Journal of Molecular and Cellular Cardiology 55 (2013) 27–30

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Rapid communication

Effects of perhexiline-induced fuel switch on the cardiac proteome and metabolome

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ARTICLE INFO

Article history:
Received 17 October 2012
Received in revised form 1 December 2012
Accepted 14 December 2012
Available online 29 December 2012

Keywords:
Metabolomics
Proteomics
Cardioprotection
Metabolism
Heart failure

ABSTRACT

Perhexiline is a potent anti-anginal drug used for treatment of refractory angina and other forms of heart disease. It provides an oxygen sparing effect in the myocardium by creating a switch from fatty acid to glucose metabolism through partial inhibition of carnitine palmitoyltransferase 1 and 2. However, the precise molecular mechanisms underlying the cardioprotective effects elicited by perhexiline are not fully understood. The present study employed a combined proteomics, metabolomics and computational approach to characterise changes in murine hearts upon treatment with perhexiline. According to results based on difference in-gel electrophoresis, the most profound change in the cardiac proteome related to the activation of the pyruvate dehydrogenase complex. Metabolomic analysis by high-resolution nuclear magnetic resonance spectroscopy showed lower levels of total creatine and taurine in hearts of perhexiline-treated mice. Creatine and taurine levels were also significantly correlated in a cross-correlation analysis of all metabolites. Computational modelling suggested that far from inducing a simple shift from fatty acid to glucose oxidation, perhexiline may cause complex rebalancing of carbon and nucleotide phosphate fluxes, fuelled by increased lactate and amino acid uptake, to increase metabolic flexibility and to maintain cardiac output. This article is part of a Special Issue entitled "Focus on Cardiac Metabolism".

1. Introduction

Perhexiline (2-(2,2-dicyclohexylethyl)piperidine) is an anti-anginal drug first used in France in the 1970's to combat manifestations of ischaemic heart disease. While withdrawn from the market in the 1980's due to the emergence of unwanted side effects, use of perhexiline is once again being revived in Australia and New Zealand and to some extent in Europe [1,2]. Perhexiline's main mode of action is ascribed to its inhibition of long-chain fatty acid oxidation by inhibition of carnitine palmitoyltransferase (CPT) 1 and 2 [3]. CPT1 and 2 are responsible for import of long-chain fatty acids into the mitochondria for β-oxidation. The predominant source of energy in the heart is derived from fatty acid metabolism. In the ischaemic heart there is a switch back to glucose metabolism reminiscent of foetal programming. However, fatty acid oxidation still predominates and leads to inefficient use of oxygen per production of ATP molecule and to uncoupling of mitochondrial respiration through expression of uncoupling proteins [1,2]. Inhibition of β-oxidation produces an oxygen sparing effect that has been accompanied by improved cardiac function [4]. Perhexiline also exhibits vasodilatory effects on coronary arteries independently of its CPT inhibition activity [5], but this effect is probably clinically not relevant. Although induction of cardioprotection through metabolic modulation is a promising therapeutic concept, the precise effects at a molecular level have yet to be elucidated. The present study investigates changes in the cardiac proteome and metabolome of perhexiline-treated mice to assess the drug effects without a priori assumptions and provide insights into potential mechanisms on how this metabolic modulator may mediate cardioprotection and myocardial salvage.

2. Materials and method

Adult male C57BL/6 mice were fed perhexiline prior to proteomic (n = 4 per group) and metabolomic (n = 5 per group) analysis of their hearts as previously described [6]. Detailed methodology is provided in the online data supplement. Protocols for proteomics are available on our website at http://www.vascular-proteomics.com.

Abbreviations: CPT, carnitine palmitoyltransferase; DIGE, difference in-gel electrophoresis; FCS, foetal calf serum; FDR, false discovery rate; GO, Gene ontology; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; LC-MS/MS, liquid chromatography tandem mass spectrometry; TCA, tricarboxylic acid.

