



Effects of epinephrine on angiogenesis-related gene expressions in cultured rat cardiomyocytes

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Abstract

Epinephrine is often used for the treatment of patients with heart failure, low cardiac output and cardiac arrest. It can acutely improve hemodynamic parameters; however, it does not seem to improve longer term clinical outcomes. Therefore, we hypothesized that epinephrine may induce unfavorable changes in gene expression of cardiomyocyte. Thus, we investigated effects of epinephrine exposure on the mediation or modulation of gene expression of cultured cardiomyocytes at a genome-wide scale. Our investigation revealed that exposure of cardiomyocytes to epinephrine in an *in vitro* environment can up-regulate the expression of angiotensinogen gene (+ 2.1 times), and down-regulate the gene expression of neuregulin 1 (−3.7 times), plasminogen activator inhibitor-1 (−2.4 times) and SPARC-related modular calcium-binding protein-2 (−4.5 times). These changes suggest that epinephrine exposure may induce inhibition of angiogenesis-related gene expressions in cultured rat cardiomyocytes. The precise clinical significance of these changes in gene expression, which was induced by epinephrine exposure, warrants further experimental and clinical investigations.

Keywords: epinephrine, angiogenesis, gene expression, cardiomyocytes, angiotensinogen, neuregulin 1, plasminogen activator inhibitor-1, SPARC-related modular calcium-binding protein

Introduction

Patients with heart failure, septic shock, low cardiac output syndrome and other clinical scenarios, will often necessitate inotropic therapy^[1]. Among those commonly used inotropic agents, epinephrine is one of the most potent and most frequently used inotropes in clinical practice, especially in cases when inotropic support is warranted perioperatively and in patients with cardiac arrest^[2–3].

Epinephrine, as an adrenergic receptor agonist, is not only a potent stimulator of myocardial contractility but also, a strong stimulator of cardiac chronotropic effect. Epinephrine is administered to improve hemodynamic parameters, such as cardiac output (CO), mean arterial blood pressure (MAP), and systemic vascular resistance (SVR). The use of epinephrine seems to improve short term clinical outcomes. Donnino's group in Boston analyzed the data from Get With the Guidelines-Resuscitation database (formerly National Registry of

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Cardiopulmonary Resuscitation, NRCPR). The database is sponsored by the American Heart Association (AHA) and contains prospective data from 570 American hospitals collected from 1 January 2000 to 19 November 2009. They found that in patients with non-shockable cardiac arrest in a hospital setting, earlier administration of epinephrine is associated with a higher probability of return to spontaneous circulation, survival in hospital, and neurologically intact survival^[4]. There have been other studies which also supported the concept that epinephrine improves survival for out of hospital arrest patients and improves survival for in-hospital cardiac arrests^[5], at least in selective groups of patients^[6-7]. However, Hiyashi *et al.* evaluated the outcomes of 3,161 adult non-traumatic bystander-witnessed cardiac arrest patients. They found that the only group which potentially benefited from epinephrine administration was those patients with cardiac arrest from ventricular fibrillation. Otherwise, epinephrine groups had a significantly lower rate of neurologically intact 1-month survival than the non-epinephrine group (4.1% vs. 6.1%, $P = 0.028$)^[3]. Furthermore, data from the European ALARM-HF study indicates that catecholamine inotropes should be used cautiously, as they have been demonstrated to increase the risk of in-hospital mortality^[2,8]. Additional studies have revealed that a combination of inotropes can cause increased hospital mortality^[9].

We, therefore, hypothesized that epinephrine administration may alter gene expression in cardiomyocytes, which may contribute to observed adverse clinical outcomes. Our present investigation was conducted to investigate gene expression changes after exposure of cardiomyocytes to epinephrine.

