



# Effects of milrinone on inflammatory response-related gene expressions in cultured rat cardiomyocytes

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## Abstract

Congestive heart failure (CHF) is defined as a cardiac dysfunction leading to low cardiac output and inadequate tissue perfusion. Intravenous positive inotropes are used to increase myocardial contractility in hospitalized patients with advanced heart failure. Milrinone is a phosphodiesterase III inhibitor and used most commonly for inotropic effect. The well-known PROMISE study investigated the effects of milrinone on mortality in patients with severe CHF, and concluded that long-term therapy with milrinone increased morbidity and mortality among patients with advanced CHF. Previous studies have suggested that phosphodiesterase inhibitors can have potential effects on inflammatory pathways. Hence, we hypothesized that milrinone may alter inflammatory gene expressions in cardiomyocytes, thus leading to adverse clinical outcomes. We used rat cardiomyocyte cell line H9C2 and studied the impact of exposing cardiomyocytes to milrinone (10  $\mu\text{mol/L}$ ) for 24 hours on inflammatory gene expressions. RNA extracted from cultured cardiomyocytes was used for whole rat genome gene expression assay (41 000 genes). The following changes in inflammatory response-related gene expressions were discovered. Genes with increased expressions included: *THBS2* (+ 9.98), *MMP2* (+3.47), *DDIT3* (+2.39), and *ADORA3* (+3.5). Genes with decreased expressions were: *SPPI* (-5.28) and *CD14* (-2.05). We found that the above mentioned gene expression changes seem to indicate that milrinone may hinder the inflammatory process which may potentially lead to adverse clinical outcomes. However, further *in vivo* and clinical investigations will be needed to illustrate the clinical relevance of these gene expression changes induced by milrinone.

**Keywords:** milrinone, gene expression, cardiomyocyte, inflammation

## Introduction

Heart failure (HF) is defined as a cardiac dysfunction

status leading to low cardiac output (CO) and inadequate tissue perfusion. The fundamental defect of HF is the decreased myocardial contractility which often requires positive inotropic therapy<sup>[1]</sup>, and/or

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impaired diastolic function. Based on 2009 American College of Cardiology/American Heart Association guidelines, intravenous positive inotropes are usually used to increase the myocardial contractility in hospitalized patients with advanced heart failure<sup>[2]</sup>. Though these inotropes could improve the hemodynamic parameters, they also have some side effects like arrhythmias, myocardial ischemia from increasing myocardial oxygen consumption and metabolic alterations<sup>[3]</sup>. Milrinone is one of the most commonly used inotropic agents. It is a type III phosphodiesterase inhibitor which acts by decreasing the degradation of adenosine monophosphate-cAMP and leads to increased  $\text{Ca}^{2+}$  influx into the sarcolemma, thus leading to increased contractile force<sup>[4]</sup>. A meta-analysis and systemic review study by Tang *et al*<sup>[5]</sup> analyzed the effect of milrinone in the management of acute HF following acute myocardial infarction (AMI) and they found that milrinone improved the cardiac function in terms of improvement in left ventricular ejection fraction (LVEF), CO and heart rate. However, the study result also showed that milrinone did not improve prognosis or survival rate of the patients with AMI. Zangrillo *et al*<sup>[6]</sup> also conducted a meta-analysis of randomized clinical studies in which intravenous milrinone was administered in patients who underwent adult cardiac surgical procedures. The analyses unveiled that milrinone was associated with significant increase in mortality as compared to placebo group and active controls. The well-known PROMISE study group also investigated the effect of oral milrinone on mortality in patients with severe chronic heart failure, and they concluded that long-term therapy with milrinone increased morbidity and mortality among patients with advanced congestive heart failure, although milrinone did offer some short term beneficial hemodynamic effects. And they stated that the exact mechanism for this phenomenon is unclear<sup>[7]</sup>. Previous studies have already suggested that phosphodiesterase inhibitors can have potential effects on the inflammatory pathways. Amrinone was found to decrease the inflammatory signaling pathway while milrinone could increase the inflammatory response<sup>[8]</sup>. Hence, we hypothesized that milrinone may alter the inflammatory gene expressions in cardiomyocytes leading to adverse clinical outcomes seen in the previous studies. And we conducted this study to investigate the effects of milrinone on gene expressions related to inflammatory responses.