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http://dx.doi.org/10.1016/j.yjmcc.2012.12.014

3. Results and discussion

3.1. Perhexiline treatment

Perhexiline was fed to C57BL/6 mice for 4 weeks to achieve steady state concentrations in plasma. The average plasma concentration of perhexiline was 0.51 ± 0.28 μg/ml, which was within the therapeutic range (0.15–0.60 μg/ml). The average concentration of its metabolite, hydroxyperhexiline, was 0.80 ± 0.24 μg/ml.

3.2. Proteomics

Proteomic analysis of perhexiline-treated hearts compared to normal hearts was performed by difference in-gel electrophoresis (DIGE) using a broad-range pH gradient (pH 3–10 non-linear, n = 4 per group) (Fig. 1A). Differentially expressed spots were excised, subjected to in-gel tryptic digestion, and identified using liquid chromatography tandem mass spectrometry (LC-MS/MS) (Supplemental Table 1). The identified proteins showed enrichment (FDR < 5%, Supplemental Table 2) for the GO Biological Process term 'gas transport' and GO terms related to the oxygen transport ('oxygen binding', 'oxygen transport', and 'oxygen transporter activity'), mainly due to an increase in haemoglobin, which may be a consequence of the vasodilatory effects of perhexiline. The larger proportion of enriched terms consisted of those relating to metabolic and catabolic processes, including the GO term 'cellular carbohydrate catabolic process' ($P = 2.48 \times 10^{-04}$).

3.3. Enzymatic changes

The most pronounced changes involved metabolic enzymes, especially those involved in glucose and lipid metabolism. A decrease in long-chain specific acyl-CoA dehydrogenase, an acyl-CoA acceptor enzyme involved in processing long chain fatty acids during β-oxidation, was accompanied by a compensatory increase in fatty acid binding protein (responsible for binding long chain fatty acids) and differential expression of the medium-chain specific acyl-CoA dehydrogenase. This enzyme is an acyl-CoA acceptor involved in β-oxidation but specific for shorter acyl chain lengths between 4–16 carbons. Differential expression was observed for adenylate kinase isoenzyme 4, which produces ATP and AMP from ADP, as well as 2-oxoisovalerate dehydrogenase subunit α. The latter is part of a complex involved in amino acid metabolism that catalyses the conversion of α-ketoacids to acyl-CoA. In terms of glucose metabolism, the most profound changes, involved the regulatory subunit of pyruvate dehydrogenase (PDH-E1α, Fig. 1B).

3.4. PDH complex

Four different pyruvate dehydrogenase kinases phosphorylate three sites on the E1 α regulatory subunit to inactivate the PDH complex. The different isoforms were visible as a charge train of spots with similar molecular weight but different isoelectric points (Fig. 1B). The observed shift from acidic to basic isoforms in response to perhexiline is consistent with decreased phosphorylation and was independently confirmed by immunoblotting (Fig. 1C).

3.5. Metabolomics

Next, cardiac metabolites were extracted and subjected to high-resolution ^{1}H NMR spectroscopy (Fig. 2A, n = 5 per group). The metabolic profiles clearly discriminated control and perhexiline-treated hearts (Fig. 2B). As expected, perhexiline treatment resulted in a decrease in acetate, the end product of lipid metabolism, although it fell short of statistical significance (p = 0.086, Supplemental Table 3). In a cross-correlation analysis, five pairs of metabolites were significantly

