ONLINE SUPPLEMENT

Contribution of $K_v7$ channels to basal coronary flow and active response to ischemia

Short title: $K_v7$ channels in coronary artery regulation

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SUPPLEMENTAL METHODS

Isometric Tension Recordings
First, second and third order branches of LAD coronary arteries from normotensive and hypertensive rats were carefully dissected and transferred to ice-cold Krebs Solution (in mmol/L: 133 NaCl, 4.6 KCl, 2.5 CaCl₂, 16.3 NaHCO₃, 1.75 Na₂HPO₄, 0.6 MgSO₄, 10 glucose) and cleaned from adherent connective tissue. These vessels were threaded on 40 µm tungsten wires and mounted in an isometric wire myograph chamber (DanishMyo Technology, Aarhus, Denmark) containing Krebs solution constantly aerated with 95% O₂/5% CO₂ at 37°C. All vessels were subjected to a standard warm-up protocol where they were repeatedly stimulated with 60 mmol/L KCl. PowerLab4/25-Chart5 and 7 acquisition systems (ADInstruments Ltd, Oxford, UK) were used to record force which was converted to tension by dividing the force with double the segment length. By cumulative addition of the synthetic analog of prostaglandin U46619 within a concentration range of 0.1-10 nmol/L a stable vessel contraction corresponding to the same contraction achieved by applying 60-100 nmol/L KCl was determined to be 1nmol/L U46619. Subsequently concentration-response relationship curves of Kᵥ7 activators ((S)-1, BMS-254352, retigabine (1-30 µmol/L) and R-L3 (1-10 µmol/L), β-adrenoceptor isoproterenol (1-30 µmol/L), the NO donor SNP (0.5-10 µmol/L) and the metabolic substrates of adenosine (1-30 µmol/L) were established by cumulative addition of drugs in presence and absence of linopirdine coronary arteries from both normotensive and hypertensive SHR rats. Effects of Kᵥ7 blockers on resting tone were addressed by cumulative addition of linopirdine, XE-991 (1-10 µmol/L) or HMR-1556 without use of U46619. Investigation of protein kinase A activity in normotensive vessels was performed by incubation of coronary arteries with 500 nmol/L PKA inhibitors KT5720 or H89. Dimethylsulfoxide (DMSO) was used as control in all experiments.

Langendorff-Perfused Hearts
Rats were anesthetized and anticoagulated by intraperitoneal injection of heparin (1000 UI/kg). The hearts were excised, placed in ice-cold perfusion solution and mounted on a perfusion system (Hugo Sachs Elektronik–Harvard Apparatus GmbH, March-Hugstetten, Germany) and perfused in the Langendorff mode at constant perfusion pressure (80 mmHg) with warm (37°C) modified Krebs-Henseleit (KH) solution (in mmol/L: 118.0 NaCl, 4.7 KCl, 2 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 2.0 sodium pyruvate, 24.9 NaHCO₃, and 10.0 glucose, pH of 7.4–7.5). KH solution was continuously aerated with 95% O₂/5% CO₂ and was filtered through an in-line 0.45 µm Sterivex-HV filter (Millipore A/S, Hellerup, Denmark) before delivery to the heart. Coronary flow was monitored via an ultrasonic flow meter probe (Transonic Systems Inc., Ithaca, USA) located above the aortic cannula. Electrical activity of the heart was assessed from volume-conducted ECG, recorded via four Ag/AgCl electrodes placed in the perfusate-filled chamber around the heart preparation. Aortic pressure, coronary flow, ECG and perfusate temperature were continuously monitored using the 16-channel Powerlab system (ADInstruments, Oxford, UK) and recorded by LabChart 7 software. All pharmacological agents ((S)-1, XE-991, linopirdine) and DMSO were infused to the heart via perfusate. Subsequently, the effect of blocking Kᵥ7 channels on tachycardia-induced vasodilation was investigated by increasing the pacing frequency from basic rate of 380 to 440, 500 and 600 bpm at 150 second intervals. Reactive hyperemic responses were induced by 30 seconds and 90 seconds of no-flow to the cannulated aorta and thereby the coronary arteries, global ischemia periods, followed by at least 8-9 minutes of reperfusion at which coronary flow returned to the pre-occlusion levels. The following
indices of reactive hyperemic responses were assessed: basal flow rate (mL/min) [mean flow rate over 2 min pre-occlusion], flow debt (mL) [basal flow rate (mL/min) multiplied by ischemia duration (min)], excess hyperemic flow (mL) [integral of flow over initial 5 min of reperfusion (mL) minus integral of flow over 5 min pre-occlusion (mL)], flow repayment (%) [excess hyperemic flow (mL)x100% divided by the flow debt (mL)], duration of reactive hyperemia (s) [time span from the start of reperfusion to the return of the flow rate to the level 5% higher than basal value], peak hyperemic flow rate (mL/min) [maximal flow at reperfusion].

