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The alpha-1A adrenergic receptor agonist A61603 reduces cardiac polyunsaturated fatty acid and endocannabinoid metabolites associated with inflammation in vivo

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Abstract

Introduction—Alpha-1-adrenergic receptors (α1-ARs) are G-protein coupled receptors (GPCRs) with three highly homologous subtypes (α1A, α1B, and α1D). Of these three subtypes, only the α1A and α1B are expressed in the heart. Multiple pre-clinical models of heart injury demonstrate cardioprotective roles for the α1A. Non-selective α1-AR activation promotes glycolysis in the heart, but the functional α1-AR subtype and broader metabolic effects have not been studied.

Objectives—Given the high metabolic demands of the heart and previous evidence indicating benefit from α1A activation, we chose to investigate the effects of α1A activation on the cardiac metabolome in vivo.

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Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Methods—Mice were treated for one week with a low, subpressor dose of A61603, a highly selective and potent α1A agonist. Cardiac tissue and serum were analyzed using a non-targeted metabolomics approach.

Results—We identified previously unrecognized metabolic responses to α1A activation, most notably broad reduction in the abundance of polyunsaturated fatty acids (PUFAs) and endocannabinoids (ECs).

Conclusion—Given the well characterized roles of PUFAs and ECs in inflammatory pathways, these findings suggest a possible role for cardiac α1A-ARs in the regulation of inflammation and may offer novel insight into the mechanisms underlying the cardioprotective benefit of selective pharmacologic α1A activation.

Keywords

alpha-1A adrenergic receptor; agonist; endocannabinoid; arachidonic acid; fatty acid elongation; anti-inflammatory

Introduction

Adrenergic receptors (ARs) are G-protein coupled receptors (GPCRs) that are activated endogenously by catecholamines and play critical roles in regulating cardiac function. The β1-AR is the most abundant AR in the heart and chronic activation of β1-ARs by catecholamines contributes to the pathobiology of heart failure. α1-ARs are less abundant, but play adaptive and protective roles in the heart, including induction of physiological cardiac hypertrophy, enhanced cardiac contractility, and protection against multiple forms of cardiac injury.[1]

Pharmacologic approaches have been used for decades to elucidate downstream effects of non-selective cardiac α1-AR activation. The endogenous catecholamines norepinephrine and epinephrine activate α1-ARs as well as β-ARs, whereas the synthetic drug phenylephrine is a non-selective α1-AR agonist used widely in experimental settings. Studies using these agents have identified important roles for α1-ARs in regulation of glucose metabolism in skeletal muscle [2], liver [3], pancreas[4], and adipocytes[5]. Non-selective α1-AR activation in the heart increases glucose metabolism[6,7] and decreases fatty acid oxidation.[8] This metabolic substrate switch from fatty acid to glucose is a critical component of the heart's adaptation to stress[9,10]. Despite the central role of catecholamines in cardiac biology, very little is known about their global metabolic effects in the heart. One previous study investigated the effects of β-AR stimulation on the rat cardiac metabolome[11], but the broad metabolic effects of α1-AR stimulation have not been investigated.

There are three highly homologous subtypes of α1-ARs: α1A, α1B, and α1D, with variable tissue expression throughout the body. The α1A and α1B are expressed in the rodent and human heart, whereas the α1D is found in the coronary vasculature and mediates vasoconstriction[12,13]. Transgenic mouse models and pharmacologic approaches have begun to delineate distinct roles for the cardiac α1-AR subtypes, indicating that the α1A is cytoprotective in vitro and cardioprotective in vivo[14]. Transgenic mice overexpressing the

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α1A in cardiomyocytes demonstrate enhanced contractility and functional recovery after ischemic challenge and pressure overload.[15-17]

Recent studies have expanded these observations using the potent and highly selective α1A agonist, A61603 (N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydro naphthalen-1-yl] methanesulfonamide hydrobromide)[18] in multiple pre-clinical studies[19-21]. A61603 protects against cardiomyocyte death due to multiple cytotoxic exposures in vitro[22], prevents doxorubicin-induced cardiotoxicity in vivo[23], and restores right ventricular contractility in a mouse model of right heart failure[19]. Importantly, these beneficial effects were achieved using a dose of A61603 that did not increase blood pressure, suggesting that vascular α1-ARs were not activated, and reinforcing the possibility that selective α1A-AR activation might represent a novel approach to treating heart muscle disease[24]. It is unknown whether metabolic alterations contribute to these beneficial effects.

In the present study, mice were treated with A61603 then heart tissue and serum were analyzed using a non-targeted metabolomics approach to discover whether broad metabolic adaptations may underlie the cardioprotective effects of activating the α1A-AR in vivo.

Materials and Methods

Animals, experimental design, drug delivery, and harvest

Mice were 12-week old C57Bl6J males. All mice underwent implantation of subcutaneous osmotic minipump (Alzet) that delivered A61603 10ng/kg/d to 8 mice and vehicle (100μM vitamin C, 0.9% NaCl) to 8 mice for one week. Mice were fed the usual animal care diet per the San Francisco VA Animal Care facility. Mice were sacrificed under deep isoflurane anesthesia, blood was collected by cardiac puncture, clotted, and 100-200μL serum was snap frozen in liquid nitrogen. The heart was dissected, the atria and great vessels were excised, and the ventricles were allowed to beat in cold PBS to clear blood. Excess liquid was blotted then the heart was snap-frozen in liquid nitrogen and shipped to Metabolon (Durham, NC).