## Materials and methods

The cell culture method and microarray technique were described in our previous publications<sup>[9-10]</sup> and

other studies<sup>[11]</sup>. Here is a brief description:

### Cell culture

Cell line H9C2 (rat cardiomyocyte, ATCC, Rockville, Maryland) was used for this study. Cardiomyocytes were seeded into 25 mL flasks at the concentration of 0.5 mol/mL and cultured at 37 °C, 5%  $\text{CO}_2$  in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum (FCS), with penicillin (100 U/mL) and streptomycin (100 mg/mL). The cardiomyocytes were cultured overnight and in the morning of the next day, milrinone was added to the culture medium in the flasks to make a final milrinone concentration at 10  $\mu\text{mol/L}$ . Then the cells were cultured for 24 hours without medium change at aforementioned conditions. The same flasks with exactly the same culture medium and identical concentration of H9C2 cells without adding milrinone served as control group. This technique is modified from the technique reported by Merten *et al*<sup>[12]</sup>. Each experiment was done in triplicates, two samples served as experimental, one served as control. At 24 hours, the cell cultures were stopped and the total RNA was extracted from the cultured cardiomyocytes and then purified by applying Trizol (Thermo Fisher Scientific, Waltham, MA, USA) and RNeasy cleanup kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturers' protocols. The total RNA yield was then quantified by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and the quality was verified by gel electrophoresis.

### Microarray

The RNA extracted from the above-mentioned cell cultures was then used for whole genome gene expression assay. The array we used contains more than 41 000 rat genes. The cDNA was synthesized from the RNA samples, and it was used to synthesize fluorescent cRNA. Labeled cRNA samples were then hybridized onto the Whole Rat Genome Oligo Microarray slides (Agilent Technologies Inc., Santa Clara, CA, USA). After hybridization, arrays were washed and scanned by a microarray scanner (Agilent Technologies).

### Data analysis

The data from this study were input into GeneSpring GX 11 software (Agilent Technologies) as 20 one-color arrays and the data were normalized to the median per chip and the median value per gene across all arrays. Parameter data were added so these 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 ( $P < 0.05$ ). Milrinone-induced gene expressional changes related to inflammatory response were identified ( $P < 0.05$ ).

## Results

After the cultured cardiomyocytes were exposed to milrinone at the concentration of 10  $\mu\text{mol/L}$  for 24 hours, the following changes in inflammatory responderelated gene expressions were discovered. The genes with increased expressions included: *THBS2* (+9.98), *MMP2* (+3.47), *DDIT3* (+2.39), *ADORA3* (+3.5). The genes with decreased expression were: *SPP1* (-5.28), *CD14* (-2.05) as shown in [Table 1](#). All these changes with  $P < 0.05$  are as also shown in [Fig. 1](#).

## Discussion

Inflammatory responses can generally be induced by various sources of stimulations. Pro-inflammatory milieu in the heart restrains cardiomyocyte differentiation from cardiac stem cells and also increases the adrenergic activation, which will probably reduce the

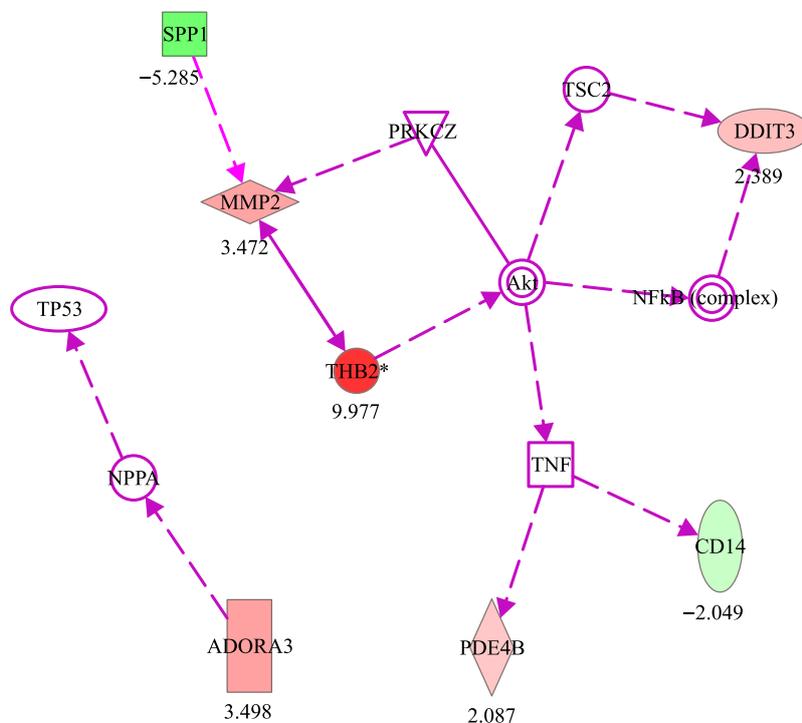
**Table 1** Effects of milrinone on inflammatory responderelated gene expressions