**Quantitative Polymerase Chain Reaction**

Total RNA was extracted from first order LAD coronary arteries from normotensive and hypertensive rats using the RNeasy Micro Kit (Qiagen, Manchester, UK) as described. RNA was quantified using a Nanodrop Spectrophotometer (LabTech International, Carlson City, U.S) and reverse transcribed with Oligo(dT)12-18 primers and Moloney Murine Leukemia Virus (M-MLV; Invitrogen, Paisley, UK). Negative controls (RT-) were carried out in the absence of M-MLV to check for genomic contamination. Quantitative mRNA analysis was determined in duplicate reactions of 10 µL volumes using Precision-iC SYBR green master mix (PrimerDesign, Ltd., Southampton, UK) with the CFX96™ Real-Time PCR Detection System (Biorad, Hertfordshire, UK). Cycle threshold (Ct) values were determined using Bio-Rad CFX96 software and the single threshold mode. To determine the optimal reference genes we used the rat geNorm Reference Gene Selection Kit (PrimerDesign, Ltd., UK), consisting of 12 commonly used reference genes, which were run on normotensive rat (n=3-6) and SHR (n=3-6) LAD coronary artery cDNA samples. The data were then analyzed with geNorm software to determine the best reference gene(s) and number of reference genes required for the most accurate gene normalization (Vandesompele et al., 2002). For the coronary artery experiments the optimal number of reference genes was 2 (where geNorm V < 0.15): β-Actin and GAPDH. In the aorta the reference genes used were CYC1 and MDH1, and in the mesenteric artery ATP5B and YWHAZ were used as reference genes, determined under our experimental conditions as the most stable in the given samples (PrimerDesign, Ltd., UK). No template controls (NTCs) were run alongside all reactions to assess contamination. To determine the change in KCNQ1-5 and KCNE1-5 expression between normotensive rat and SHR arteries, the 2-ΔΔCt method was used (Livak and Schmittgen, 2001). Primer sequences are listed in Suppl. Table S1.

**Western Blot Analysis**

LAD coronary arteries from normotensive (n=3-9) and hypertensive rats (n=3-9) were homogenized in 200 µL lysis buffer (2 mmol/L EDTA, 20 mmol/L Tris base, 1% NP40, 137 mmol/L NaCl, 10% glycerol, 10 µL/mL protease inhibitor cocktail; Sigma-Aldrich, Brøndby, Denmark), centrifuged to remove cell debris, and denatured at 95°C for 5 minutes in the presence of reducing agent and sample buffer (Invitrogen, Nærum, Denmark). Proteins were separated on 4-12% SDS-polyacrylamide gels (Invitrogen) and transferred to immunoblot PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 5% milk in PBS-Tween (0.1%) and incubated with the primary antibody against Kv7.4 (1 µg/mL; Santa Cruz, sc-50417, UK). The membrane was washed, reprobed for β-actin (0.4 µg/mL; Sigma-Aldrich, A1978, Germany) and visualized by enhanced chemiluminescence (ECL) staining. All bands were normalized against β-actin.
SUPPLEMENTAL REFERENCES

