

Metabolomic Profiling of Cellular Responses to Carvedilol Enantiomers in Vascular Smooth Muscle Cells

Mingxuan Wang, Jing Bai, Wei Ning Chen*, Chi Bun Ching

School of Chemical and Biomedical Engineering, College of Engineering, Nanyang Technological University, Singapore, Singapore

Abstract

Carvedilol is a non-selective β -blocker indicated in the treatment of hypertension and heart failure. Although the differential pharmacological effects of individual Carvedilol enantiomer is supported by preceding studies, the cellular response to each enantiomer is not well understood. Here we report the use of GC-MS metabolomic profiling to study the effects of Carvedilol enantiomers on vascular smooth muscle cells (A7r5) and to shed new light on molecular events underlying Carvedilol treatment. The metabolic analysis revealed alternations in the levels of 8 intracellular metabolites and 5 secreted metabolites in A7r5 cells incubated separately with *S*- and *R*-Carvedilol. Principal component analysis of the metabolite data demonstrated the characteristic metabolic signatures in *S*- and *R*-Carvedilol-treated cells. A panel of metabolites, including L-serine, L-threonine, 5-oxoproline, myristic acid, palmitic acid and inositol are closely correlated to the vascular smooth muscle contraction. Our findings reveal the differentiating metabolites for A7r5 cells incubated with individual enantiomer of Carvedilol, which opens new perspectives to employ metabolic profiling platform to study chiral drug-cell interactions and aid their incorporation into future improvement of β -blocker therapy.

Citation: Wang M, Bai J, Chen WN, Ching CB (2010) Metabolomic Profiling of Cellular Responses to Carvedilol Enantiomers in Vascular Smooth Muscle Cells. PLoS ONE 5(11): e15441. doi:10.1371/journal.pone.0015441

Editor: Carlo Gaetano, Istituto Dermopatico dell'Immacolata, Italy

Received: August 8, 2010; **Accepted:** September 21, 2010; **Published:** November 24, 2010

Copyright: © 2010 Wang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Financial support was provided by Academic Research Funds, Ministry of Education, Singapore. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: WNChen@ntu.edu.sg

Introduction

β -blockers, also known as β -adrenergic receptor antagonists, are a class of drugs that block catecholamines by binding to β -adrenergic receptors. There are three known types of β -adrenergic receptors, designated β_1 (located mainly in heart and kidneys), β_2 (located mainly in lungs, uterus, liver, gastrointestinal tract, vascular smooth muscle and skeletal muscle) and β_3 (located in fat cells). The β -adrenergic receptors are a class of G-protein-coupled receptors characterized by seven transmembrane spanning domains forming a pocket in which agonists and antagonists compete for their binding sites [1]. Carvedilol, a nonselective β -antagonist (β_1 , β_2), has proven potent antihypertensive activity and also effective in the treatment of coronary artery disease and congestive heart failure [2]. It also possesses calcium channel blocking effect by inhibiting voltage-dependent L-type Ca^{2+} in vascular smooth muscle cells [3]. Carvedilol has an asymmetric carbon atom, which gives rise to *S*- and *R*-enantiomers. The racemic mixture of Carvedilol is administered clinically although it results predominantly from *S*-Carvedilol [4,5]. Although each enantiomer exhibits differential pharmacological effects, the cellular response to individual enantiomer is not well understood. Since the metabolite concentrations represent sensitive responses of cells to external stimuli [6,7], the analysis of metabolic profiling of A7r5 cells underlying Carvedilol enantiomers treatment will greatly facilitate the understanding on Carvedilol-cell interactions.

We have previously developed LC-MS/MS-based proteomics system to study the cellular responses to the treatments by different generations of beta-blockers [8,9,10]. Our study identified several differentially expressed proteins mediating the vascular smooth muscle contraction and also highlighted the differences associated with the

treatment of individual enantiomer. While proteomics data has provided important insights into the active effects of *S*-enantiomers, metabolomics may shed new light on the understanding of drug-cell interactions as it reveals the downstream changes of proteome.

Recent advances of instrumentation and computation has enabled the simultaneous analysis of a large number of metabolites. Gas chromatography coupled with mass spectrometry (GC-MS) has proven to be an effective combination for metabolites identifications and quantifications in mammalian cell lines [11,12,13,14] due to its excellent resolution and sensitivity.

The aim of the current study was to obtain a systematic view of the cellular responses to Carvedilol enantiomers. We wished to identify multiple metabolites that could facilitate the understanding of the *in vitro* actions of Carvedilol and aid their incorporation into future improvement of β -blocker therapy. In this study, we employed GC-MS-based metabolomic profiling system to functionally assign Carvedilol enantiomers induced changes in the metabolism of A7r5 cells. This study identifies the differential effects of Carvedilol enantiomers on both intracellular and secreted metabolites including those involved in the vascular smooth muscle contraction pathway. Our results support the fact that *S*-Carvedilol possesses more biological activity. Moreover, our study demonstrates the feasibility of GC-MS-based metabolic profiling as an analytical tool to understand drug-cell interactions.

Results

GC-MS profiles depict the intracellular response of A7r5 cells to *S*- and *R*-Carvedilol

We performed GC-MS-based metabolomic profiling in A7r5 cells incubated with individual enantiomer of Carvedilol. A total of

32 intracellular metabolites were identified using the NIST library and SHIMADZU GC/MS Metabolite Mass Spectra Database with similarity index above 90%. Out of the 32 metabolites, 8 displayed significantly different levels ($p < 0.05$) in A7r5 cells incubated with *S*-Carvedilol, while the changes of most metabolites in *R*-Carvedilol-treated cells are less significant relative to control cells (Figure 1). For example, the levels of palmitic acid and myristic acid displayed up-regulated in *S*-Carvedilol-treated cells while the changes in *R*-Carvedilol-treated cells is not significant compared with control cells.

Multivariate statistical modeling using principal component analysis (PCA) was performed on the GC-MS spectra of four independently grown replicates. PCA has been successfully applied to build classification models from metabolomics data [15,16,17,18]. The scores plot (Figure 2a) resulting from PCA revealed clear discrimination between the intracellular metabolome of A7r5 cells incubated with *S*- and *R*-Carvedilol, indicating that A7r5 cells displayed distinctly metabolic characteristics under individual Carvedilol enantiomer treatment. It was also found out that L-serine, palmitic acid and myristic acid contributed more significantly to distinguishing A7r5 cells incubated with *S*- or *R*-Carvedilol after loadings plot analysis (Figure 2b).