Methods and materials

Cell culture

Rat cardiomyocyte cell line H9C2 (ATCC, Rockville, Maryland) was utilized and cardiomyocytes were inoculated at 0.5 M/mL and cultured at 37°C, 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) in 25 mL flasks, with penicillin (100 U/mL), and streptomycin (100 µg/mL). The cells were kept overnight and the next morning,

epinephrine was added to the culture medium to make a final concentration of epinephrine of 1 µmol/L. The cells were then cultured for 48 hours without medium change. Flasks with similar culture medium and identical concentration of H9C2 cells without epinephrine served as controls; this technique is modified from the technique reported by Merten^[10]. Each test was done in triplicates, two serves as experimental, one serves as control. At 48 hours, the cell culture was stopped and total RNA was extracted from cultured cardiomyocytes and purified by using Trizol (Therma Fisher Scientific, Waltham, MA, USA) and RNeasy cleanup kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. The total RNA yield was quantified by NanoDrop 1,000 Spectrophotometer (Therma Fisher Scientific, Waltham, MA USA) and the quality was verified by gel electrophoresis.

Microarray

The RNA was then used for whole genome gene expression evaluation. This array contains more than 41,000 rat genes. The cDNA was synthesized from the RNA samples and used to synthesize fluorescent cRNA. Labeled cRNA samples were hybridized to the Whole Rat Genome Oligo Microarray slides (Agilent Technologies, Santa Clara, CA, USA). After hybridization, arrays were washed and scanned.

Data analysis

Data were imported into GeneSpring GX 11 software (Agilent Technologies, Santa Clara, CA) as 20 one-color arrays and normalized to the median per chip and the median value per gene across all arrays. Parameter data were added in order that microarrays could be grouped by time and treatment. Guided workflow returned several gene lists. These were analyzed for significant Gene Ontology and pathway hits based on passing P value criterion ($P < 0.05$). Epinephrine-induced gene expressional changes related to angiogenesis were identified ($P < 0.05$).

Results

The results of the present investigation are listed in **Table 1** and **Fig. 1**. Up-regulated genes included

Table 1 Genes with altered expressions in cultured rat cardiomyocytes exposed to epinephrine.

Increased gene expressions	Decreased gene expressions
<i>ANGPT2</i> (2.12 times)	<i>NRG1</i> (-3.653 Times)
	<i>SERPINE1</i> (-2.42 times)
	<i>SMOC2</i> (-4.52 times)

Note: (ANGPT2 (Angiopoietin-2); NRG1 (Neuregulin1); SERPINE1 (plasminogen activator inhibitor-1; PAI-1 is a serine protease inhibitor); SMOC2 (SPARC-related modular calcium-binding protein-2). $P < 0.05$).