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)

All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. Samples were processed as previously described[25,26], sample extract reconstituted in solvents containing a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. The extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 μm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, however it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall

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higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5 mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH amide 2.1x150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10mM ammonium formate, pH 10.8. The MS analysis alternated between MS and data-dependent MS^n scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z.

Raw data were extracted, peak-identified by comparison to library entries of purified standards or recurrent unknown entities based on authenticated standards that contain the retention time/index (RI), mass to charge ratio (*m/z*), and chromatographic data (including MS/MS spectral data). Biochemical identifications are based on retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, misassignments, and background noise. Peaks were quantified using area-under-the-curve. For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. All data used in this analysis has been archived in the UCSD Metabolomics Workbench (http://www.metabolomicsworkbench.org/), accession # (Pending Assignment/Uploaded 17 April 2016).

Metabolomic Statistical Analyses

Metaboanalyst (v3.0) run on the statistical package R (v2.14.0) used metabolite peak areas (as representative of concentration)[27,28]. These data were first normalized to a pooled average sample from the relevant vehicle-treated group, and scaled using Pareto scaling feature. To detect systemic metabolic signature of A61603 treatment, a Pairwise One-Way Analysis of Variance (ANOVA) and Fisher LSD post-hoc test across the tissues (heart, serum) and experimental groups (vehicle treated, A61603-treated) were performed using Metaboanalyst v3.0. ANOVA significant metabolites (FDR<0.05) were matched to metabolomics pathways using the Pathway Analysis and enrichment analysis features in Metaboanalyst 3.0. Only metabolites identified and detected in all groups were included in the one-way ANOVA. Up to three missing values in each group were imputed. If four or more values of each metabolite were missing in a given group, the entire metabolite was removed from the analysis. Only metabolites significantly altered between tissue-matched control and A61603 treatment groups were included. All data from this study are available in Supplemental Table 1. Heat maps were generated using the GENE- E software (http://www.broadinstitute.org/cancer/software/GENE-E/index.html) and Microsoft Excel 2016. All data are shown as mean ± SEM, unless otherwise indicated.

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Results

Heart Tissue

We compared heart tissue from mice treated for 7 days with the selective α1A-AR agonist, A61603, or vehicle. We used a very low A61603 dose, 10 ng/kg/d, that has no detectable effect on blood pressure, heart rate, heart size, or heart function, but does increase phosphorylation of cardiac ERK, a canonical α1A signaling partner. (data not shown) All comparisons used a one-way ANOVA to identify significantly altered metabolites. Of the 503 total metabolites identified in heart, 381 were adequate for the one-way ANOVA (Supplemental Figures 1and 2), and 30 were ANOVA-significant with a false discovery rate (FDR) < 0.05 (Figure 1A). Metaboanalyst recognized 23 of these 30 metabolites and applied pathway analysis to identify biosynthesis of unsaturated fatty acids as the most affected pathway (Figure 1B, $p=1.29E^{-11}$, $FDR=1.06E^{-9}$). Metaboanalyst enrichment analysis for pathways identified alpha linoleic acid and linoleic acid metabolism, ammonia recycling, and urea cycle with the lowest p values, enriched 3 to10 fold (Figure 1C). Enrichment analysis for location identified the peroxisome with the lowest p values, enriched 1.5-2.0 fold expected (data not shown).

Of the 7 metabolites not recognized by Metaboanalyst for pathway analysis using multiple synonymous chemical names (i.e. *N-Palmitoyl taurine*,* 1-(1-enyl-stearoyl)-GPE, *Palmitoylethanolamide*, N-Stearoyltaurine*, Oleoyl Ethanolamide*,* 1-palmitoyl GPG, 10-Nonadecenoic acid, 15-Methylpalmitate), 4 are involved in the endocannabinoid (EC) Pathway. The EC system consists of cannabinoid receptors and their endogenous neuromodulatory lipid ligands that collectively play long-recognized roles in the neurological and immunological systems[29] and recently have been identified in the heart[30]. We found a roughly 50% decrease in the abundance of the biologically active EC metabolites oleoyl ethanolamide, palmitoyl ethanolamide, N-stearoyltaurine, and N-palmitoyltaurine after treatment with A61603 (Figure 2).

Review of specific metabolites within these pathways revealed a 25% decrease in cardiac arachidonic acid, an omega (ω)-6 polyunsaturated fatty acid (PUFA) (Figure 2 Figure 3B). Similarly, we found decreases of ~25-45% in eight metabolites involved in the synthesis of ω3 (Figure 3A) and ω6 fatty acids (Figure 3B). Biosynthesis of PUFAs, including arachidonic acid, occurs through multiple desaturation and elongation steps (Figures 3 and B), each controlled by distinct enzymes.[31] Our findings suggest that activation of the α1A broadly reduces ω3 and ω6 fatty acid synthesis, possibly through regulation of one or more of these desaturates and elongases.

There was no clear signal that α1A activation affected glucose metabolism, though pyruvate levels were consistently and significantly decreased in hearts of A61603-treated mice (Figure 1A). Collectively, these findings suggest that A61603-treatment significantly affects the biosynthesis of biologically active PUFAs and EC receptor ligands.