GC-MS profiles depict the secreted metabolome of A7r5 cells in response to the treatment of *S*- and *R*-Carvedilol

The secreted metabolites in culture medium from A7r5 cells were analysed using GC-MS system. Analysis detected 9 secreted metabolites. A representative GC-MS total ion chromatograms is shown in Figure 3 and the up-regulated metabolite L-Alanine was observed in *S*-Carvedilol-treated cells. The culture medium incubated at the same conditions was set as negative control. From four independent experiments, 4 metabolites were identified as significantly changes between samples incubated with *S*-Carevdilol and control, while the changes in *R*-Carevdilol-treated cells is less significant (Figure 4). PCA was performed on the GC-

MS spectra of four biological replicates from A7r5 cells incubated with control, *S*- and *R*-Carvedilol. The PCA scores plot shown in Figure 5a demonstrates that exposure of A7r5 cells to *S*- or *R*-Carvedilol caused distinct changes in the metabolome. Loadings plot identified L-alanine, L-leucine, L-valine and succinic acid as the major discriminators between *S*- and *R*-Carvedilol-treated cells (Figure 5b).

Intracellular Calcium concentration variations in response to Carvedilol enantiomers treatment

To investigate the calcium inhibition effect of each Carvedilol enantiomer, the intracellular Ca^{2+} concentration was measured. The results indicated that the intracellular concentration in *S*-Carvedilol-treated A7r5 cells was lower than that in control cells (S:C, 0.848 ± 0.018 , $p < 0.05$), while the variations were less significant in *R*-Carvedilol-treated cells (R:C, 0.935 ± 0.012 , $p < 0.05$). Student's t-test was performed to assess the significance of the variance.

Discussion

In pharmacology, the importance of drug chirality and its role in explaining the dramatic differences of individual enantiomers in biological activities is increasingly appreciated [19]. Our previous studies investigated the intracellular proteome in A7r5 cells incubated with individual enantiomers of β -blockers and identified differentially expressed proteins mediating the vascular smooth muscle contraction [8,9,10]. In the current study, we have applied GC-MS-based metabolomic profiling approach to investigate the cellular response to the treatment of Carvedilol enantiomers. We wished to investigate whether a metabolomic approach could identify signatures associated with *S*- and *R*-Carvedilol treatment and whether these signatures could shed new light into the *in vitro* actions of Carvedilol. To our knowledge, this is the first study to apply a metabolic profiling system to characterize chiral drug-cell interactions. The GC-MS-based metabolic profiling platform paired with multivariate statistical

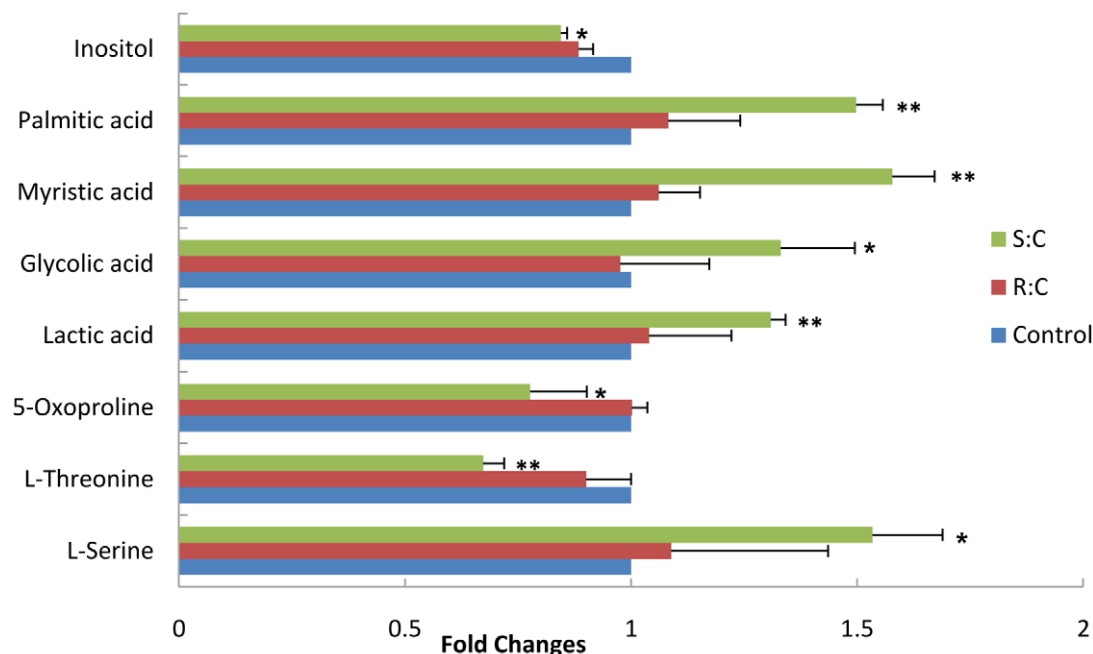
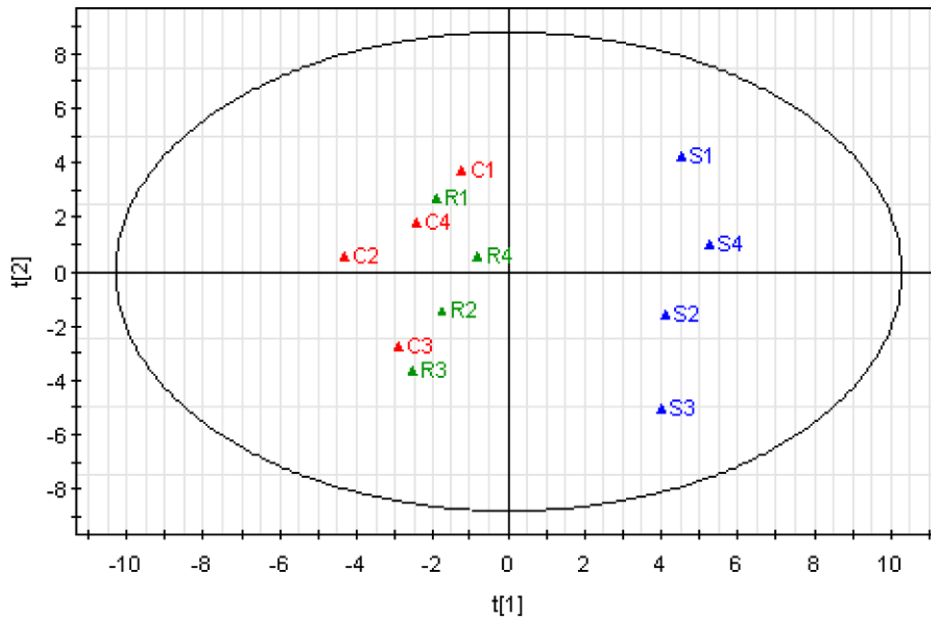


