

Supplementary Materials for

Inhibition of aquaporin-1 prevents myocardial remodeling by blocking the transmembrane transport of hydrogen peroxide

Virginie Montiel, Ramona Bella, Lauriane Y. M. Michel, Hrag Esfahani, Delphine De Mulder, Emma L. Robinson, Jean-Philippe Deglasse, Malte Tiburcy, Pak Hin Chow, Jean-Christophe Jonas, Patrick Gilon, Benjamin Steinhorn, Thomas Michel, Christophe Beauloye, Luc Bertrand, Charlotte Farah, Flavia Dei Zotti, Huguette Debaix, Caroline Bouzin, Davide Brusa, Sandrine Horman, Jean-Louis Vanoverschelde, Olaf Bergmann, Dimitri Gilis, Marianne Rooman, Alessandra Ghigo, Simonetta Geninatti-Crich, Andrea Yool, Wolfram H. Zimmermann, H. Llewelyn Roderick, Olivier Devuyst, Jean-Luc Balligand*

*Corresponding author. Email: jl.balligand@uclouvain.be

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Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/12/564/eaay2176/DC1)

Data file S1 (Microsoft Excel format). Individual subject-level data for main figures.

Data file S2 (Microsoft Excel format). Individual subject-level data for supplementary figures.

Materials and Methods

- *Animal experiments*

On the day of surgery, each mouse was anesthetized with ketamine hydrochloride (84 mg/kg; intraperitoneally, IP) and xylazine hydrochloride (10 mg/kg, IP), and received analgesia after surgery (Buprenorphine 0.1 mg/kg, IP). Ascending aorta constriction (TAC) was performed on 16-week-old male mice as follows. After anesthetizing the mouse, a constrictive band was placed and tightened around the aorta to the diameter of a 27 G needle. The ligature was not tightened in sham-operated mice. Animals were treated with 0.1 mg/kg of Buprenorphine after the surgery. Doppler measurements of trans-stenotic gradients were systematically performed at day 1, then at weeks 3 (and in some cases 9 weeks) post surgery. Only mice with a velocity higher than 2.5 m/s were entered into the experiment. Osmotic minipumps were implanted in similarly anesthetized 16-week-old male mice and Ang II (2 mg/kg/d) or saline control infused for 14 days.

Blood pressure telemetry recording in vivo

Implants were surgically inserted as previously reported (10). The use of miniaturized chronically implanted instruments with a recovery period of 10 days reduced the confounding effects of stress on the animals. After the 10-day recovery period, osmotic minipumps were implanted in mice and Ang II (2 mg/kg/d) infused for 14 days. Recordings were carried out during 24 hours at the end of the 14-day treatment with Ang II (i.e. at day 14).

Experiments on patient samples

Cardiac biopsies were immediately frozen in liquid nitrogen and stored at -80°C. After obtaining myocardial ventricles, the right and left ventricles were dissected, removing epicardial fat and visible blood vessels. The left ventricle myocardial tissue, without the septum, was snap-frozen and stored at -80°C until further processing.

- *Reverse transcription and real-time quantitative polymerase chain reaction in mouse tissue and human cardiac biopsies*

Total RNA was extracted from heart tissue of *Aqp1*^{+/+} and *Aqp1*^{-/-} mice sacrificed 9 weeks after TAC or Sham surgery using TRI-Reagent (Fermentas, Alost, BE) according to the manufacturer's instructions. Genomic DNA contamination was prevented by treatment with RQ1 RNase-free DNase (Promega, Inc.). 1.5 µg of total RNA from each sample was reverse transcribed. Total mRNA from patient biopsies was extracted with an RNA extraction kit

(RNeasy mini-kit Qiagen 74106) and subjected to on-column treatment with RNase-free DNase set (Qiagen 79254). Subsequently, 400 ng of RNA was reverse-transcribed with RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, Alost, BE) using OligodT and random hexamers (Thermo Scientific).

Determination of BNP (*Nppb*), α -MHC (*Myh6*), β -MHC (*Myh7*) and hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) expression was carried out by qPCR on murine cDNAs diluted 1:25. cDNAs prepared from the micro biopsies diluted 1:10 were used to determine the expression of Aquaporin 1 (*AQP1*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Both qPCRs were performed using low ROX Takyon (Eurogentec) on a Viia 7 Real time PCR detection system (Life technologies). The exon-overlapping amplicons were amplified using the calibrated primers sets presented in Table S2. qPCRs were performed in triplicate for each sample. Results are expressed as $2^{-\Delta\Delta C_t}$ normalized on housekeeping gene (*HPRT* or *GAPDH*) expression according to the Livak method. LV mass of corresponding patients were determined by echocardiography before surgery for aortic stenosis.

- *RNA profiling including RT-qPCR in human myocardial tissue*

Individual patient details are listed in table S1.

Nuclear isolation: To minimize handling times and to control potential inter-experimental artefacts, nuclei were isolated and sorted from 2 left ventricular samples in parallel per experiment, one hypertrophic sample and one corresponding control sample. The method for CM nuclear isolation were based on a previously described method (20, 69). All buffers were precooled, supplemented with dithiothreitol (DTT, 1 μ M), actinomycin D (98 %, HPLC, resuspended in water, Sigma Aldrich, 1mg/l), *cOmplete* protease inhibitor cocktail (Roche; 0.5 μ L/mL) and SUPERase In RNase Inhibitor (Ambion; 200 U/ml), and buffered with 10 mM Tris-HCl to pH 8.0. Frozen hearts were thawed in 10 ml lysis buffer (0.32 M sucrose, 5 mM CaCl_2 , 5 mM EDTA, 0.5 mM EGTA, 3 mM MgAc) and homogenized using an IKA Ultra-Turrax (20,000 RPM, 15 s). After addition of 30 ml lysis buffer, the homogenate was then dounced 8 times on ice with a hand-held dounce homogenizer (Sigma Aldrich, Pestle A), filtered consecutively over mesh, a 100 μ m and a 70 μ m cell strainer and spun for 10 min (700 x g at 4 °C). The resulting pellet was dissolved in 10 ml centrifuge buffer (2.1 M sucrose, 3 mM MgAc, and 5 mM EDTA), and laid upon 5 ml centrifuge buffer in an autoclaved Thinwall Ultra-Clear Tube (Beckman Coulter). Tubes were loaded in a precooled rotor (SW32.1Ti) and centrifuged for 60 min (26,000 x g at 4 °C) in an OPTIMA MAX Ultracentrifuge (both from Beckman Coulter). The resulting pellet was dissolved in 0.5 ml sorting buffer (0.43 M sucrose, 70 mM KCl, 2 mM

MgCl₂ and 5 mM EDTA) and labeled for 1 h on ice using 3 µl anti-PCM1 antibody (Sigma Aldrich Prestige Antibodies, HPA023374) that was pre-incubated for 15 min on ice with 2 µl Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) Antibody (Life Technologies).

Flow sorting: Nuclei were sorted on an Influx streaming air high-speed cell sorter or FACSaria III (BD Biosciences) using a gating strategy as described (69, 70). Nuclei were sorted into DNA LoBind tubes (Eppendorf) on a cooled tube holder. Tubes were preloaded with 50 µl EDTA (10 mM) and SUPERase In RNase Inhibitor (100 U) and 100,000 PCM-1 positive nuclei were collected per tube for RNA extraction. The solution of sorted nuclei for RNA extraction was mixed with 0.75 ml TRIzol LS reagent (Ambion Life Technologies) and stored at -80°C until further processing.

RNA-seq Library Preparation: The use of nuclear RNA omitted the need for ribosomal RNA depletion. Strand-specific RNA-seq libraries were prepared using the following protocol.

RNA extraction: Total nuclear RNA was extracted using TRIzol LS by phenol-chloroform extraction following the manufacturer's instructions, including 1 µl glycogen as an inert RNA carrier, and dissolved in 20 µl nuclease free water (Ambion Life Technologies).

Fragmentation and Priming: Nuclear RNA was used in its entirety and extracted. RNA was fragmented and primed as follows: 3 µg Random Primers (mostly hexamers; Life Technologies) were added to 10 µl RNA and 8 µl 5X First-Strand Buffer. This mixture was heated to 94 °C for 3 min for RNA fragmentation, and incubated on ice for at least 1 min.

