Sirtuin 1 ablation in endothelial cells is associated with impaired angiogenesis and diastolic dysfunction

Julien MAIZEL, Sandhya XAVIER, Jun CHEN, Chi Hua Sarah LIN, Radovan VASKO,
Michael S GOLIGORSKY

1Medical Intensive Care Unit, Department of Nephrology and INSERM U-1088, University of Picardie, Amiens, France, 2Department of Nephrology and Rheumatology, University Medical Center, Gottingen, Germany and 3Department of Medicine, Renal Research Institute, New York Medical College, Valhalla, New York, USA

Address correspondence/reprint requests to:
Dr Julien Maizel
Medical Intensive Care Unit
Amiens University Hospital
80000 Amiens
France
Phone: +33 3 22455664
Fax: +33 3 22455854
Email: julien.maizel@u-picardie.fr

Running Title: Sirtuin 1 in cardiomyopathy of aging

Key words: Hypoxia, Echocardiography, VEGF, Angiogenesis, Adriamycin cardiomyopathy, Transverse Aortic Constriction

Copyright © 2014 by the American Physiological Society.
Abstract

The discordant myocardial growth and angiogenesis can explain left ventricular hypertrophy progresses toward heart failure with aging. Sirtuin-1 expression declines with age, therefore we explored the role played by angiogenesis and Sirtuin 1 in the development of cardiomyopathy. We compared the cardiac function of 10-15 weeks old (wo), 30-40 wo and 61-70 wo endothelial Sirtuin-1-deleted (Sirt1\(^{\text{endo-/-}}\)) mice and their corresponding knockout controls (Sirt1\(^{\text{Flox/Flox}}\)). After 30-40 weeks, Sirt1\(^{\text{endo-/-}}\) animals exhibited diastolic dysfunction (DD), decreased mRNA expression of Serca2a in the left ventricle (LV) and decreased capillary density compared to the control animals despite a similar VEGFα mRNA expression. However the LV fibrosis and the HIF1α expression were not different. The creation of a transverse aortic constriction (TAC) provoked a more severe DD and LV fibrosis in the Sirt1\(^{\text{endo-/-}}\) compared to control TAC animals. Although the VEGFα mRNA expression was not different and the protein expression of HIF1α was higher in the Sirt1\(^{\text{endo-/-}}\) TAC, the capillary density remained reduced. In cultured endothelial cells administration of Sirtuin-1 inhibitor decreased mRNA expression of VEGF receptors FLT 1 and FLK 1. The ex-vivo capillary sprouting from aortic explants showed the impaired angiogenic response to VEGF in the Sirt1\(^{\text{endo-/-}}\) mice. In conclusion, data demonstrates a) defect in angiogenesis preceding development of DD; b) dispensability of endothelial sirtuin-1 under unstressed conditions and during normal aging; and c) impaired angiogenic adaptation and aggravated DD in Sirt1\(^{\text{endo-/-}}\) mice challenged with LV overload.
The development of left ventricular hypertrophy (LVH) during pathological situations (hypertension, myocardial ischemia) is considered to be an adaptive response to the increased wall stress exerted on the left ventricle (LV). Although the normal cardiomyocytes grow during childhood or exercise and such a growth is not associated with heart failure, however, in aging the LVH becomes a high risk factor for heart failure. The reasons why cardiac growth can progress toward heart failure are incompletely understood. However the cardiac function has been shown to depend on the angiogenic capacity of the cardiac vessels. The disruption of coordinated myocardial growth and matching it angiogenesis leads to the progression to heart failure (27). With aging the angiogenic capacity decreases, which could explain at least in part why LVH in older patients is associated with cardiac failure (16). In aging animals, angiogenic competence is decreased. Mouse aortic rings obtained from these animals show 40% reduction in vascular sprouting induced by VEGF compared to young animals (22). A similar defect occurs in prematurely senescent Klotho-mice (26). Of note, caloric restriction regimen rescues angiogenic competence of aortic rings obtained from 24-months-old rats by 45-63% (9).

One of the downstream targets of caloric restriction, sirtuin-1, is robustly expressed in endothelial cells, tends to decline with age and following application of cardiovascular stressors (5).

Sirtuin 1 is a (NAD+)-dependent deacetylase residing in the nucleus and the cytoplasm of mammalian cells. It has been demonstrated that SIRT1 plays an essential role in responses to stressors, like reactive oxygen species (10). SIRT1 knockout mice are not viable; on the other hand, SIRT1 transgenic mice show phenotypes resembling calorie restriction. Deletion of SIRT1 in hepatocytes leads to hepatic steatosis and inflammation (10). The role of SIRT1 in cell fate and metabolic regulation is based on its effects augmenting FOXO-regulated stress resistance, interaction with p53, E2F1, PGC-1a, PPAR-γ, eNOS, to name a few (12), thus underwriting its function as a sensor of the metabolic and cellular energy state of cells, deacetylating many key-proteins and per se regulating a wide range of functions (i.e., senescence, apoptosis, longevity). Angiogenesis is one of these functions regulated by sirtuin 1 (21). The loss of sirtuin 1 expression in endothelial cells leads to impaired angiogenesis, premature senescence and downregulation of several pro-angiogenic genes (5).

