The effect of metformin therapy on cardiac function and survival in volume-overload model of heart failure in rats


To cite this version:
The effect of metformin therapy on cardiac function and survival in volume-overload model of heart failure in rats

Short title: Metformin therapy in volume-overload heart failure in rats

Authors: Jan Benes 1,2,3, Ludmila Kazdova 2, Zdenek Drahota 5, Josef Houstek 5, Dasa Medrikova 5, Jan Kopecky 5, Nikola Kvarova 5, Marek Vrbacky 5, David Sedmera 5,6, Hynek Strnad 7, Michal Kolar 7, Jiri Petrak 8,9, Oldrich Benada 10, Petra Skaroupkova 2, Ludek Cervenka 2,3,4, Vojtech Melenovsky 1,2,3

Affiliations:
1Department of Cardiology, Institute for Clinical and Experimental Medicine – IKEM, Prague, Czech Republic
2Center for Experimental Medicine, Institute for Clinical and Experimental Medicine – IKEM, Prague, Czech Republic
3Center for Cardiovascular Research, Prague, Czech Republic
4Department of Physiology, 2nd Faculty of Medicine, Charles University, Prague, Czech Republic
5Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic
6Institute of Anatomy, 1st Faculty of Medicine, Charles University, Prague, Czech Republic
7Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic
8Institute of Pathological Physiology, 1st Faculty of Medicine, Charles University, Prague, Czech Republic
9Institute of Hematology and Blood Transfusion, Prague, Czech Republic
10Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Prague, Czech Republic

Corresponding author:
Jan Benes, MD
Department of Cardiology, Institute for Clinical and Experimental Medicine - IKEM
Videnska 1958/9, Prague 4, 140 21, Czech Republic
jan.benes@ikem.cz, phone: 420-236-055-006, fax: 420-261-362-985
Abstract
Aims: Advanced heart failure (HF) is associated with altered substrate metabolism. Whether modification of substrate use improves the course of HF remains unknown. Antihyperglycemic drug metformin (MET) affects substrate metabolism and its use might be associated with improved outcome in diabetic HF. The aim of the study was to examine whether MET would improve cardiac function and survival also in non-diabetic HF.

Methods: Volume overload HF was induced in male Wistar rats by creating aorto-caval fistula (ACF). Animals were randomized to placebo/MET (300mg/kg/day, 0.5% in food) groups and underwent assessment of metabolism, cardiovascular and mitochondrial functions (n=6-12/group) in advanced HF stage (21st week). A separate cohort served for survival analysis (n=10-90/group).

Results: The ACF group had marked cardiac hypertrophy, increased LVEDP and lung weight confirming decompensated HF, increased circulating free fatty acids (FFA), intraabdominal fat depletion, lower glycogen synthesis in the skeletal muscle (diaphragm), lower myocardial triglyceride content and attenuated myocardial $^{14}$C-glucose and $^{14}$C-palmitate oxidation, but preserved mitochondrial respiratory function, glucose tolerance and insulin sensitivity. MET therapy normalized serum FFA, decreased myocardial glucose oxidation, increased myocardial palmitate oxidation, but it had no effect on myocardial gene expression, AMPK signalling, ATP level, mitochondrial respiration, cardiac morphology, function and long-term survival despite reaching therapeutic serum level (2.2 ± 0.7 μg/ml).

Conclusion: MET-induced enhancement of myocardial fatty acid oxidation had neutral effect on cardiac function and survival. Recently reported cardioprotective effects of MET may not be universal to all forms of HF and may require AMPK activation or ATP depletion. No increase in mortality on MET supports its safe use in diabetic HF.

Keywords: heart failure, metformin, survival, energy metabolism, AMP-activated protein kinase
Introduction

Advanced heart failure (HF) is characterized not only by a depression of heart mechanical performance but also by altered myocardial metabolism - attenuated expression of fatty acid oxidation genes [1,2] and by diminished oxidation of long chain fatty acids [1,3-5], which may contribute to diminished metabolic flexibility and to energetic deficiency that further promotes worsening of HF [6]. Targeting energetic substrate metabolism might thus serve as a target for novel therapeutic approaches to HF [7,8].