Serum

Of the 543 total metabolites identified in serum, 381 were adequate for one-way ANOVA (Supplemental Figures 4 and 5), and 34 of them were ANOVA significant with an FDR <

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0.05 (Figure 4A). Pathway analysis of these 34 metabolites identified pyrimidine metabolism as the most likely affected pathway (Figures 4B and 4C, p=0.026, FDR =1). The pyrimidine salvage pathway (Figure 5) recovers core pyrimidine components which are recycled for use as nucleic acids or for recovery of nitrogen to the general nitrogen metabolism[32]. Further analysis of metabolites in the pyrimidine salvage pathway revealed that A61603 treatment is associated with significant decreases in serum levels of deoxycytidine, deoxyuridine, and ureidopropionate (Figure 5). All three of these metabolites are intermediates in β-alanine synthesis, but A61603 treatment did not affect abundance of β-alanine in either serum or heart.

Of note, treatment with A61603 had no effect on serum glucose, cholesterol, or creatinine levels—three commonly used markers of cardiovascular risk in the clinical setting. Collectively, these findings suggest relatively minimal systemic effects from the administration of an α1A agonist.

Heart and Serum

We next determined if there were A61603-altered metabolites in the serum that might reflect A61603-altered metabolites in the heart and might serve as a circulating marker of cardiac response to A61603. Since there were no metabolites found in our initial stringent cut-off of an FDR<0.05, we decreased the stringency to FDR<0.10 and identified A61603-induced metabolites compared to the vehicle controls (Figure 6A). Only 5-hydroxy-lysine overlapped in both compartments (Figure 6A, top row).

Since both A61603-induced heart and serum 5-hydroxy-lysine levels were significantly different by FDR (defined as <0.10), we next compared these groups using one-way ANOVA (Figure 6B). There was a statistically significant change in A61603-treated heart compared to its vehicle control (p<0.02)(Figure 6B), and the A61603-treated serum had a p value trending to significance (p=0.128)(Figure 6B). 5-Hydroxylysine is a hydroxylated derivative of the amino acid lysine present in certain collagens and belongs to a class of organic compounds known as hydroxyl-fatty acids[33]. While the changes in the serum reflected those in the heart, a more sensitive marker or set of serologic markers would be needed to follow A61603 activity in the heart. As such, the metabolite subset recognized in the current study did not identify a reliable serum metabolite marker for the cardiac effects of A61603.

Discussion

In this study, we used non-targeted metabolomics analysis to identify previously unrecognized links between pharmacological α1A-AR activation and biosynthesis of PUFAs and ECs in the heart. These findings expand our limited understanding of adrenergic regulation of the cardiac metabolome and generate novel hypotheses regarding the activity of cardiac α1A-ARs. In particular, PUFAs and ECs both play central roles in inflammation, suggesting that regulation of inflammation may contribute to the demonstrated cardioprotective effects of the selective α1A agonist, A61603.

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We discovered that systemic treatment with A61603 broadly reduced the cardiac content of ω3, ω6, and ω9 PUFAs without affecting serum PUFA levels. PUFAs play critical and complex roles in the heart, acting act as both biosynthetic intermediates and signaling molecules[34]. Observational studies have linked higher serum ω3-PUFA levels and dietary PUFA intake with lower mortality due to cardiovascular disease[35]. However, the biological relevance of PUFA abundance in cardiac tissue is uncertain and rodent models have demonstrated that dietary PUFA content is not correlated with heart content[36]. Previous reports indicate that treatment with PUFAs leads to a decrease in α1-AR mediated contractility in cultured cardiomyocytes[37,38], but the role of cardiac α1-ARs in regulating PUFA biosynthesis has not been explored.

We found decreases in ostensibly beneficial ω3 PUFAs such as docosapentanoic acid (DPA) and docosahexanoic acid (DHA)[39], as well as more cardiotoxic PUFAs such as the ω9 PUFA, erucic acid[40,41]. Arachidonic acid (AA), among the most extensively studied of PUFAs, was decreased to 0.74-fold relative abundance in the hearts of mice treated with A61603. Previous studies have reached variable conclusions about the function of AA in the heart, in part because AA metabolites play conflicting roles—some beneficial[42] and some maladaptive[43]. AA is released from membrane phospholipids by phospholipases. α1-AR stimulation activates phospholipase C[44] and phospholipase D[45,46], which cleave phospholipids to diacyl glycerol (DAG) and phosphatidic acid respectively. These lipid products are then enzymatically converted to AA. In our study, A61603 treatment increased total DAG content by 24% ($p<0.05$). The observed decrease in AA content could potentially result from regulation of DAG lipase—the enzyme that converts DAG to AA--or through an increase in conversion of AA to downstream metabolites.

PUFAs have not been studied widely in injured heart tissue. PUFA abundance in the heart is not affected by activation of the angiotensin I receptor[47], and treatment with the β-AR agonist isoproterenol increases AA 3.4-fold and the ω6 PUFA linoelaidic acid 2.5-fold[11]. Chronic activation of both the angiotensin I receptor and the β-AR lead to maladaptive cardiac hypertrophy and heart failure. Consistent with these findings, AA and DHA increase in failing human heart tissue as well as hypertrophied and failing rat hearts[48]. In contrast, sustained α1A activation leads to a decrease in PUFA levels and cardioprotection, though the mechanistic connection between these findings is uncertain. Collectively, these studies illustrate the complexity of ascribing inherent benefit or harm to tissue PUFA levels.

Our results also indicate that pharmacological activation of the α1A-AR leads to decreased levels of ECs in the heart. ECs are metabolites in the AA biosynthetic pathway, formed by activation of fatty acid amid hydrolase[49]. ECs participate in the regulation of cellular metabolism and inflammation through activation of CB1, CB2, and TRPV1 channels. The effects of ECs in the heart are either beneficial[50] or maladaptive[51,52], depending on context and selective receptor activation[53].