Figure 1. Differential expression levels of intracellular metabolites in *S*- and *R*-Carvedilol-treated cells. The "asterisk" indicated the statistical significance of the metabolite changes by Student's t-test: *, $p < 0.05$; **, $p < 0.01$. doi:10.1371/journal.pone.0015441.g001

A



R2X[1] = 0.366261 R2X[2] = 0.267868 Ellipse: Hotelling T2 (0.95)

B

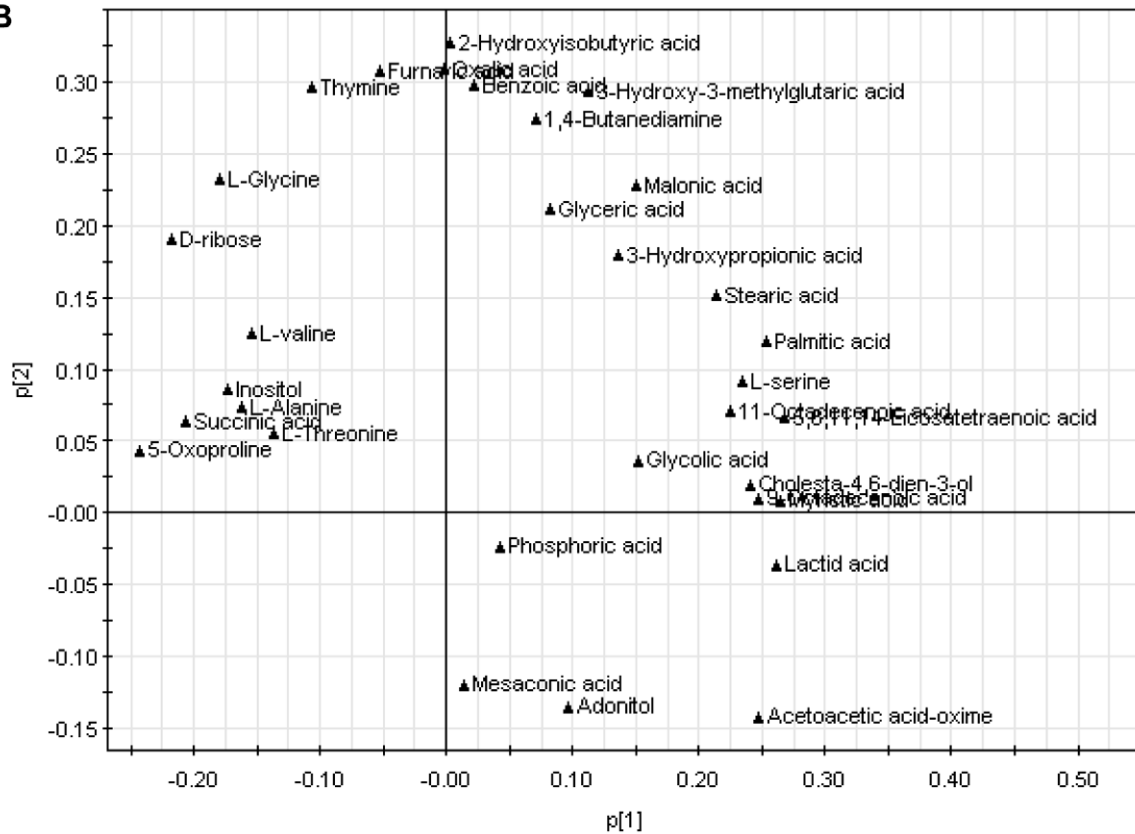


Figure 2. Principal component analysis (PCA) of the GC-MS spectra from intracellular metabolites in A7r5 cells. A) PCA scores plot. Solvent control (red), S-Carvedilol (blue) and R-Carvedilol (green) are included. B) PCA loading plot.
doi:10.1371/journal.pone.0015441.g002