Therefore, the objective of the present study was to better understand the role played by angiogenesis and one of its key regulators Sirtuin 1 in the development of cardiomyopathy, hence, we conducted a study to explore the cardiac consequences of the inactivation of Sirtuin 1 in coronary endothelial cells.
Materials and Methods

Mice with endothelial Sirt1 deletion
The animal study protocol was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Endothelial Sirt1-deleted mouse model was created by cross-breeding of B6;129-Sirt1tm1Ygu/J (homozygous for targeted allele Sirt1co/co, viable and fertile, containing a loxP-flanked neomycin cassette upstream and downstream of exon 4 of the targeted gene) (4) with Tie2-Cre transgenic mice (B6.cg-tg(tek-cre)1ywa/J) expressing cre recombinase in vascular endothelial cells (both from Jackson Lab) (14). The resulting Sirt1endod-/ mice were mated with Sirt1Flox/Flox mice to obtain endothelial-deleted Sirt1 mutant mice. Endothelial Sirt1endod-/ knockout (and corresponding knockout-control Sirt1Flox/Flox) were obtained at Mendelian ratios and used in the experiments. Genotyping was performed by tail DNA PCR analysis. Tail DNA was isolated using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St Louis, USA). Primer sequences used for genotyping floxed Sirt1 allele were as follows: Sirt1 forward: 5’GGT TGA CTT AGG TCT TGT CTG3’; SIRT1 reverse: 5’CGT CCC TTG TAA GTT TTC CC3’ and for SIRT1 null allele 5’AGG CGG ATT TCT GAG TTC GA3’. Tie2 transgene was detected using following primers: forward 5’GCG GTC TGG CAG TAA AAA CTA TC3’ and reverse 5’ GTG AAA CAG CAT TGC TGT CAC TT3’. The PCR products were analyzed on 1.5% TAE acrylamide gels. Male and female mice were used in the same proportion in the presented experiments.

Consequences of aging for Sirt1endod-/ cardiac functions
We compared three groups of young (10 to 15 weeks old) (n=10), middle age (30 to 40 weeks old) (n=15) and old (61 to 70 weeks old) (n=11) Sirt1endod-/ and control (Sirt1flox/flox) animals. Each group was examined by echocardiography followed by exsanguination under ketamine/xylazine anesthesia. The heart was washed with cold PBS in situ than carefully harvested and sections of LV were used for histology, western blot and Q PCR studies.

Left ventricular pressure overload in Sirt1endod-/ mice
We compared two groups composed of 30-40 weeks old controls (Sirt1flox/flox) (n=7) and Sirt1endod-/ (n=5) exposed to a LV pressure overload and one group of the same age controls without surgery (n=5). The pressure overload was created by transverse aortic constriction (TAC) under general anesthesia 7 weeks before the euthanasia. The procedure for TAC has been described elsewhere (7). Briefly, under general anesthesia with isoflurane, oral intubation and ventilation, the animal’s sternum was opened at the first 2 ribs and the aortic arch was exposed. A 271/2G needle was placed aside the aortic arch and a suture (silk suture 6/0) was ligated around the aorta and the needle. The needle was removed, then chest and skin were closed with an auto-resorbent sutures. Transthoracic echocardiography was performed before, 1 week and 7 weeks after the surgery. Following the last TTE, the animals were euthanized by exsanguination under ketamine/xylazine anesthesia. The heart was washed with cold PBS in situ than carefully harvested and sections of LV were subjected to histologic studies, western blotting and qPCR studies.

Adriamycin cardiomyopathy in Sirt1endod-/ mice
Similar studies were conducted in mice with Adriamycin cardiotoxicity, a model of a
distrophic myocardial injury. Thirty weeks old Sirt1<sup>endo-/</sup> (n=5) and control (n=5) mice
were treated with Doxorubicin hydrochloride (Sigma Chemical Co, St Louis, MO) according
to a procedure described previously (2). Doxorubicin was dissolved in saline and injected
intraperitoneally (IP) at 4 mg/Kg (10ml/Kg of body weight) twice a week (every Monday
and Thursday) for a total of 10 injections. Animals were not treated for 2 weeks between
the first 4 injections and the last 6 injections. Transthoracic echocardiography was
performed at baseline, 5 weeks after the initiation of treatment and one week before
euthanasia (9 weeks after the beginning of treatment). Animals were euthanized after the
last injection (10 weeks after the beginning of treatment). The heart was washed with cold
PBS <i>in situ</i> than carefully harvested and sections of LV were subjected to histologic studies.

**Echocardiography**

Transthoracic echocardiography examinations were performed under isoflurane inhalation
general anesthesia with a heart rate of approximately 400-450 beats per minute (bpm).
Isoflurane was administered with a vaporizer. Following induction with 3.5–4.5%
isoflurane in an isolated chamber, anesthesia was maintained with 1–1.5% isoflurane
delivered through a small nose cone. Transthoracic echocardiography measurements were
performed using a commercially available echocardiograph (770, Visualsonic, Canada) with
a 30 MHz transducer. M-mode echocardiography of the LV was performed in order to
measure the following parameters: LV end-diastolic diameter (LVDD), LV end systolic
diameter (LVSD), diastolic posterior wall thickness (PW) and diastolic septal wall thickness
(SW), according to the American Society of Echocardiography guidelines (24). Left ventricle
mass was calculated using the following formula: LV mass = 1.04 x [(LVDD+PW+SW)<sup>3</sup>-
LVDD<sup>3</sup>]. The shortening fraction (SF) was calculated as the difference between the LVDD
and the LVSD divided by the LVDD. Mitral pulsed Doppler was recorded at the tip of the
mitral valve with an apical view, in order to analyze the isovolumic relaxation time (IVRT),
the mitral flow duration, the duration of diastole and the ejection time. The Tei index was
calculated as the difference between the duration of diastole and the mitral flow duration,
divided by the ejection time (29).