### SUPPLEMENTAL TABLES AND FIGURES

#### Supplemental Table S1: KCNQ and KCNE primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>GenBank Accession Number</th>
<th>Amplicon (bp)</th>
<th>Region Spanned (nt)</th>
</tr>
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<tbody>
<tr>
<td>KCNQ1</td>
<td>(+) 5’-CCATCTTTGTTCATCCCCCATCT-3’ (-) 5’-CCAGTTGTGTCACCTTGCTTT-3’</td>
<td>NM_032073</td>
<td>100</td>
<td>1797-1896</td>
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<td>KCNQ2</td>
<td>(+) 5’-GGTGTCCTCATCTTCGTCTCTT-3’ (-) 5’-TCCGCGTTTTCTCAAAGTG-3’</td>
<td>NM_133322</td>
<td>100</td>
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<tr>
<td>KCNQ3</td>
<td>(+) 5’-ATACACATTTATCTGCTTTCTTTITA-3’ (-) 5’-TGCTTCAGTTTATCCGAATCAA-3’</td>
<td>NM_031597</td>
<td>122</td>
<td>3299-3420</td>
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<td>KCNQ4</td>
<td>(+) 5’-GCTCATCTTCCGCTTTTCC-3’ (-) 5’-GCCAATGGTCGTCAGTGTAAT-3’</td>
<td>XM_233477</td>
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<td>KCNQ5</td>
<td>(+) 5’-CCTGGCGTACACAGAGATAT-3’ (-) 5’-TTTGACTGGGCGAACTGAAC-3’</td>
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<td>KCNE1</td>
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<td>NM_012973</td>
<td>114</td>
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<td>KCNE2</td>
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<td>KCNE3</td>
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<td>Antisense Primer</td>
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<td>KCNE5</td>
<td>5’-GTCAACCGCGTCCTGGAG-3’</td>
<td>5’-CAGCAGCAAGCGGTTCAA-3’</td>
<td>NM_00110100</td>
<td>96</td>
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(+): sense, (-): antisense; bp: base pair, nt: nucleotide. Information on proprietary primers used in the geNorm Reference Gene Selection Kit is not available.
### Supplemental Table S2. Indices of coronary reactive hyperemia

<table>
<thead>
<tr>
<th>Parameter/Treatment group</th>
<th>DMSO (n=8)</th>
<th>linopirdine / XE-991 (n=9)</th>
<th>SHR (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal flow rate (mL/min)</td>
<td>12.7±0.3</td>
<td>11.5±0.2,*</td>
<td>7.79±2.1,*</td>
</tr>
<tr>
<td>Flow debt (mL)</td>
<td>19.0±0.47</td>
<td>17.2±0.35,*</td>
<td>9.78±2.3,*</td>
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<tr>
<td>Excess hyperemic flow (mL)</td>
<td>19.8±1.8</td>
<td>13.5±0.9,*</td>
<td>0.97±2.4,*</td>
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<tr>
<td>Flow repayment (%)</td>
<td>105.7±11.6</td>
<td>78.8±5.3,*</td>
<td>14.5±8.8,*</td>
</tr>
<tr>
<td>Duration of reactive hyperemia (s)</td>
<td>324.6±31.9</td>
<td>219.9±18.0,*</td>
<td>187.6±8.0,*</td>
</tr>
<tr>
<td>Peak hyperemic flow rate (mL/min)</td>
<td>30.2±0.3</td>
<td>27.4±0.7,*</td>
<td>19.4±1,*</td>
</tr>
</tbody>
</table>