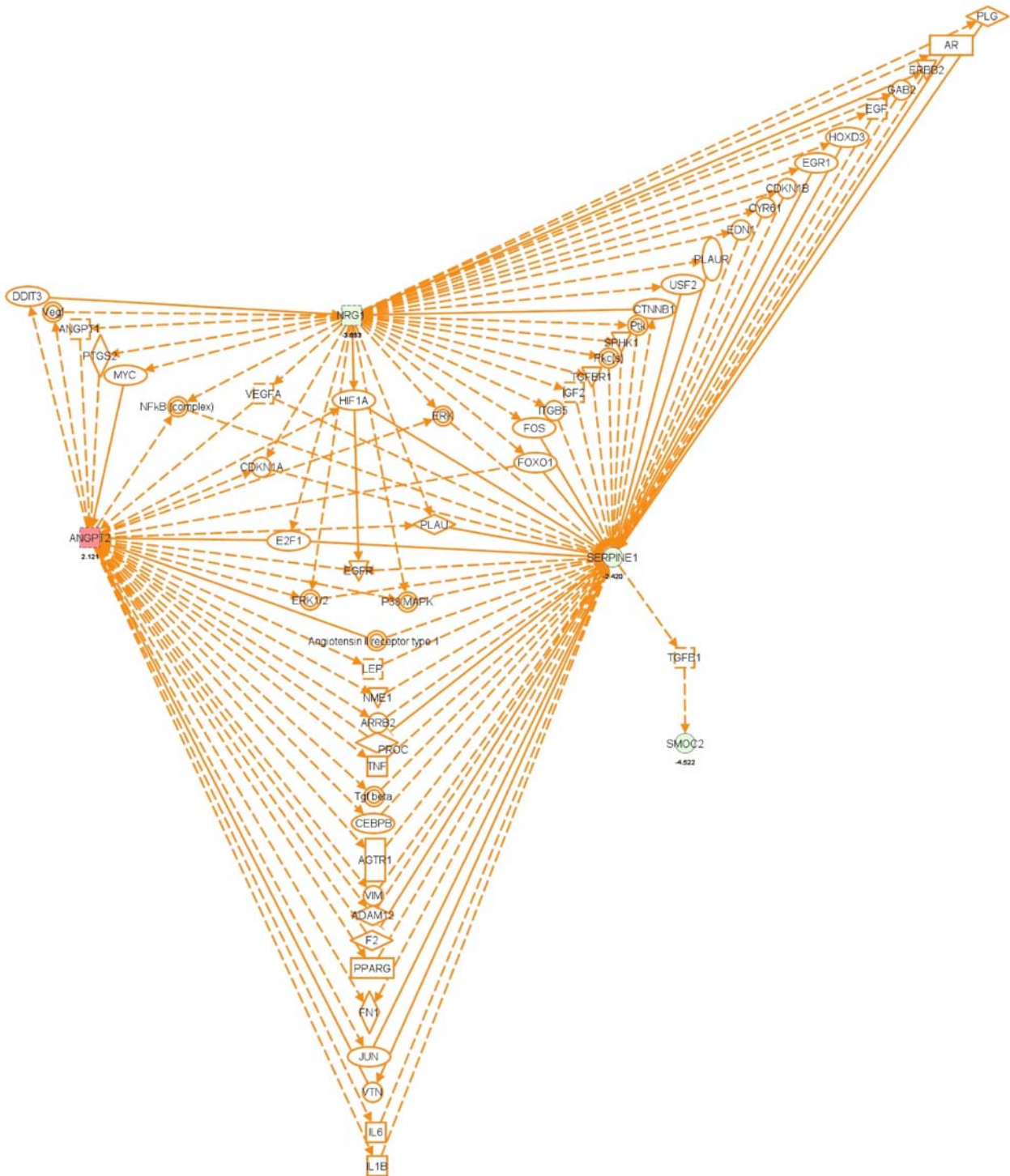


Fig. 1 Gene expression changes related to angiogenesis after exposure of cardiomyocytes to epinephrine for 48 hours. Red-highlighted indicates increased expression while green-highlighted indicates decreased expression. The numbers show the times of the gene expression changes.

ANGPT2 (Angiopoietin-2, a natural antagonist for *ANGPT1* and *TIE*), which was increased by 2.12 times. Down-regulated gene expression included *NRG1* (Neuregulin1, a peptide essential for the development of heart), which was decreased by -3.653 times; *SERPINE1* (plasminogen activator inhibitor-1; PAI-1 is a serine protease inhibitor), expression was decreased 2.42 times. *SMOC2* (SPARC-related modular calcium-binding protein-2) expression was down-regulated by 4.52 times.

Discussion

Epinephrine remains a widely used pharmacological agent in clinical practice. The results of the present investigation revealed significant changes in gene expression that may provide a link in understanding the clinical effects of epinephrine administration in different pathological settings.

ANGPT2 was significantly up-regulated, increasing by 2.1 times. *NRG1* had significantly down-regulated gene expression by 3.7 times, *SERPINE1* gene expression was down-regulated by 2.4 times, and *SMOC2* gene expression was down-regulated by 4.5 times. In this regard, it remains controversial as to the precise effects of epinephrine on short and long-term clinical outcomes in patients with cardiac arrest, heart failure and/or other clinical scenarios which necessitate inotropic support^[2-3].