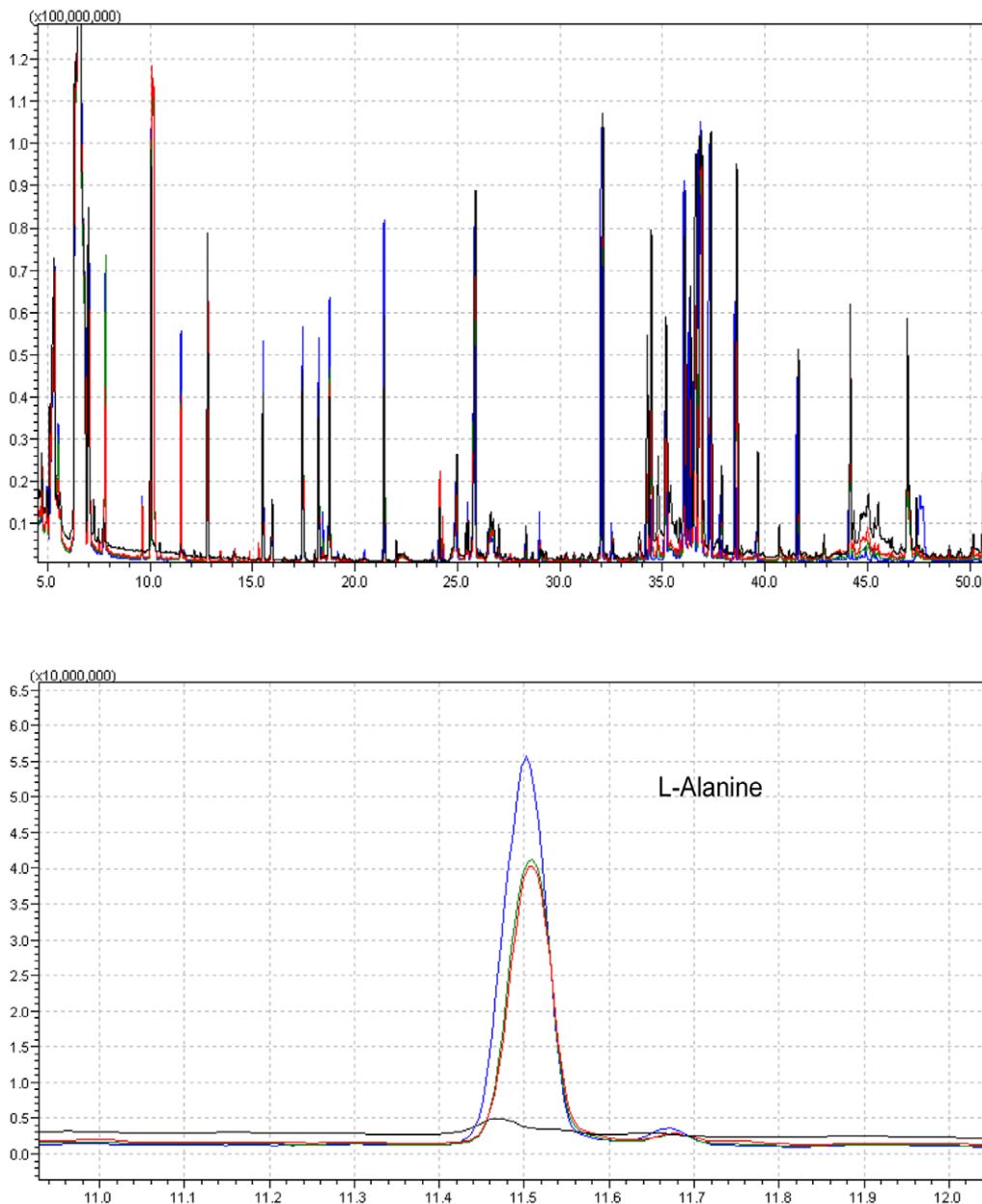


Figure 3. The representative GC-MS spectra of L-Alanine derived from total ion chromatograms. Culture medium (black), untreated cells (red), *R*-Carvedilol-treated cells (green) and *S*-Carvedilol-treated cells (blue).
doi:10.1371/journal.pone.0015441.g003

method (PCA) has enabled us to highlight the distinct cellular response to individual enantiomers of Carvedilol and several metabolites altered in the treatment of *S*-Carvedilol.

To obtain the information about the metabolic variations underlying the treatment of Carvedilol enantiomers, we first analysed the intracellular metabolome in A7r5 cells. From four independent experiments, 8 metabolites were consistently identified with statistically significant changes and closely relevant with the cell functions associated with the treatment of *S*-Carvedilol and will be discussed as follows.

The intracellular Ca^{2+} has been a central player in regulating vascular smooth muscle contraction and relaxation [20,21,22,23]. A decreased of intracellular Ca^{2+} concentration will elicit relaxation. The previous study has suggested that Carvedilol exhibited

the inhibitory effects on Ca^{2+} mobilization and this unique action may contribute to its antihypertensive effect [3]. Using KEGG database and literature mining, we have linked the intracellular metabolic variations to the vascular smooth muscle relaxation pathway (Figure 6). Changes in amino acid levels were variable. L-serine, a precursor of central neurotransmitters, was found to be up-regulated in cells incubated with *S*-Carvedilol compared with control cells and those incubated with *R*-Carvedilol. The up-regulated L-Serine has been reported to exhibit antihypertensive effect by evoking vasodilatation in vascular smooth muscles cells [24]. The vasodilatation effect is mediated by activating Ca^{2+} -activated K^{+} channel [25], which induces the hyperpolarization in vascular smooth muscle cells and promotes the closure of voltage-operated calcium channels (VOC). The inactivated vascular

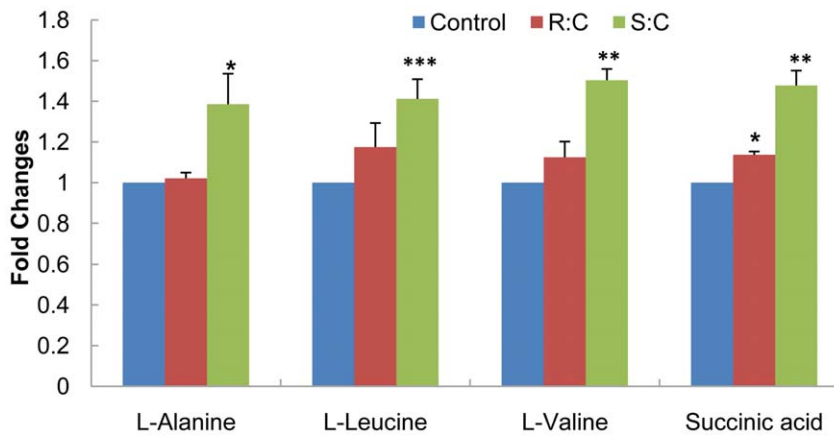


Figure 4. Differential expression levels of secreted metabolites in *S*- and *R*-Carvedilol-treated cells. The “asterisk” indicated the statistical significance of the metabolite changes by Student’s t-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. doi:10.1371/journal.pone.0015441.g004

smooth muscle VOC thus reduces Ca^{2+} influx, which results in the Ca^{2+} reduction and subsequent relaxation or vasodilatation. In contrast, levels of L-threonine and 5-oxoproline were reduced in *S*-Carvedilol-treated cells. L-threonine is an essential amino acid that could be used for serine biosynthesis [26]. 5-oxoproline is converted into glutamate by 5-oxoprolinase [27]. Glutamate activates glutamate receptor that inhibits calcium channel, which induces muscle relaxation. Despite great effort, we were not able to identify glutamate in this study.

