Pheochromocytoma-Induced Cardiomyopathy is Modulated by the Synergistic Effects of Cell-Secreted Factors

Hector R. Mobine. Cell-Secreted Factors in Pheo-Induced CM

Hector R. Mobine M. Eng.1, Aaron B. Baker, Ph.D.1, Libin Wang M.D., Ph.D.2, Hiroko Wakimoto M.D.2, Ph.D., Kurt C. Jacobsen M.S.2, Christine E. Seidman M.D.2,3, J.G. Seidman Ph.D.2, and Elazer R. Edelman M.D., Ph.D.1,4

1Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA.
2Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA.
3Howard Hughes Medical Institute, Boston, Massachusetts, USA.
4Cardiovascular Division, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts, USA.

Address correspondence to: Hector R. Mobine, Massachusetts Institute of Technology, 77 Massachusetts Avenue, E25-442, Cambridge, MA 02139. Tel.: 617-258-8895; Fax: 617-253-2514; E-mail: mobine@mit.edu

Word Count = 5346

Manuscript ID: CIRCULATIONAHA/2008/813261

The Journal Subject Codes: 11,130.
Abstract

**Background:** Pheochromocytomas are rare tumors derived from the chromaffin cells of the adrenal medulla. While these tumors have long been postulated to induce hypertension and cardiomyopathy through the hypersecretion of catecholamines, catecholamines alone may not fully explain the profound myocardial remodeling induced by these tumors. We sought to determine whether changes in myocardial function in pheochromocytoma-induced cardiomyopathy result solely from catecholamines secretion or from multiple pheochromocytoma-derived factors.

**Methods and Results:** Isolated cardiomyocytes incubated with pheochromocytoma-conditioned growth media contracted at a higher frequency than cardiomyocytes incubated with norepinephrine only. Sprague-Dawley rats and Black-6 mice were implanted with agarose-encapsulated pheochromocytoma (PC12) cells, DOPA decarboxylase knock-out PC12 cells deficient in norepinephrine (PC12-KO), or norepinephrine-secreting pumps. PC12 cell implantation increased left ventricular dilation by 35±6 and 9.6±1.4%, and reduced left ventricular fractional shortening by 20±3 and 28±4%, in rats and mice compared to animals dosed only with norepinephrine. Elimination of norepinephrine secretion in PC12-KO cells induced neither cardiac dilation (3.9±1.8% increase vs. control) nor changes in (1.9±0.4% reduction) fractional shortening compared to controls.

**Conclusions:** Pheochromocytomas induce a greater degree of cardiomyopathy than equivalent doses of norepinephrine, suggesting pheochromocytoma-induced cardiomyopathy is not solely mediated by norepinephrine, rather pheochromocytoma secretory factors in combination with catecholamines act synergistically to induce greater cardiac damage than catecholamines alone.

Key words: Norepinephrine, Cardiomyopathy, Catecholamines, Heart Failure
Introduction

Pheochromocytomas are rare but devastating tumors arising from chromaffin cells of the adrenal medulla or extra-adrenal paraganglia. These tumors often induce alterations in myocardial structure and function, leading to eventual development of severe cardiomyopathy\(^1\)-\(^5\). Pheochromocytomas are characterized by hypersecretion of catecholamines, namely norepinephrine (NE) and epinephrine, which are most often hypothesized to be the primary cause of tumor-induced alterations in cardiac function. Excessive adrenergic stimulation can induce and exacerbate cardiovascular disease\(^6\)-\(^8\). Exogenous epinephrine\(^9\), \(^10\) and norepinephrine\(^11\), \(^12\) are cardiotoxic in a dose-dependent fashion\(^13\), \(^14\). Cardiomyocyte viability is decreased as a function of norepinephrine concentration\(^15\) mediated by beta-adrenergic receptor ($\beta$AR) stimulation, increased cAMP, and calcium influx\(^15\). Selective stimulation of $\beta$ARs mimics NE cardiotoxicity, and $\beta$AR blockade significantly attenuates these toxic effects\(^16\).

Infusion of NE increases systolic blood pressure (SBP), down-regulates $\beta$AR, and alters LV contractility. LV hypertrophy is characterized by multifocal mixed inflammatory infiltrates, acute myocyte degeneration\(^13\), \(^17\), and increased interstitial fibrosis\(^18\)-\(^20\).