Metformin (MET), a widely used antihyperglycemic drug with insulin-sensitizing properties, could be a suitable candidate for metabolic HF therapy. MET lowers serum glucose by inhibiting liver gluconeogenesis, lowers circulating free fatty acids (FFA) and improves insulin sensitivity. Some effects of metformin can be explained by an activation of AMP-activated protein kinase (AMPK) [9] - the enzyme that senses and regulates cellular energetic homeostasis, but it is not likely the only mechanism of MET effects [10,11]. Administration of MET might also favorably affect mitochondrial function and increase mitochondrial biogenesis by activating peroxisome proliferator-activated receptor-α (PPARα)/PPAR-γ coactivator-1α (PGC-1α) [12]. Although MET is one of the most widely prescribed medication in human medicine, its effects on the heart are not well characterized.

Until recently, MET use in patients with HF was contraindicated due to theoretical risk of lactic acidosis. Non-randomized observational studies had suggested that MET-treated diabetics with HF may have lower mortality than those on other antidiabetic regimes [13,14]. Because non-diabetic HF patients also have insulin resistance [15] and FFA elevation [16], MET might be helpful in wider HF population. The use of MET for metabolic therapy of HF needs to be established in experimental settings.

Volume overload represents clinically relevant condition leading to HF, for example in aortic or mitral valve insufficiency. The rat model of chronic HF due to volume overload induced by aorto-caval fistula has been previously well characterized [17-19]. It shares many similarities with the natural course of human HF including gradual development of the disease that proceeds through a stage of compensated hypertrophy followed by gradual decompensation into the overt HF [19], neurohumoral activation, cardiac output redistribution [20], fluid retention with pulmonary congestion and impairment of myocardial efficiency [21]. On the other hand, volume overload-induced HF has several features distinct from other HF models including a lack of myocardial fibrosis and inflammation [22,23] and involvement of different signalling pathways (upregulation of Akt and Wnt signalling) compared to experimental myocardial infarction or pressure overload [23]. The aim of this study was to test the hypothesis that chronic MET therapy would correct HF-induced metabolic abnormalities, improve cardiac performance and survival in the volume-overload HF rat model.

Materials and methods (for details see online supplement)

Animal HF model

HF was induced by volume overload from aorto-caval fistula (ACF) using needle technique [17,18]. Sham-operated controls underwent similar procedure but without the creation of ACF. MET groups received 0.5% metformin (Teva Pharmaceuticals, Opava, Czech Republic) mixed into the standard diet (normal salt/protein diet - 0.45% NaCl, 19-21% protein, SEMED, Prague, Czech Republic), placebo groups received identical diet but without MET. The study examined 3 rat cohorts and each cohort had four randomly-allocated groups: sham-placebo (SH+PL), sham-metformin (SH+MET), ACF-placebo (ACF+PL), ACF-metformin (ACF+MET). The first cohort (n=6-10/group) served for cardiac and mitochondrial function assessment, the second cohort (n=6-8/group) served for organ metabolic studies and both...
cohorts were terminated after 21st week after ACF procedure. The third cohort (n=10/SH groups, n=90/ACF groups) was left free of any procedures and served for a survival analysis till 52nd week. The investigation conformed to the NIH Guide for the care and use of laboratory animals (NIH Publication No. 85-23, 1996), Animal protection law of the CR (311/1997) and was approved by the ethics committee of IKEM.

Echocardiography and hemodynamics
Animals were anesthetized with ketamine/midazolam i.p. injection (50mg/5mg/kg). Echocardiography was performed using 7.5 MHz probe (Vivid System 5, GE, Indianapolis, USA), end-systolic and end-diastolic sizes of the left ventricle together with wall thicknesses were measured in parasternal long (PLAX) and short (PSAX) axis projection, the size of the right ventricle in apical four chamber projection (A4C). Invasive hemodynamics evaluation was performed by F2 Millar catheter inserted into aorta and LV via the carotid artery. After the hemodynamic assessment, rats were killed by exsanguination, coronary tree was flushed with ice-cold cardioplegic solution and LV free wall samples were instantly flash-frozen in liquid nitrogen for biochemical analyses or used for mitochondrial function assessment or electron microscopy.