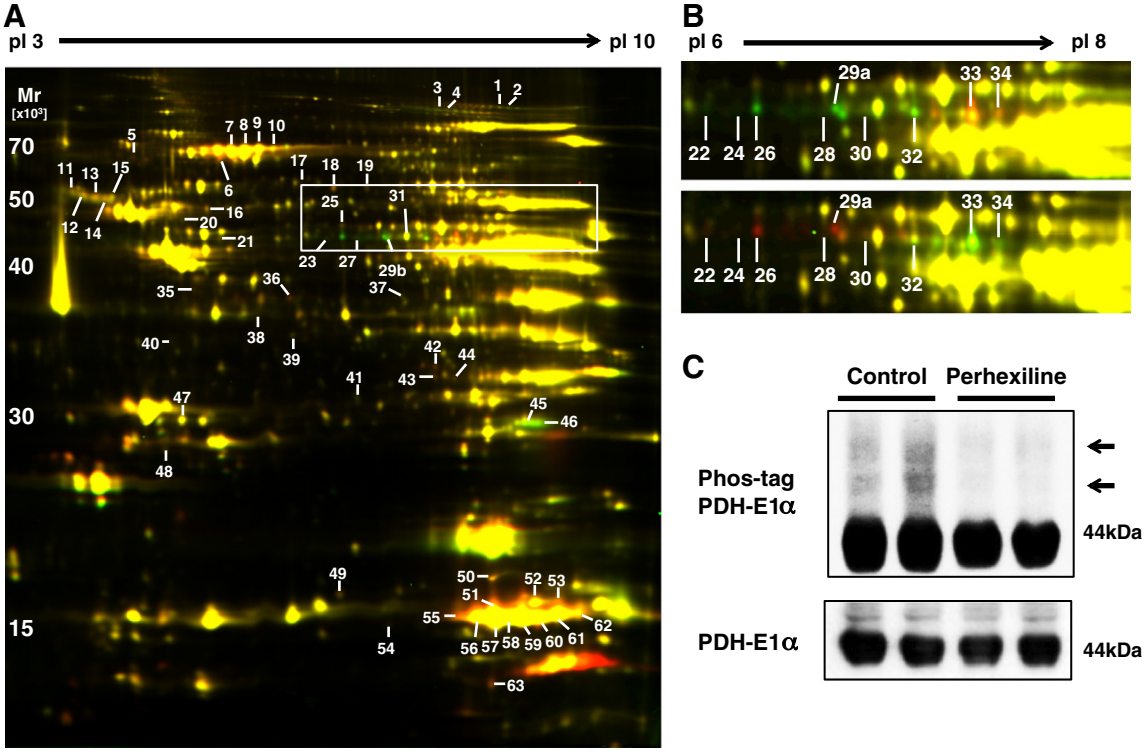

Fig. 1. Effect of perhexiline on cardiac protein expression. A) Cardiac protein extracts from control (green colour) and perhexiline-treated (red colour) mice were quantified using DIGE (n = 4 per group). Differentially expressed spots were numbered and identified by LC-MS/MS (Supplemental Table 1). B) Enlargement of the boxed area in panel A to highlight different isoforms of the α subunit of the pyruvate dehydrogenase E1. The observed decrease of the more acidic forms (green colour) with a corresponding increase of the more basic isoforms (red colour) is consistent with dephosphorylation of the regulatory subunit (upper panel). Results were reproduced with different biological replicates using a dye-swap (lower panel). C) Phosphate-affinity gel electrophoresis for mobility shift detection of phosphorylated proteins. Separation of phosphorylated and non-phosphorylated isoforms of the α subunit of the pyruvate dehydrogenase E1 (upper panel, phosphorylated bands marked with an arrow) without differences in protein abundance (lower panel).

correlated after multiple testing corrections (FDR<5%): Leucine was correlated with valine, β-hydroxybutyrate and alanine. Alanine and beta-hydroxybutyrate were also significantly correlated as were taurine and creatine (Supplemental Table 4). Both showed a significant reduction in perhexiline-treated hearts (Supplemental Table 3). The hierarchical clustering in Fig. 2C illustrates that while many of the metabolites are closely correlated, indicated by the short branches of the tree, taurine has the largest separation from the rest with lactate and creatine together showing the next largest separation ($p<0.01$). The clustering also revealed that glutamate, alanine and succinate cluster together, suggesting that these metabolites may play a role in the same metabolic pathway [7]. Fig. 2D highlights the loss of mutual information for features like taurine, a degradation product of cysteine, and fumarate, a TCA cycle metabolite that has been proposed to be cardioprotective [8], in perhexiline-treated hearts (Fig. 2D).