However, it is still unclear whether catecholamine excess alone can explain the severity of cardiomyopathy with pheochromocytoma and heart failure in the absence of blood pressure effects. Only one-third of patients with these tumors are persistently hypertensive and onset of cardiomyopathy does not correlate with blood pressure or circulating catecholamine\(^27\). Previous experiments with pheochromocytoma implants did not control cell growth and consequently NE secretions were excessively high, nor did they directly compare pheochromocytoma effects to equivalent effects of NE alone\(^13\), \(^22\), \(^23\). The lack of dose control makes direct comparison of cell and drug models problematic. It may be that these tumors secrete other factors that exacerbate
catecholamine-induced damage or are cardiotoxic themselves. To determine the factors secreted by pheochromocytoma cells responsible for cardiomyopathy induction at low levels of NE, the secretion of NE by pheochromocytoma cells must be replicated in vivo at a concentration and rate equal to that of pheochromocytoma-bearing animals. To investigate the development of dilated cardiomyopathy in the presence of a pheochromocytoma, we engineered a novel polymeric encapsulation system enabling the implantation of a pheochromocytoma cell line into a murine model, allowing for the control of tumor cell growth and subsequent factor secretion. The effects of pheochromocytoma cells on cardiac and cellular function and remodeling were compared to the effects of NE alone. Our successful development of a new animal model of pheochromocytoma-induced cardiomyopathy (PICM) allowed us to demonstrate differential effects of, and responses to, complete pheochromocytoma secretions versus catecholamines, yielding new insight into the etiology, pathogenesis, and approaches for treating PICM.
Methods

Cell Culture and Encapsulation

Rat pheochromocytoma cells (PC12, ATCC, VA) were maintained in F12K media (ATCC) with 10% horse serum (Hyclone, Logan, UT), 5% FBS (Hyclone) and 100 units/ml penicillin/streptomycin (Invitrogen, CA)\textsuperscript{25,26}. Cells were grown at 37°C and 5% CO\textsubscript{2}, scraped and re-suspended in a preheated solution of 2.5% (w/w) agarose (Sigma–Aldrich, Type VII, MO) in 0.9% NaCl. The mixture was drawn into an Eppendorf Repeater Pipette with a 0.5 mL Combitip (VWR, MO) and 10 μL aliquots sheared into mineral oil (~600 mL) forming cell-encapsulating agarose beads as described\textsuperscript{29}. The beads were separated using a 1,000 μm pore size mesh (Small Parts, FL) and washed with PBS. Cell number rose from $10^4$ to $1.8 \times 10^4$ cells per bead over 21 days and following growth kinetics of PC12 cells on tissue culture polystyrene plates. Each bead secreted 460 pg/day of norepinephrine by ELISA. Dopamine levels in PC12 cells were undetectable\textsuperscript{30}, as were epinephrine levels given the low or non-existent expression of phenylethanolamine-N-methyltransferase, the enzyme responsible for converting norepinephrine to epinephrine\textsuperscript{31}. The lack of epinephrine or dopamine secretion makes these cells then an ideal side-by-side comparison to the NE-secreting pumps.

Cardiomyocyte Contractility

Cardiomyocytes were obtained from 2-day-old neonatal Sprague-Dawley rats (Taconic). Cardiac ventricles were minced, incubated in trypsin (0.6 mg/mL in HBSS) for 16 hrs at 4°C and digested with collagenase type II (Sigma–Aldrich), 1 mg/mL. Cells were re-suspended in DMEM supplemented with 10% FBS, 25 mM HEPES and penicillin [100 U/mL]). Each ventricle yielded ~ $6 \times 10^5$ cells with viability between 88% and 94%. Myocytes were plated on 60 mm culture dishes and incubated with varying concentrations of control media, NE media, or PC12-conditioned media for 20 minutes. PC12 media was collected from separate dishes.
containing varying numbers of beads, the media was assayed for NE and matched with newly prepared NE media. Contractility was recorded in a temperature-controlled chamber mounted with a digital video camera (Olympus, DP70, NY). Cardiomyocyte contractility was quantified with MatLab (Mathworks, MA). All in vitro contractile studies were performed at constant temperature and CO₂ to reduce environmental impact on contractile function.