The fatty acids level treaded toward increase in cells incubated with *S*-Carvedilol. The up-regulated myristic acid and palmitic acid have been reported to stimulate nitric-oxide Synthase, which in turn activates the production of nitric oxide (NO) [28]. In the cardiovascular system, NO plays an essential role in hemodynamics and controlling vascular remodeling [29,30,31]. For example, in vascular smooth muscles cells NO stimulates soluble guanylyl cyclase (s-GC) to generate the second messenger cGMP, which results in activation of cGMP-dependent protein kinase G (PKG) [32,33]. Inositol triphosphate receptor (IP_3R) is a membrane glycoprotein complex acting as Ca^{2+} release channel activated by the second messenger inositol triphosphate (IP_3). The activated PKG is recruited by the IP_3R -associated cGMP kinase substrate (IRAG), resulting in IP_3R phosphorylation and inhibition of Ca^{2+} release from endoplasmic reticulum (ER) [34]. The reduced Ca^{2+} concentration in vascular smooth muscle cells induced by increased NO production thus results in muscle relaxation. The Ca^{2+} concentration measurement assay was conducted to prove our hypothesis and the results indicated that the Ca^{2+} concentration in *S*-Carvedilol-treated cells is down-regulated compared with control cells and those incubated with *R*-Carvedilol. In addition, our observation of a decreased level of inositol in cells incubated with *S*-Carvedilol may be due to the decrease of IP_3 caused by the phosphorylation of IP_3R .

Lactic acid, an end product of glycolysis, was up-regulated in cells incubated with *S*-Carvedilol. A higher concentration of lactic acid is produced in vascular smooth muscle cells when faced with low levels of oxygen. Intaking of Carvedilol may cause some common side effects in clinic such as shortness of breath, dizziness [35,36]. Our observation of increased level of lactic acid could be related with the side effects induced by *S*-Carvedilol. Glycolic acid is the smallest α -hydroxy acid which is able to exfoliate dead skin cells and encourage cell regeneration. The function of glycolic acid in vascular smooth muscle cells is unclear. The increased glycolic acid could be possibly involved in the inhibition of vascular smooth muscle thickening that spontaneously happens in hypertensive rats [37].

Metabolites secretion by a cell reflects the biochemical response to external stimuli. We have previously investigated the secreted proteome in A7r5 cells incubated with β -blockers and a common secreted protein T-kininogen was identified as a potential drug marker [38,39]. Since the metabolites are the end products of cellular regulatory processes, the quantitative analysis of the secreted metabolites in A7r5 cells upon Carvedilol enantiomers treatment should provide us a complementary understanding on overall cellular response. A panel of 4 metabolites (L-alanine, L-leucine, L-valine, succinic acid) showed up-regulated in cells incubated with *S*-Carvedilol compared with control cells and those incubated with *R*-Carvedilol. L-leucine and L-valine are essential amino acids with three branched-chains, which could be used as an energy source by muscle tissue. L-alanine can be produced from branched chain amino acids, such as L-leucine and L-valine. The possible reason that the high secreted levels of these amino acids is to keep them in balance in the more relaxed cells. PCA analysis demonstrates the characteristic signatures of cells incubated with individual enantiomers of Carvedilol. Succinic acid secretion was strongly enhanced with *S*-Carvedilol treatment, which may result from the inactivation of succinate dehydrogenase. The highly produced NO in *S*-Carvedilol-treated cells may selectively inhibit succinate dehydrogenase activity [40]. Since the secreted metabolites variations in a cell reflect its internal metabolic state, the distinct secretion profiles further confirm the differences of intracellular metabolome of A7r5 cells in response to the *S*- and *R*-Carvedilol treatment. However, no specific production or secretion pathways were found to connect secreted metabolic variations to the changes in intracellular metabolic states due to the limited secreted metabolites seen. The possible explanations for this: 1) Metabolites are very instable and degraded rapidly in culture medium. 2) Partial of the secreted metabolites may not be amenable to derivatization for GC-MS analysis. The complementary analytical technologies (for example, LC-MS, NMR) without such derivatization may become alternative ways to investigate the secreted metabolome in A7r5 cells. This should be dressed in our further study.

In conclusion, our results highlight the potential of GC-MS-based metabolic profiling of A7r5 cells in response to Carvedilol enantiomers treatment. The cells incubated with individual enantiomers demonstrate characteristic metabolic signatures, which is consistent with the fact that each enantiomer exhibits differential biological activities. Specifically, the metabolic changes observed in A7r5 cells in response to *S*-Carvedilol treatment were