First strand and second strand cDNA synthesis: Reverse transcriptase was then performed by adding 2 µL dNTP Mix (10 mM) (Life Technologies) as well as 4 µl DTT (100 mM), 40 U SUPERase In RNase Inhibitor, 400 U SuperScript III Reverse Transcriptase (Thermo Life Technologies) and 8 µg actinomycin D. Water was added to make the volume up to 40 µl, and the sample was incubated at 25 °C for 5 min 50 °C for 30 min and inactivated at 70 °C for 15 min. cDNA-RNA duplexes were purified using 72 µl (1.8X) Agencourt AMPure XP (Beckman Coulter) and eluted in 20 µl of the following second-strand synthesis reaction mix: 0.33 mM dATP, dCTP and dGTP (Life Technologies), 0.33 mM dUTP (Sigma Aldrich), 0.25 U Ribonuclease H (Ambion Life Technologies), 10 U DNA polymerase I (New England Biolabs), 1X NEB buffer 2. The reaction was allowed to proceed for 2.5 h at 16 °C.

End repair, indexing and amplification: Second strand synthesis reactions were purified using 1.8X Agencourt AMPure XP (Beckman Coulter) and eluted in 15 µL dA-tailing mix (NEBNext dA-Tailing Module, New England Biolabs). This reaction was incubated at 37 °C for 30 min, purified using 1.8 X volume Agencourt AMPure XP (Beckman Coulter) and eluted in 16.25 µl nuclease free water. Adaptor ligation was then performed by adding 5 µl 5X Quick ligation reaction buffer (New England Biolabs), 2.5 µl Quick T4 DNA ligase and 1.25 µl Universal NEBNext adaptor (750 nM and incubated at 20 °C for 15 min. 3 µl USER (Uracil-Specific Excision Reagent, New England Biolabs) enzyme was then added. The latter mix was incubated at 37 °C for 30 min before purification with 1.8X Agencourt AMPure XP beads and elution in 20 µl nuclease free water.

To then amplify libraries, 25 µl NEBNext 2X High-Fidelity PCR Master Mix (New England Biolabs) was added to each sample, together with 2.5 µl NEBNext universal primer and 2.5 µl of an indexed primer (500 nM final concentration each primer, NEBNext indexed primer set 1 or 2, New England Biolabs). The PCR amplification was performed as follows: 98 °C for 30 s, 15 cycles of 98 °C 10 s, 65 °C 30 s followed by 72 °C for 30 s and then a final 72 °C for 7 min. For size selection and purification, amplified libraries were separated on 1.5 % agarose gel (Ultrapure, Life Technologies) alongside Hyperladder II (Bioline), visualized using Molecular Imager Gel Doc XR+ System (BIO-RAD) and bands excised from the agarose gel as required using a surgical scalpel and UV safety shield. Libraries were purified using the QIAquick gel extraction kit (Qiagen) according to manufacturers' instructions, with the modification of using MinElute columns (Qiagen) and the final elution in 15 µl nuclease free water.

RNA-seq library QC and sequencing: DNA fragment size and the absence of adaptor dimers was verified on a 2100 Bioanalyzer Instrument using the High Sensitivity DNA Analysis Kit (Agilent Technologies), and DNA concentration was verified using the Quantus and QuantiFluor dsDNA quantification kit (both Promega).

Libraries were sequenced on an Illumina HiSeq 2500 (Illumina).

RNA-seq analysis: RNA-seq reads were aligned using Bowtie2 against the human genome (GRCh37/hg19) and processed using Tophat and Cufflinks (71). Read counts of genomic features of interest were determined using the SeqMonk Mapped Sequence Data Analyser (www.bioinformatics.babraham.ac.uk/projects/seqmonk, Version 0.35.0) and differential gene expression analysis performed using DESeq2 (72). Analysis of gene expression was based on

strand-specific read counts, with only reads mapping to the strand opposite of the annotated gene orientation being counted. Differential RNA expression was assessed based on differences between control/healthy samples and hypertrophic samples, grouped as biological replicate sets from 4 independent experiments.

Gene ontology analysis was performed using the DAVID Bioinformatics Resources 6.7 tool (<https://david.ncifcrf.gov/>) online tool (73-75). Gene lists were uploaded, converted to UCSC IDs and functional clustering analysis performed according to biological function. Biological classifications are presented with a fold enrichment score and adjusted *P* value, generated using Benjamini Hochberg correction and FDR of 5 %.

Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) for AQPs and hypertrophy markers in hypertrophic human samples (Fig. 1, D and fig. S1, E to G): Total nuclear RNA was extracted using TRIzol LS by phenol-chloroform extraction following the manufacturer's instructions, including 1 µl glycogen as an inert RNA carrier, and dissolved in 20 µl nuclease free water (Ambion Life Technologies).

cDNA synthesis: 150 ng RNA was primed with 2 µg random hexamers (Roche) and reverse transcribed using SuperScript III Reverse Transcriptase (Life Technologies) following the manufacturer's instructions.

Gene expression analysis: cDNA was analyzed by real-time quantitative PCR for the expression of genes of interest on a CFX384 (BIO-RAD) using the Platinum SYBR Green qPCR SuperMix-UDG (Thermo Life Technologies) and oligonucleotide primers (200 nM final concentration, Integrated DNA Technologies). Gene expression was normalized to the geometric mean of the expression of multiple reference genes (*GAPDH*, *ACTB* and *β2-M* for control and hypertrophic (LV CH) samples following the $\Delta\Delta C_t$ method, and expressed relative to control/healthy samples (76). Primer sequences can be found in table S2.

- *Human myocardial tissue immunohistochemistry*

For AQP1 detection on human heart paraffin sections, after deparaffinization, endogenous peroxidases were inhibited 20 min with 3% H₂O₂ in methanol. Sections were then subjected to antigen retrieval in 10 mM citrate buffer pH 5.7 and to blocking of aspecific antigen binding sites (Tris buffered saline (TBS) + powder milk (2% in

TBS) + Bovine Serum Albumin (BSA) 5% + Human IgG 1% + 1% Tween20). Anti-AQP1 primary antibody (Rabbit Anti-Aquaporin 1 antibody, AB2219, Millipore) was incubated overnight at 4 °C at a 1/5000 dilution. This was followed by an incubation with Envision anti-rabbit secondary antibodies (Dako K4003) for 30 min at room temperature. This reaction was visualized using Alexa488-conjugated tyramide (ThermoFisher B40953). Membranes were stained with rhodamine-conjugated wheat-germ agglutinin (Vector, RL-1022). Finally, nuclei were stained with Hoechst33342 and slides were mounted with Dako Fluorescent Mounting medium. Images were acquired using a Zeiss AxioImager equipped with an ApoTome module.

- *Human red blood cells*

Human isolated red blood cells were resuspended at 0.1% hematocrit in HBSS, preincubated with bacopaside II (15 µM) or vehicle for 30 min at 18 °C, washed once (350 x g, 5 min) with HBSS and incubated with CM-H2-DCFDA (1 µM; Invitrogen C6827) for 30 min at 37 °C. Samples were then washed twice and treated with the catalase inhibitor, NaN₃ (1 mM; Sigma S-8032) for 10 min before H₂O₂ (10 µM) addition. Data acquisition was performed with a FACSCantoII flow cytometer (BD Biosciences) and data analysis with FlowJo software (BD Biosciences).

- *Antibodies*

For western blot analysis, we used the following primary antibodies at the indicated dilutions: monoclonal rabbit anti-Phospho-p38 MAPK (Thr180/Tyr182) antibody (#4511, Cell Signaling, 1/1000), rabbit anti-p38 MAPK antibody (#9212, Cell Signaling, 1/1000), rabbit anti-Phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204) antibody (#9101, Cell Signaling, 1/1000), rabbit anti-p44/42 MAPK antibody (#9102, Cell Signaling, 1/1000), purified mouse anti-HSP-90 antibody (610419, BD Transduction Laboratories, 1/2500), rabbit anti-Aquaporin-1 antibody (AB2219, Millipore, 1/1000). Secondary antibodies were all from Jackson ImmunoResearch (1/5000).