Transfection of DOPA decarboxylase Short Hairpin RNA (shRNA)

Phoenix cells (Orbigen, CA) were transfected with a single DOPA decarboxylase shRNA construct (Origene, MD) and were selected for 3 - 4 weeks in 2 µg/ml of puromycin to generate stably transfected, retrovirus-producing cells. Constructs used for shRNA were GGTGTATGGCTGCACATTGATGCTGCATA. PC12 cells were exposed to retrovirus expressing DOPA decarboxylase shRNA or empty vector in the presence of 0.5 µg/ml polybrene (Sigma) for 4 - 6 hours. The media was replaced with media containing retrovirus and the transfectants were incubated overnight. Transfected cells were then selected with 1 µg/ml puromycin for 4-5 weeks and used in experiments.

Animal Experiments

Female Sprague-Dawley Rats (8 weeks old, 250 g) and female Black-6 mice (7-8 weeks old, 15-20 g) were obtained from Taconic. All animal studies were performed in accordance with protocols approved by the MIT Institutional Animal Care and Use Committee and Harvard Medical School's IACUC and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Osmotic pumps (Alzet, Model 2004 (rats) and Model 1004 (mice), CA) or agarose-encapsulated PC12 cells were implanted in the retro-peritoneal cavity, mimicking the spatial release of pheochromocytoma cells. A total of 20 agarose-encapsulated PC12 or PC12-KO beads were implanted. The 9.2 ng/day NE secretion rate of the 20 agarose-PC12 beads was matched with
osmotic pumps (Alzet) delivering 0.25 μL/hr (rats) and 0.11 μL/hr (mice) loaded with 9.06 and 21 μM solution of NE in acidic saline (0.1mg/mL ascorbic acid in saline) for rats and mice, respectively. The ascorbic acid solution retards catecholamine oxidation. Catecholamine levels were tracked through weekly blood draws via the retro-orbital plexus and quantified by ELISA (Rocky Mountain Diagnostics). Control animals received osmotic pumps loaded with acidic saline carrier solution alone. Animals implanted with empty agarose beads and non-surgical, non-implanted animals had statistically identical cardiac dimensions and mRNA levels.

**Echocardiographic and Hemodynamic Measurements**

Echocardiography of anesthetized rats (pentobarbital 30 mg/kg IP) was performed at the 56-day endpoint with a linear array probe (Visual Sonics, RMV710B, Toronto, Canada) and a Visual Sonics Vevo 770. Cardiac dimensions were obtained from M-mode tracings using measurements averaged from three separate cardiac cycles by an echocardiographer blinded to the rat’s genotype. Arterial pressure was recorded by inserting a pressure-conductance catheter (Millar Instruments, SPR-878, TX) into the LV via the right internal carotid artery. The catheter was connected to a pressure-conductance unit (Millar Instruments, MPVS-400) and waveforms were recorded using ChartV5 software (AD Instruments, CO). Data were analyzed with Millar PVAN 3.4 (Millar Instruments). Four randomly selected rats from each group were chosen for analysis.

Transthoracic echocardiography was performed in anesthetized mice (2% isoflurane) using a 12-MHz probe and a Sonos 5500 ultrasonograph (Hewlett-Packard, MA). Left ventricular parameters and heart rates were obtained from M-mode interrogation in a short-axis view, averaged from three separate cardiac cycles at heart rates greater than 400 beats/minute. The echocardiographer was blinded to mice genotypes. Cardiac contractile function was
represented by the parameter LV fractional shortening (percentage), calculated as \[\frac{(LV \text{ diastolic diameter} - LV \text{ systolic diameter})}{LV \text{ diastolic diameter}} \times 100.\]

**Histological Analyses**

Animals were euthanized, hearts excised, rinsed in PBS, weighed, then pressure perfused (100 mmHg) with PBS for 5 minutes followed by 10% neutral buffered formalin (NBF) until visibly firm and pale. The hearts were placed in 10% NBF overnight, processed, paraffin fixed (Polysciences Inc., PA), and serial coronal sections cut and stained with hematoxylin and eosin (Sigma–Aldrich) and Gomori’s Trichrome (American Histolabs Inc., MD). A pathologist blinded to the treatment groups graded the tissues. TUNEL assay was performed with an apoptosis kit (Millipore, MA) according to manufacturer’s instructions. Six images per heart were acquired on Leica microscope. Results were expressed as the number of apoptotic nuclei per total nuclei per image field.