Myocardial biochemistry and ultrastructure
Myocardial ATP content was measured in flash-frozen tissue using HPLC [24]. The content of total and phosphorylated form of AMPK was assessed by the Western blot as described before [25]. Briefly, phosphorylated AMPK (pAMPK) was assessed using rabbit anti pAMPK antibody (Cell Signaling), total AMPK (tAMPK) using goat anti alpha1+alpha2 AMPK antibody (Santa Cruz). Acetyl-CoA carboxylase (ACC) is a downstream target for AMPK. The ratio of phosphorylated ACC (pACC) at Ser-79 to total ACC (tACC) is a robust assay of AMPK activation [26]. Phosphorylated ACC was quantified by the Western blot using phospho-specific rabbit antibodies against Ser-79 (Abcam). Total ACC was measured by IR-Dye-800-conjugated Streptavidine (Rockland). The mitochondrial respiratory-chain complexes CI-CV were quantified by the Western blot [27]. Specific activities of cytochrome c oxidase and citrate synthase were determined spectrophotometrically in the myocardial homogenate [28]. Myocardial ultrastructure was studied on LV samples fixed in glutaraldehyde, post-fixed with osmium tetroxide, stained with uranyl acetate and examined with transmission electron microscope (Philips CM100, FEI, formerly Philips EO, Netherlands) with 25000x magnification. Image area occupied by mitochondria, myofibrils and cytoplasm was quantified by grid point-counting method [29] and expressed as percentage.

Mitochondrial function
In the myocardial tissue homogenate, maximal ADP-stimulated oxidative capacity of mitochondria was determined as oxygen consumption rate with palmitoylcarnitine (12.5μM)+malate (3mM)+glutamate (10mM)+succinate (10mM) using high-resolution oxygraph-2k (OROBOROS, Austria) [30]. Respiratory control index that indicates the tightness of the coupling between respiration and phosphorylation was calculated as the ratio of glutamate+malate+ADP (1.5mM) respiration without and with oligomycin (6μM).

Myocardial gene expression
Total RNA was isolated by RNeasy Micro Kit (QIAGEN Inc., Valencia, CA, USA), 200 ng of total RNA was used for the amplification procedure and 1.5 μg of amplified RNA was hybridized on the chip according to the manufacturer’s procedure.
Microarray analysis: The raw data (.TIFF image files) was analyzed using beadarray package [31] of the Bioconductor [32] within the R environment (http://www.r-project.org) [33]. The gene set enrichment analysis (GSEA) was performed on gene sets defined by the KEGG pathways [34]. Lists of genes assigned to the KEGG pathways were downloaded from the KEGG (release 48.0).

Systemic and organ metabolic analyses
MET serum level was checked in tail-vein serum at 11th week in ACF+MET (n=12) and SH+MET (n=18) groups. The MET level was measured using HPLC method with separation on silica column (ThermoQuest, Runcorn, UK) with a spectrophotometric detection. Oral glucose tolerance tests (oGTT) were performed in all groups at 20th week using an oral glucose load of 300mg/100g BW as gavage after overnight fasting. Serum glucose was measured by the glucose-oxidase assay, serum FFAs were determined by a colorimetric assay (Roche, Mannheim, Germany). Serum insulin was determined using the rat insulin ELISA kit (Mercodia, Uppsala, Sweeden). Tissue triglycerides were measured in liquid nitrogen-powdered tissues after chloroform/methanol extraction using the enzymatic assay (Pliva-Lachema, Brno, Czech Republic), used also for serum triglycerides. The glycogen in the heart was measured after KOH extraction [35].

Glycogen synthesis and glucose oxidation in the heart and muscle - Basal and insulin-stimulated 14C-glucose incorporation into glycogen and CO2 was determined ex vivo in isolated diaphragm [36]. Similarly, 1-mm thick, cross-sectional slices of the left ventricle at mid-papillary level were analyzed [37].