**Western blotting**

Expression of proteins of interest was quantified using Western blot analysis. Briefly, LV
tissues were homogenized twice for 10 s using a Polytron homogenizer (Pro Scientific Inc.)
at 4°C in RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mM
NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF and a protease inhibitor cocktail tablet). Homogenates were
centrifuged at 14,000 rpm for 15 min at 4°C and the supernatants were assayed. The
protein concentration was determined in a Bradford assay (Bio-Rad). Samples were
denatured in Laemmli buffer (125 mM Tris HCl, 2% SDS, 1% β-mercaptoethanol, 10%
glycerol, 0.005% bromophenol blue, pH 6.8) and heated at 95°C for 5 min. Twenty µg of
total protein per sample were separated on 4%-20% acrylamide/bisacrylamide SDS-PAGE
under constant current conditions. Proteins were then transferred to polyvinylidene
difluoride (PVDF) 0.45 µm membranes (Millipore) in a transfer buffer at 90 V for 90 min.
Membranes were then blocked with 5% non-fat milk in Tris-buffered saline (TBS) with
0.1% Tween 20 for 1 h at room temperature and then immunoblotted with the desired
primary antibody in TBS BSA 0.1% overnight at 4°C. The source and concentration of HIF1α antibody was Millipore, 1/1000. After several washings in TBS 0.1% Tween 20, membranes were incubated with HRP-conjugated goat anti-mouse antibody (1:5000). Each secondary antibody was incubated for 1 h at room temperature prior to development using the enhanced chemiluminescence detection kit (Millipore, USA). Bands were visualized and quantified using the Image J software (Version 1.45; NIH). Protein levels were normalized against endogenous β-tubulin levels.

**Immunoprecipitation**

One hundred µg of total LV protein extract prepared for western blot analyses were used for immunoprecipitation. Proteins were incubated with HIF1α antibody (Millipore, 1/500) overnight at 4°C and then for 2 hours at 4°C with 20µl of protein A/G plus-Agarose immunoprecipitation reagent (Santa Cruz Biotechnologies, USA). Immune complexes were collected by centrifugation and washed three times with ice-cold phosphate-buffered saline (PBS). After a final wash, the supernatant was discarded and the pellet was resuspended in SDS lysis buffer, and then boiled in 5× SDS loading dye for 5 min. Protein was separated by SDS–PAGE and transferred on PVDF membranes. Immunoprecipitated proteins were then detected with anti-acetyl lysine antibody (1/1000, Abcam, USA) or HIF1α antibody (Millipore, 1/800) overnight at 4°C. Bands were visualized and quantified using the Image J software (Version 1.45; NIH).

**Quantitative PCR (qPCR)**

Total RNA was extracted from left ventricles using RNA Purification kit (Denville Scientific Inc, USA), and total RNA (1 µg) was transcribed into complementary DNA using the “High Capacity cDNA Reverse Transcription Kit” according to the manufacturer’s protocol (Applied Biosystems, USA). The following primers were used: Serca 2a (Forward, TGA ATC TGA CCC AGT GGC TGA; Reverse, ACT CCA GTA TTG CAG GCT CCA), PGC1a (Forward, AGC CTA TGA GCA CGA AAG GCT CAA; Reverse, TGG CCC TTT CAG ACT CCC GCT), Sirtuin 3 (Forward, TCA CAA CCC CAA GCC CTT TT, Reverse GTG GGC TTC AAC CAG CTG TT), Sirtuin 6 (Forward, GAC CTG ATG CTC GCT GAT GA, Reverse CTG GGC GTC ATG TTT TGT GG), Short endoglin (Forward, TGA GTA TCC CAA GCC TCC ACC CCA T, Reverse CTG AGG GGC GTG GGT GAA GGT CAG), Long endoglin (Forward, GCA CTC TGG TAC ATC TAT TCT CAC ACA GTG GG, Reverse GGG CAC TAC GCC ATG CTG CTG GTG G), Collagen 1 (Forward CTG CTG GCA AAG ATG GAG A; Reverse ACC AGG AAC ACC CTG GAA TC), VEGFA (Forward, TCC ACC ATG CCA AGT GTT CCC AGG CTG CAC CCA; Reverse, GAC GTG GCC ACG CAC TCC AGG) and 18S (Forward, AAG GAG ACT CTG GCA TGC TAA C; Reverse, CAG ACA TCT AAG GGC ATC ACA GAC). SYBR Green chemistry was used to perform quantitative determinations of the mRNAs. The cDNAs were amplified using a Stratagene Mx3005P (Agilent Technologies) sequence detection system (Applied Biosystems, USA). 18S was used to normalize differences in RNA isolation, RNA degradation, and the efficiency of the reverse transcription reaction. Changes in mRNA expression were determined using the comparative threshold method (17). RNA expression was also expressed in relation to the levels observed in control mice.

**Histochemical studies**

Hearts were fixed in 4% PFA for 24 hours, embedded in paraffin and sectioned (8µm). The
Heart sections were stained with hematoxylin eosin, Masson’s trichrome and anti CD31 (Abcam ab28364, USA). Analysis of 5 to 8 randomly selected fields (400x magnification) was conducted. Masson’s trichrome images were quantified using a digital method by Photoshop software previously described (6). This technique allows measurement of the percentage of Trichrome’s positive pixels (blue) within the total number of tissue pixels (blue and red).

For CD31 staining, sections were first deparaffinized, rehydrated and then exposed to an antigen retrieval solution (Target retrieval solution, Dako, USA). Slides were stained following the streptavidin biotin procedure using the LSAB system HRP (Dako, USA). Briefly, slides were incubated with the primary antibody (CD31, Abcam, 1/100 dilution) overnight at 4°C following peroxydase blocking. Slides were then exposed to the streptavidin peroxydase solution followed by chromogen solution. Counterstain was performed with hematoxylin.