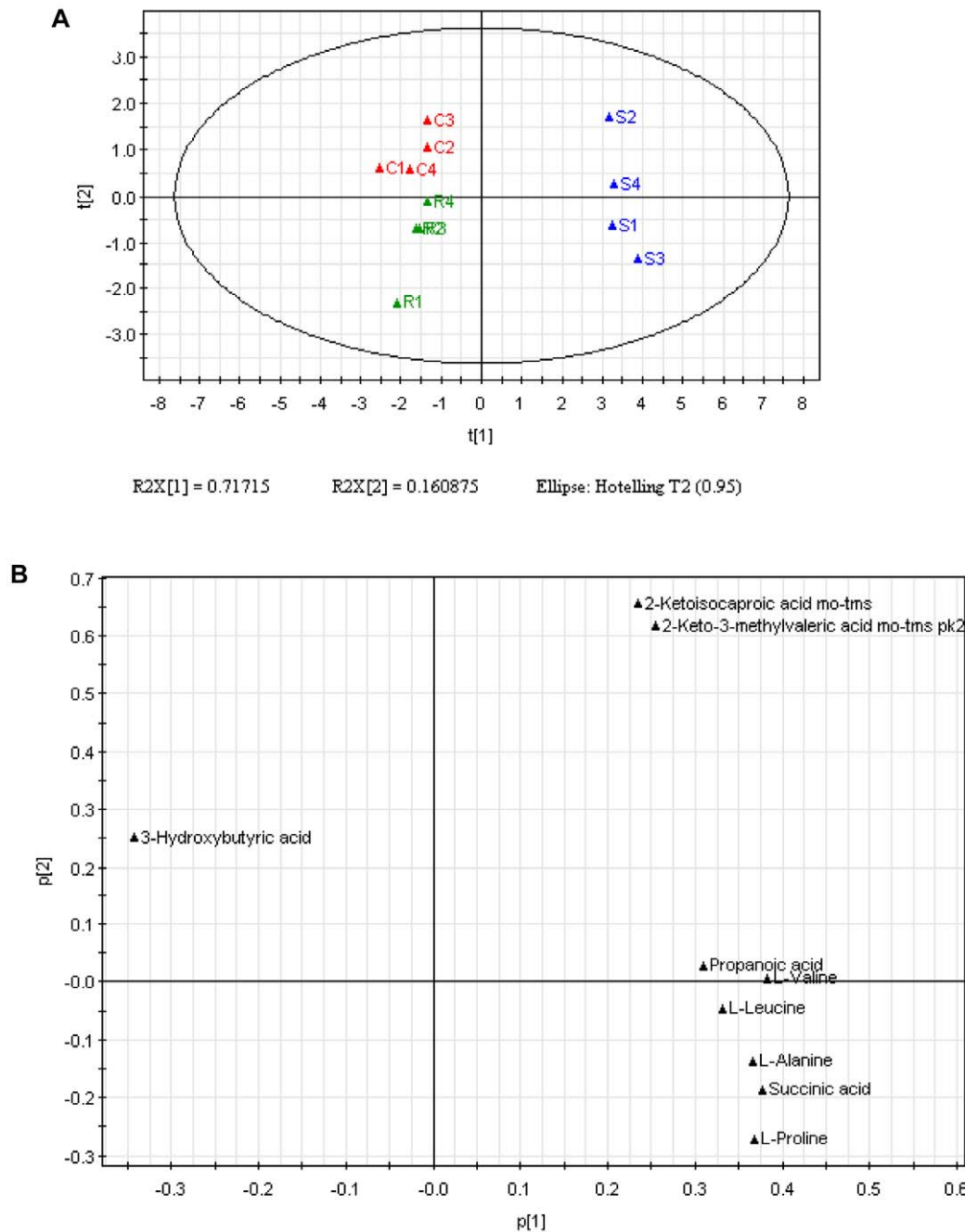


Figure 5. Principal component analysis (PCA) of the GC-MS spectra from secreted metabolites in A7r5 cells. A) PCA scores plot. Solvent control (red), *S*-Carvedilol (blue) and *R*-Carvedilol (green) are included. B) PCA loading plot.
doi:10.1371/journal.pone.0015441.g005

closely involved in the vascular smooth muscle relaxation pathway. These findings enhance our understanding of Carvedilol-cell interactions, which may in turn aid their incorporation into future improvement of β -blocker therapy.

Materials and Methods

GC-MS sample preparation

Vascular smooth muscle cell line (A7r5) was obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL

streptomycin (Life Technologies). Cells were maintained at 37°C in an atmosphere of 5% CO₂ with the medium replaced every 24 hours.

1.5×10^7 cells were treated with either *S*- or *R*-Carvedilol at a concentration of 5 μ M due to MTT reduction analysis [10]. Cells treated in parallel with equal amounts of dimethyl sulfoxide (DMSO) were used as control. After 48 hours, culture medium was collected, filtered through a 0.2 μ m filter (WHATMAN), and immediately frozen in liquid nitrogen. Cells were washed with cold PBS twice, harvested and snapped frozen in liquid nitrogen. Carvedilol enantiomers were previously separated from their racemic form using High Performance Liquid Chromatography (HPLC) in our lab [10]. Four independent experiments were conducted.

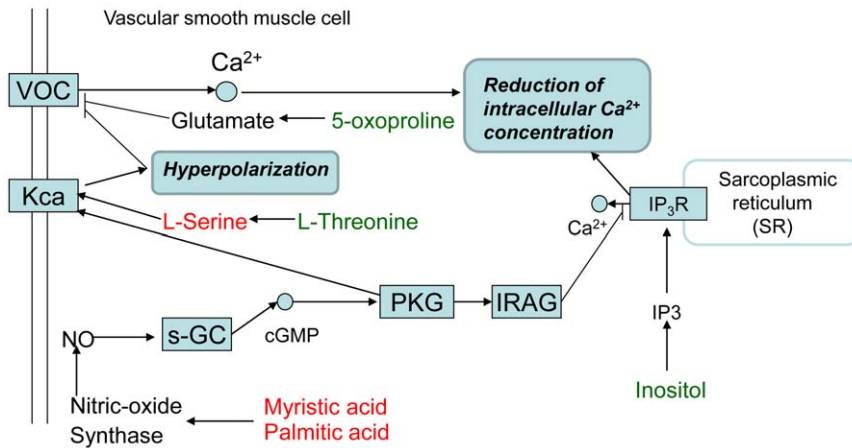


Figure 6. Schematic representation of the vascular smooth muscle relaxation pathway showing the most relevant metabolic changes induced by 5-Carvedilol. Metabolites in red/green display increased/decreased concentrations upon 5-Carvedilol treatment. doi:10.1371/journal.pone.0015441.g006

The extraction of intracellular metabolites from A7r5 cells was performed using a modified Mainak Mal [41] and Hindrik Mulder [11] procedure. Cell pellets were dissolved in a mixture of chloroform/methanol/water in ratio of 2:5:2 (v/v/v). Ribitol (2 mg/mL dissolved in water, 10 μ L) was added into the extraction solvent as internal standard to correct for metabolites losses during sample preparation. The samples were ultrasonicated in an ice bath ultra-sonicator for 30 min and subsequently centrifuged at 18000 g for 10 mins at 4°C. 0.8 mL supernatant was collected and evaporated to complete dryness overnight using a TurboVap® LV Concentration Workstation. The culture medium samples were prepared by spiking 500 μ L of each culture medium with 10 μ L internal standard solution (ribitol, 2 mg/mL dissolved in water) and lyophilised. Immediately prior to GC-MS analysis, derivatization was performed in two steps: Firstly, methoximation was carried out by dissolving the sample in 50 μ L of 20 mg/mL solution of methoxyamine hydrochloride in pyridine to protect the carbonyls. The incubation was kept at 37°C for 60 min. Silylation was then carried out by adding 100 μ L of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) to each sample for 30 min at 70°C. After incubation, samples were shaken for 2 hours at room temperature and then transferred to vials for GC-MS analysis.