Fatty acid oxidation in the heart - Fatty acid oxidation in the heart tissue muscles and heart slices was determined by measuring the incorporation of 14C-palmitic acid into CO2 [38].

Statistics
Two-way ANOVA with Bonferroni post hoc adjustment was used to compare the effects of surgery and metformin treatment. Survival analysis was performed using Gehan-Breslow-Wilcoxon test. P-values < 0.05 were considered statistically significant.
Results

**MET serum assessment**
MET serum level at 11\textsuperscript{th} week was 2.2 ± 0.7 μg/ml (13 ± 4.15 nmol/ml) in ACF+MET group (n=12) and 1.9 ± 2.7 μg/ml (11.6 ± 16.1 nmol/ml) in SH+MET group (n=18) (p=0.68), being within the range of human therapeutic dose (corresponding to daily 2000 mg MET dose in an average adult) [39] and within the range of no observable adverse effect for rats [40].

**Organ morphometry, hemodynamics and echocardiography**
All groups had similar body weights and tibial lengths. Both ACF groups had marked heart hypertrophy (Table 1) and increased lung weight/body weight indicating pulmonary congestion. ACF animals had a depletion of intraabdominal adipose tissue in epididymal and perirenal fat body.

ACF animals had elevated left ventricular end-diastolic pressure (LVEDP), indicating decompensated heart failure but still preserved LV contractility (dP/dt\textsubscript{max}) and relaxation (dP/dt\textsubscript{min}, tau constant). The systolic duration/cycle length ratio was higher in both ACF groups. No effect of MET on hemodynamics was observed (Table 2).

ACF animals had marked enlargement of both ventricles (Table 3), a twofold increase in stroke volume and cardiac output due to fistula and diminished left ventricular fractional shortening and ejection fraction. Both LV anterior and posterior walls in all groups showed similar thicknesses. No effect of MET on echocardiographic parameters was observed.

**Metabolic assessment**

*Glucose and glycogen metabolism* - When assessed using oral glucose tolerance test (oGTT), all the groups showed similar glucose levels throughout the test and preserved postprandial glycemic regulation (Fig. 1A, B). ACF animals had lower insulin levels at the baseline (Fig. 1C) and a trend towards lower levels post-load (Fig. 1D). Myocardium of all animals showed similar contents of glycogen (Fig. 2D) and the rate of glycogen synthesis (data not shown). Myocardial glucose oxidation was significantly lower in both ACF groups and metformin treatment induced further lowering (Fig. 2E), independently of ACF procedure. ACF animals had a lower rate of glycogen synthesis in the skeletal muscle (diaphragm) than SHAM (Fig. 3A) but all groups had a similar insulin-stimulated increment of glycogen synthesis (Fig. 3B), which is a measure of skeletal muscle insulin sensitivity [36,41]. There was no difference in skeletal muscle (diaphragm) glucose oxidation (data not shown).

*Lipid metabolism* - Serum and liver triglycerides were similar in all groups (Fig. 2A, C). In contrast, myocardial triglyceride content was markedly decreased in ACF animals (Fig. 2B). ACF animals had increased serum FFA concentration in fasted state and even more after glucose loading compared to sham-operated animals. MET treatment reduced FFA levels in both metformin-treated groups compared to placebo (Fig. 1E, F). Myocardial palmitate oxidation was reduced in ACF+PL group by 37% (p<0.001) compared to SH+PL. MET treatment (ACF+MET) increased myocardial palmitate oxidation to the level similar in SH+PL group (Fig. 2F).

**Mitochondrial function**
Cytochrome c oxidase (CIV) and citrate synthase activities (Fig. 5D, E) and the protein content of mitochondrial respiratory chain complexes CI-V (Fig. S4 in online supplement) did not show any difference between groups. Maximal ADP-stimulated respiratory chain capacity, measured as O\textsubscript{2} consumption with palmitoyl-carnitine+malate+glutamate+succinate (Fig. 5C), as well as respiratory control index (Fig. 5B) were again similar in all groups,
reflecting that the resting respiration was largely intact and coupled. Analysis of myocardial ATP content showed similar level of ATP (Fig. 5A) indicating undisturbed resting energetic state in HF animals.

