TRPA1 channel is a cardiac target of mIGF-1/SIRT1 signaling

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Running Head: TRPA1 and cardiac mIGF-1/SIRT1 signaling

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Abstract

Cardiac overexpression of locally acting insulin growth factor isoform (mIGF-1) and the consequent downstream activation of NAD^+ dependent protein deacetylase SIRT1 trigger potent cardiac anti-oxidative and anti-hypertrophic effects. TRPA1 belongs to the transient receptor potential (TRP) ion channel family of molecular detectors of thermal and chemical stimuli that activate sensory neurons to produce pain. Recently, it has been shown that TRPA1 activity influences blood pressure, but the significance of TRPA1 in the cardiovascular system remains elusive. Using a genomic screening in mice hearts, we found here that TRPA1 is a target of mIGF-1/SIRT1 signaling. TRPA1 expression is increased in the heart of cardiac-restricted mIGF-1 transgenic (Tg) mice, both in the cardiomyocytes and non-cardiomyocyte cells. In wild type mice, SIRT1 occupies TRPA1 promoter inhibiting its expression, whereas in presence of cardiac mIGF-1 transgene, SIRT1 is displaced from TRPA1 promoter, leading to an increase in its expression. Cardiac specific ablation of SIRT1 (CKO) in mIGF-1 Tg mice paradoxically did not increase TRPA1 expression. We recently reported a systemic “hormetic” effect in mIGF-1 Tg mice, a mild hypertension, which was depleted upon SIRT1 CKO. Administration of the selective TRPA1 antagonist HC-030031 to mIGF-1 Tg mice restored blood pressure to basal levels. We identified TRPA1 as a functional target of the cardiac mIGF-1/SIRT1 signaling pathway, which may have pharmacological implications for the management of cardiovascular stress.

Keywords:
TRPA1; IGF-1; SIRT1; heart.
Introduction

Cardiovascular diseases are a leading cause of mortality, representing one third of all global deaths. Insulin like growth factor-1 (IGF-1) and Sirtuin-1 (SIRT1) are crucial mediators of cell homeostasis and of cardiovascular stress [7]. In mammals a complex IGF-I signaling system with multiple alternative spliced isoforms displaying distinct effects on cardiovascular function is in place [7]. These variants have a common core peptide, flanked by varying termini (Class 1 and 2 N-terminal peptides, and E peptides). IGF-I is both a systemic growth factor produced by the liver in response to growth hormone and a local growth factor acting in an autocrine/paracrine manner in the skeletal and heart muscle. mIGF-I is a locally acting isoform that comprises Class 1 N-terminal and Ea C-terminal peptides [7]. mIGF-I boosts antioxidative cell defenses by up-regulating skeletal muscle or cardiac gene programs with regenerative, anti-oxidant and anti-apoptotic properties [21, 27, 28]. Moreover, mIGF-I repairs the heart from injury through production of specific cytokines that recruit endothelial-primed cells for de novo vascularization of the myocardial tissue, indicating that cardiomyocyte (CM) specific overexpression of this transgene might have profound systemic effects [20].

The Sirtuin family of nicotinamide adenine dinucleotide (NAD\(^+\))-dependent protein deacetylases is deeply implicated in the regulation of organism healthspan [9]. SIRT1 is the largest and best characterized member: its enzymatic activation trigger pleiotropic beneficial effects [9]. In fact, pharmacological or nutritional interventions capable of activating SIRT1 have been shown to increase life span in most model organisms [9]. On the contrary, SIRT1 knock-out (KO) mice die at birth or soon after due to developmental defects of the retina and heart [26]. Moderate SIRT1 cardiac-specific overexpression was shown to protect mice from cardiac oxidative stress and
postponed the onset of age-dependent cardiac fibrosis and cell death, with increased expression of antioxidants, such as catalase, through forkhead box O (FoxO)-dependent mechanisms [1]. *In vitro* and *in vivo* findings reinforced these cardio-protective effects of SIRT1, suggesting that its activation might be of benefit for the treatment of cardiac diseases [9]. IGF-I and SIRT1 actually impinge on the same signaling pathways [7, 25]. In this respect, we have shown that the liver-produced circulating IGF-I isoform and mIGF-I display distinct effects in cardioprotection [29].