- *Preparation of isotypic cultures of cardiac myocytes and fibroblasts from mice*

Adult mice were anesthetized with ketamine/xylazine. Hearts were quickly excised, cannulated and mounted onto a Langendorff system. Subsequently, hearts were perfused at 37 °C with a Liberase-containing solution (113 mM NaCl, 4.7 mM KCl, 0.6 mM KH₂PO₄, 1.2 mM MgSO₄, 12 mM NaHCO₃, 10 mM KHCO₃, 30 mM Taurine, 25 µg/ml Liberase TH, 10 mM BDM, 5.5 mM Glucose and 10 mM HEPES/NaOH, pH 7.46) at a constant rate of 2,5 ml/min. Once digested, cells were mechanically separated and filtered. Thereafter, cytosolic Ca²⁺ levels were gradually restored. Viable rod-shaped cardiac myocytes were separated by sedimentation and plated on laminin-

coated wells in M199 medium containing 10 mM BDM, 5% FBS and Penicillin/ Streptomycin. After one hour, plating medium was replaced by culture medium (M199 medium, 10 mM BDM, 1X Insulin-transferrin-selenium, ITS, 1 mg/mL BSA and 10 mM HEPES).

In experiments with pro-hypertrophic agonists, culture medium was supplemented with either angiotensin II at 1 μ M, endothelin 0.6 μ M, isoproterenol 60 μ M or phenylephrine 50 μ M without ITS for 18 h; the latter experiment was repeated with addition of Bacopaside II (Sigma-Aldrich, product number 44698) or its solvent control.

Neonatal mouse ventricular fibroblasts and myocytes were isolated from 10-15 hearts of neonatal pups following a protocol adapted from (77). Heart ventricles were removed from neonatal pups, minced into 1-mm³ pieces, and subjected to consecutive digestions with collagenase type II (Worthington Biosciences) and trypsin (Sigma). Cells were separated by using Percoll gradient centrifugation and selective attachment of fibroblasts. Fibroblasts were cultured in DMEM (GIBCO), supplemented with 10% fetal bovine serum (FBS) and 1 % of penicillin/streptomycin, and myocytes in DMEM supplemented with 10% fetal bovine serum (FBS), 10 μ M AraC and 1% of penicillin/streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. Fibroblasts were used at passage 2. Antibodies against vimentin were used for the positive identification of cardiac fibroblasts in each preparation. Approximately 36 h post-isolation, myocytes were transferred to serum-free maintenance media. The myocytes were then treated with 20 μ M phenylephrine (PE) in serum-free conditions. Conditioned media from myocytes after 24 h of PE treatment were collected, centrifuged at 1000 $\times$ g for 15 min to remove debris and directly used for incubation on fibroblasts or stored at -80°C for later use. Cardiac fibroblasts at passage 2 were gradually serum-starved, then incubated in serum-free media for 6 h, and subsequently incubated with the conditioned media from myocytes for 24 h prior to fixation for immunocytochemistry.

- *Preparation of CDRI08 (B. monnieri) extracts for oocyte experiments*

CDRI08 extracts (KeenMind, Flordis) were purchased at a local pharmacy in Adelaide, Australia. A stock solution was prepared following the protocol of Dowell (35). Powdered content of 7 capsules of KeenMind (totaling 3.03g) was mixed into 25 ml of 70% methanol (17.5 ml HPLC-grade methanol with 7.5 ml reverse-osmosis MilliQ-filtered water), sonicated 30 minutes, and centrifuged at 12 k at 18° C for 15 min. The supernatant was transferred into sterile microfuge tubes and stored at 4 °C. This stock methanol extract of KeenMind (120 mg/ml) was diluted to

final concentrations up to 0.45 mg/ml in isotonic Na⁺ saline (1:267 dilution) for use in oocyte swelling experiments; Na⁺ saline with equivalent methanol alone served as the vehicle control.

- *Oocyte preparation and quantitative osmotic swelling assays*

Human AQP1 cRNA from BamHI-linearized AQP1 cDNA (NCBI GenBank: BC022486.1) was synthesized with T3 polymerase (T3 mMessage mMachine; Ambion, Austin, TX). Unfertilized oocytes were harvested from *Xenopus laevis* frogs with protocols approved by the University of Adelaide Animal Ethics Committee (M2018-016) in accord with Australian National Guidelines for animal use, defolliculated with collagenase type 1A (2 mg/ml) in OR-2 saline (82 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, and 5 mM HEPES; pH 7.6) for 1.5 hours, washed in OR-2 saline, and then incubated in Frog Ringers saline (96 mM NaCl, 2 mM KCl, 0.6 mM CaCl₂, 5 mM MgCl₂, and 5 mM HEPES supplemented with 10% horse serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 mg/ml tetracycline, pH 7.6) at 16 to 18 °C. Oocytes were injected with 1 ng of AQP1 cRNA in 30-50 nl of sterile water and incubated for 2 to 3 days at 16–18 °C to allow protein expression. Isotonic Na⁺ saline (Frog Ringers without serum or antibiotics) was used for experimental assays. Osmotic swelling rates were evaluated by introducing AQP1-expressing oocytes into 50% hypotonic saline (isotonic Na⁺ saline diluted with an equal volume of water) at time zero and monitoring cross-sectional area as a function of time, with a grayscale video camera (Cohu, California, USA) on a dissecting microscope (Olympus SZ-PT; Olympus, Arizona USA). Images were captured at 0.5 Hz for 60 s using NIH ImageJ (National Institutes of Health; Maryland USA). Swelling rates were calculated from linear regression slope values using GraphPad Prism (Calif, USA). Each oocyte served as its own control, and was tested first without drug treatment, and then incubated 2 hours in isotonic Na⁺ saline with vehicle (methanol), Bacopaside II (5 to 50 µM) or KeenMind extract (110 to 450 µg/ml), and then reassessed with a second swelling assay.

- *Ultracentrifugation protocol for membrane extracts of murine isolated adult cardiac myocytes*

Extracts from freshly isolated adult cardiac myocytes were incubated in 1 ml of buffer solution containing Tris– HCl 20 mM (pH 7.4), dithiothreitol 0.1 mM, saccharose 250 mM, and a mixture of protease inhibitors (Sigma-Aldrich) 1/1,000 and fractionated by ultracentrifugation (105,000 × g for 20 min at +4 °C). The supernatant (corresponding to soluble cytosolic fraction) was removed and the pellet was suspended in 0.5 ml of buffer solution containing beta-glycerophosphate 80 mM, EGTA 20 mM, MgCl₂ 15 mM, Triton X-100 0.5 %, glycerol 10 %, and a mix of protease

inhibitors. After sonication and further centrifugation to remove cellular debris, the final supernatant (enriched in membrane fragments) was recovered.

- *Western blot analysis*

Denatured proteins were loaded and separated on 15 % SDS polyacrylamide gels (Mini-protean III, Bio-Rad) after which they were transferred to a polyvinylidene difluoride membrane (PerkinElmer). After blocking with 5% BSA in Tris buffered saline supplemented with 0.1 % Tween 20 (TBST, Sigma), membranes were incubated and hybridized with the antibodies cited above (overnight for all the primary antibodies), in TBST containing 5 % BSA. After four washes (15 minutes each), membranes were incubated for 1 hour with the respective secondary antibody in TBST containing 1 % BSA. After four additional washes in TBST, membranes were incubated with a chemiluminescent reagent (ECL, Amersham for HSP90, and Immobilon Western, Millipore for all the others proteins) and exposed to Amersham X-ray or to classic X-ray film. For phosphoproteins, denatured proteins were transferred to nitrocellulose membranes.

- *Hearts and isolated murine adult cardiac myocyte immunohistochemistry*

Murine hearts were dissected, embedded in Neg-50 frozen section compound and subsequently frozen in liquid nitrogen vapors. To quantify the cardiac myocyte area and the number of vessels, heart cryosections were labeled with rhodamine-conjugated–wheat germ agglutinin (WGA), as a membrane marker, and with biotinylated-Isolectine B4, revealed with fluorescein-avidin, as an endothelial marker (all reagents from Vector). For isolated murine adult cardiac myocyte immunocytochemistry, a monoclonal anti- α -Actinin (sarcomeric) antibody (A7811, Sigma-Aldrich, 1/300) was used and revealed with Alexa Fluor-conjugated secondary antibodies (Invitrogen, 1/500). Nuclei were then stained with DAPI and samples were imaged by structured illumination microscopy (Zeiss AxioImager equipped with an ApoTome module). Cardiac myocyte transverse area was determined with Axiovision software.

Fibrosis quantification

Heart cryosections were fixed overnight in 4 % formaldehyde, embedded in paraffin, sectioned at 5 μ m, and stained with picosirius red according to a standard protocol. Stained sections were digitized with a SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany). Quantification was performed using Tissue IA software (Leica Biosystems, Dublin, Ireland). After elimination of artifacts and perivascular fibrosis, the area occupied by interstitial fibrosis was expressed as a percentage of total tissue area. Collagen content as well as relative collagen thickness and

packing/alignment was quantitatively determined by additional microscopic examination using polarized light. Mean results obtained from three different histological sections/heart were calculated.