**RNA Preparation and RT-PCR**

Excised hearts were perfused with PBS and a biopsy (8x 8mm) taken. Total RNA was isolated with the use of Qiashredder and RNeasy spin columns (Qiagen, CA). cDNA was synthesized using Taqman RT-PCR kit (Applied Biosystems, CA). Specific primers were designed using Primer3. Real-time polymerase chain reaction (PCR) was performed with an Opticon Real Time PCR Machine (Biorad, CA) using SYBR Green PCR Master Mix Reagent Kit (Applied Biosystems). All samples (n=5 per group) were measured in triplicate and the expression level was normalized to GAPDH expression.

**Statistical Analysis**

Comparisons of data from two groups used Student’s t test and for multiple groups, one-way ANOVA or two-way ANOVA for repeated measurements (Minitab, PA). Contractility and LV mRNA data were analyzed for statistical significance using two way ANOVA followed by a
TUKEY post-hoc test to determine significance. A value of $P < 0.05$ was considered statistically significant. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.
Results

Cardiomyocyte Contractility

Freshly isolated neonatal cardiomyocytes were used to determine whether PC12-conditioned media induced a greater effect at the single cell level than cardiomyocytes dosed with identical concentrations of only NE. PC12-conditioned media caused cardiomyocytes to contract at a higher frequency than those incubated with identical NE doses and with nearly two-fold greater contractility at 0.07 nM (p<0.01) (Figure 1). The increased beating frequency induced by PC12-conditioned media compared to identical doses of NE occurred in a dose-dependent fashion.

Rat and Mouse Models, Cardiac Morphology, and Function

Experiments were performed in two species to verify the nature of the effects. NE secretion in pheochromocytoma-implanted (Pheo) animals was matched with the implantation of NE secreting pumps (Figure 2). Implanted pumps and PC12 cells in rats raised NE plasma value by 0.4 and 0.3 ng/mL, respectively. Implanted pumps, PC12 cells, and PC12-KO cells (Pheo-KO) in mice raised NE plasma values by 4, 3, and 0.7 ng/mL, respectively. To ensure the cardiac pathology observed was not a result of the host-PC12 cell interaction instead of PC12 secreted factors, empty agarose beads were implanted and their effects on cardiac pathology were compared to control rats. The heart weight normalized to body weight of rats implanted with empty agarose beads (6.0±0.5 µg/g) was statistically identical to control rats (6.3±0.4 µg/g). There was no detectable histological difference in the cellular and tissue response, indicating that host-cell interactions would not be a factor cardiomyopathy development.

Cardiac dilation was more pronounced in Pheo rats and mice (p<0.01 vs. control, p<0.01 vs. NE) compared to NE rats and mice (Figure 3A). The hearts of Pheo rats were 47% larger...
than controls (p<0.01) and NE rats (p<0.01), the latter statistically indistinguishable from controls (Table 1). Similarly, Pheo mice developed a 22% greater degree of dilation over NE mice (p<0.01), 17% dilation over Pheo-KO mice and 61% greater than controls (p<0.01). Furthermore Pheo mice experienced a greater loss of cardiac function, evident in the 10.8±1.9% (p<0.01 vs. control, p<0.05 vs. NE) decrease in fractional shortening (FS) compared to NE mice that experienced a 6.3±2.9% (p<0.01) decrease in FS. These changes occurred despite the absence of significant increases in the SBP or heart rates, and both were statistically indistinguishable from Pheo, Pheo-KO and NE mice (Figure 3, B-C). Of note, Pheo-KO mice did exhibit slight increase in cardiac dilation but no discernible loss of FS compared to controls (Table 2). Left ventricular end-systolic volume scaled linearly with norepinephrine levels (R²=0.916, p<0.0001) in control, pheo and pheo-KO mice but not in NE pump animals whose catecholamine levels were all at the upper limit and without correlative effect. Histologic analyses of cardiac tissue showed little microscopic difference between Pheo, NE, Pheo-KO and control animals (Figure 4). TUNEL-positive apoptotic cells were identified in greatest density in Pheo animals (1.53±0.90 apoptotic nuclei/total nuclei, p=0.25 vs. NE, p=0.10 vs. control), intermediate density in NE animals (0.97±0.60 apoptotic nuclei/total nuclei, p=0.79 vs. control), and lowest density in control animals (0.86±0.60 apoptotic nuclei/total nuclei).