Using hypertrophic (Angiotensin II)/oxidative (paraquat) stressors, we identified a signaling pathway that protects CM and that relies on mIGF-1 dependent SIRT1 activation [29, 30]. For *in vivo* studies in mice, we generated cardiac-specific mIGF-I transgenic mice in which SIRT1 was excised from adult CM in an inducible and conditional fashion (mIGF-1 Tg x SIRT1 CKO). Functional and molecular analyses of these animals confirmed that mIGF-I-induced SIRT1 activity is required to protect the heart from oxidative stress-induced cell damage and lethality [30]. Moreover, we uncovered unexpected systemic roles for the cardiac mIGF-1/SIRT1 pathway in mice, among which a mild hypertension [3]. The robust physiological responses obtained with mIGF-I-induced SIRT1 activity suggested a potential mechanistic basis for strategies to improve the outcome of heart disease. To explore this more in depth at the molecular level, using a high throughput sequencing approach (ChIP-Seq) we identified new direct putative genomic binding targets of nuclear SIRT1 in the heart [3]: among these, TRPA1. This molecule belongs to the transient receptor potential (TRP) ion channel family of molecular detectors of thermal and chemical stimuli that activate sensory neurons to produce pain [12]. TRPA1 is the smallest of the mammalian TRP superfamily of cation channels that comprises 28 members assigned to six subfamilies based on sequence homology. These channels have been strongly
linked to the pathogenesis of clinical disorders, such as cancer and inflammation [2, 13]. TRPA1 activity might also be implicated in the pathogenesis of cardiovascular disorders: it is highly expressed in cerebral endothelial cells and in smooth muscle cells, and pharmacological or genetic modulation of its activity influences blood pressure [6]. However, the significance of TRPA1 in the cardiovascular system is not understood. For this reason, in this work we explored the relevance of TRPA1 as a target of cardioprotective mIGF-1/SIRT1 signaling using mouse genetics as a tool and blood pressure as functional read-out. We found that cardiac TRPA1 expression is modulated by mIGF-1 and can translate the effects of this growth factor on blood pressure levels in a SIRT1-dependent manner.

Methods

Animal models

Transgenic FVB mice carrying a rat mIGF-1 cDNA driven by the mouse αMyHC promoter (αMyHC/mIGF-1) were generated and maintained as previously described [21]. SIRT1 floxed (Fl/Fl) mice were previously described [4] and acquired from The Jackson Laboratory. Tamoxifen-inducible αMyHC/mER-CRE-mER transgenic mice were crossed to SIRT1 floxed (Fl/Fl) mice to deplete SIRT1 expression in adult CM [22] upon tamoxifen administration. Mice were placed on tamoxifen-containing chow (Harlan Special Diet TD.55125) at 4 months of age, for two weeks, leading to efficient and reproducible gene recombination [14]. The mIGF-1 transgene was introduced by three-way crosses to generate αMyHC/mIGF-1 Tg x αMyHC/mER-CRE-mER; SIRT1Fl/Fl mice (referred to as mIGF-1 Tg x SIRT1 CKO) [30]. PCR genotyping was performed using genomic DNA from tail biopsies. For in vivo inhibition of TRPA1 activity mice were injected with TRPA1 antagonist HC-030031
(20-50-100 mg/kg i.p., dissolved in 10% DMSO) (Sigma-Aldrich) for 30 minutes, an effective time for this drug adopted from previous studies and upon our preliminary experiments.