The number of CD31-positive structures per image (400x magnification) were counted and normalized to the cardiomyocyte area. The cardiomyocyte area was quantified per image using the Adobe Photoshop software v8.0 (Adobe Systems, San Jose, CA, USA). First, each picture was adjusted to obtain the same pixel dimensions per image. Than the area of blank pixel (interstitial species) was automatically measured. The cardiomyocyte area was calculated as the total area of the image minus the blank areas (interstitial spaces) (31).

**Cell culture**

Immortalized human umbilical vein endothelial cells (HUVEC) (ATCC, Manassas, USA), were maintained in DMEM supplemented with 10% FBS and 1% penicillin under conditions of 37°C and 5% CO₂. The cells were cultured in 35mm dishes. Half of the dishes were daily treated with Sirtuin 1 inhibitor III (Calbiochem, Millipore, USA) 10μmol per well for 5 days. At day 5, the cells were harvested and total cell RNA was isolated using the RNA Purification kit (Denville scientific inc, USA) for QPCR following the same procedure exposed above. The following primers were used: Human FLT1 (Forward, GCA CCA TAC CTC CTG CGA AA; Reverse, TGG TGG CTT TGC AGT GAT AGA), Human FLK1 (Forward, CGT GTC TTT GTG GTG CAC TG; Reverse, GGT TTC CTG TGA TCG TGG GT) and Human 18S (Forward, GAG GAT GAG GTG GAA CGT GT; Reverse, TCT TCA GTC GCT CCA GGT CT).

**Aortic sprouting angiogenesis assay**

Thoracic aortas were obtained from 12-week-old controls and Sirt1endo-/− mice. Aortic rings sectioned with 1-mm interval were embedded in 3D matrigel in 96-well plate and cultured at 37°C in EGM2 without VEGF or supplemented with VEGF. Newly formed capillary cords in explant cultures were imaged after 6 days using Nikon TE2000 microscope equipped with a CCD camera (Hamamatsu Photonics) at a magnification of 40x. Quantitative angiogenesis assays were performed according to previously published protocol (3).

**Statistical Analyses**

All experiments were repeated at least three times. Values are given as mean± SE. Data were analyzed using U Mann Whitney test for comparison of two groups or ANOVA (with or without repeated measurements) with post-hoc analysis for multiple group comparisons using Bonferroni method. A p value of less than 0.05 was considered statistically significant.
Results

Characterization of the Sirt1\textsuperscript{endo-/} mouse model

Our strategy to delete SIRT1 in endothelial cells, similar to that used by Potente et al. (21), has been previously reported (5, 32). We cross-bred B6;129-Sirt1tm1Ygu/J mice (homozygous for targeted allele Sirt1co/co, viable and fertile, containing a loxP-flanked neomycin cassette upstream and downstream of exon 4 of the targeted gene) with Tie2-Cre transgenic mice. The offspring thrive as their wild-type littermates (Mendelian distribution), are normotensive and show normal blood counts (data not shown). Endothelial and endothelial progenitor cells isolated from SIRT1\textsuperscript{endo-/} mice, however, showed increased proportion of senescent and apoptotic cells under basal conditions and reduced resistance to stress, as shown by our group (5). En face aortic preparations stained for the expression of senescence-associated β-galactosidase revealed a dramatic increase in the frequency of senescent endothelial cells already at the age of 12 weeks (5).

Consequences of aging for Sirt1\textsuperscript{endo-/} cardiac functions

The body weight of the animals increased with age but remained similar between the controls and Sirt1\textsuperscript{endo-/} animals. On echocardiography, the Sirt1\textsuperscript{endo-/} animals, after 30-40 weeks of age, developed progressively a diastolic dysfunction with a higher IVRT and Tei index. The LV mass and the systolic function remained similar (Figure 1). The diastolic dysfunction was in accord with the decreased mRNA expression of Serca2a in LV (Figure 2). We also found a lower capillary density (number of CD31 positive structures per cardiomyocyte) in the Sirt1\textsuperscript{endo-/} group compared to the controls animals (Figure 3) whereas the quantification of Masson’s trichrome staining in the LV and the mRNA expression of collagen 1 were similar between the control and Sirt1\textsuperscript{endo-/} groups (Figure 4A and 4B). The Sirtuin 6 mRNA expression in the myocardium was decreased in the Sirt1\textsuperscript{endo-/} animals compared to the control animals after 30-40 weeks of age and preceded the decreased Sirtuin 3 mRNA expression (Figure 5A and 5B). But the PGC1a mRNA expression remained similar between groups (Figure 5C). The Sirt1\textsuperscript{endo-/} animals presented a higher short-to-long endoglin mRNA expression in 30-40 weeks-old animals and older (Figure 5D). To explain the decreased angiogenesis in our Sirt1\textsuperscript{endo-/} animals we explored the protein expression of HIF1α, an angiogenic factor activated by a sirtuin 1 deacetylation. We did not detect any difference in the protein expression and in the proportion of acetylated HIF1α in the myocardium of the different groups of animals (Figure 6A). The VEGFa mRNA expression was also similar at 30-40 weeks old and younger mice, but decreased in the 60-70 weeks-old Sirt1\textsuperscript{endo-/} animals compared to the controls (Figure 6B). Thus, the above studies showed that the cardiac phenotype of Sirt1\textsuperscript{endo-/} animals is mild indicating that sirtuin 1 is dispensable under basal conditions. This finding necessitated further investigation of the cardiac phenotype of these mice under the applied stress.