Gene expression up-regulated	Gene expression down-regulated
<i>THBS2</i> (+9.98)	<i>SPP1</i> (-5.28)
<i>MMP2</i> (+3.47)	<i>CD14</i> (-2.05)
<i>DDIT3</i> (+2.39)	
<i>ADORA3</i> (+3.5)	

*THBS2*: thombospondin-2; *MMP2*: matrix metalloproteinase-2; *DDIT3*: DNA damage inducible transcript-3; *ADORA3*: adenosine A3 receptor; *SPP1*: secreted phosphoprotein-1; *CD14*: CD14 molecule.

endogenous cardiac repair<sup>[3]</sup>. Our study unveiled some of the gene expression changes related to inflammatory response in cultured rat cardiomyocytes after exposure to milrinone for 24 hours. The expressions of *THBS2*, *MMP2*, *DDIT3* and *ADORA3* genes were significantly increased, while the expressions of *SPP1* and *CD14* genes were significantly decreased.

*THBS2* gene encodes a glycoprotein that modulates the cell-matrix interactions. *THBS2* is normally expressed in extracellular matrix, developing blood vessels and basal epidermal keratinocyte layer. *THBS2* is also expressed during tissue remodeling, foreign body reaction, carcinogenesis, tissue ischemia and



**Fig. 1** Inflammatory response-related gene expression changes induced by exposure to milrinone in cultured rat cardiomyocytes. *THBS2*: thombospondin-2; *MMP2*: matrix metalloproteinase-2; *DDIT3*: DNA damage inducible transcript-3; *ADORA3*: adenosine A3 receptor; *SPP1*: secreted phosphoprotein-1; *CD14*: CD14 molecule; *PRKCZ*: protein kinase C zeta type; *TP53*: tumor protein P53; *NPPA*: natriuretic peptide precursor A variant; *AKI*: adenosine kinase; *TNF*: tumor necrosis factor; *PDE4B*: phosphodiesterase 4B; *NFkB*: nuclear factor kappa B subunit; *TSC2*: tuberous sclerosis 2.

inflammation<sup>[14]</sup>. The expression of *THBS2* is significantly increased in myocardium under stress<sup>[15]</sup>. Hanatanis *et al*<sup>[15]</sup> investigated the correlations between the *THBS2* and cardiovascular mortality. They found a positive association between *THBS2* and cardiovascular disease severity. Hence, they believe that *THBS2* can possibly be used as a new biomarker for the prediction of cardiovascular disease severity and mortality in patients with compromised left ventricular ejection fraction. Endogenous expression of *THBS1* in the bordering area of myocardial infarct functionally limits the expansion of granulation tissue into the non-infarcted myocardium and thus prevents the extension of inflammatory process into the neighboring remodeling myocardium<sup>[16]</sup>. This seems to be a beneficial process for the preservation of myocardial function. Interestingly, an experimental animal study by Schroen *et al*<sup>[17]</sup> found that *THBS2* was significantly increased in rats with heart failure and it can potentially be used as an early indicator for the future development of heart failure. Our study found that the gene expression of *THBS2* was increased by 9.98 times after exposure to milrinone; does this indicate a bad prognosis or other clinical implications?

*MMP2* encodes matrix metalloproteinase-2 which belongs to Zinc-binding proteolytic enzymes. It is involved in the breakdown of extracellular matrix in normal physiological processes such as tissue remodeling, and some disease processes as atherosclerosis, arthritis and tumor metastasis<sup>[18]</sup>. Excessive *MMP2* activation can potentially increase collagen-I synthesis through FAK phosphorylation in cardiac fibroblasts<sup>[19]</sup>. *MMP2* is usually expressed in the early phase after myocardial infarction possibly as a compensatory mechanism for the myocardial regeneration process to be initiated<sup>[20]</sup>. Gao *et al*<sup>[21]</sup> reported that *MMP2* is increased during the post-myocardial infarction period and *MMP2* product cleaves the myosin light chain kinase which phosphorylates the myosin light chain required for the myosin and actin interaction. Hence *MMP2* can potentially impair myocardial function by decreasing the myosin light chain kinase. Also depletion of collagen and other extracellular matrix by MMPs from the core and fibrous cap overlying plaques may lead to atheromatous plaque rupture which may induce myocardial infarction and stroke<sup>[22-23]</sup>. There are reports that *MMP2* and *MMP9* are significantly increased in prehypertensive patients, in whom the extracellular matrix turnover is increased leading to worsening arterial stiffness<sup>[24]</sup>. And induction of *MMP2* by Calpain-1 may cause degradation of elastic

fibers leading to calcification of arterial wall<sup>[25]</sup>. All these may suggest that increase in gene expression of *MMP2* after exposure to milrinone can potentially lead to adverse cardiovascular events in patients who are treated with milrinone for long-term therapy.