Cardiac function was further characterized by left ventricular (LV) catheterization 56 days after cell implantation or NE infusion. At the doses tested, NE increased myocardial function above control, with a steeper slope of the end-systolic pressure-volume relationship (ESPVR) (p<0.05), decreased end diastolic pressure (p<0.05), and a higher maximum ventricular elastance (p<0.01) (Figure 5, D-F). In contrast, at the same doses of NE released, pheo cells reduced all indices of cardiac function. There was a 73% reduction in the slope of the ESPVR (p<0.01 vs. NE rats, p<0.01 vs. control), a 24% decrease in end diastolic pressure (p>0.05 vs. NE rats, p<0.01 vs. controls), and a 70% decrease in ventricular elastance (p=0.017
vs. NE rats, p< 0.01 vs. controls) (Table 3). The decreased ESPVR slope reflects a decrease in the heart’s inotropic capabilities. To maintain stroke volume under these circumstances, the ventricle will often operate at higher volumes in a process of compensatory dilation. This mechanism is further validated by the 96% and 36% increase in stroke volume for Pheo rats over controls and NE rats, respectively.

**Myocardial Gene Expression**

Cardiac extracellular matrix biomolecules play a central role in maladaptive myocardial remodeling and cardiac decompensation. Clinical studies over the past decade have shown increased levels of CC chemokine ligand 2 (CCL2, MCP-1), matrix metalloproteinase 3 (MMP3), collagen-1 and decreased levels of tissue inhibitor of matrix metalloproteinases 3 (TIMP3) to be associated with cardiovascular disease and cardiac dysfunction. Pheo-implants raised MMP3 (p<0.01), collagen-1 (p<0.05), CCL2 (p < 0.01) and reduced TIMP3 (p <0.01) mRNA levels compared to NE rats (Figure 6). BNP mRNA levels were elevated 2.8±0.2 fold above control animals 28 days after implantation and remained elevated for the duration of the experiment. It took twice as long for NE rats to exhibit the same level of elevation. Levels of mRNA were identical to controls (100±20% over controls) at 28 days and became elevated (290±50% over controls) only at 56 days. These statistically significant changes highlight the ability of pheochromocytomas to induce cardiac pathology with accelerated kinetics versus NE alone. The changes in gene expression values bear a strong correlation to the heart’s morphological and functional changes as diagnosed echocardiographically. Specifically, increasing CCL2 levels correlate strongly with increasing left ventricular end diastolic diameter (LVEDD) (R²= 0.92, p<0.0001) and decreasing fractional shortening (R² = -0.84, p=0.000). Similarly, increases in MMP3 and collagen correlate with increasing LVEDD (R²= 0.86, p<0.0001 for MMP3 and R²= 0.73, p=0.003 for collagen), with increases in collagen highly
correlative with decreases in fractional shortening ($R^2 = -0.94$, $p<0.0001$). TIMP3 mRNA levels further correlate with LVEDD ($R^2 = -0.81$, $p<0.0001$) and fractional shortening ($R^2 = 0.66$, $p=0.010$).
Discussion

While catecholamines may dominate the cardiotoxic effects of late-stage pheochromocytomas, the secretion of low levels of catecholamines during early tumor development is insufficient to induce hemodynamic effects. Indeed, only 29% of all pheochromocytoma patients are persistently hypertensive, and only another 30% demonstrate episodic hypertension\(^1\). Previous research has attributed the cardiotoxicity of pheochromocytomas to catecholamines but has not addressed whether other tumor secreted factors act to induce myocardial remodeling\(^2\). Here we employ agarose-encapsulation of PC12 cells that enables quantifiable and reproducible control of cell growth and NE release allowing for the matched secretion of NE by the PC12-agarose beads with implanted norepinephrine releasing osmotic pumps. We focused on the impact of low-grade, low-NE secreting tumors to separate the effects of non-catecholamine secretory products of early-stage pheochromocytomas from those of late-stage tumors with NE hypersecretion.