Cardioprotective mechanisms induced by EC modulation include enhanced Na^+/Ca^{2+} exchange current[54], activation of HSP72, PKA, PLC, PKC, eNOS, iNOS, and ERK1/2[55-57], and regulation of myosin heavy chain isoform switching[50]. Interestingly, α1-AR activation regulates each of these processes in the heart as well, suggesting that the EC system may

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participate broadly in the mechanisms underlying α1-mediated cardioprotection. Of particular significance to our current study, ECs have been shown to decrease responsiveness to AR agonist stimulation in the vasculature[58,59], to mediate α1-induced glutamate neurotransmission in the central nervous system[60], and to regulate peripheral norepinephrine release[61]. However, our study is the first to show an effect of α1-AR activation on EC biosynthesis.

Given the complex functions of both PUFAs and the EC system in the heart, it is unclear how our findings might help to explain the cardioprotective effects of A61603. However, one reasonable hypothesis is that α1A activation regulates inflammation, insofar as many of the metabolites that were reduced in A61603-treated hearts are involved in inflammatory pathways. ECs are well-recognized mediators of systemic inflammatory diseases such as rheumatoid arthritis[61], and also regulate inflammation in the heart[62]. PUFAs regulate T lymphocyte function[63] and modify inflammatory diseases[64] and AA and its metabolites provide critical positive and negative regulation of systemic and cardiac inflammation[65]. Inflammation is central to the biology of acute myocardial injury[69] and to the pathophysiology of chronic heart failure[70] and α1A activation is protective in both settings. The role of inflammation in the heart is complex, however, and inflammatory mediators released from both circulating cells or any of the resident cell cardiac types play both adaptive and maladaptive roles.

The role of α1-ARs in regulating cardiac inflammation has not been explored to any significant extent. Transgenic mice overexpressing a constitutively active α1A have increased IL-6 expression in the heart, but no evidence of increased cardiac inflammation[66]. These mice are protected against ischemia-reperfusion injury, a condition that typically is worsened by inflammation. One other study found that non-selective α1-AR activation suppresses lipopolysaccharide-induced cardiomyocyte TNF-α expression and improves cardiac dysfunction during endotoxemia through ERK activation and NF-κB suppression[67]. α1-ARs are found on monocytes and macrophages, and their activation appears to alter production of inflammatory mediators[68]. Our analysis of the serum of mice treated with A61603 revealed no clear evidence of systemic inflammatory modulation, though reduction in pyrimidine salvage intermediates (Figure 5), would favor a reduction in inflammatory cell function.[69,70] The role of the α1A-AR in regulating cardiac inflammation will be the subject of future work in our lab.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard abbreviations

A61603	alpha-1 adrenergic receptor agonist: N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydro naphthalen-1-yl] methanesulfonamide hydrobromide
α1-AR	alpha1-adrenergic receptor
AA	arachidonic acid
CB1/2	cannabinoid receptor type 1/2

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DAG	diacylglycerol
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
ECs	endocannabinoids
GPCR	G-protein coupled receptor
PUFA	poly-unsaturated fatty acid

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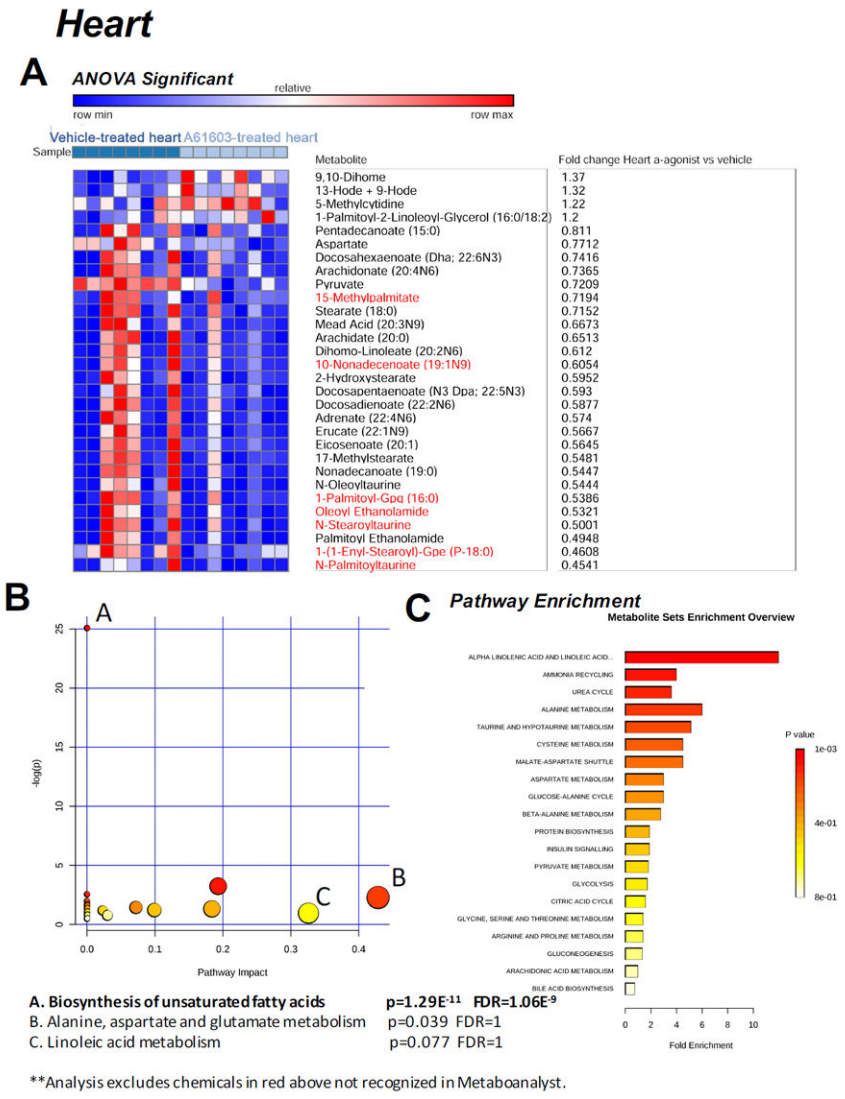