Immunofluorescence staining and imaging

Hearts were fixed for 24 hours at 4 °C in 4% formaldehyde, embedded in paraffin and sectioned at three levels of the tissue. After deparaffinization, 5 µm tissue sections were processed according to the protocol described in Aboubakar et al. (78). Endogenous peroxidases were inhibited 20 min with 3 % hydrogen peroxide in methanol. Sections were then subjected to antigen retrieval in 10 mM citrate buffer pH 5.7 and to blocking of aspecific antigen binding sites (Tris buffered saline (TBS) + Bovine Serum Albumine (BSA) 5% + 0,1% Tween20). Anti-Collagen I primary antibody (Abcam ab34710) was incubated for 1 hour 30 minutes at RT at a dilution of 1/300 in TBS containing 1% BSA and 0.1% Tween20 and detected by anti-rabbit horseradish peroxidase (HRP)-conjugated polymer secondary antibodies (Agilent) for 1h at room temperature. HRP was then visualized by tyramide signal amplification (TSA) using AlexaFluor647-conjugated tyramides (Thermo Fisher Scientific B40958). After a washing step in PBS, nuclei were finally stained with Hoechst 33342 (Thermo Fisher Scientific) diluted in TBS containing 10 % BSA and 0.1 % Tween 20, washed in TBS containing 0.1 % Tween 20, and mounted with Dako fluorescence mounting medium (Agilent). Slides were digitized using a Panoramic 250 FlashIII scanner (3DHitech) at x20 magnification.

Quantitative evaluation of immunostaining in whole tissue sections

Stainings were quantified on multiplex-stained paraffin sections of entire tissue sections (3 sections/heart) with software applications ("APP"s) using the image analysis tool Author version 2017.2 (Visiopharm, Hørsholm, Denmark). Tissue sections were first delineated at a low digital magnification using a thresholding classification method based on the autofluorescence of the tissue. Macrovasculature and pericardium were excluded (APP2) as well as blurred areas, damaged tissue and artefacts. Manual correction was applied when required. Collagen I staining was detected in the delineated tissue with a thresholding classification method on the AF647 fluorescence. The same parameters were kept constant for all slides. Results were expressed as % of staining in tissue $\{(\text{collagen I area above threshold in tissue area} / \text{tissue area}) * 100\}$.

- Detection of cell water volume changes in adult mouse cardiac myocytes

Cardiac myocytes were isolated from *Aqp1*^{+/+} and *Aqp1*^{-/-} mice as described before and plated on coverslips. 2 hours after isolation cells were loaded with 25 μ M of CellTracker™ Green CMFDA-AM (acetoxymethyl derivative, converted to 5-chloromethylfluorescein by intracellular esterases, then trapped intracellularly; from Thermo Fisher Scientific), for 30 minutes at 37 °C. New medium was added after incubation and cells were cultured overnight. The next day the coverslip was mounted in a perfusion chamber maintained at 37 °C and placed on a spinning disk confocal microscope (CSU10/Nikon Eclipse TE2000-E) equipped with a 20 X objective. The cells were perfused at a flow rate of ~1 ml/minute with an initial iso-osmotic solution (Tyrode solution containing 137 mM NaCl, 5.3 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 10 mM Hepes, pH 7.4, 300 mOsmol l⁻¹) for 5 minutes, followed by a hypo-osmotic challenge (200 or 100 or 50 mOsmol l⁻¹) for 20 minutes and again the isosmotic solution for 5 minutes. A second experiment was performed where cells were perfused with Tyrode solution for 5 minutes followed by the same solution supplemented with phenylephrine (PE; 50 μ M) for 30 minutes.

After trapping of the membrane-permeant dye inside the cell, its content (Qm) remains stable while fractional changes in its concentration over time (Ct) occur if the cell undergoes changes in its water volume (Vt), where $Vt = Qm/Ct$. Accordingly, the fluorescence measured through a region of interest (ROI) in each cell is directly proportional to the dye's concentration (Ct) and can be used to calculate Vt. Indeed, in a perfect osmometer $F_0/F_t = \pi_0/\pi_t$, where F_0 is the fluorescence measured in the cell's ROI during iso-osmotic perfusion having an osmotic pressure π_0 and F_t is the fluorescence recorded in the same area during application of a hypo-osmotic challenge having an osmotic pressure π_t . So, $C_0/C_t = F_0/F_t = \pi_0/\pi_t = V_t/V_0$. Therefore, a ROI was drawn around each cell and fluorescence in that ROI was measured every 4 s. by exciting the CellTracker at 495 nm and recording its emission at 503-552 nm with a Prime 95B camera. All the fluorescence values were normalized to the fluorescence mean obtained during the first 5 minutes of iso-osmotic control solution (osmotic pressure π_0) and Vt was calculated using the previous equation, transforming the fluorescence data F_0/F_t in volume data V_t/V_0 as illustrated in the supplementary Fig S3 (79).

- *In vitro determination of water membrane permeability by ¹H-NMR relaxometric procedure*

Adult cardiac myocytes freshly isolated from one mouse heart (wild type or *Aqp1* knockout) were incubated at 37°C for 1 h in the M199 cell culture medium containing 1% Penicillin/Streptomycin, BDM 10mM, BSA 0.1 mg/ml

(0.001%). Then cells were divided in two parts and one resuspended in 0.5 ml of iso-osmolar Tyrode's buffer (300 mOsm/L) and the other in 0.5 ml of a hypo-osmolar Tyrode's buffer (100 mOsm/L) both containing BDM 10 mM and Gd-HPDO3A 15 mM. Gd-HPDO3A (Prohance) was kindly provided by Bracco S.p.A. (Milan, Italy). Then cells were transferred in 5 mm NMR tubes (after 5 minutes incubation in the case of the hypo-osmolar condition), centrifuged 4 minutes at 0.1 g and the supernatant was discarded. In order to avoid the internalization of the metal complex, the relaxometric measurements were carried out within 15 minutes. Water proton T_{1s} of the cellular pellet were measured at 0.5 T and 25°C on a Stellar spectrometer (Stellar, Mede, Italy) by means of the inversion-recovery (IR) pulse sequence with 32 τ increments and four averages. A biexponential recovery of the magnetization was observed. The intracellular water residence time (τ_{in}) was determined by analysing the IR data in terms of the 2SX model (80, 81). Briefly, in a cell suspension, water molecules distribute in the extracellular (ex) space and in the intracellular (in) cytoplasm showing characteristics residence times τ_{in} , and τ_{ex} in the two compartments, respectively. These parameters are related by the equilibrium mass balance $\tau_{ex} \cdot V_{in} = \tau_{in} \cdot V_{ex}$, where V_{in} and V_{ex} ($=1 - V_{in}$) are the intracellular and extracellular volume fraction, respectively. From the relaxometric point of view, each environment is characterized by its own longitudinal relaxation rate, the extracellular (R_{1ex}) and the intracellular one (R_{1in}), respectively. In cell suspensions, without the addition of the paramagnetic contrast agents (i.e. Gd-HPDO3A), water exchange between the two compartments occurs much faster than the recovery of the longitudinal magnetization, i.e. $k_{ex} + k_{in} \gg |R_{1ex} - R_{1in}|$ (fast exchange limit). In this condition, water molecules visit both compartments multiple times over the magnetization recovery and the time evolution of the magnetization recovery in the IR experiment is monoexponential. On the contrary, in the presence of Gd-HPDO3A, confined into the extracellular space, the condition $(k_{ex} + k_{in}) \sim |R_{1ex} - R_{1in}|$ is met and the water exchange modulates the observed relaxation behavior. The recovery of longitudinal magnetization after inversion is no longer monoexponential and better described by the 2SX model, which allows to obtain the τ_{in} , having introduced this parameter in the fitting equation. In the fitting procedure the relaxation rate of the cytosolic compartment, and the GdHPDO3A millimolar relaxivity ($4.5 \text{ s}^{-1} \text{ mM}^{-1}$) were fixed. The relaxation rate of the extracellular compartment in the absence of the paramagnetic complex was fixed to 0.4 s^{-1} . V_{in} and V_{ex} were assessed via the measurement of Gd concentrations by inductively coupled plasma mass spectrometry (ICP-MS). Since the metal complexes distribute only in V_{ex} , this external volume fraction can be determined by the relation:

$$V_{ex} = nGd(\text{mol}) / (MGd(\text{mol/l}) * Volp(l))$$

were nGd is the number of Gd moles determined by ICP-MS; and M is the concentration of the GdHPDO3A solutions added to the cells, and Volp is the volume of the wet cellular pellets.