3.6. Computer simulations

Finally, we simulated the effects of perhexiline on myocardial metabolism using a well-validated proteome-scale network model (Supplemental Fig. 1, Supplemental Table 5): In our simulations, median lactate uptake increased more than threefold contributing to a fivefold increase in median PDH flux (Supplemental Fig. 2). This is in line with a previous report [9] that perhexiline alters metabolic substrate use from free fatty acids to lactate through altered PDH complex function. Similarly, our proteomic data showed unchanged or even reduced glycolytic enzymes but an increase in lactate dehydrogenase and PDH activation (Supplemental Table 1). There was also a 60% increase in pyruvate kinase flux (and thus increase in substrate-level phosphorylation [7]), driven by increased phosphoenolpyruvate synthesis by phosphoenolpyruvate carboxykinase (up 80%). However, these increases were insufficient to maintain acetyl-CoA delivery and flux through the TCA cycle to alpha-ketoglutarate (AKG) was below control rates. Increased amino acid uptake (e.g. median glutamate uptake increased threefold) supplemented the TCA cycle at several points, such that flux from AKG to citrate was increased well above control levels (Supplemental Fig. 2). Thus, perhexiline may cause complex rebalancing of carbon and nucleotide phosphate fluxes, fuelled by increased lactate and amino acid uptake, to increase metabolic flexibility and to restore energy homeostasis to maintain cardiac output [10,11].

3.7. Limitations

Further studies are required to assess the effects of perhexiline on PDH activity, in order to determine if PDH is a direct or indirect drug target. It has been shown that perhexiline accumulates within cardiac mitochondria due to its ability to form tight but reversible interactions with mitochondrial phospholipids. Within the mitochondrial matrix perhexiline molecules may donate their protons thus altering the