Figure 1. Analysis of non-targeted metabolomics of hearts from vehicle-treated and A61603-treated mice

A. Summary heat map of pairwise One-way ANOVA significant metabolites, determined by Fisher-LSD post hoc test results. Fisher LSD post hoc comparisons were made between A61603-treated and vehicle-treated heart groups (N=8/group). **B**. Pathway enrichment analysis for A61603–treated heart group compared to vehicle-treated heart group, determined by ANOVA significant metabolites in the heart. *a-c* indicates top pathways identified **C**. Enrichment analysis of cardiac A61603-mediated alterations compared to pathway metabolite sets

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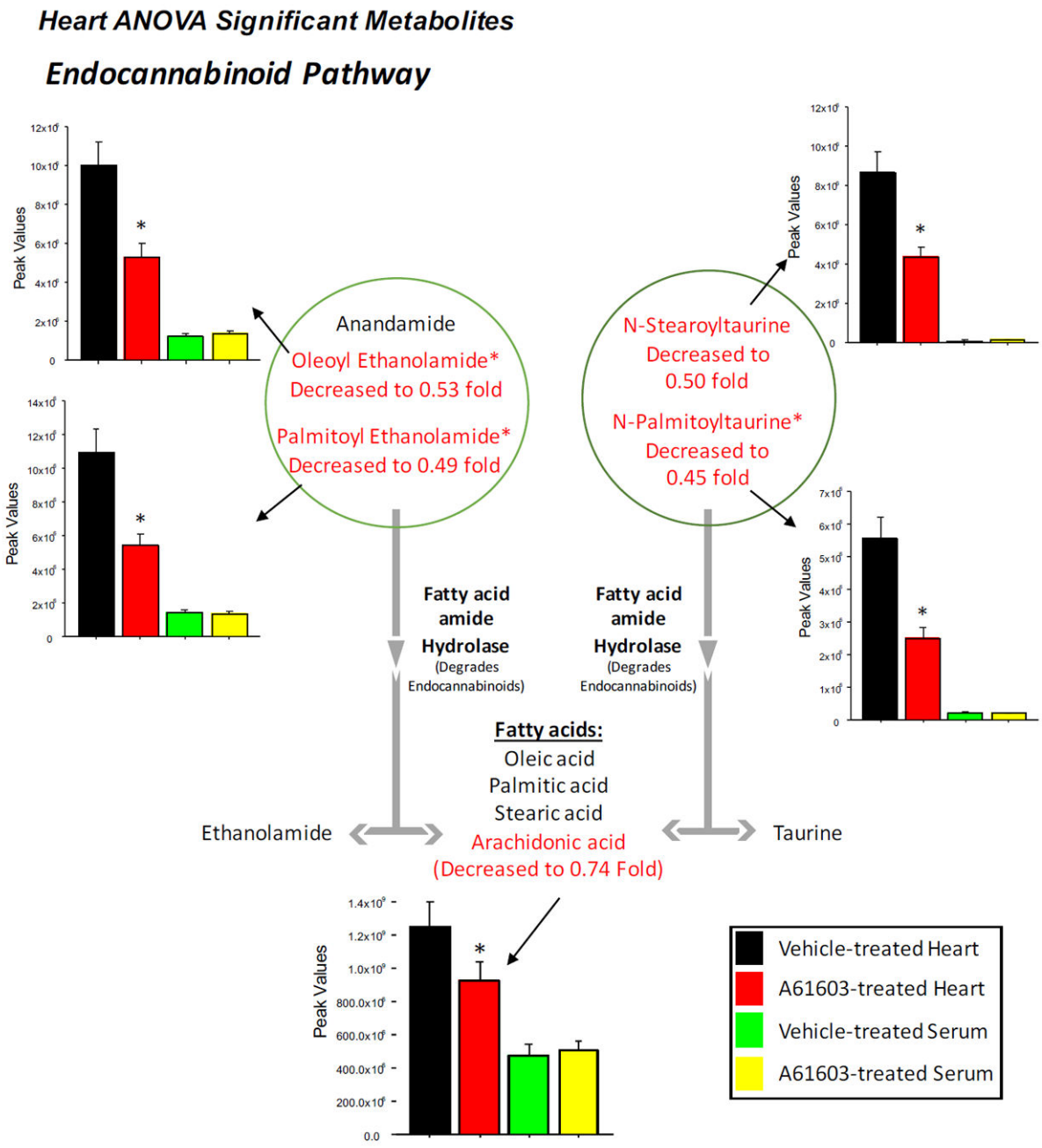

Figure 2. Functional annotation of A61603-mediated alterations in cardiac metabolites in vivo

Overview of the endocannabinoid pathway and the intermediates altered in vivo by A61603 treatment, determined by One-way ANOVA test results in the heart ($p<0.05$). Data represent mean ± SEM. (N=8/group). *$p<0.05$.