V_{in} is then given by:

$$V_{in} = 1 - V_{ex}$$

- *Inductively coupled plasma mass spectrometry (ICP-MS)*

The final Gd concentration in cardiac myocytes was assessed by using inductively coupled plasma mass spectrometry (ICP-MS; Element-2; Thermo-Finnigan, Rodano (MI), Italy). Sample digestion was performed by means of microwave heating for 14 min at 160 °C in 1 mL of concentrated HNO₃ (70%) (Milestone, Ethos Up Microwave Digestion System, Bergamo, Italy).

- *ROS detection by H2-DCFDA*

Isolated adult cardiac myocytes were obtained by the Langendorff method described above and placed in culture medium for 2 hours. Then culture medium was replaced by HBSS Ca²⁺ containing HEPES 10 mM. Myocytes were then loaded with H2-DCFDA (1 µM – Thermo Fisher Scientific) for 30 minutes at 37 °C and washed twice with HBSS. Myocytes were subsequently imaged with a fluorometer (SpectraMax i3 Multi-Mode Detection Platform), and fluorescence of DCFDA was quantified. Different reagents (H₂O₂ or Phenylephrine) were added just after the first measurement of fluorescence (time 0); fluorescence was subsequently analyzed each minute during 5 minutes and then each 5 minutes up to 25 minutes. Fluorescence measurements were normalized by the total amount of protein per well. For the experiments using Bacopaside II, cells were incubated during 30 minutes with Bacopaside II or its solvent control (at different concentrations) before loading with H2-DCFDA and exposure to H₂O₂.

- *Proximity Ligation Assay (PLA)*

Isolated adult cardiac myocytes were obtained by the Langendorff method described above and placed in culture medium for 2 hours to allow them to adhere to coverslips. They were fixed with paraformaldehyde (4%) and permeabilized with Triton X-100 (0.2% in PBS), and placed in a blocking solution at 37 °C. Cardiac myocytes were then incubated overnight at 4 °C with primary antibodies as follows: (i) mouse anti-Aqp1 antibody (sc-32737, Santa Cruz, 1/1000) and rabbit anti-p47^{phox} antibody (07-001, Millipore, 1/1000); or (ii) rabbit anti-AQP1 antibody (AB2219, Millipore, 1/1000) and mouse anti-CAV3 antibody (610421, BD Transduction Laboratories, 1/1000).

Species-specific anti-IgG secondary antibodies coupled with “reporter” oligonucleotides (Duolink In Situ Red Starter Kit Mouse/Rabbit (DUO92101, Sigma-Aldrich) were then added according to the manufacturer’s protocol. Co-localized proteins bound to the specific antibodies were detected after “reporter” DNA circularization with a size-limiting linker oligonucleotide, ligation and rolling circle amplification with a polymerase. Amplified “reporter” DNA was then detected using complementary fluorescent probes. After nuclear staining with DAPI, images were analyzed by structured illumination microscopy (Zeiss AxioImager equipped with an ApoTome module), as described (82). Red fluorescent dots were quantified (AxioVision 4.9.1) in a blinded fashion.

- *Amino acids incorporation into proteins measured by [¹⁴C]-Phenylalanine incorporation*

[¹⁴C]-Phenylalanine (Perkin Elmer) incorporation into proteins of myocytes from *Aqp1*^{+/+} and *Aqp1*^{-/-} mice, as index of hypertrophy, was measured as follows. [¹⁴C]-Phenylalanine (1 µCi/mL) was added to plated myocytes from both genotypes with or without simultaneous treatment with ET-1 (0.6 µM). After 18 h incubation, myocytes were harvested and were washed twice with ice cold phosphate–buffered saline (PBS) and lysed as described above. Proteins were precipitated during 20 minutes by adding trichloroacetic acid to a final concentration of 10 % (vol/vol). Pellets containing proteins were washed with 100 mM NaOH and then dissolved with formic acid. [¹⁴C]-Phenylalanine incorporation into myocytes was measured by liquid scintillation counting (measurement of degradation per minute, DPM). Results are expressed as the percentage increase of DMP compared to untreated control groups and normalized to the total amount of protein per dish.

- *Echocardiography*

Two-dimensional echocardiography was performed with a high resolution ultrasound system (Vevo2100 VisualSonics; 40 Mhz probe) in anesthetized animals (1-3 % isoflurane) placed in supine position and fixed on limb electrodes to monitor heart rate (HR). LV parasternal long axis view was recorded in B-mode to measure LV internal volumes at end of diastole (LVEDv) and systole (LVESv), used to calculate the ejection fraction (EF; B-mode). M-mode view was performed to measure LV posterior wall (PW) and interventricular septum (IVS) thicknesses, LV internal dimensions (LVID), allowing the assessment of the fractional shortening (FS). The relative wall thickness index (RWT) was calculated as $RWT = (IVSd + PWd) / LVIDd$ (d = in diastole) and LV mass as $1.053 * [(LVIDd + PWd + IVSd)^3 - LVIDd^3]$.

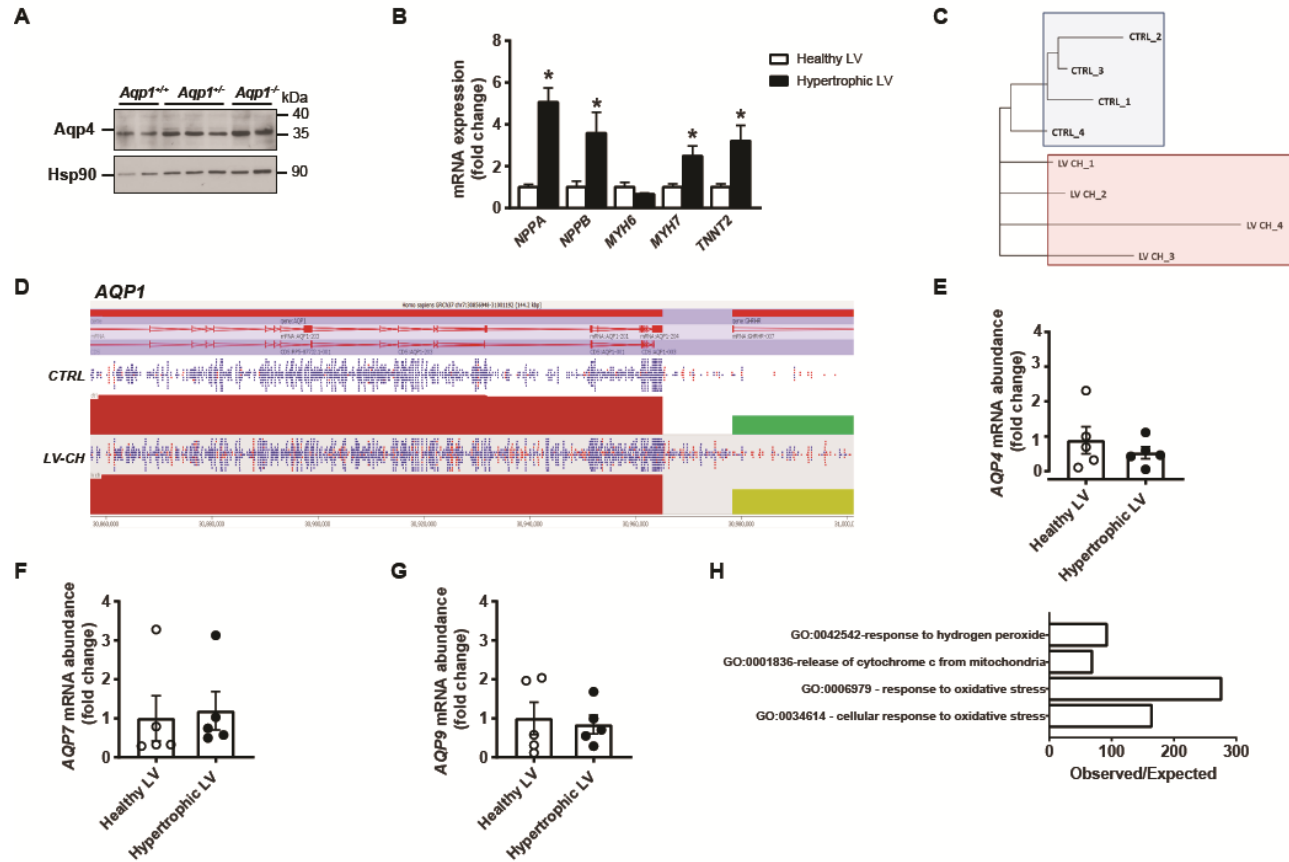


Fig. S1. Expression of AQP4 in mouse cardiac myocytes and AQP in pericentriolar material 1-positive nuclear RNA from control and hypertrophic human myocardium.