Consequences of left ventricular pressure overload for cardiac functions of Sirt1\textsuperscript{endo-/} mice

The LV mass increased after surgery in a similar manner between both strains of mice. Also
the systolic function decreased with surgery but did not differ between controls and Sirt1endo-/-(Figure 7). In contrast, a diastolic dysfunction (IVRT and Tei index) appeared 7 weeks after the TAC in the Sirt1endo-/animals. At that time the mRNA expression of Serca2a in the LV was decreased in the TAC animals but no difference was found between the Sirt1endo-/ TAC and the Controls TAC (Figure 8). Similar to that, Sirtuin 3 mRNA expression decreased in the myocardium of both TAC groups, but we did not find any differences of Sirtuin 6 mRNA expression (Figure 9A and 9B). The PGC1α mRNA expression decreased similarly in both TAC groups (Figure 9C).

The capillary density in the TAC Sirt1endo-/animals remained lower than in the control TAC mice (Figure 10). The development of fibrosis and the mRNA expression of Collagen 1 were significantly higher in the Sirt1endo-/ TAC compared to the 2 other groups (Figure 11A and 11B).

The aortic constriction increased the expression of the HIF1α protein in the LV and was more prominent in the Sirt1endo-/ TAC animals. However, the proportion of acetylated to total HIF1α remained constant among mice with and without TAC (Figure 12A). This indicated that the abundance of acetylated HIF1α was the highest in the Sirt1endo-/ mice subjected to TAC. The VEGFa mRNA expression increased similarly in both TAC groups (Figure 12B).

**Consequences of adriamycin injections**

Similar studies were conducted in mice with Adriamycin cardiotoxicity, a model of a distrophic myocardial injury. No differences between Sirt1endo-/ and control adriamycin-treated with animals were detectable on echocardiography (Figure 13). All animals that received adriamycin injections developed a systolic and diastolic dysfunctions associated with an increased LV mass. This cardiomyopathy was nonetheless similar between Sirt1endo-/ and controls animals. On morphologic examination, adriamycin treatment was associated with a similar increase in fibrosis and decrease in capillary density in the LV of both Sirt1endo-/ and control animals (Figure 14A and 14B).

**Cell culture and ex vivo aortic angiogenesis assay**

To understand why the capillary density remained lower in the Sirt1endo-/ TAC mice despite the higher VEGF-a mRNA expression in this group, we analyzed the mRNA expression of VEGF receptors in HUVEC exposed to Sirt1 inhibitor. The administration of Sirt 1 inhibitor during 5 days to HUVEC was associated with a significant decrease of the VEGF receptors FLT1 and FLK1 mRNA expression (Figure 15). This can explain why the angiogenesis remained altered in the Sirt1endo-/ animals despite the higher expression of VEGF in the aortic constriction groups.

In the aortic sprouting assay, the angiogenesis of the control aortic rings showed a trend (p=0.08) toward increase of the number of capillary sprouts with the addition of VEGF in the culture medium, but this increase was absent in the Sirt1endo-/ aortic rings (Figure 16). Moreover, in the VEGF-enriched medium the number of capillary sprouts was significantly higher in controls than in the Sirt1endo-/ rings. This confirms the impaired angiogenic response to VEGF in the Sirt1endo-/ mice.

**Discussion**
The decline of the angiogenic capacity is a hallmark of the aging process and has also been described in many cardiac diseases (11, 18). Here, we show for the first time the complex consequences on the myocardium of the angiogenic failure due to the decreased expression of the Sirtuin 1 protein in coronary endothelial cells. In our model the decreased expression of the VEGF receptors observed in vitro in endothelial cells exposed to Sirtuin 1 inhibitor can explain the impaired capillary density observed in our Sirt1endo-/− animals. The decreased number of capillaries has been previously described as a mechanism of cardiac failure (18, 27). Especially in pathological situations increasing the LV work (e.g. hypertension), the development of LVH requires the matching level of angiogenesis (13, 25). The disruption of coordination between cardiomypocyte growth and angiogenesis leads to the development of heart failure (27). The angiogenesis is controlled by several pathways among them VEGF and its receptors (FLT1 and FLK1) play important roles. The knockout of Sirtuin 1 in the coronary endothelial cells is associated with a decreased expression of FLT1 and FLK1 compromising the angiogenesis (21). In the absence of aortic constriction, the Sirt1endo-/− animals develop diastolic dysfunction without any significant myocardial fibrosis or increased expression of the HIF1α protein in the myocardium. Once aortic banding is created, the increased oxygen demands required by the development of LVH unmask the inability of those animals to adapt normally to left ventricular pressure overload. Those animals showed worsened diastolic dysfunction, cardiac fibrosis and overexpression of the HIF1α protein. Despite the overexpression of HIF1α and VEGF, the capillary density remained insufficient, perhaps, due to the impaired expression of the VEGF receptors FLT1 and FLK1. Interestingly those observations in Sirt1endo-/− animals with a hypertrophic cardiomypathy (aortic banding) were not observed in the dystrophic model of adriamycin injections in the Sirt1endo-/− animals.

Although Sirutin 1 is genetically deleted in endothelial cells, young 10-15 week-old animals do not exhibit any cardiac abnormalities, as judged by echocardiography, histology and molecular biology studies. The cardiac alterations appear later after 30 weeks of age. This could mean several things. First, this could be related to the insufficient sensitivity of our technological platform (echocardiography, molecular biology and histology), which however is a gold standard for such investigations. Second, this could mean that the absence of sirutin 1 expression in endothelial cells is not sufficient to provoke cardiac alterations. In accord with this view, we show here that aging and pressure overload are required to unmask cardiac abnormalities. Therefore, sirtuin expression in endothelial cells is mandatory for the adaptation of cardiac functions to aging and pressure overload. In addition, the aortic ring experiments performed in our study were conducted using rings obtained from 12 weeks-old animals. At this age the animals do not show any differences in aortic sprouting without VEGF application (Figure 16). In contrast, exposing rings to VEGF provokes the discrepant response of aortic sprouting between control and Sirt1endo-/− animals. This finding emphasizes the role played by the endothelial Sirutin 1 in the angiogenesis induced by VEGF.