Angiogenesis is a physiological process through which new vascular structures form from preexisting blood vessels^[11]. Angiogenesis proceeds through sprouting, endothelial cell migration, proliferation, and vessel destabilization and stabilization. This process is different from vasculogenesis, the latter is the formation of endothelial cells from mesoderm cell precursors^[11]. Angiogenesis is responsible for most blood vessel growth during development and in disease^[12]. Angiogenesis is a critical process in wound healing and survival of ischemic myocardium, pro-angiogenic therapy has emerged as having promising potential in cardiac repair^[13]. Therapeutic angiogenesis, neovascularization of ischemic myocardium, can hamper the progression of heart failure^[14].

ANGPT2 is a gene which encodes angiopoietin-2, a natural antagonist for *Ang1* (angiopoietin-1) and *Tie-2* (Tyrosine kinase receptor-2), both are stimulants for angiogenesis. The angiopoietins are protein growth factors which regulate the process of angiogenesis, the formation of new blood vessels. There are at present four identified angiopoietin subtypes: *Ang1*, *Ang2*, *Ang3*, and *Ang4*. *Ang1* and *Ang4* function as agonistic or activating ligands for *Tie2*. *Tie-2/Ang1* signaling

pathway activates β_1 -integrin and *N-cadherin* in *LSK-TIE-2* + cells and promotes hematopoietic stem cell (HSC) interactions with extracellular matrix and its cellular components. Although *Ang2* and *Ang3* behave as competitive antagonists, they function by binding to their prospective physiologic receptors, *Tie-1* and *Tie-2*. Though it is still controversial which of the *Tie* receptors mediate functional signals downstream of *Ang1* stimulation, it is clear that at least *Tie-2* is capable of physiologic activation as a result of binding the angiopoietins^[15-16]. Up-regulation of *Ang2* by 2.1 times may potentially inhibit the angiogenic effects of *Ang1* stimulation and can be harmful to ischemic myocardial survival.

NRG1 encodes neuregulin1, which is a peptide essential for cardiac development^[17]. Neuregulin 1 is one of the four proteins in the neuregulin family which acts on the epidermal growth factor receptor (EGFR) family of receptors^[18]. Neuregulin 1 is produced in many isoforms by alternative splicing, which allows it to perform a wide variety of functions. These isoforms include heregulins (HRGs), glial growth factors (GGFs), and sensory and motor neuron-derived factor (SMDF). These isoforms are tissue-specific and significantly differ in their structures. All HRG isoforms contain immunoglobulin (Ig) and epidermal growth factor-like (EGF-like) domains. GGF and GGF2 isoforms also contain a kringle-like sequence plus immunoglobulin and EGF-like domains; and the SMDF isoform shares only the EGF-like domain with other isoforms^[19]. The receptors for all *NRG1* isoforms are the *ERBB* family of tyrosine kinase transmembrane receptors [the *ERBB* family includes *Her1* (EGFR, *ErbB1*), *Her2* (Neu, *ErbB2*), *Her3* (*ErbB3*), and *Her4* (*ErbB4*)]^[18]. Through they displayed interaction with *ERBB* receptors, *NRG1* isoforms induce the growth and differentiation of epithelial, neuronal, glial, and potentially other types of cells. Neuregulin-1, as a cardioactive growth factor released from endothelial cells, is necessary for cardiac development, structural maintenance, and functional integrity of the heart. *NRG-1* and its receptor family *ErbB* can play a beneficial role in the treatment of chronic heart failure (CHF) by promoting survival of cardiomyocytes, improving sarcomeric structure, balancing Ca^{2+} homeostasis, and enhancing cardiac pumping function^[18]. The subsequent effectors of *NRG-1/ErbB* can include cardiac-specific myosin light chain kinase (cMLCK), Protein phosphatase type 1 (PP1), sarcoplasmic reticulum Ca^{2+} -ATPase 2 (SERCA2), and focal adhesion kinase (FAK). The beneficial effects of neuregulin-1 make recombinant human neuregulin-1 (rhNRG-1) a potential treatment of CHF^[20]. Decreased expression of neuregulin1 (

–3.7 Times) may potentially hamper angiogenesis.

