+				+
41	+			+	+			+		
42					+					+

Supplemental Table 1 Drug therapy of patients.

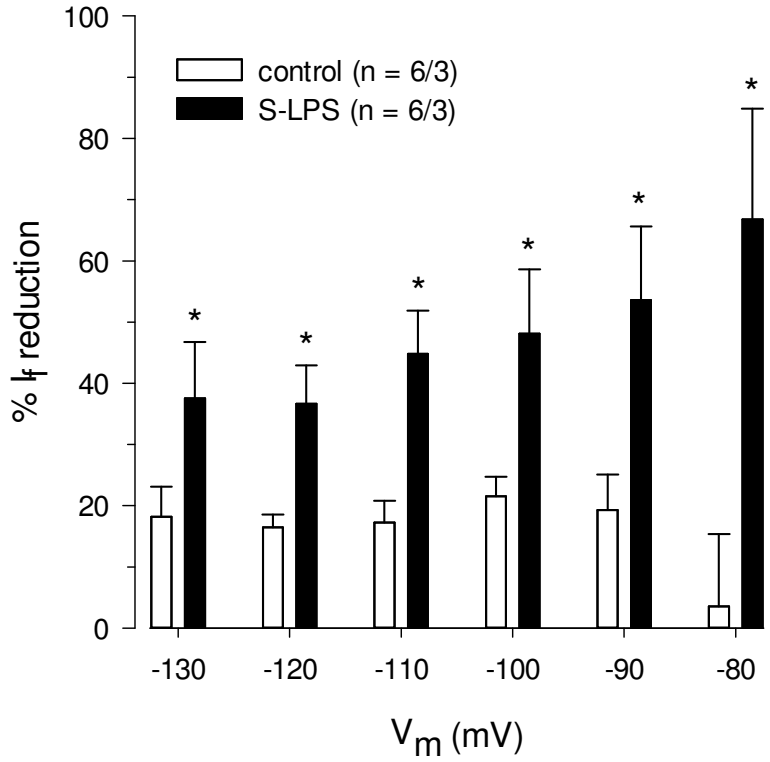
Supplemental Fig. S1



I_f steady-state activation of human atrial myocytes incubated with different S-LPS preparations.

Cells were incubated with S-LPS extracted from *E. coli* or *S. enterica* serotype Minnesota (10 µg/mL, 6 h). Normalized conductances were fitted by a Boltzmann function. *E. coli*: $V_{1/2}$=-93.0±2.1 mV, *k*=6.4±0.5 mV; Salmonella e.: $V_{1/2}$=-90.6±7.9 mV, *k*=6.3±0.5 mV.

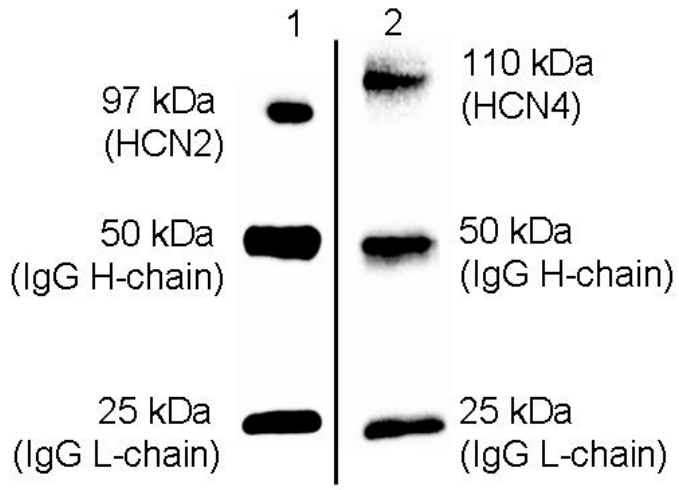
Supplemental Fig. S2



Time-dependent I_f current rundown of human atrial myocytes.

I_f current densities were measured before and after superfusion (6 min) using either I_f-Tyrode solution or I_f-Tyrode solution containing LPS (10 µg/mL) and percentage of I_f reduction was calculated. *p≤0.05 vs. control.

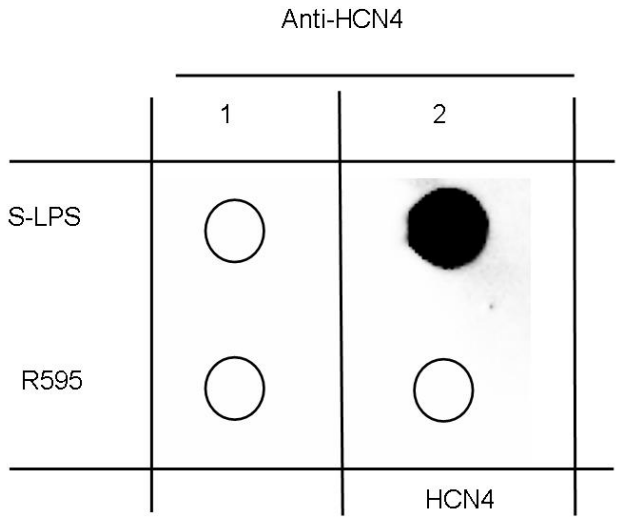
Supplemental Fig. S3



Immunoprecipitation of HCN2 and HCN4 proteins and Western blot experiments.

Immunoprecipitation of HCN2 and HCN4 proteins was performed by incubating mouse heart homogenates with anti-HCN2 or anti-HCN4 antibody. Immunoprecipitates were separated by SDS-PAGE and HCN2 (1) as well as HCN4 (2) were visualized by Western blot experiments. One representative experiment out of 3 is shown.

Supplemental Fig. S4



LPS-HCN4 interactions: Dot-blot analysis for LPS-HCN4 binding.

S-LPS and R595 (10 µg) were cross-linked with methanol prewetted PVDF membranes. Membranes were dried and dots were incubated with PBS (1) or 5 µl of immunoprecipitated HCN4 protein (2). After all incubation steps membranes were incubated with anti-HCN4 antibody and immunoreactive dots were visualized; empty circles represent no immunoreactivity. One representative experiment out of 3 is shown.