In-vitro, pheochromocytoma cell conditioned media induced more frequent cardiomyocyte contractions than NE alone suggesting that catecholamines are not solely responsible for the effects of pheochromocytomas on cardiomyocyte physiology. Animals implanted with PC12 cells exhibited far greater structural and functional impairment than animals with NE implants, all without changes in blood pressure or heart rate. In these similar hemodynamic domains, only pheochromocytoma cells induced significant cardiomyopathy in mice and rats. These changes closely mimic the human consequences of these tumors, as LV chamber size was enlarged, fractional shortening was reduced, and hemodynamic function altered, all to a far greater degree than with equivalent NE doses. These effects were absent with the elimination of NE secretion from PC12-KO cells, whose implantation failed to induce a cardiomyopathic state. The induction of cardiomyopathy therefore likely results from the
confluence of pheochromocytoma-secreted factors, rather than from a single factor alone. Though NE is necessary to induce cardiomyopathy, it is not sufficient to explain the full force of the effects of pheochromocytoma on the heart.

The altered balance of the cytoskeletal proteins, MMP3, TIMP3, and collagen, creates maladaptive myocardial remodeling that mediates the transition from compensated to decompensated heart failure. MMP3 elevation and reduction in its inhibitor, TIMP3, exacerbates ventricular dilation by disintegrating the collagen network. High dose NE infusion increased myocardial collagen, myocyte diameter, and fibrosis with elevated TIMP3 and MMP3 mRNA levels. In line with these observations, collagen and MMP3 mRNA levels were significantly increased and TIMP3 levels decreased in pheochromocytoma implanted animals. The hearts of animals exposed to low doses of NE alone expressed minimal alterations in collagen, cardiac function, MMP3 levels, and TIMP3 levels. Other inflammatory mediators involved in the pathogenesis and progression of heart failure followed suit to provide further mechanistic insight. CCL2 mediates myocardial remodeling by promoting the attraction and invasion of activated leukocytes into damaged or inflamed tissue. Circulating CCL2 levels rise significantly in advanced dilated cardiomyopathy, congestive heart failure, and ischemic reperfusion injury. Similarly, CCL2 levels increased significantly in Pheo implanted rats at day 28 and continued to rise at day 56. In contrast, NE rats expressed normal levels of CCL2 at day 28 compared to controls, with levels only rising at 56 days. Our data demonstrating that CCL2 mRNA levels were upregulated to a greater extent in Pheo versus NE rats coincides with the changes seen in a variety of clinical structural pathologies.

Taken together, our findings suggest that pheochromocytoma tumor cells in combination with their catecholamines act synergistically to induce greater cardiac damage than catecholamines alone. Numerous factors secreted by pheochromocytoma cells can directly or
indirectly alter heart function\textsuperscript{43, 44}. Our work suggests that it is the confluence of these factors that enable the synergistic induction of myocardial injury. The implications of our work lie not only in a better understanding of pheochromocytomas and their secretory products but also in the differences between cell-secreted substances and their exogenous analogues in isolation. Given that secondary factors are sufficient to induce PICM in early-stage patients, these data further highlight the need for understanding the fundamental development of early pathology for the purpose of basic insight into cardiomyopathies, developing novel therapeutic approaches, and identifying pheochromocytoma screening tools other than catecholamine metabolites.
Acknowledgements

We are grateful to Philip Seifert, James Stanley, and Gee Wong for their valuable assistance with histological preparation and pathological analysis.

Funding Sources

This work was supported by the National Institutes of Health (R01 49039 to E.R.E.), a Pre-Doctoral Fellowship (to H.R.M.) and a Philip Morris postdoctoral research fellowship (to A.B.B.)