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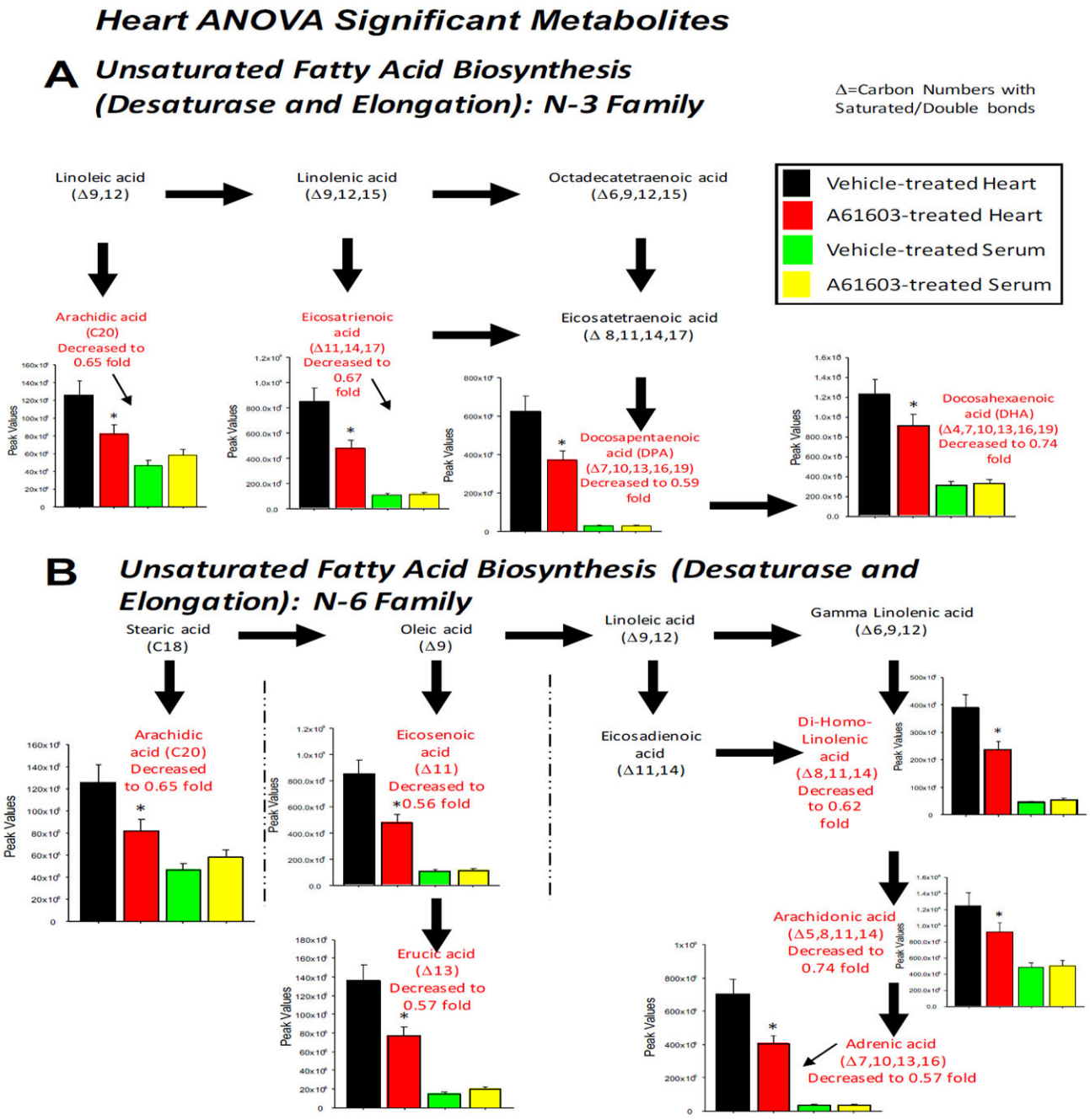

Figure 3. Functional annotation of N-3 and N-6 unsaturated fatty acid biosynthesis including intermediates altered in heart tissue by in vivo A61603

α1A-mediated alterations in **A.** N-3 poly-unsaturated fatty acids (PUFAs) and **B.** N-6 poly-unsaturated fatty acids (PUFAs) in the heart, determined by One-way ANOVA ($p<0.05$). Δ (delta) references the specific double bonds present in fatty acids. Data represent mean ± SEM. (N=8/group). *$p<0.05$.