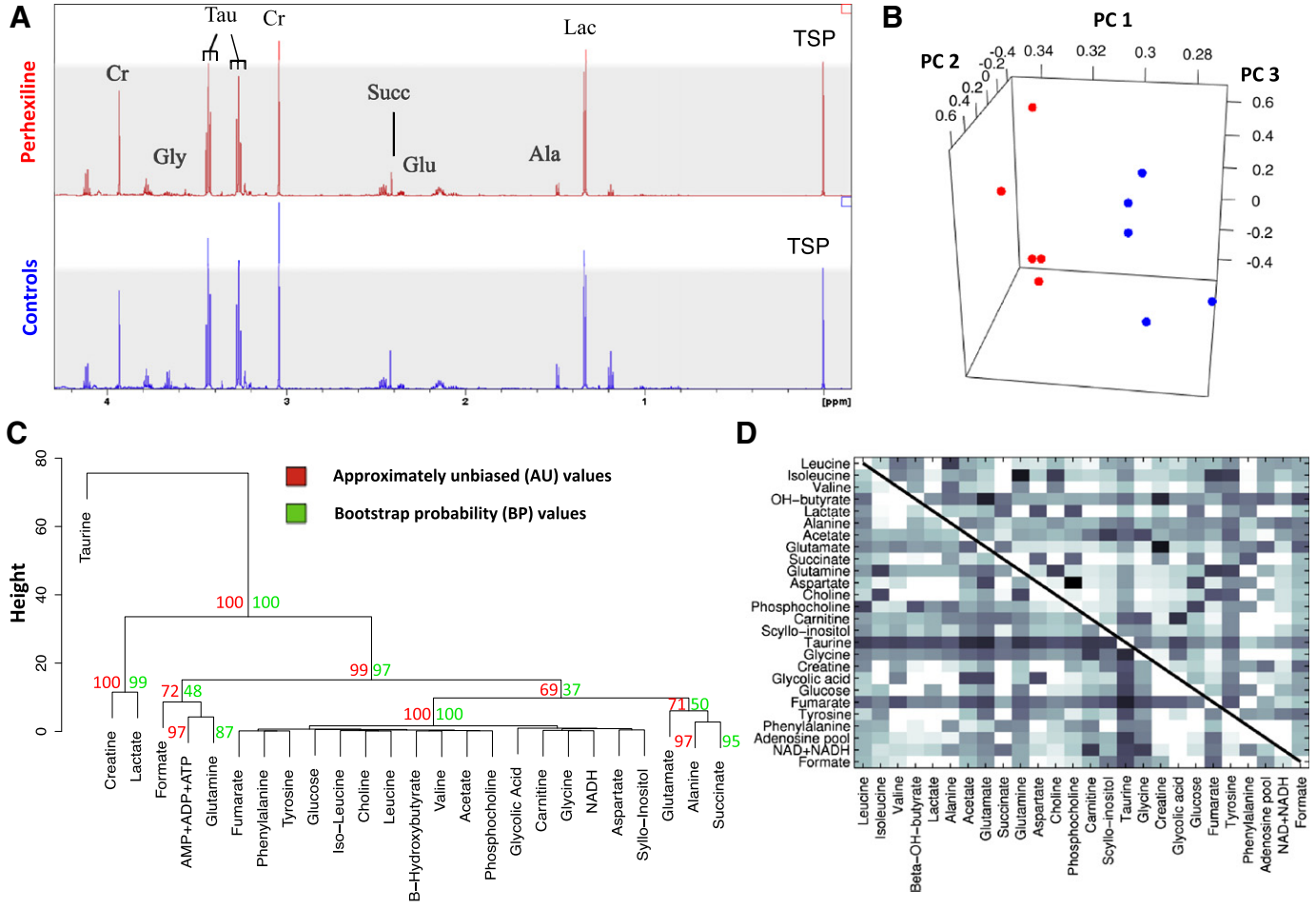

Fig. 2. Effect of perhexiline on cardiac metabolite expression. A) Cardiac metabolite expression in control and perhexiline-treated mice (n = 5 per group) was quantified using ^{1}H NMR spectroscopy. Sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP) was added to the samples for chemical shift calibration and quantification. Quantitative data are provided in Supplemental Table 3. B) Principal Component Analysis on the set of metabolites quantified by ^{1}H NMR spectroscopy allowed a clear discrimination of control (blue) and perhexiline-treated hearts (red). C) Clustering of cardiac metabolites in the dendrogram. D) Mutual information heatmaps between all metabolites, calculated using the context likelihood of relatedness algorithm. The lower and upper triangles of each heatmap correspond to metabolite similarities in the control and perhexiline-treated hearts, respectively. Thus, comparison of the lower and upper triangles visualizes model-based differences in relatedness of metabolite profiles in response to perhexiline treatment.

redox environment and changes in the redox environment could activate the PDH complex [12,13]. Similar changes may occur with other cardioprotective agents, such as etomoxir, trimetazidine and ranolazine, and constitute part of the Randle cycle [14]. Also, an analysis of the mitochondrial subproteome may have revealed additional molecular targets, i.e. CPTs that were not identified in our analysis.

4. Conclusion

This study is the first proteomic and metabolomic evaluation of cardiac effects induced by a metabolic modulator. Our data suggest that besides suppressing fatty acid metabolism, perhexiline is likely to have more wide-ranging and complex systemic effects than previously thought (due to stoichiometric constraints) and shows that computational modelling combined with proteomics and metabolomics [15] provides a unique view of systemic metabolism and drug action.

Disclosures

None declared.

Acknowledgment

The set-up of technology was supported by grants from the British Heart Foundation (BHF) and Oak Foundation. The research was supported by the National Institute of Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College Londonin partnership with King's College Hospital and by a Senior Fellowship of the BHF (M.M.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2012.12.014.

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