GC-MS analysis

All samples were analysed using a Shimadzu QP2010Plus GC-MS system (Shimadzu, Kyoto, Japan) equipped with a DB-5 fused-silica capillary column (30 m \times 250 μ m i.d.; film thickness: 0.25 μ m; Agilent J&W Scientific, Folsom, CA, USA). Each 1 μ L of the derivatised samples was injected into GC-MS system by Shimadzu AOC-20i+s auto-sampler in the splitless mode. The solvent cutoff time was set at 3.5 min. Helium was used as carrier gas at 1.1 mL/min. The injection and ion source temperatures were kept at 280 and 200°C, respectively. The oven temperature was kept at 100°C for 4 min and increased at 4°C/min to 320°C where it remained for 1.56 min. Detection was implemented in electron impact (EI) ionization mode at 70eV. Mass spectra were recorded from 35 to 600 m/z with a scan time of 0.3 s. Chromatogram acquisition and mass spectra identification were processed using the Shimadzu GCMSsolution (version 2.5) software. To allow normalization of retention times, a retention index (RI) solution (C-10 to C-40) was injected into the GC-MS

system and performed by AART (Automatic Adjustment of Retention Time) function of GCMSsolution software. All chromatograms were subjected to noise reduction and baseline correction prior to peak area integration. Chromatographic peak deconvolution was performed using the following parameters: peaks width = 2 s, smoothing = 3. For those peaks detected, the peak area was calculated and normalized using the internal standard generating a response ratio. Chemical identification of detected metabolite peaks was performed by searching NIST mass spectral library and SHIMADZU GC-MS Metabolite Mass Spectra Database (Release 1.0). Identification was regarded as preliminary for spectra matched in the NIST library with a similarity above 90%. Identifications were regarded as definitive if the mass spectrum and retention index matched those of a metabolite present in the SHIMADZU GC-MS Metabolite Mass Spectra Database, which was prepared by analysis of authentic chemical standards on the instrument employed in this study. All samples were analysed within 24 hours in a random order.

Intracellular Ca²⁺ concentration Measurements

The intracellular Ca²⁺ concentration was measured using a Fluo-4 NW Calcium Assay Kit (Invitrogen, Eugene, OR, USA) according to the manufacturer's protocol. Briefly, each type of A7r5 cells was incubated at 37°C for 30 min in the Fluo-4 dye solution dissolved in assay buffer (Component C, Invitrogen) in a 96-well plate. Following incubation, cells were equilibrated at room temperature for an additional 30 min. The assay was performed using a fluorometer (Tecan) at 494 nm for excitation and 516 nm for emission.

Statistical analysis

The significance of intracellular and secreted metabolite level changes from A7r5 cells in response to Carvedilol enantiomers treatment was calculated using the Student's *t*-test, where all detected metabolites were tested and a threshold of $p < 0.05$ was used all this study. Principle component analysis (PCA) was performed to determine whether intracellular and secreted patterns of metabolite changes in A7r5 cells under treatment of *S*- and *R*-Carvedilol, in addition to the altered levels of individual metabolites. PCA analysis is an unsupervised clustering method aiming at summarizing data with reduced dimensions. In this study, four biological replicates were used to perform PCA analysis

for each sample using the SIMCA-P+ 12 (Umetrics, Umea, Sweden) software.

Acknowledgments

We thank Dr. Wang Xiujuan for her technical advice and assistance.