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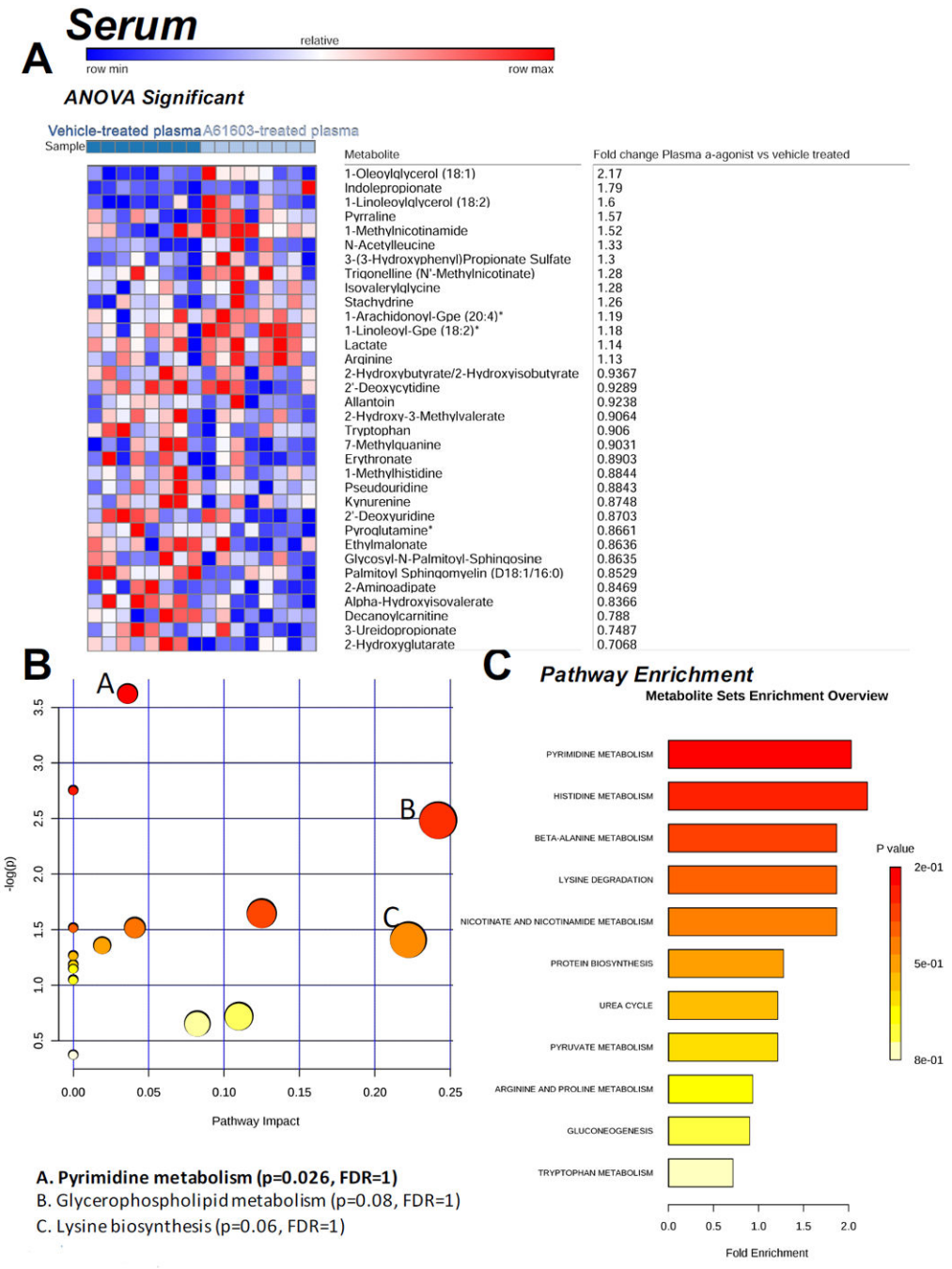

Figure 4. Analysis of non-targeted metabolomics of serum from vehicle-treated and A61603-treated mice

A. Summary heat map of pairwise One-way ANOVA significant metabolites, determined by Fisher-LSD post hoc test results (significance defined as FDR<0.05). Fisher LSD post hoc comparisons were made between A61603-treated and vehicle-treated serum groups (N=8/group). Only significantly altered metabolites are shown. Significance was defined as FDR <0.05. **B**. Pathway enrichment analysis for A61603-treated serum group compared to

vehicle-treated serum group. *a-c* indicates top pathways identified. **C**. Enrichment analysis of A61603-mediated alterations in serum compared to pathway metabolite sets.

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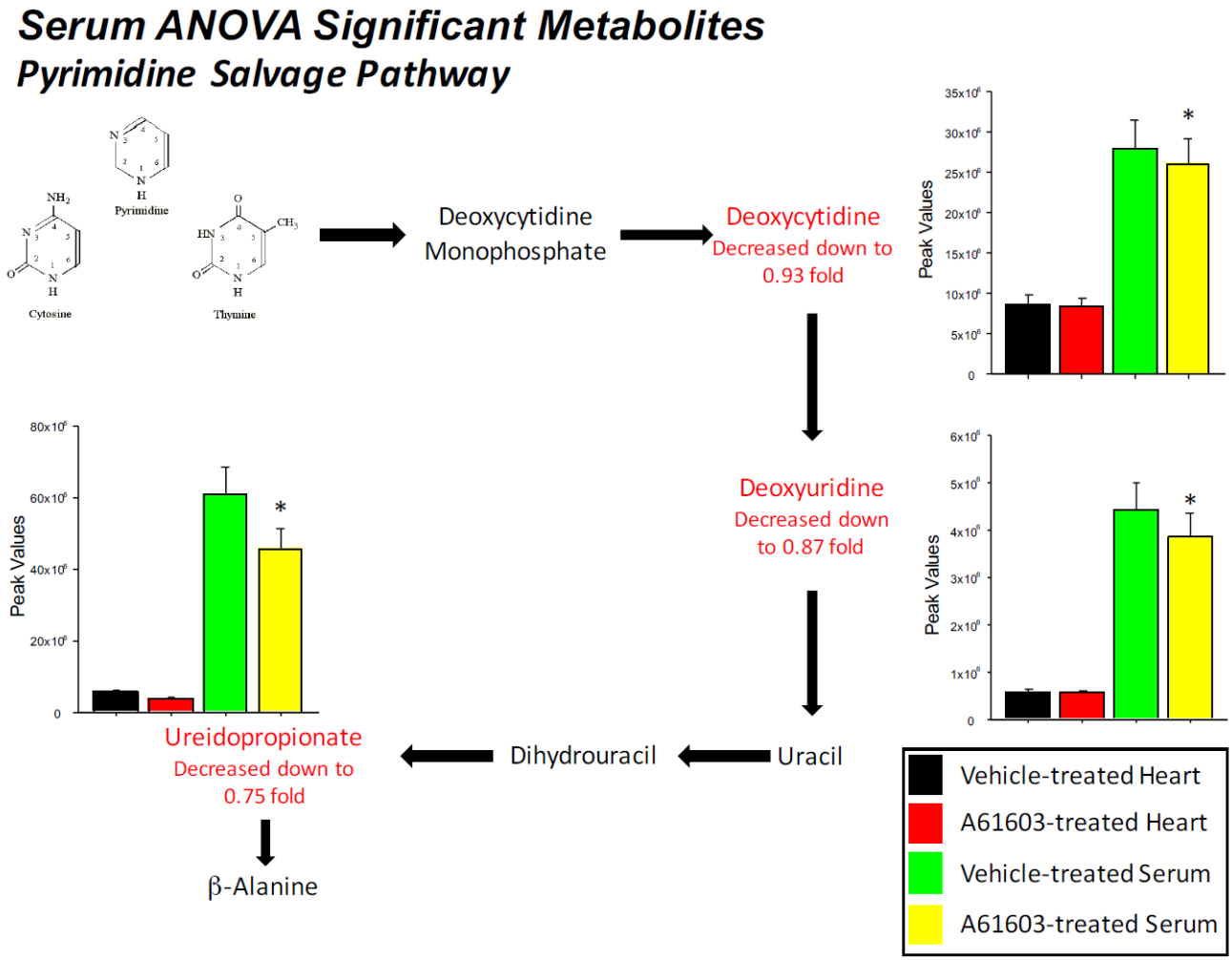