The only age-dependent cardiac modification observed in our control animals is the mild increase of cardiac fibrosis on the Masson’s trichrome staining. However, this increase is similar in control and Sirt1endo-/− animals and the area of fibrosis is rather small even in the older group. It is therefore likely that this mild increase of fibrosis cannot be responsible for the observed changes in the cardiac function of our animals. It is also possible that 60-70 weeks of age is not sufficiently advanced to reveal any significant modifications. The
absence of alterations in echocardiography and in molecular biology parameters in the
heart of the 60-70 weeks-old control animals do not preclude later abnormalities.
The mRNA expression of Serca 2a is decreased in the myocardium of our Sirt1 endo/- animals,
but not in the control animals. This is in accordance with the diastolic dysfunction observed
on echocardiography. The protein Serca 2a is known to be related to the relaxation of the
cardiomyocyte by sequestering Ca2+ from the cytosol into the sarcoplasmic reticulum (20).
Serca 2a is also involved in the angiogenesis. The protein Serca 2a is a key step in the redox-
sensitive Ca2+ store regulation via a S-glutathionylation. This glutathionylation of Serca 2a
is induced by the accumulation of reactive oxygen species and enhances the ATP-dependent
Ca2+ uptake, decreases cytoplasmic Ca2+ levels and promotes endothelial angiogenic
responses in vitro (8). Therefore the decreased expression of Serca2a in the myocardium of
our aging or pressure overloaded Sirt1 endo/- animals can be also responsible for the
decreased angiogenesis (30).
The sirtuin 3 and 6 expressions do not decrease with aging in our control animals, but they
are decreased with aging in the Sirt1 endo/- myocardium. The sirtuin 3 is expressed in the
mitochondria and plays a role in the response to low energy input in mitochondria (23).
Sirtuin 6 is localized to the nucleus and its expression has been related to life span and the
protection against the development of cardiac hypertrophy (28). Little is known about the
regulation of Sirtuin 3 and 6 expression in heart. Along with sirtuin 1, sirtuin 3 and 6 levels
increase in response to calorie restriction. With aging (59 to 70 years old subjects) the
sirtuin 3 expression in the vastus lateralis muscle of humans was shown to decrease (15),
but in the myocardium it has never been described. Our results show that sirtuin 3 and 6
mRNA expression is decreased with aging (mostly in 60 to 70 weeks-old animals) in the
Sirt1 endo/- animals, but not in controls. This finding suggests that in the myocardium the
sirtuin 3 and 6 expression during ageing are decreased due to the absence of sirtuin 1 in the
endothelial cells.
The mRNA expression of PGC1a was not different between the experimental groups,
although PGC1a is a known target of Sirt 1. The PGC1a regulates the angiogenesis in the
cardiac tissue by driving the expression of VEGF (1). The knocked down expression of
PGC1a in cardiac tissue leads to an impaired angiogenesis and the development of a
peripartum cardiomyopathy (19). The administration of VEGF in those animals, partly
rescued the peripartum cardiomyopathy. The sirtuin 1 gene in our model is knocked out
only in the endothelial cells. Therefore, it is possible that the expression of sirtuin 1 and
PGC1a in the myocardium undergo compensatory changes, which are reflected in the
results obtained from the left ventricle.
In conclusion, a model of hypertrophic cardiomyopathy, as opposed to the dystrophic
cardiomyopathy, demonstrates the inability of endothelial cells deficient in Sirtuin 1 to
create new vessels and adapt to a challenging condition, like pressure overload, leading to
the development of diastolic dysfunction.

Acknowledgements. Studies were supported in part by NIH grants, and Westchester
Artificial Kidney Foundation. We are indebted to Drs. Leonard and Carol Eisenberg for help
using echocardiography equipment. This work was supported by the Societe Francaise de
Nephrologie, la Societe de Reanimation de Langue Francaise, le Conseil Regional de Picardie
and the University Hospital of Amiens (JM).
References


FIGURE LEGENDS

Figure 1: Cardiac consequences of the endothelial Sirtuin 1 inactivation at different ages. Comparisons of left ventricular (LV) weight on body weight, shortening fraction, isovolumic relaxation time and Tei index at different ages between control and Sirt 1<sup>endo-/-</sup> measured by echocardiography. Echocardiography shows a diastolic dysfunction after 30-40 weeks of age with an increased isovolumic relaxation time (IVRT) and Tei index. Systolic fraction and left ventricular (LV) weight / body weight were similar between the groups of experimental animals.

Figure 2: Cardiac expression of Serca2a measured by quantitative PCR in the left ventricles of control and Sirt 1<sup>endo-/-</sup> animals at different ages. The expression of Serca2a mRNA in the LV of Sirt1<sup>endo-/-</sup> animals decreased after 30-40 weeks of age compared to controls.

Figure 3: CD-31 staining (brown stain) in the left ventricle of control and Sirt 1<sup>endo-/-</sup> animals at different ages. Quantification of the CD31 staining is presented in the bar graph as the number of vessels per mm<sup>2</sup>. Note that the capillary density in the LV myocardium of the Sirt1<sup>endo-/-</sup> mice was decreased after 30-40 weeks of age compared to controls.