Chromatin immunoprecipitation

Protein-DNA complexes were captured by fixing heart homogenates from adult wild type or mIGF-1 Tg mouse at resting state in 1% (v/v) formaldehyde (Sigma) for 10 min at room temperature with gentle shaking. The reaction was quenched by adding 0.125M glycine for 5 min at room temperature. Cells were washed (x3) with cold PBS and resuspended in Cell Lysis Buffer (CLB) (50mM Heps, 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP40, 1x proteinase inhibitor; 1 x10^7 cells/ml) on ice for 10min to release nuclei. Nuclei were pelleted (4000 rpm, 10 min, 4 C) and resuspended in Nuclei Lysis Buffer (NLB) (140mM NaCl, 10mM Tris-Cl (pH 8), 1% NP40, 1x proteinase inhibitor) on ice for 10 min. Cells were sonicated using the Bioruptor Sonicating Waterbath (Diagenode). 300 ml of lysate was sonicated for 40 pulses of 30s ON 30s OFF in 1.5 ml TPX eppendorf tubes, replacing ice regularly to minimize overheating of samples. Chromatin was pooled and centrifuged for at 13000 rpm, 10 min, 4 C to remove debris and single use aliquots (300ul) were used for immunoprecipitation or stored at -80 C. Chromatin was immunoprecipitated by bringing each aliquot of chromatin to 1ml with NLB proteinase inhibitor. Chromatin was precleared with 80ul of ChIP-grade Protein Agarose A beads (Upstate) for 4 hr at 4 C with rotation and incubated overnight with 4 µg of SIRT1 monoclonal antibody (10E04, Millipore), or unrelated rabbit IgG polyclonal antibody (Chemicon) at 4 C with rotation. Immunoprecipitated complexes were collected by incubation with 100 µl of ChIP blocked Protein A agarose beads (Upstate) for 4hr at 4 C with rotation and
non-specific complexes were removed by washing beads twice with High Salt Buffer (0.1% SDS, 1% Triton-X, 2mM EDTA, 20mM Tris-Cl (pH 8.1), 500mM NaCl), Low Salt Buffer (0.1% SDS, 1% Triton-X, 2mM EDTA, 20mM Tris-Cl (pH 8.1), 150mM NaCl), LiCl Buffer (0.25M LiCl, 1% NP40, 1% deoxycholic acid, 1mM EDTA, 10mM Tric-Cl (pH 8.1) and TE Buffer. Washes were performed for 5min at 4 C with rotation and beads were collected by centrifugation at 3000 rpm, 4 C 3 min. DNA was eluted with 100 µl of Elution Buffer (1% SDS, 100mM NaHCO3). Beads were vortexed for 30 s and incubated at room temperature with rotation for 15min and the process was repeated and eluates combined (200 µl). Crosslinks were reversed with 0.3M NaCl by incubating at 65 C overnight in a hybridization oven and RNA was removed with RNase A (Upstate) at 37 C for 30 min. Protein was removed by incubating samples with Proteinase K (Upstate) for 1 hr at 45 C. DNA was purified using Qiagen PCR Purification Kit (QIAGEN) and eluted in 30 µl of dH2O. For ChIP individual SIRT1 ChIP samples were pooled and concentrated using a SpeedVac. PCR against regions (19-118) (77-176) (285-384) from transcription start site (TSS) was performed in SIRT1 pulled down samples, input and no template control, using Taq DNA Polymerase (New England BioLabs, cat n. MO273) and processed for gel electrophoresis. Primer sequences were designed to amplify a region of 100 bp and are available upon request.

Blood pressure measurement

Non-invasive assessment of BP was performed by tail-cuff measurements (Visitech Systems, Apex, NC, USA) as described previously [17].
Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from hearts using TRIzol (Invitrogen). After RNA quality verification, 1-2 mg was used to prepare cDNA (Ready-To-Go, T-Primed First-Strand Kit, Amersham Bioscience). Quantitative polymerase chain reaction (PCR) for SIRT1 was performed using the SYBR Green (SIGMA) in a Light-Cycler (Roche) in a 25 μl volume with the following amplification program: 1. 50°C 2 min, 1 cycle; 2. 95°C 10 min, 1 cycle; 3. 95 °C 15 s -> 60 °C 30 s -> 72 °C 30 s, 40 cycles; 4. 72°C 10 min, 1 cycle. Primer sequences for TRPA1 were Forward: AAGCGGAGACTTGGACATGA, Reverse: TAACGAGGCTCTGTGAAGCA. UbiC, Rn18S and GAPDH transcripts were used as internal controls, according to the GeNorm method [29]. Primer sequences were as follows: UbiC Forward AGCCCAGTGTACCACCAAG, Reverse: GCAAGAACTTTATTCAAAGTGCAA; GAPDH Forward: AACTTTGGCATTGTGGAAGG, Reverse: ACACATTGGGGGTAGGAACA; Rn18S Forward: CGCGGTTCTATTTTGTTGGT, Reverse: AGTCGGCATCGTTATGGTC

Primary cardiomyocyte isolation procedure

Excised mouse hearts were perfused using a Langendorff perfusion apparatus with calcium-free KRB solution containing collagenase (1 mg mL⁻¹) until they became flaccid. The hearts were then chopped finely, and the mince was agitated gently in the same medium to dissociate individual cells. The resulting cell suspension was filtered to remove undigested material, and the CM were separated from noncardiomyocytes (NCM) by sedimentation of CM at 500 g for 2 min. Supernatant containing NCM was separated and further centrifuged at 1500 g for 5 min to pellet cells. Calcium tolerance
of sediment containing CM was restored gently by resuspending it in KRB containing a progressively higher concentration of calcium ion to a final concentration of 1 mM. Both CM and NCM sediments were stored in TRIzol (Invitrogen, Monza, Italy) for RNA analyses.