Reactive hyperemia in isolated, perfused NT hearts infused either with DMSO or a K<sub>v7</sub> blocker and reactive hyperemia in SHRs as compared to its control. Data are mean ± SEM. An un-paired t-test is used for the comparison and * indicates P<0.05.
Supplemental Figure S1: Diameter size and force of first, second and third order LAD branches in normotensive rats and comparison of basal force between normotensive and hypertensive LAD. No differences were found in diameter size (A) and basal force (B) of first, second and third order LAD coronary artery branches in normotensive rats upon normalization. No differences were found in basal force in comparison of normotensive and hypertensive arteries (C). An one-way ANOVA test was used in the panel A-B and an unpaired t-test in the panel C.
Supplemental Figure S2. Effect of HMR-1556 used to specifically inhibit the function of $\text{K}_7.1$ channels in non-pre-constricted coronary arteries. (A) Isometric tension responses to accumulative application of HMR-1556 in a concentration range of 1-10 μmol/L as compared to dimethyl sulfoxide (DMSO) control vehicle in normotensive rats. Statistical significance was found using a two-way ANOVA followed by a Bonferroni post-test between HMR and DMSO vehicle group, **$P<0.01$ and ***$P<0.001$. 
Supplemental Figure S3. Contraction to high K⁺ concentrations and prostaglandin agonist U46619 in normotensive and hypertensive rats. (A) Comparison of isometric tension responses to 60 mmol/L KCL and 1nmol/L U46619 in LAD coronary arteries of normotensive and hypertensive rats. (B) Dose-dependent responses to cumulative application of the prostaglandin agonist U46619 in normotensive and hypertensive SHR vasculature.
Supplemental Figure S4. Relaxation to Kv7 activator in vessels with intact and denuded endothelium and in different branch orders of LAD arteries in normotensive rats. (A) Relaxation to 10 μmol/L Carbachol was significantly impaired in denuded coronary arteries as compared to LAD vessels with intact endothelium. (B) Relaxation to (S)-1 was comparable between vessels with intact endothelium and endothelium-denuded LAD coronary arteries. (C) No significant dilative response differences were when comparing the effect of (S)-1 in first, second and third order branches of LAD coronary artery from normotensive rats. Statistical analysis was performed by two-way ANOVA test.
Supplemental Figure S5. Relaxation to nicardipine and pinacidil in normotensive and hypertensive rats. Both nicardipine (10 nmol/L) (A) or pinacidil (10 µmol/L) (B) had a vasodilatory effect on preconstricted vessels of both normotensive and hypertensive vessels. No differences in percentage relaxation were found between normotensive and hypertensive arteries as assessed with unpaired t-test. (C) illustrates the vasorelaxant effect of 10 µmol/L K<sub>ATP</sub> opener pinacidil in presence of 10 µmol/l K<sub>V7</sub> channel blocker linopirdine on preconstricted LAD coronary arteries of normotensive rats. Similarly, the K<sub>V7</sub> activator (S)-1 has a vasorelaxant effect on preconstricted coronary arteries from normotensive rats in the presence of the K<sub>ATP</sub> channel blocker glibenclamide. Significant difference were found by *** p<0.001 by ANOVA Bonferroni post-test which was done on mean values of 4-9 vessels ±SEM.
Supplemental Figure S6. Effect of PKA activation and inhibition in LAD of normotensive rats. (A) Concentration-dependent effects of adenosine 2A receptor agonist, cyclic AMP analog 8-Br-cAMP, on normotensive coronary arteries. (B) Concentration-dependent effects of adenosine on LAD of normotensive rats upon incubation with PKA inhibitors KT5720 or H89 (1 μmol/L) for 1 hour. Significant difference in responses to 8-Br-cAMP in presence and absence of 10 μmol/L linopirdine upon incubation with PKA inhibitors by ***p<0.001 by ANOVA Bonferroni post-test which was done on mean values of 9 vessels ±SEM.
Supplemental Figure S7. Isometric tension responses of coronary arteries to β-adrenergic stimulation. (A) Application of isoproterenol resulted in vasodilation of coronary arteries in normotensive rats. The vasodilatory effect was significantly attenuated upon linopirdine. (B) However, coronary arteries of hypertensive SHR rats were resistant to isoproterenol-mediated vasorelaxation. (C) The same attenuated effect of isoproterenol was found in normotensive coronary arteries upon PKA inhibition. Two-way ANOVA and a Bonferroni post-test was used, * P<0.05, ** P<0.01 and ***P<0.001.
Supplemental Figure S8. Isometric tension responses of coronary arteries to NO-donor SNP. Application of SNP resulted in vasodilation in normotensive rats and this effect was not changed upon linopirdine application. Vasodilation to 10 and 30 μmol/L SNP was attenuated in hypertensive as compared to normotensive vasculature. PKA inhibition had no effect on vasodilatory responses to SNP in normotensive vasculature. Two-way ANOVA and a Bonferroni post-test was used and § indicated $P<0.05$, ** $P<0.01$ and *** $P<0.001$