DDIT3, also named as CCAAT/enhancer binding protein, belongs to a family of transcription factors. It has the capability to cause cellular growth arrest and apoptosis<sup>[26]</sup>. DDIT3 is induced by DNA alkylation, nutritional deprivation and radiation. It was reported that decreased expression of DDIT3 exists in different myeloid diseases<sup>[26-27]</sup>. Studies also found an increased DDIT3 expression in patients with non-small cell lung carcinoma<sup>[28]</sup>. Our study found that DDIT3 expression is increased by 2.39 times after exposure of cardiomyocytes to milrinone.

Another gene which had elevated expression due to exposure of cardiomyocytes to milrinone is *ADORA3*. *ADORA3* encodes adenosine A3 receptor. Adenosine is generally believed to have cardioprotective effect. Some studies showed that adenosine A3 receptor activation reduces the intracellular Ca<sup>2+</sup> level which may offer some cardioprotective effects, especially during reperfusion phase. Reperfusion cardiac injury is believed due to excessive intracellular Ca<sup>2+</sup><sup>[29]</sup>. Also, adenosine A3 has been shown to protect the patients from doxorubicin-induced myocardial toxicity by preventing inflammatory response to doxorubicin<sup>[30]</sup>. However, Lu *et al*<sup>[31]</sup> reported that adenosine A3 receptor counteracts the cardioprotective effect of adenosine. And they found that knocking out the gene for Adenosine A3 receptors leads to protective effects on the heart. We found *ADORA3* gene expression increased by 3.5 times, but we are not sure what its clinical implications are.

SPP1, also known as osteopontin, is a glycosylated phosphoprotein with high amino acid content. It has multiple physiological functions in bone resorption and calcification, cell adhesion, apoptosis, tumorigenesis, wound healing, immune-modulation, chemotaxis and inflammation. Osteopontin is believed to be a proinflammatory protein which likely protects against inflammation. Singh *et al*<sup>[32]</sup> found that neutrophil accumulation at the site of infection is reduced significantly in osteopontin-deficient mice. Their study supports the concept that protective effect of osteopontin results from enhanced initial neutrophil accumulation at sites of infection leading to optimal bacterial killing. This indicated that osteopontin participates in immune modulation and inflammatory responses. Decreased gene expression of SPP1 may suggest that milrinone likely hinders inflammatory responses and inflammation process. Monocytes in

humans are classified based on lipopolysaccharide (LPS) receptors CD14 and FcγIII R CD16. Recognition of LPS by Toll like receptor initiates inflammatory responses<sup>[33]</sup>. Monocytes are involved in the process of inflammation and there is recruitment of especially CD14 monocytes in the lymph nodes 24 hours after the infection<sup>[34]</sup>. CD14 is a glycosylphosphatidylinositol-anchored membrane glycoprotein, which is expressed in immune cells related to innate immunity. When a pathogen invades the human body, inflammatory reaction occurs *via* the toll like receptor (TOL) pathway by binding to LPSbinding protein and CD14 receptors<sup>[35]</sup>. CD14 is also involved in the development of primary sclerosing cholangitis, pathogenesis of rheumatoid arthritis and other autoimmune disorders<sup>[36-37]</sup>. The down-regulated gene expression of *CD14* in our study with milrinone may indicate that milrinone exposure may be related to reduced launch of inflammation and impaired body's immune responses to foreign invasions.

In summary, as a commonly used inotrope, milrinone induced inflammation-related gene expression changes in cultured rat cardiomyocytes. We found that the gene expressions of *THBS2*, *MMP2*, *DDIT3*, and *ADORA3* were significantly increased, while the gene expressions of *SPP1* and *CD14* were significantly down-regulated after the cultured cardiomyocytes were exposed to milrinone *in vitro* for 24 hours. The clinical implications of these gene expression changes were unclear, but our results seem to indicate that milrinone exposure may hinder the inflammatory process. Further *in vivo* experimental and clinical investigations will be needed to illustrate the clinical relevance of these gene expression changes induced by milrinone.

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