**RNA in situ hybridization**

In situ hybridization was performed as previously described, using the coding region of TRPA1 to generate RNA probe [10].

**Statistical analysis**

Results are expressed as means ± S.E. Comparisons were made by using Student’s t test. Differences were considered as significant when p<0.05 (*), p<0.01 (**) or p<0.001 (***)

**Results**

SIRT1 displacement from TRPA1 promoter in the myocardium of mIGF-1 mice is associated to increased TRPA1 expression levels.

ChIP-Seq screening for differential SIRT1 genomic binding sites in the whole hearts from wild type versus cardiac-restricted mIGF-1 Tg mice led to the identification of a few dozens of differential SIRT1 genomic binding sites, 33 specific for the WT and 32 specific for mIGF-1 [3]. In this ChIP-Seq data set, SIRT1 showed a 3 fold enrichment in the occupancy of TRPA1 gene body in WT hearts compared to mIGF-1 Tg hearts [3]. Here, we sought to validate these highthroughput sequencing data by canonical ChIP using a specific ChIP-grade anti-SIRT1 antibody followed by qPCR amplification of 3 distinct selected promoter regions, (19-118) (77-176) (285-384), from transcription start site (TSS), respectively. As shown in Figure 1, in presence of
the mIGF-1 transgene, the binding of SIRT1 to these promoter regions is almost undetectable, indicating SIRT1 removal and suggesting that TRPA1 is a molecular target of the mIGF-1/SIRT1 pathway. TRPA1 is a cation channel involved in several clinical disorders; preliminary evidence link TRPA1 activity to vasodilation and vasoconstriction [18], although its cardiac expression levels and its role in cardiovascular biology are not clear. Here, using RNA in situ hybridization we found that TRPA1 transcript is massively upregulated in the heart of mIGF-1 Tg versus WT mice (Figure 2A). TRPA1 signal localized to the myocardial fibers (Figure 2A). To understand if TRPA1 was differently expressed in CM or in NCM cell types (endothelial cells, fibroblasts, macrophages, smooth muscle cells), which comprises 50% of total cardiac cell number, WT and mIGF-1 Tg hearts were fractionated to separate the CM from NCM [14, 30], and TRPA1 mRNA levels were analyzed by qPCR. TRPA1 mRNA was found ~5.5 times more expressed in the CM fraction compared to the NCM fraction (Figure 2B) in WT hearts. A markedly higher expression in CM versus NCM fraction for TRPA1 was also found in presence of the cardiac mIGF-1 transgene (Figure 2B). In mIGF-1 Tg hearts a ~12.7 fold increase in TRPA1 mRNA expression in the CM fraction was observed, without evident changes in NCM, which are significantly higher expression values compared to WT hearts (Figure 2B). These data are consistent with the imaging in situ (Figure 2A), and demonstrate that i) TRPA1 is expressed more robustly in CM compared to NCM and that ii) TRPA1 is markedly upregulated by mIGF-1 in the heart, specifically in the CM and not in the NCM (Figure 2A, B).
Cardiomyocyte specific inducible genetic ablation of SIRT1 in mIGF-1 mice lowers TRPA1 expression