Disclosures: None
References


Figure Legends

Figure 1. Conditioned media from PC12 cells (dashed line) induces a greater change on cardiomyocyte contractility than norepinephrine media (solid line). The data at each time point is the mean ± SE of n= 4 separate culture plates. **P < 0.01.

Figure 2. Norepinephrine levels secreted by pheochromocytoma cells were matched via the implantation of osmotic pumps. (A) The plasma NE levels were statistically equal in mice implanted with NE pumps and PC12 cells and elevated in comparison to control and PC12-KO mice. Control and PC12-KO mice NE levels were statistically indistinguishable. (n=5 mice per group, n=4 control mice). (B) Plasma NE levels were statistically equal in rats implanted with NE pumps and PC12 cells and elevated in comparison to control rats. (n=5 rats per group). *P < 0.05, **P < 0.01 versus controls.

Figure 3. Implanted pheochromocytoma cells induce greater cardiac dysfunction than equivalent doses of NE alone. (A) Change in fractional shortening (white) and ejection fraction (black) from Day 0 to Day 56 for Control, NE, Pheo, and Pheo-KO mice. (B) Low doses of NE secreted by the implanted NE pumps (black), PC12 cells (red) and PC12-KO cells (blue) had no effect on SBP. (C) The heart rates of mice implanted with NE pumps (black), PC12 cells (red), and PC12-KO cells (blue) were indistinguishable. (n=5 per group, n=4 control mice).

Figure 4. Low doses of NE released by pheochromocytoma cells and NE secreting pumps result in dimensional changes in cardiac tissue without attaining irreversible damage at subcellular
level. Representative images from the hearts of rats (A) and mice (B) stained with Hematoxylin and Eosin and Gomori’s Trichrome. Histopathology of NE, Pheo and Pheo-KO animals exhibited no myocyte disarray or fibrosis. All images taken at 40x magnification. (n=5 animals per group, n= 4 control animals per group).

Figure 5. Rats implanted with pheochromocytoma cells experience greater cardiac dysfunction than equivalent doses of NE alone. Representative images of the hearts of Control (A), NE pump (B) and Pheo (C) Rats. (D-F) Representative left ventricular pressure-volume loops obtained at the 56-day endpoint for Control (D), NE (E) and Pheo (F) rats. End-systolic pressure-volume relationship (ESPVR) during preload reduction is indicated by the black line (n=5 rats per group).

Figure 6. Rats implanted with pheochromocytoma cells increase mRNA levels of cardiac dysfunction associated proteins. Quantification of left ventricular mRNA levels for Pheo and NE rats at 28- and 56-day. The mRNA levels were normalized to percent controls. CCL2, MMP3 and collagen mRNA levels were upregulated to a greater degree in rats implanted with pheochromocytomas than those implanted with NE pumps. TIMP3 levels were down regulated to a greater degree in pheo rats than NE rats (n=5 rats per group). *P < 0.05, **P < 0.01 versus NE rats; †P < 0.05, ††P < 0.01 versus controls.
### Table 1. Cardiac Morphology and Function of Control, NE and Pheo rats.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>NE</th>
<th>Pheo</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>7</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Age, weeks</td>
<td>10±2</td>
<td>10±2</td>
<td>10±2</td>
</tr>
<tr>
<td>HW:TL (g:cm)</td>
<td>0.17±0.04</td>
<td>0.19±0.02†</td>
<td>0.25±0.02**,††</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>317±15</td>
<td>360±45</td>
<td>362±19</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>104±17</td>
<td>84±18</td>
<td>94±30</td>
</tr>
<tr>
<td>LVWT, mm</td>
<td>1.16±0.12</td>
<td>1.18±0.16</td>
<td>1.00±0.19</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>5.5±0.1</td>
<td>6.0±0.6††</td>
<td>8.1±0.3**,††</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.4±0.6</td>
<td>3.4±0.6††</td>
<td>5.3±0.3**,††</td>
</tr>
<tr>
<td>FS, %</td>
<td>49±4</td>
<td>43±2</td>
<td>34±3*,††</td>
</tr>
<tr>
<td>EF (%)</td>
<td>39±6</td>
<td>36±4</td>
<td>27±4††</td>
</tr>
</tbody>
</table>