(A). AQP4 protein expression is conserved in adult mouse cardiac myocytes between *Aqp1*^{+/+}, *Aqp1*^{+/-} and *Aqp1*^{-/-} mice; (B). Hypertrophy marker expression as measured by RT-qPCR from PCM1-positive nuclear RNA. Marker expression levels were normalized to the geometric mean of reference genes *GAPDH*, *ACTB* and *B2M*. N = 5; Mean±SEM; *P<0.05 by Student two-tailed t-test, ctrl vs. LV hypertrophy for each gene. (C-D). RNA-seq reads aligning to *AQP1* in PCM1-positive nuclear RNA from control and hypertrophic human myocardium (C). Datastore similarity tree showing clustering of control and left ventricle hypertrophic (LV CH) PCM1-positive nuclear transcriptomes. DEseq analysis performed in SeqMonk 35.0 from all reads quantified to genes. (D). A representative example of reads aligning to the *AQP1* locus (GRCh38 chr7:30911855-30625516) in control vs LV CH PCM1-positive RNA-seq data. RNA-seq data was visualized, analysed and exported from SeqMonk 35.0. The second track from the top in red indicates annotated introns (thinner line) and exons (thick line). (E-G). *AQP4* (E), *AQP7* (F) and *AQP9* (G) mature mRNA abundance by RT-qPCR from control and left ventricle hypertrophic PCM1-positive nuclear RNA (N=5 each). (H) Gene ontology analysis of differentially expressed genes (607) that are upregulated in LV hypertrophy compared with control PCM1-positive RNA-seq. Figure shows the biological processes that are significantly enriched for and related to oxidative stress only and presented as observed/expected ratio. Data presented with a fold enrichment score; P<0.05, generated using Benjamini-Hochberg correction (N= 4 independent experiments).

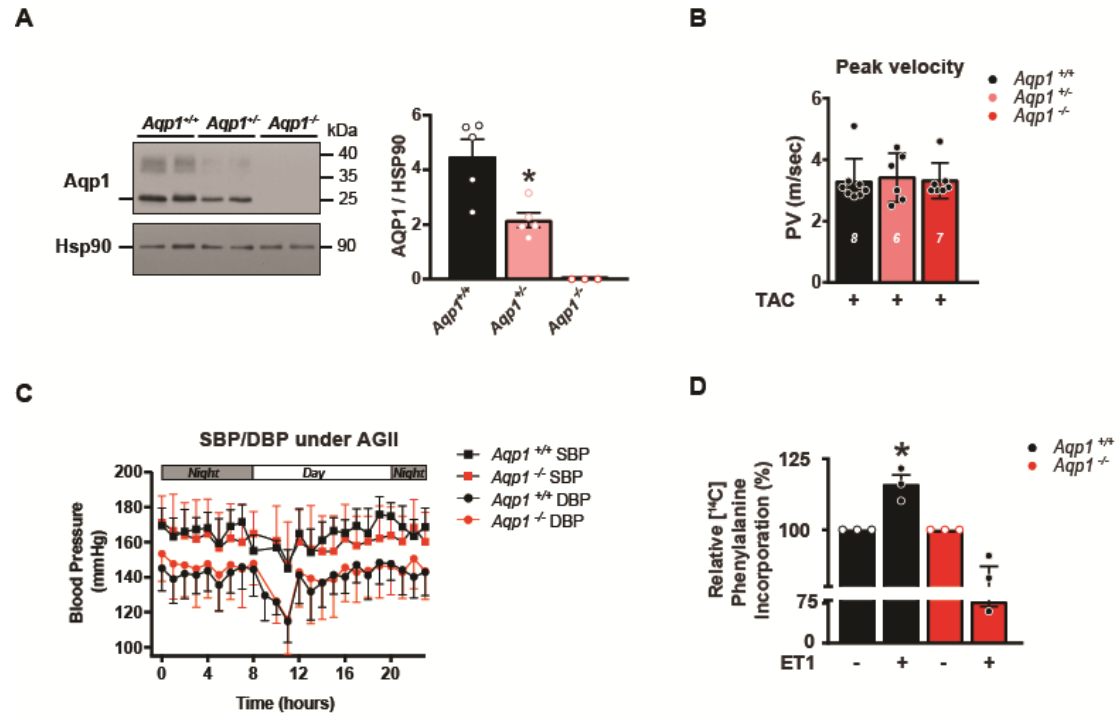


Fig. S2. Afterload control in *Aqp1* mice under TAC or Ang II infusion and effect of endothelin-1 on [¹⁴C]-phenylalanine incorporation in adult cardiac myocytes from *Aqp1*^{+/+} and *Aqp1*^{-/-} mice.

(A) Comparative abundance of AQP1 proteins between *Aqp1*^{+/+}, *Aqp1*^{+/-} and *Aqp1*^{-/-} mice by Western blot (left); densitometric data (right); N=5, 5 and 3 mice respectively; Mean±SEM; *, *P*<0.05 compared with *Aqp1*^{+/+} (B,C). Afterload control in *Aqp1* mice under transverse aortic constriction or Ang II infusion. (B). Peak velocity registered by Doppler echography in adult male *Aqp1*^{+/+}, *Aqp1*^{+/-} and *Aqp1*^{-/-} mice 3 weeks after Transverse Aortic Constriction (TAC). N= 8 *Aqp1*^{+/+}, 6 *Aqp1*^{+/-} and 7 *Aqp1*^{-/-} mice. (C). 24-hour systolic blood pressure (SBP) and diastolic blood pressure (DBP) in adult male *Aqp1*^{+/+} and *Aqp1*^{-/-} mice treated with osmotic minipump of Angiotensin II (AGII; 2.0 mg/kg/day for 2 weeks); N= 4 *Aqp1*^{+/+} and 5 *Aqp1*^{-/-} randomly chosen mice of each genotype; (D). [¹⁴C]-Phenylalanine incorporation in adult cardiac myocytes from *Aqp1*^{+/+} and *Aqp1*^{-/-} mice incubated with endothelin-1 (ET-1) during 20 hours (0.6 μM); N= 3 different preparations from 3 mice of each genotype; Mean±SEM; * *P*<0.05 vs ctl by Kruskal-Wallis followed by Dunn's test for multiple comparisons.

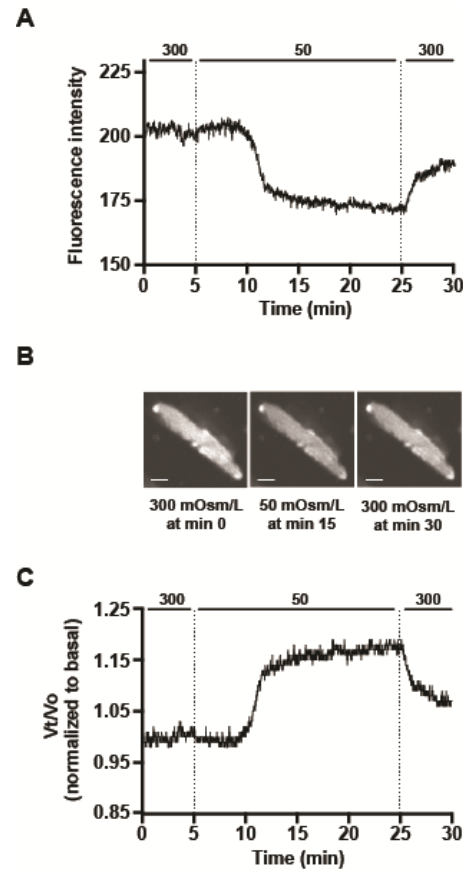


Fig. S3. Fluorescence-based measurements of cell water intake in adult mouse cardiac myocytes exposed to hypo-osmotic buffer.