Figure 5. Functional annotation of A61603-mediated alterations in serum intermediates from the pyrimidine salvage pathway

Overview of the pyrimidine salvage pathway and the serum intermediates altered in vivo by A61603 treatment, determined by One-way ANOVA. Data are shown as mean ± SEM. (N=8/group)

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A

Significantly Altered Heart Metabolites with A61603-treatment (vs. Vehicle) FDR<0.10	Significantly Altered Serum Metabolites with A61603-treatment (vs. Vehicle) FDR<0.10
5-Hydroxylysine	***5-Hydroxylysine***
Sphingosine	Orotate
Choline phosphate	5-Methyl-2'-deoxycytidine
C-glycosyltryptophan	Homoserine
1-Stearoyl-2-arachidonoyl-GPE (18:0/20:4)	N-Acetyl-aspartyl-glutamate (NAAG)
Tiglylcarnitine	Alpha-hydroxyisocaproate
Propionylcarnitine	Thymine
Sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0)	Sphingomyelin (d18:1/18:1, d18:2/18:0)
S-Methylcysteine	1-Methylimidazoleacetate
N-Acetyltaurine	Palmitoylcarnitine
3-Methylhistidine	Homostachydrine*
N1-Methyladenosine	N-Acetylglycine
Pyrraline	Leucine
Dihomo-linolenate (20:3n3 or n6)	Beta-sitosterol
1-Oleoyl-2-linoleoyl-glycerol (18:1/18:2)	1-Palmitoleoylglycerol (16:1)*
Docosatrienoate (22:3n3)	Guanidinosuccinate
Margarate (17:0)	Hexanoylglycine
Palmitoylcholine	3-Hydroxybutyrate (BHBA)
Palmitate (16:0)	N-Formylmethionine
1-Arachidonylglycerol (20:4)	Gamma-glutamyl-epsilon-lysine
1-Dihomo-linoleoylglycerol (20:2)	10-Nonadecenoate (19:1n9)
Ribitol	Glycerate
Docosapentaenoate (n6 DPA; 22:5n6)	Urate
N-Acetylaspartate (NAA)	
1-Docosahexaenoylglycerol (22:6)	

B

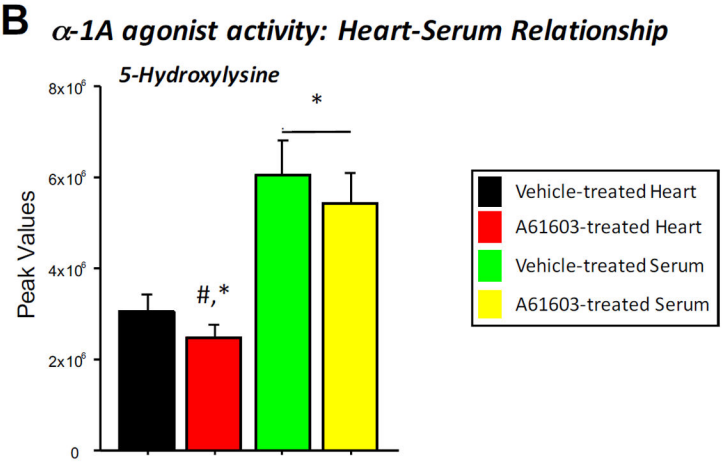

Figure 6. A61603 responsive metabolite found in heart and plasma

Metabolites significantly altered by A61603 in **A**. Heart and serum. A pairwise One-way ANOVA was performed to identify significant metabolites using a Fisher-LSD post-hoc test results against either vehicle-treated heart (left column) or vehicle-treated serum (right column), which included those identified in Figure 1A (heart) or Figure 4A (serum), using a slightly lower stringency (significance defined as FDR<0.10) to identify metabolites altered in both. Data represent mean ± SEM. (N=8/group). *FDR<0.10, #p<0.02.