Figure 4: (A) Masson's trichrome staining (blue stain) in the left ventricle of control and Sirt 1<sup>endo-/-</sup> animals at different ages. Quantification of the fibrosis is presented in the bar graph as the percentage of Masson's trichrome positive area. No evidence of exaggerated fibrosis in Sirt1<sup>endo-/-</sup> animals. Masson's trichrome staining did not show any significant differences of LV fibrosis between Sirt1<sup>endo-/-</sup> and control animals at different ages. (B) Cardiac expression of Collagen 1 measured by quantitative PCR in the left ventricles of control and Sirt 1<sup>endo-/-</sup> animals at different ages. The mRNA expression of collagen 1 in the left ventricle of sirt1<sup>endo-/-</sup> and controls was not different.

Figure 5: Cardiac expression of metabolic (Sirtuin 3, Sirtuin 6 and PGC1a) and senescence markers (short : long endoglin) measured by quantitative PCR in the left ventricles of control and Sirt 1<sup>endo-/-</sup> animals at different ages. The mRNA expression of (A) Sirtuin 6 and (B) Sirtuin 3 decreased with age in the myocardium of Sirt1<sup>endo-/-</sup> animals. PGC1a mRNA abundance was not different from controls (C). Compared to controls the mRNA expression of (D) short : long endoglin increased in the Sirt1<sup>endo-/-</sup> mice with aging.

Figure 6: (A) Expression of the total protein HIF1a and the immunoprecipitated acetylated HIF1a measured by immunoblotting in the in the left ventricles of control and Sirt 1<sup>endo-/-</sup> animals. (B) Cardiac expression of VEGFa measured by quantitative PCR in the left ventricles of control and Sirt 1<sup>endo-/-</sup> animals at different ages.

Figure 7: Comparisons of body weight, left ventricular (LV) weight on body weight, shortening fraction, isovolumic relaxation time and Tei index between controls without surgery, controls with transverse aortic constriction (TAC) and Sirt 1<sup>endo-/-</sup>.
TAC measured by echocardiography. Measurements were performed before the surgery (baseline), 1 and 7 weeks after the surgery. The inactivation of Sirtuin 1 in the coronary endothelial cells worsens the cardiac consequences due to pressure overload provoked by transverse aortic constriction (TAC). The diastolic function (isovolumic relaxation time and Tei index) of the Sirt1endo-/ animals subjected to TAC was more profound on echocardiography than in control TAC animals. The LV weight/body weight increased and the systolic fraction decreased similarly after surgery between the 2 groups.

Figure 8: Cardiac expression of Serca2a measured by quantitative PCR in the left ventricles (LV) of controls without surgery, controls with transverse aortic constriction (TAC) and Sirt 1endo-/ TAC animals 7 weeks after the surgery. The expression of Serca2a mRNA in the LV decreased in both constricted TAC groups, but was not different between the Sirt1endo-/ and the control animals.

Figure 9: Cardiac expression of metabolic (Sirtuin 3, Sirtuin 6 and PGC1a) markers measured by quantitative PCR in the left ventricles (LV) of controls without surgery, controls with transverse aortic constriction (TAC) and Sirt 1endo-/ TAC animals 7 weeks after the surgery. (A) The mRNA expression of Sirtuin 3 in the LV decreased in both TAC groups. (B) Sirtuin 6 mRNA expression levels in the LV remained similar between the different groups. (C) The mRNA expression of PGC1a in the LV decreased in both constricted groups.

Figure 10: CD-31 staining (brown stain) in the left ventricle of controls without surgery, controls with transverse aortic constriction (TAC) and Sirt 1endo-/ TAC animals 7 weeks after the surgery. Quantification of the CD31 staining is presented in the bar graph as the number of vessels per mm². Note that the capillary density in the control TAC animals increased, but the opposite occurred in the Sirt1endo-/ TAC group of mice.

Figure 11: (A) Masson’s trichrome staining (blue stain) in the left ventricle of controls without surgery, controls with transverse aortic constriction (TAC) and Sirt 1endo-/ TAC animals 7 weeks after the surgery. Quantification of the fibrosis is presented in the bar graph as the percentage of Masson’s trichrome positive area. The LV fibrosis increased in both groups of TAC animals, but the extent of fibrosis was significantly higher in the Sirt1endo-/ animals. (B) Cardiac expression of Collagen 1 measured by quantitative PCR in the left ventricles of controls without surgery, controls with transverse aortic constriction (TAC) and Sirt 1endo-/ TAC animals 7 weeks after the surgery. The mRNA collagen 1 expression in the myocardium of the Sirt1endo-/ constricted animals significantly increased, but there was no significant difference with the control group.

Figure 12: (A) Expression of the total protein HIF1a and the immunoprecipitated acetylated HIF1a measured by immunoblotting in the in the left ventricles of controls without surgery, controls with transverse aortic constriction (TAC) and Sirt 1endo-/ TAC animals 7 weeks after the surgery. Immunoblotting of HIF1a in the LV showing the
increased expression of total HIF1a in the LV of Sirt1endo-/ of TAC animals. The expression of lysine acetylated HIF1a was similar between both TAC groups, therefore the proportion of deacetylated HIF1a was higher in the Sirt1endo-/ animals. (B) Cardiac expression of VEGFa measured by quantitative PCR in the left ventricles of controls without surgery, controls with transverse aortic constriction (TAC) and Sirt1endo-/ TAC animals 7 weeks after the surgery. The mRNA expression of VEGFa increased in the LV of both constricted groups.

Figure 13: Comparisons of left ventricular (LV) weight on body weight, shortening fraction, isovolumic relaxation time and Tei index measured by echocardiography between Sirt1endo-/ and controls animals subjected to adriamycin injections. Echocardiography was performed at baseline, 5 weeks and 9 weeks after the beginning of treatment.