(A). Changes in fluorescence intensity in a representative isolated adult cardiac myocyte from *Aqp1*^{+/+} mice loaded with a fluorescent dye from iso-osmotic (300 mOsmol l⁻¹) to hypo-osmotic solution (50 mOsmol l⁻¹; from 5-25 min), then back to iso-osmotic solution (>25 min). (B): Representative image of fluorescence change in an isolated adult cardiac myocyte from *Aqp1*^{+/+} mouse loaded with a fluorescent dye before (left; min 0) and after (middle; min 15) application of a hypo-osmotic solution (50 mOsmol l⁻¹), then back to iso-osmotic solution (right)(scale 10 μ m). (C): Calculated change in cell volume over time (V_t , normalized to basal volume) as deduced from quantitative changes in dye fluorescence upon dilution under hypo-osmotic solution (50 mOsmol l⁻¹) inside a delimited cell area (see Methods for details).

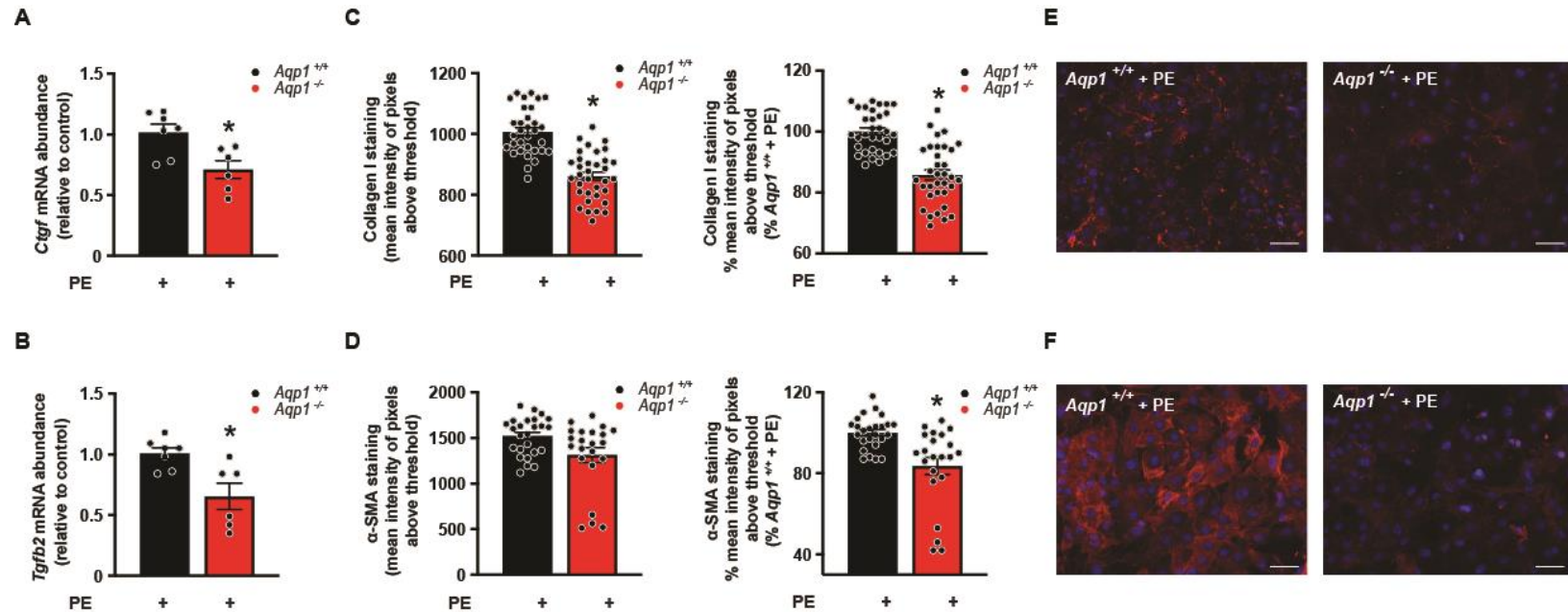


Fig. S4. Reduced expression of profibrotic mediators in *Aqp1*^{-/-} cardiac myocytes and of their paracrine effect in cardiac fibroblasts.

(A,B). RT-qPCR quantification of *Ctgf* and *Tgfb2* transcripts abundance in phenylephrine (PE)-treated (20 μ M, 18 h) cardiac myocytes from *Aqp1*^{+/+} and *Aqp1*^{-/-} mice; independent preparations from N=7 *Aqp1*^{+/+} and 6 *Aqp1*^{-/-} mice; Mean $\pm$ SEM; **P*<0.05 by student t-test. (C,D). Quantitative immunostaining for COL1A1 (collagen 1) and ACTA2 (alpha-smooth muscle actin, α -SMA) in cardiac fibroblasts after incubation (24 h) in the medium conditioned by cardiac myocytes cultured as in A,B. Each dot is the quantification of immunostaining from randomly chosen microscopic fields of fibroblast cultures; from N=3 independent preparations of conditioned media from each genotype. Mean $\pm$ SEM; **P*<0.05 by unpaired student t-test (C) or Mann-Whitney test (D). (E,F) Representative immunostaining for COL1A1 (E) and ACTA2 (F) in cardiac fibroblasts incubated with medium conditioned by PE-treated cardiac myocytes from *Aqp1*^{+/+} (left) and *Aqp1*^{-/-} (right) mice (scale 50 μ m).

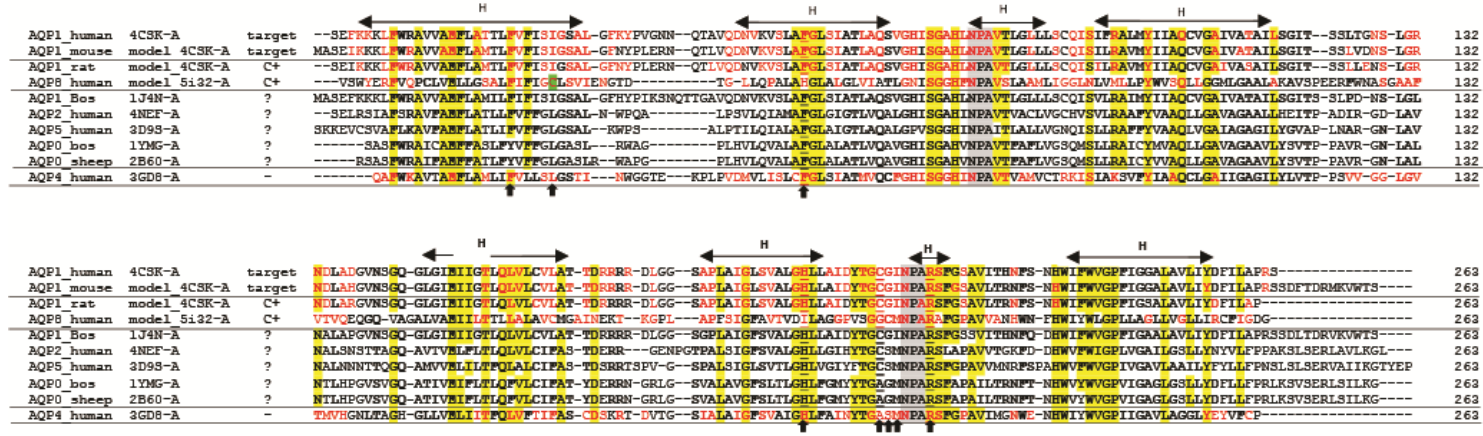


Fig. S5. Protein sequence alignment using AQP1 as target.

The first column indicates the conventional name of the aquaporins, the second column indicates the PDB code of their X-ray structure if available, or otherwise the PDB code of the template used for comparative modeling. In the third column, information about H₂O₂ accessibility is reported (« C+ »: positive control, « - »: negative control, « ? »: unknown). Helix regions are indicated by horizontal arrows. Residues of the aromatic/arginine (ar/R) constriction region are underlined, Cys53 in AQP8 is highlighted in green, conserved amino acids are in bold and highlighted in yellow, and residues with similar physico-chemical properties between the different structures are in bold. Pore lining residues are in red for human AQP1, AQP4, AQP8, mouse AQP1 and rat AQP1 sequences. The conserved NPA motif is highlighted in gray. Black arrows on the bottom indicate the position of important amino acids located at the constriction level at the extracellular side of the pore, which are depicted in Fig. 7A.