Electron microscopy showed no apparent structural abnormalities and the proportions occupied by myofibrils (my), mitochondria (mi) and cytosol were similar in all groups (Fig. S3 in online supplement).

**AMP-activated protein kinase (AMPK) signalling**

To characterize the activity of the AMPK-regulatory cascade, total content and phosphorylation of both AMPK and its target, acetyl-CoA carboxylase (ACC), were assessed by the Western blot. At the level of AMPK, ACF animals showed significantly higher contents of both total (tAMPK) and phosphorylated AMPK (pAMPK) than sham groups. However, the ratio between phosphorylated and total AMPK (pAMPK/tAMPK) was similar independent of ACF procedure or metformin treatment (Fig. 4 A-C). At the level of ACC, no differences neither in total ACC (tACC), phosphorylated ACC (pACC) nor in pACC/tACC ratio were observed between groups (Fig. 4 D-F). These data suggest a modest upstream AMPK activation due to ACF procedure, but the absence of a significant functional consequence at the level of the downstream target of AMPK neither due to ACF procedure nor to MET treatment.

**Myocardial gene expression analysis**

Out of 23401 detected transcripts, we observed no difference between ACF+MET and ACF+PL, which was in striking contrast to fistula-induced transcriptional changes (ACF+PL vs. SH+PL), where 128 transcripts were differentially expressed (99 upregulated and 29 downregulated, Storey's q-value < 0.05 and two-fold or greater change in intensity). Fig. S1 in online supplement depicts a heatmap with all differentially expressed transcripts. KEGG pathway analysis revealed the downregulation of pathways involved in fatty acid metabolism in response to ACF (Fig. S2 in online supplement).

**Survival**

None of the control animals died throughout the study. First deaths in ACF groups occurred between 10th and 15th week and 77.2% of ACF+PL (80.5% of ACF+MET) animals were dead by the end of the study. Median survival was 45.5 weeks in ACF+PL group and 44.5 weeks in ACF+MET group. MET therapy had no effect on survival in ACF animals (Fig. 6).
Discussion

The study shows that chronic volume overload-induced heart failure (HF) is associated with lower glycogen synthesis in the skeletal muscle (diaphragm), lower heart triglyceride content, higher plasmatic free fatty acids (FFA), lower plasmatic insulin level and depressed myocardial glucose and palmitate oxidation. Long-term administration of antihyperglycemic drug metformin (MET) normalized elevated FFA, further decreased myocardial glucose oxidation and increased myocardial palmitate oxidation, but had no effect on myocardial AMPK activation, ATP content, mitochondrial function or morphology. No relevant improvement in cardiac performance or long-term survival was observed in MET-treated HF animals. Despite several recent studies reported beneficial effect of MET in other non-diabetic HF models [42-44], our study indicates that this improvement is not common to all HF forms. Decreased cellular energetic charge [10] and/or AMPK activation by MET may be required for improvement of cardiac performance in HF. Conversely, prolonged exposure of symptomatic HF animals to high-dose MET led to no increase in mortality, supporting safety of MET use in diabetic HF.

Peripheral and systemic MET effects

At the systemic level, MET lowered basal and postprandial circulating FFA due to increased FFA utilization and perhaps also due to diminished FFA release from adipose tissue because of known inhibitory effects of MET on catecholamine-stimulated lipolysis [45]. ACF rats had a depletion of intraabdominal adipose tissue, indicative of enhanced fat mobilization due to neurohumoral activation from HF, but MET did not reverse fat depletion. MET also had no effect on insulin-mediated glycogen synthesis in skeletal muscle, which is a measure of insulin sensitivity.