Figure 14: (A) Masson’s trichrome staining (blue stain) in the left ventricle of controls without adriamycin, controls with adriamycin and Sirt 1endo-/ adriamycin animals 9 weeks after the beginning of treatment. Quantification of the fibrosis is presented in the bar graph as the percentage of Masson’s trichrome positive area. (B) CD-31 staining (brown stain) in the left ventricle of controls without adriamycin, controls with adriamycin and Sirt 1endo-/ adriamycin animals 9 weeks after the beginning of treatment. Quantification of the CD31 staining is presented in the bar graph as the number of vessels per mm².

Figure 15: Expression of the VEGF receptors Flt1 and Flk1 measured by quantitative PCR in cultured immortalized human umbilical vein endothelial cells (HUVEC) exposed to 5 days of Sirtuin 1 inhibitor or placebo.

Figure 16: Ex-vivo capillary sprouting from aortic explants of control and Sirt1endo-/ mice after 6 days of culture in media with or without VEGF. The numbers of capillary sprout per aortic explant are presented in the bar graph.
Table 1: Body weight and left ventricular echocardiographic parameters in mice of different ages

<table>
<thead>
<tr>
<th></th>
<th>10-15 wo</th>
<th></th>
<th>30-40 wo</th>
<th></th>
<th>60-70 wo</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Sirt1 <em>endo/-</em></td>
<td>Controls</td>
<td>Sirt1 <em>endo/-</em></td>
<td>Controls</td>
<td>Sirt1 <em>endo/-</em></td>
</tr>
<tr>
<td>Body weight</td>
<td>19.1±1.5</td>
<td>19.5±1.6</td>
<td>24.1±1.2</td>
<td>25±1.4</td>
<td>34.1±1.6</td>
<td>32±3</td>
</tr>
<tr>
<td>LVEDD</td>
<td>3.67±0.15</td>
<td>3.8±0.19</td>
<td>4±0.14</td>
<td>3.9±0.17</td>
<td>4.28±0.1</td>
<td>4.17±0.1</td>
</tr>
<tr>
<td>LVESD</td>
<td>2.53±0.16</td>
<td>2.74±0.24</td>
<td>2.81±0.12</td>
<td>2.77±0.19</td>
<td>3±0.1</td>
<td>3±0.11</td>
</tr>
<tr>
<td>IVS</td>
<td>0.63±0.05</td>
<td>0.63±0.04</td>
<td>0.65±0.02</td>
<td>0.7±0.04</td>
<td>0.74±0.03</td>
<td>0.66±0.04</td>
</tr>
<tr>
<td>PW</td>
<td>0.58±0.03</td>
<td>0.59±0.05</td>
<td>0.63±0.02</td>
<td>0.68±0.04</td>
<td>0.8±0.03</td>
<td>0.76±0.04</td>
</tr>
</tbody>
</table>

LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; IVS, inter ventricular septum; PW, posterior wall
Figure 1

- LV weight on body weight
- Shortening fraction, %
- Isovolumic relaxation time, ms
- Tei index

Control vs Sirt 1^{endo/-}
Figure 2

The diagram shows the relative Serca2a mRNA levels over different weeks (10-15 wo, 30-40 wo, 60-70 wo) for both control and Sirt 1<sup>endo/-</sup> groups. The data points indicate a decrease in relative mRNA levels over time for the Sirt 1<sup>endo/-</sup> group compared to the control group.
Figure 3

Control

Sirt 1<sup>endo-/-</sup>

10-15 wo

30-40 wo

60-70 wo

Number of vessels per mm<sup>2</sup>

0 500 1000 1500 2000 2500 3000 3500 4000 4500

10-15 wo 30-40 wo 60-70 wo

0.07 0.02 0.03

Control  Sirt 1<sup>endo-/-</sup>
Figure 5

A. Relative Sirtuin 6 mRNA levels

B. Relative Sirtuin 3 mRNA levels

C. Relative PGC1α mRNA levels

D. Relative Short: Long endoglin mRNA levels

- **Control**
- **Sirt 1<sup>endo-/-</sup>**
Figure 9

(A) Relative Sirtuin 3 mRNA levels

(B) Relative Sirtuin 6 mRNA levels

(C) Relative PGC1a mRNA levels
Figure 10

The bar chart shows the number of vessels per mm² for different groups: Control, Control + TAC, and Sirt1<sup>endo⁻/⁻</sup> + TAC. The chart indicates a significant decrease in vessel count in the Sirt1<sup>endo⁻/⁻</sup> + TAC group compared to the Control and Control + TAC groups, with p-values of 0.02 for both comparisons. The images below illustrate the tissue samples for each group, visually confirming the observed differences.
Figure 11

A  Masson's trichrome positive area, %

- Control
- Control
- Sirt1<sup>endo-/−</sup>

B  Relative Collagen 1 mRNA levels

- Control
- Control
- Sirt1<sup>endo-/−</sup>
Figure 12  A

![Graph showing HIF1α / Tubulin levels](image)

![Western blots showing HIF1α and B Tubulin](image)

B

![Graph showing Relative VEGFα mRNA levels](image)
Figure 13

- LV weight/body weight
- Shortening fraction, %
- Isovolumic relaxation time, ms
- Tei index

Graphs showing changes over time for different conditions.

Controls vs. Adriamycin vs. Sirt1^endo/- Adriamycin.
Figure 15

![Graph showing relative mRNA levels of FLT1 and FLK1 in control and Sirtuin 1 inhibitor conditions.](image)

- **FLT1 mRNA levels**
  - Control: 0.8 (0.2 SD)
  - Sirtuin 1 inhibitor: 0.6 (0.2 SD)
  - p-value: 0.02

- **FLK1 mRNA levels**
  - Control: 1.0 (0.2 SD)
  - Sirtuin 1 inhibitor: 0.8 (0.2 SD)
  - p-value: 0.02