References

- Wallukat G (2002) The beta-adrenergic receptors. *Herz* 27: 683–690.
- Ruffolo RR, Jr., Feuerstein GZ (1997) Pharmacology of carvedilol: rationale for use in hypertension, coronary artery disease, and congestive heart failure. *Cardiovasc Drugs Ther* 11 Suppl 1: 247–256.
- Nakajima T, Ma J, Iida H, Iwasawa K, Jo T, et al. (2003) Inhibitory effects of carvedilol on calcium channels in vascular smooth muscle cells. *Jpn Heart J* 44: 963–978.
- Bartsch W, Sponer G, Strein K, Muller-Beckmann B, Kling L, et al. (1990) Pharmacological characteristics of the stereoisomers of carvedilol. *Eur J Clin Pharmacol* 38 Suppl 2: S104–107.
- Gagyi L, Gyeresi A, Kilar F (2008) Role of chemical structure in stereoselective recognition of beta-blockers by cyclodextrins in capillary zone electrophoresis. *J Biochem Biophys Methods* 70: 1268–1275.
- Rainaldi G, Romano R, Indovina P, Ferrante A, Motta A, et al. (2008) Metabolomics using ¹H-NMR of apoptosis and Necrosis in HL60 leukemia cells: differences between the two types of cell death and independence from the stimulus of apoptosis used. *Radiat Res* 169: 170–180.
- Mayr M, Zampetaki A, Sidibe A, Mayr U, Yin X, et al. (2008) Proteomic and metabolomic analysis of smooth muscle cells derived from the arterial media and adventitial progenitors of apolipoprotein E-deficient mice. *Circ Res* 102: 1046–1056.
- Sui J, Tan TL, Zhang J, Ching CB, Chen WN (2007) iTRAQ-coupled 2D LC-MS/MS analysis on protein profile in vascular smooth muscle cells incubated with S- and R-enantiomers of propranolol: possible role of metabolic enzymes involved in cellular anabolism and antioxidant activity. *J Proteome Res* 6: 1643–1651.
- Sui J, Zhang J, Tan TL, Ching CB, Chen WN (2008) Comparative proteomics analysis of vascular smooth muscle cells incubated with S- and R-enantiomers of atenolol using iTRAQ-coupled two-dimensional LC-MS/MS. *Mol Cell Proteomics* 7: 1007–1018.
- Wang M, Wang X, Ching CB, Chen WN (2010) Proteomic profiling of cellular responses to Carvedilol enantiomers in vascular smooth muscle cells by iTRAQ-coupled 2-D LC-MS/MS. *J Proteomics* 73: 1601–1611.
- Fernandez C, Fransson U, Hallgard E, Spegel P, Holm C, et al. (2008) Metabolomic and proteomic analysis of a clonal insulin-producing beta-cell line (INS-1 832/13). *J Proteome Res* 7: 400–411.
- Takeda S, Sim PG, Horrobin DF, Sanford T, Chisholm KA, et al. (1993) Mechanism of lipid peroxidation in cancer cells in response to gamma-linolenic acid (GLA) analyzed by GC-MS(I): Conjugated dienes with peroxyl (or hydroperoxyl) groups and cell-killing effects. *Anticancer Res* 13: 193–199.
- Filipiak W, Sponring A, Filipiak A, Ager C, Schubert J, et al. (2010) TD-GC-MS analysis of volatile metabolites of human lung cancer and normal cells in vitro. *Cancer Epidemiol Biomarkers Prev* 19: 182–195.
- Danielsson AP, Moritz T, Mulder H, Spegel P (2010) Development and optimization of a metabolomic method for analysis of adherent cell cultures. *Anal Biochem* 404: 30–39.
- Tiziani S, Lodi A, Khanim FL, Viant MR, Bunce CM, et al. (2009) Metabolomic profiling of drug responses in acute myeloid leukaemia cell lines. *PLoS One* 4: e4251.
- Fiehn O, Kopka J, Dormann P, Altmann T, Trethewey RN, et al. (2000) Metabolite profiling for plant functional genomics. *Nat Biotechnol* 18: 1157–1161.
- Devantier R, Scheithauer B, Villas-Boas SG, Pedersen S, Olsson L (2005) Metabolite profiling for analysis of yeast stress response during very high gravity ethanol fermentations. *Biotechnol Bioeng* 90: 703–714.
- Chrysanthopoulos PK, Goudar CT, Klapa MI (2010) Metabolomics for high-resolution monitoring of the cellular physiological state in cell culture engineering. *Metab Eng* 12: 212–222.
- Smith DF (1989) The stereoselectivity of drug action. *Pharmacol Toxicol* 65: 321–331.
- Filo RS, Bohr DF, Ruegg JC (1965) Glycerinated Skeletal and Smooth Muscle: Calcium and Magnesium Dependence. *Science* 147: 1581–1583.
- Webb RC (2003) Smooth muscle contraction and relaxation. *Adv Physiol Educ* 27: 201–206.
- Bolton TB (1979) Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol Rev* 59: 606–718.
- van Breemen C, Saida K (1989) Cellular mechanisms regulating [Ca²⁺]_i smooth muscle. *Annu Rev Physiol* 51: 315–329.
- Jalil JE (2008) Blood pressure reduction, potassium channels, and the endothelium: insights from L-serine. *Hypertension* 51: 626–628.
- Mishra RC, Tripathy S, Desai KM, Quest D, Lu Y, et al. (2008) Nitric oxide synthase inhibition promotes endothelium-dependent vasodilatation and the antihypertensive effect of L-serine. *Hypertension* 51: 791–796.
- Ravnikar PD, Somerville RL (1987) Genetic characterization of a highly efficient alternate pathway of serine biosynthesis in *Escherichia coli*. *J Bacteriol* 169: 2611–2617.
- Kumar A, Bachhawat AK (2010) A futile cycle, formed between two ATP-dependant gamma-glutamyl cycle enzymes, gamma-glutamyl cysteine synthetase and 5-oxoprolinase: the cause of cellular ATP depletion in nephrotic cystinosis? *J Biosci* 35: 21–25.
- Zhu W, Smart EJ (2005) Myristic acid stimulates endothelial nitric-oxide synthase in a CD36- and an AMP kinase-dependent manner. *J Biol Chem* 280: 29543–29550.
- Naseem KM (2005) The role of nitric oxide in cardiovascular diseases. *Mol Aspects Med* 26: 33–65.
- Takahashi S, Lin H, Geshi N, Mori Y, Kawarabayashi Y, et al. (2008) Nitric oxide-cGMP-protein kinase G pathway negatively regulates vascular transient receptor potential channel TRPC6. *J Physiol* 586: 4209–4223.
- Bian K, Doursout MF, Murad F (2008) Vascular system: role of nitric oxide in cardiovascular diseases. *J Clin Hypertens (Greenwich)* 10: 304–310.
- Lincoln TM, Dey N, Sellak H (2001) Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J Appl Physiol* 91: 1421–1430.
- Feil R, Lohmann SM, de Jonge H, Walter U, Hofmann F (2003) Cyclic GMP-dependent protein kinases and the cardiovascular system: insights from genetically modified mice. *Circ Res* 93: 907–916.
- Koller A, Schlossmann J, Ashman K, Uttenweiler-Joseph S, Ruth P, et al. (2003) Association of phospholamban with a cGMP kinase signaling complex. *Biochem Biophys Res Commun* 300: 155–160.
- Taccetta-Chapnick M (2002) Using carvedilol to treat heart failure. *Crit Care Nurse* 22: 36–40, 42–36, 48 passim.
- (1996) Effect of carvedilol on mortality and morbidity in patients with chronic heart failure. *Circulation* 94: 592.
- Ohlstein EH, Douglas SA, Sung CP, Yue TL, Loudon C, et al. (1993) Carvedilol, a cardiovascular drug, prevents vascular smooth muscle cell proliferation, migration, and neointimal formation following vascular injury. *Proc Natl Acad Sci U S A* 90: 6189–6193.
- Sui J, Zhang J, Ching CB, Chen WN (2008) Comparative proteomic analysis of extracellular proteins reveals secretion of T-kininogen from vascular smooth muscle cells in response to incubation with S-enantiomer of propranolol. *Mol Pharm* 5: 885–890.
- Sui J, Wang M, Zhang J, Ching CB, Chen WN (2010) Identification of differentially secreted proteins using two-dimensional liquid chromatography/tandem mass spectrometry in vascular smooth muscle cells incubated with S- and R-atenolol. *Rapid Commun Mass Spectrom* 24: 1717–1719.
- Oddis CV, Finkel MS (1995) Cytokine-stimulated nitric oxide production inhibits mitochondrial activity in cardiac myocytes. *Biochem Biophys Res Commun* 213: 1002–1009.
- Mal M, Koh PK, Cheah PY, Chan EC (2009) Development and validation of a gas chromatography/mass spectrometry method for the metabolic profiling of human colon tissue. *Rapid Commun Mass Spectrom* 23: 487–494.

Author Contributions

Conceived and designed the experiments: MXW WNC CBC. Performed the experiments: MXW. Analyzed the data: MXW WNC CBC. Contributed reagents/materials/analysis tools: MXW JB. Wrote the paper: MXW WNC CBC.