Since SIRT1 is displaced from TRPA1 promoter in the myocardium in presence of mIGF-1 transgene, we investigated if genetic ablation of SIRT1 could elicit the same effects. As reported, to ablate SIRT1 activity in the CM compartment, we crossed CM-specific, tamoxifen-inducible αMyHC/mER-CRE-mER transgenic mice with conditional SIRT1^{Fl/Fl} knock-out (KO) mice, to produce SIRT1 CKO mice [30]. Cardiac function and SIRT1 expression in these mice were unaltered during growth and adulthood [30]. After 2 weeks on a tamoxifen-enriched diet, 4-month-old SIRT1 CKO mice displayed efficient CM-specific SIRT1 inactivation [30]. This did not occur in tamoxifen-fed wild-type, αMyHC/mER-CRE-mER, and SIRT1^{Fl/Fl} mice and was cardiac specific. KO of cardiac SIRT1 in the adult did not generate evident functional perturbations [30]. Using a three-way crossing of mIGF-1 Tg mice with SIRT1 CKO we next generated mIGF-1 Tg × SIRT1Fl/Fl mice, born at expected ratio and without any abnormalities [30]. Thus, we further sought to analyze four groups of 4 months old mice for TRPA1 mRNA expression: wild-type, mIGF-1 Tg × SIRT1^{Fl/Fl}, SIRT1 CKO, and mIGF-1 Tg × SIRT1 CKO mice. Upon feeding these four groups of mice a tamoxifen-enriched diet for 2 weeks, SIRT1 deletion occurred specifically in the heart of SIRT1 CKO and mIGF-1 Tg × SIRT1 CKO mice [30]. qPCR analysis in CM and NCM compartments revealed that ablation of SIRT1 alone in the CM had no effects on TRPA1 expression in CM and NCM as compared to WT (SIRT1 CKO, Figure 3). Moreover, this gene expression analysis confirmed increased TRPA1 mRNA levels in the CM of mIGF-1 Tg; SIRT1^{Fl/Fl} mice (similar to mIGF-1 Tg mice) compared to WT (Figure 3); however, TRPA1 mRNA expression returned to basal levels in the CM compartment of mIGF-1 Tg × SIRT1 CKO mice (Figure 3).
These data demonstrate that SIRT1 is required for the mIGF-1 dependent upregulation in TRPA1 expression in CM.

**mIGF-1/SIRT1 dependent mild hypertension is prevented by systemic administration of TRPA1 antagonist HC-030031**

Consistent with previous studies, non invasive measurement [17] of the diastolic and systolic blood pressure (DBP and SBP, respectively) in WT, mIGF-1 Tg and mIGF-1 Tg x SIRT1 CKO mice showed no difference between WT and mIGF-1 Tg x SIRT1 CKO mice, while a ~25% significant increase in both diastolic blood pressure (DBP) and systolic blood pressure (SBP) in mIGF-1 Tg mice compared to WT littermates was observed in the basal state [Figure 4 and [3]]. In mIGF-1 Tg x SIRT1 CKO mice SBP, and to a lesser extent DBP, was found restored to WT levels (Figure 4). Given that TRPA1 activity can modulate DBP and SBP in rodents [6], we aimed to assess its potential impact on the mild hypertension induced by cardiac overexpressing of mIGF-1 Tg in a SIRT1 dependent fashion in mice. To this purpose, three cohorts of mice (WT, mIGF-1 Tg and mIGF-1 Tg x SIRT1 CKO) were administered i.p. with vehicle (DMSO) or with HC-030031, a selective TRPA1 channel blocker, for 30 min at different doses (20, 50, 100 mg/kg) prior to DBP and SBP measurements. As shown in Figure 4, administration of HC-030031 at 50 and 100 mg/kg blunted the high blood pressure, both DBP and SBP, induced by cardiac mIGF-1 transgene in mice, while it had no significant inhibitory effects on the other groups. These findings suggest that increased TRPA1 expression is instrumental for mIGF-1-induced mild hypertension in mice, since pharmacological inhibition of this channel restores the blood pressure to basal levels.
Discussion