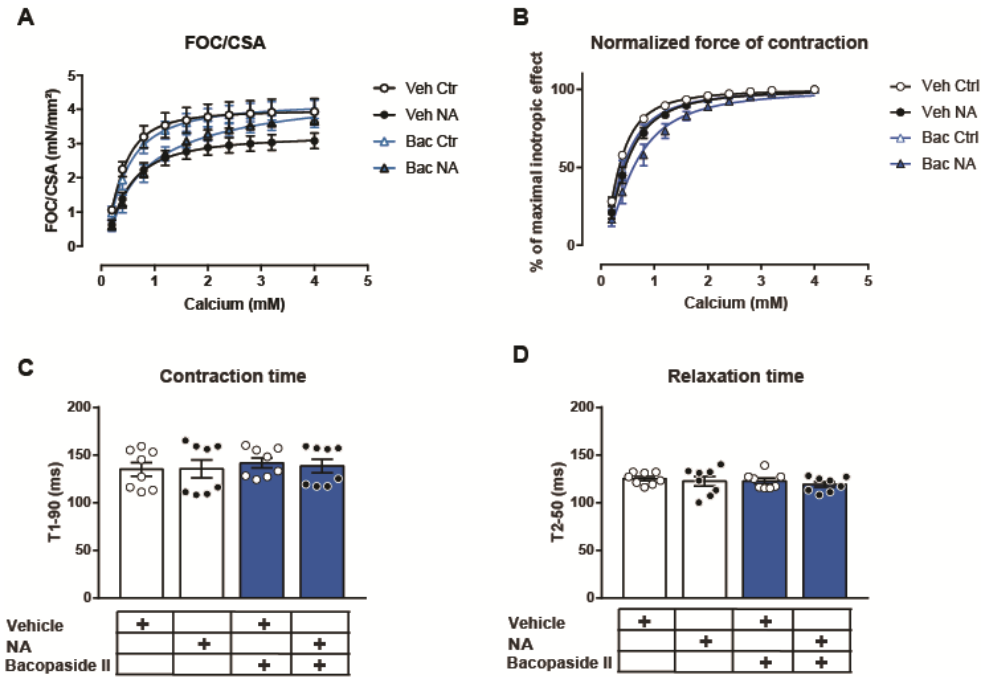


Fig. S6. Effect of Bacopaside II on contractile properties of EHM with or without noradrenaline.

(A). Concentration-dependent increase in contraction force (FOC) in response to calcium and normalized to the tissue cross sectional area (CSA) ; (B). Same data as in (A) after normalization to maximum developed force per EHM in the different treatment conditions; (C). Contraction time (from 10 to 100 % contraction); (D). Relaxation time (from 0 to 50% relaxation); NA: Noradrenaline; Bac: Bacopaside II; 8 EHM per condition, from 2 separate experimental series.

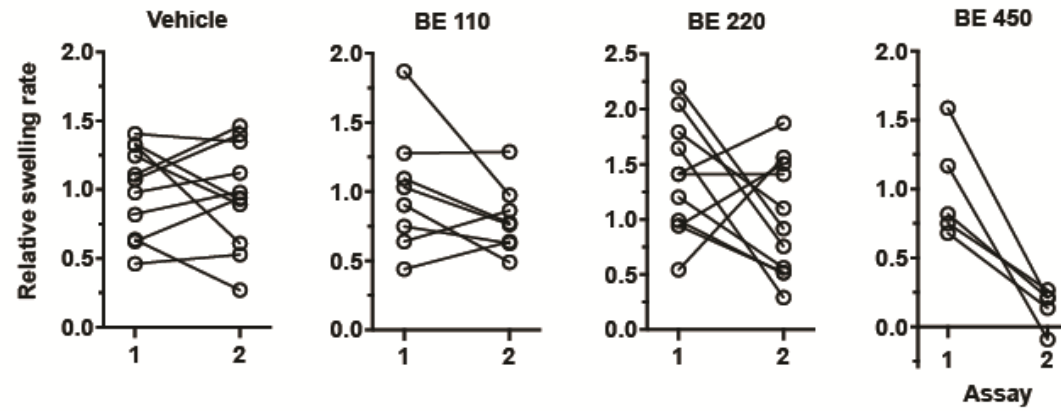


Fig. S7. Inhibitory effect of the standardized extract of *B. monnieri* on AQP1-mediated water permeability in *Xenopus* oocytes.

Trend plots illustrate the swelling rates measured for each oocyte in the first swelling assay in untreated condition (1) and second swelling assay post-incubation in vehicle or Bacopa extract (BE) (2).

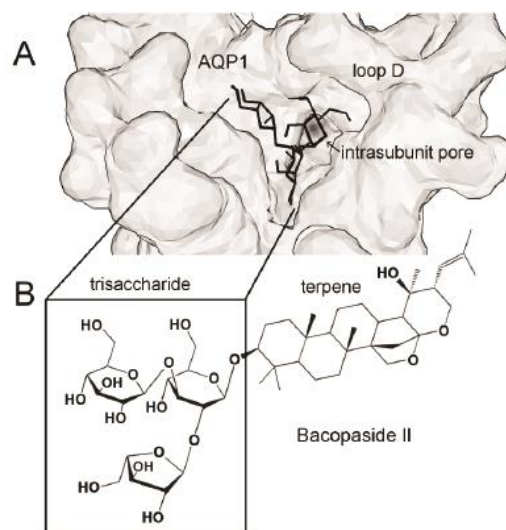


Fig. S8. Schematic diagram summarizing the proposed mechanism of AQP1 inhibition by Bacopaside II.

(A) One subunit of a tetrameric AQP1 channel, seen from the intracellular side to show the predicted interaction site. The trisaccharide domain of bacopaside II appears to occlude the water channel pore (the terpene moiety is removed for clarity). (B) Structure of bacopaside II.

Table S1. Clinical characteristics of donors with normal versus hypertrophic left ventricular remodeling.

cardiac examination	age [y]	BMI	cause of death
normal	31	22	suicide
normal	39	34	intoxication
normal	57	22	suicide
normal	57	22	accident
normal	60	24	intoxication
left ventricular hypertrophy	37	20	natural
left ventricular hypertrophy	43	32	natural
cardiomyopathy	57	31	intoxication
left ventricular hypertrophy	61	29	natural
left ventricular hypertrophy	63	30	intoxication

Table S2. Sequences of primers used in RT-qPCR.

Name	Species	Target	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
<i>Nppb</i>	Mouse	brain type natriuretic peptide (BNP)	GCCAGTCTCCAGAGCAATTC	AGCTGTCTCTGGGCCATTT
<i>Myh6</i>	Mouse	alpha myosin heavy chain	TGTGGTGCCTCGTTCCA	TTTCGGAGGTACTGGGCTG
<i>Myh7</i>	Mouse	beta myosin heavy chain	ACCAACCTGTCCAAGTTCCG	ACTCCTCATTGAGGCCCTTG
<i>Ctgf</i>	Mouse	Connective Tissue Growth Factor	TGACCCCTGCGACCCACA	TACACCGACCCACCGAAGACACAG
<i>Tgfb2</i>	Mouse	Transforming Growth Factor Beta 2	AGAGCTCGAGGCGAGATTTG	GATGTGGGGTCTTCCCACTG
<i>Hprt</i>	Mouse	hypoxanthine-guanine phosphoribosyl transferase	TTGGATACAGGCCAGACTTTG	CGTGATTCAAATCCCTGAAG
<i>AQP1</i>	Human	Aquaporin-1	TATGCGTGCTGGCTACTACC	CAGGGTTAATCCCACAGCCA
<i>AQP4</i>	Human	Aquaporin-4	GCCATCATTGGAGCAGGAATCC	GCCATCATTGGAGCAGGAATCC
<i>AQP7</i>	Human	Aquaporin-7	GAGGAAGATGGTGCGAGAGTTC	CAAGTTGACACCAAGGTAGCTCC
<i>AQP9</i>	Human	Aquaporin-9	CTGAACAGTGGCTGTGCCATGA	CCACTACAGGAATCCACCAGAAG
<i>GAPDH</i>	Human	Glyceraldehyde-3-phosphate dehydrogenase	GAGTCAACGGATTGGTCGT	GACAAGCTTCCCGTTCTCAG
<i>ACTB</i>	Human	β -Actin	GCGGCGCCCTATAAAACC	TCATCATCCATGGTGAGC
<i>B2M</i>	Human	β 2-microglobulin	GCTATCCAGCGTACTCCAAA	GATGGATGAAACCCAGACAC