SBP, systolic blood pressure; LVWT, LV wall thickness at end diastole; LVEDD, LV end diastolic diameter; LVESD, LV end systolic diameter; FS, fractional shortening; EF, ejection fraction. Values are means ± SD. *P < 0.05, **P < 0.01 versus NE rats; †P < 0.05, ††P < 0.01 versus controls.
Table 2. Cardiac Morphology and Function of Control, NE, Pheo and Pheo-KO mice.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>NE</th>
<th>Pheo</th>
<th>Pheo-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Mice</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>7±2</td>
<td>7±2</td>
<td>7±2</td>
<td>7±2</td>
</tr>
<tr>
<td>ΔLVWT (mm)</td>
<td>-0.03±0.01</td>
<td>-0.13±0.03†</td>
<td>-0.09±0.03</td>
<td>-0.04±0.02</td>
</tr>
<tr>
<td>ΔLVEDD (mm)</td>
<td>-0.03±0.01</td>
<td>0.24±0.09†</td>
<td>0.34±0.04††</td>
<td>0.26±0.08*†</td>
</tr>
<tr>
<td>ΔLVESD (mm)</td>
<td>-0.01±0.01</td>
<td>0.43±0.11††</td>
<td>0.63±0.07*,††</td>
<td>0.2±0.07†</td>
</tr>
<tr>
<td>ΔFS (%)</td>
<td>-1.2±0.3</td>
<td>-6.3±2.9</td>
<td>-10.8±1.9*,††</td>
<td>-1.0±1.5</td>
</tr>
<tr>
<td>ΔEF (%)</td>
<td>3.4±0.6</td>
<td>-8.8±3.8</td>
<td>-21.5±4.2</td>
<td>-1.6±1.9</td>
</tr>
</tbody>
</table>

ΔLVWT, change in LV wall thickness at end diastole; ΔLVEDD, change in LV end diastolic diameter; ΔLVESD, change in LV end systolic diameter; ΔFS, change in fractional shortening; ΔEF, change in ejection fraction. Values are means ± SD. *P < 0.05, **P < 0.01 versus NE rats; †P < 0.05, ††P < 0.01 versus controls.
Table 3. Hemodynamic Measurements of Control, NE and Pheo rats.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>NE</th>
<th>Pheo</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESPVR Slope (mmHg/L)</td>
<td>1.49±0.16</td>
<td>1.04±0.33</td>
<td>2.58±0.20**</td>
</tr>
<tr>
<td>Stroke Volume (uL)</td>
<td>53.3±1.4</td>
<td>76.4±6.0††</td>
<td>104.3±7.6**††</td>
</tr>
<tr>
<td>End Diastolic Pressure (mmHg)</td>
<td>4.51±0.14</td>
<td>3.47±0.61†</td>
<td>3.41±0.25††</td>
</tr>
<tr>
<td>End Diastolic Volume (uL)</td>
<td>275.5±3.5</td>
<td>284.3±3.4††</td>
<td>392.2±28.3**††</td>
</tr>
<tr>
<td>Elastance (mmHg/uL)</td>
<td>2.19±0.32</td>
<td>0.954±0.18††</td>
<td>0.64±0.10**††</td>
</tr>
</tbody>
</table>

Values are means ± SD. (n=5 for all groups). *p<0.05, **p<0.01 versus NE rats; †p<0.05, ††p<0.01 versus controls.
Figure 1.
Figure 2.
Figure 3.
Figure 4.

A & B: H&E and Trichrome stained images of control, NE, PC12, and PC12-KO samples.
Figure 6.

LV mRNA Levels (% Control)

CCL2  MMP3  TIMP3  Collagen

NE - 28d  Pheo - 28d  NE - 56d  Pheo - 56d

**,**  **,**  **,**  **,**
Pheochromocytoma-Induced Cardiomyopathy is Modulated by the Synergistic Effects of Cell-Secreted Factors

Hector R. Mobine, Aaron B. Baker, Libin Wang, Hiroko Wakimoto, Kurt C. Jacobsen, Christine E. Seidman, Jonathan G. Seidman and Elazer R. Edelman

Circ Heart Fail. published online February 10, 2009;
Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/early/2009/02/10/CIRCHEARTFAILURE.108.813261