In several experimental models, activation of mIGF-1 and SIRT1 signaling pathways has proven effective in protecting against cardiovascular stresses and ischemic insults [3, 7, 21, 29, 30]. SIRT1 pharmacological activators have been developed and showed pre-clinical efficacy in cardio-protection against infarct. We recently assessed that SIRT1 is located within the nuclei of CM [3], in contrast with other reports [23, 24], whereas it is located in the cytoplasm of NCM cell types. ChIP-Seq offered us a powerful tool to determine how SIRT1 interacts with CM DNA [3], and we have provided the first SIRT1 genome-wide DNA binding data set reported, after the one obtained by Oberdoerfer et al. in embryonic stem (ES) cells with a ChIP-on-ChIP approach [16]. This report described a few hundreds of genes bound by SIRT1 in the promoter region in ES cells under basal conditions, and in conditions of oxidative stress (H$_2$O$_2$ treatment) a massive displacement of SIRT1 that goes to occupy other promoters was observed [16]. In mice hearts, we found SIRT1 similarly bound to 302 gene promoters, but there were only few dozens of promoters exclusively bound by SIRT1 either in the WT heart or in the mIGF-1 Tg heart [3]. This distribution indicates that the mIGF-1 transgene induces only subtle alterations in SIRT1 DNA binding patterns. Most interestingly these variations in the mIGF-1/SIRT1 genomic (together with transcriptomic) effects in unchallenged mouse hearts were related to genes regulating functions beyond CM specific homeostatic mechanisms, and relating to global body functions. Interestingly, we found genes implicated in blood pressure control [3]. Among these we focused on TRPA1, since its gene body was occupied abundantly by SIRT1 binding in WT but not in mIGF-1 Tg hearts. TRPA1 was first isolated in 1999 in a screen for transformation-sensitive proteins in cultured fibroblasts [11], and it was subsequently shown to detect cold and chemical stimuli.
that activate sensory neurons to produce pain [12]. More recent studies extended our understanding of TRPA1 functions, and it appears that its activity might also be implicated in cardiovascular disorders and in modulating pressure [6], making it a novel target in cardiovascular research. TRPA1 is expressed in endothelial cells and in smooth muscle cells, and here we report for the first time its abundant expression at the level of mRNA in the CM cell fraction, versus the NCM fraction, of the murine heart. We found that TRPA1 mRNA is massively increased by mIGF-1 cardiac transgene in a SIRT1 dependent manner, since CM specific inducible genetic ablation of this deacetylase in the adult heart restored TRPA1 to basal levels. An outstanding question opened by our results is: if displacement of SIRT1 from TRPA1 promoter in mIGF-1 Tg mice increased cardiac expression of this cation channel, why genetic ablation of SIRT1 in the heart paradoxically does not have similar effects? Cardiac epigenome and transcriptome are intertwined in a very complex manner, and the increase in SIRT1 activity/expression and global changes in genome occupancy in presence of mIGF-1 [3, 29, 30] might affect a myriad of unidentified transcriptional co-factors [5] that may contribute to activate TRPA1 transcription. Moreover, mIGF-1 has multiple downstream effectors, such as SGK, PDK1, NF-κB and others [7, 21] that are able to cross-talk with SIRT1: the disruption of this signaling interactome by genetic depletion of SIRT1 might be involved in the absence of TRPA1 upregulation in mIGF-1 Tg x SIRT1 CKO. The regulation of TRPA1 gene transcription by growth factors- and stress- responsive signaling pathways is an untapped research field. Also, mIGF-1 activates an anti-oxidant gene program in a SIRT1-dependent manner [1,2]. It has been demonstrated that the transcription factor hypoxia-inducible factor-1α (HIF1α) protects the heart against oxidative and hypoxic stresses [3] and, interestingly, activates TRPA1 gene transcription [4]; HIF1α activity is inhibited by
SIRT1 through direct contact and deacetylation [5]. Further studies should determine if in the absence of SIRT1 occupancy on TRPA1 promoter in the heart of mIGF-1 Tg mice, as we have shown, active HIF1α could be crucial in determining TRPA1 upregulation. It is surprising that mIGF-1 Tg mice are mildly hypertensive and that inhibition of TRPA1 can restore blood pressure to basal levels, considering that blood pressure is only partially determined by the heart, and mostly by peripheral vascular resistance. Our current study is limited by the lack of phenotyping the level of blood pressure by telemetry; further studies will be necessary to assess the role of peripheral vascular resistance. The fact that mIGF-1 cardiac transgene can elicit other systemic effects such as increase in immune cells count and behavioral modification (better performance in a fear conditioning test) [3], underlie profound differences in the “circulome” of mIGF-1 Tg animals, depending on molecules secreted by CM in an autocrine and/or paracrine fashion. In this respect, it was shown that cardiac mIGF-1 transgene is pro-angiogenic: it can trigger the production of cytokines that mobilize stem cells in the bone marrow to form new blood vessels [20]. Here we found that the effect of mIGF-1 on blood pressure mediated by TRPA1 could be rescued by CM specific depletion of SIRT1, which in turn is not associated to a decreased cardiovascular performance [30]. Of note, plasma measurement of vasoactive stress hormones associated to elevated blood pressure, such as corticosterone and vasopressin, did not show any change (*data not shown*), indicating a lack of overt stress in the animal. This study is does not rule out how the blood pressure lowering effect of the inhibition of the mIGF-1/SIRT1/TRPA1 pathway is related to the development of cardio-cerebrovascular diseases. However, although we lack an integrated physiological understanding of cardiac mIGF-1 systemic function on multiple distant target cell types, we propose that the moderate stress inducing
properties of cardioprotective mIGF-1 pathway are coherent with the theory of hormesis, i.e. exposure to mild stress should result in an adaptive response with various benefits [8, 19].