SERPINE1 encodes plasminogen activator inhibitor-1 (PAI-1). PAI-1 is a serine protease inhibitor (serpin) that functions as the key inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), the two important activators of plasminogen. Besides PAI-1 which functions as a serine protease inhibitor (serpin) protein (*SERPINE1*), protease nexin also acts as an inhibitor of tPA and urokinase. PAI-1 is mainly produced by the endothelium but also secreted by other tissue types (such as adipose tissue). PAI-1 inhibits the serine proteases tPA and uPA/urokinase, and thus it is an inhibitor of fibrinolysis, the physiological process that degrades blood clots^[21]. PAI-1 also inhibits the activity of matrix metalloproteinases. Congenital deficiency of PAI-1 can lead to a hemorrhagic diathesis (a tendency to hemorrhage)^[22]. PAI-1 is present in increased levels in many disease states, as well as in obesity and the metabolic syndrome. It has been linked to the increased occurrence of thrombosis in patients with these conditions. In inflammatory conditions in which fibrin is deposited in tissues, PAI-1 appears to play a significant role in the progression to fibrosis. Lower level of PAI-1 would presumably lead to less suppression of fibrinolysis and conversely a more rapid degradation of fibrin. A recent study reported that inhibition of PAI-1 may decrease angiogenesis^[23]. Thus, decreased expression of *SERPINE1* (–2.42 times) seems create a disadvantageous environment for angiogenesis in myocardium.

SMOC2 encodes SPARC (secreted protein acidic and rich in cysteine)-related modular calcium-binding protein-2, which is a facilitator of angiogenesis^[24]. *SMOC-2* consists of two thyroglobulin-like domains, a follistatin-like domain and a novel domain found only in the homologous *SMOC-1*. Phylogenetic analysis of the calcium-binding domain sequences showed that *SMOC-1* and *SMOC-2* form a separate group within the *BM-40* (basement-membrane protein 40) family. Analysis of recombinantly expressed proteins unveiled that *SMOC-2* is a glycoprotein with a calcium-dependent conformation. Molecular biology analysis (Northern blots and reverse transcription PCR) revealed that *SPARC*-related modular calcium-binding protein-2 has a widespread expression in many different tissues^[25]. Down-regulation of *SMOC2* (4.5 times) will very likely be unfavorable for angiogenesis.

Our study revealed that exposure to epinephrine for 48 hours significantly altered the gene expressions of cultured cardiomyocytes. These gene expression changes induced by epinephrine exposure suggest that epinephrine may have significant unfavorable impact on angiogenesis. This inhibition of angiogenesis can potentially be detrimental in some selective patients

that could benefit from the angiogenic process to revive dysfunctional or dying myocardium.

Whether these epinephrine-induced gene expression changes lead to clinically observed long-term and/or short-term adverse outcomes in patients treated with epinephrine is not clear yet. Further studies, especially in vivo and human studies, will be needed to clarify the importance of these gene expression changes and their clinical implications in mediation or modulation of important complex pathways that ultimately influence morbidity or mortality.

In conclusion, our present investigation utilized cultured cardiomyocytes to study the effects of epinephrine on the angiogenesis-related gene expressions. The results of our study demonstrated epinephrine-induced upregulation of *ANGPT2* and downregulation of *angiopoietin-2*, *NRG1*, and *SMOC2*. The results suggest that epinephrine exposure may inhibit the angiogenic process, potentially devastating to injured or ischemic myocardium. Whether these changes lead to clinically observed adverse outcomes is not clear. Further studies will be needed to illustrate the importance of these gene expression changes.

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