The role of TRPA1 in cardiovascular aging is unknown. TRPA1 KO mice have been generated and characterized: homozygous mice display behavioral deficits in response to cold and mechanical stimuli [15]. These animals have been employed mostly for pharmacological and neurological studies, showing that TRPA1 can also influence changes in blood pressure in connection to alterations in blood flow, vascular reactivity and autonomic system reflexes [18]. This existing model could be valuable to study TRPA1 activity regulation by growth hormones-dependent signals and cardiac ischemic insults. Elucidating in detail the systemic effects of TRPA1 and of other targets of the mIGF-1/SIRT1 pathway may help to develop new cardiac and anti-aging strategies.

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References


Figure Legends

Figure 1. SIRT1 occupancy of TRPA1 promoter in the myocardium of WT and mIGF-1 mice. Chromatin was isolated from whole hearts of WT and mIGF-1 Tg mice and processed for ChIP using a SIRT1 antibody. PCR products of three distinct regions of TRPA1 gene promoter (19-118), (77-176) and (285-384), of 100 bp of length each, were separated by gel electrophoresis. Input was used as control. Two representative WT and mIGF-1 Tg mice of 8 in total are shown.

Figure 2. TRPA1 mRNA expression in the cardiac muscle of WT and mIGF-1 Tg mice. A. mRNA hybridization in situ to detect TRPA1 mRNA expression. Hearts
from 4 months old adult animals were dissected, fixed overnight in 4%
paraformaldehyde, and processed for whole mount in situ hybridization. Detection of
transcripts on paraffin sections was performed using digoxigenin-labeled
hybridization probes against TRPA1 coding region. Two serial sections of a
representative animal per group are shown. B. The expression levels of TRPA1
mRNA were examined by qRT–PCR in the cardiomyocytes and non cardiomyocytes
fractions of wild-type (WT) and mIGF-1 Tg mice hearts. UbiC, Rn18S and GAPDH
transcripts were used as internal controls, according to the GeNorm method. Results
are means ± SE of 10 animals (***=p>0.001 versus WT).

Figure 3. Cardiomyocyte specific inducible genetic ablation of SIRT1 in mIGF-1
mice lowers TRPA1 expression. All mice were 4 months old and placed for 2 weeks
under a tamoxifen diet. Upon sacrifice, total RNA was extracted from the
cardiomyocytes and non cardiomyocytes fractions of the hearts of wild-type, SirT1
CKO, mIGF-1 Tg; SirT1^{F/F}, and mIGF Tg × SirT1 CKO. TRPA1 mRNA levels were
determined by qPCR. UbiC, Rn18S and GAPDH transcripts were used as internal
controls, according to the GeNorm method. Results are expressed as arbitrary units
and are means ± SE of 8 animals (**=p>0.01; ***=p>0.001).

Figure 4. mIGF-1/SIRT1 dependent mild hypertension is prevented by systemic
administration of TRPA1 antagonist HC-030031. Noninvasive blood pressure
values in conscious mice were measured using the tail-cuff method [diastolic and
systolic blood pressure (DBP and SBP, respectively)] in a cohort of WT, mIGF-1 Tg
and mIGF-1 Tg x SIRT1 CKO mice. Mice were injected i.p. with vehicle (DMSO) or,
for blockage of TRPA1 activity, HC-030031 was administered for 30 min at different
doses (20, 50, 100 mg/kg) prior to DBP and SBP measurements. Results are means ± SE of 8 animals for each genotype (*=p>0.01 versus WT mice).
ChIP: SirT1
PCR: TRPA1 promoter

WT  mIGF-1  WT  mIGF-1  WT  mIGF-1  Input
Reg 1 (19-118)  Reg 2 (77-176)  Reg 3 (285-384)
A

**A** TRPA1 mRNA *in situ*

mIGF-1 Tg

WT

B

**B** TRPA1 mRNA expression (arbitrary units)

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*0.5 mm*
DBP

SBP

HC-030031

20 mg/kg

50 mg/kg

100 mg/kg

mm Hg