Cardiac effects of MET

In the heart, MET treatment significantly increased the palmitate oxidation that was attenuated in the ACF+PL group. Diminished oxidation of long chain fatty acids and downregulation of enzymes of fatty acid oxidation in the heart have been repeatedly described both in HF patients [1] and in animal HF models [3,4,46] including rats with ACF [5,47,48], reflecting probably a reactivation of fetal-like gene transcriptional programme [2,4]. Whether this change is adaptive or not is still discussed. Functional consequences of drug-induced reversal of diminished FA oxidation are unknown. Our study indicates that drug-induced enhancement of FA oxidation has a neutral effect on survival and cardiac function in HF due to volume overload supporting the view that diminished myocardial FA oxidation does not play the causal role in HF progression. Similar findings were reported also in pacing-induced HF, where long-term therapy with PPAR-α agonist fenofibrate increased FA utilization, but did not delay the HF onset [49]. MET had no effect on mitochondrial respiratory function, myocardial gene transcription profile, and the size of mitochondrial compartment within the cardiomyocyte, speaking against the specific activation of mitochondrial biogenesis [50]. Myocardial gene expression profile in MET-treated ACF animals was unchanged, indicating non-genomic mechanisms involved in FA oxidation increase after MET. Moravec et al. had showed that isolated cardiac mitochondria from ACF rats have impaired activation and transport of palmitoyl group across outer mitochondrial membrane (via palmitoyl-CoA synthase and carnitine-palmitoyl-CoA transferase-1), leading to attenuated oxidation of palmitate, but preserved oxidation of palmitoyl-carnitine [5]. Kinetic studies indicated that the cooperation between those two enzymes deteriorates with increasing FFA concentrations. Thus, the improvement of FA oxidation after MET treatment in ACF group can be a consequence of the mere reduction of circulating FFAs in serum [5]. The apparent opposite responses of myocardial fatty acid oxidation and glucose oxidation can be explained either by
a metformin-induced inhibition of glycolysis [11] or by a Randle effect- increased fatty acid oxidation increases the concentration of acetyl-CoA in the Krebs cycle leading to an inhibition of pyruvate dehydrogenase with a subsequent inhibition of glycolysis and glucose oxidation [51].

Comparison to other HF studies
The absence of benefit of MET on cardiac function or survival in ACF-induced HF is in contrast with other recently published studies in other HF models. Gundewar et al. [44] examined the effect of very low dose MET (125 µg/kg/day i.p.) on cardiac function and survival in mice subjected to LAD ligation. MET extended the survival at 4 weeks by 47%, improved LV remodeling and corrected MI-induced defects in mitochondrial respiration and ATP synthesis. Despite the administered MET-dose (per kg BW/day) was lower by three orders of magnitude than in our study (i.e. 300 mg MET/kg/day) or than is normally used in humans, authors were able to detect increased phosphorylation of AMPK, eNOS and increased expression of PGC-1α in the heart. In another study, Sasaki et al. examined the effect of 4-week oral MET therapy (100 mg/kg/day) in the tachypacing-HF model in dogs. Compared to placebo, MET improved LV ejection fraction, slowed HF progression and decreased myocardial apoptosis via AMPK-dependent mechanism [42]. Xiao et al. [52] documented an improvement of cardiac diastolic function upon metformin treatment in pressure overload HF model. Dose used in this study (200mg/kg/day) was similar to ours.

Lack of the protecting effect of MET in volume-overload HF model
Mechanism of MET action is still incompletely understood. One possibility expects an activation of AMP-activated protein kinase (AMPK) that turns on energy-providing and turns off energy-consuming metabolic pathways [9,53], but non-AMPK dependent MET effects have also been described [10,11]. The reason why MET therapy provided no hemodynamic or survival benefit in ACF-induced HF model in contrast to other models is not clear. We excluded the possibility of underdosing or poor absorption, because MET serum levels were adequate and within the therapeutic range observed in humans [40]. The effect of MET could be affected by varying intracellular import, which depends on the capacity of organic cation transporter (OCT-1). Genetic polymorphisms in OCT-1 gene (SCL22A1) were shown to affect a clinical response to MET therapy in humans [54]. In our study, MET administration had no effect on either mitochondrial functions or on mitochondrial and ultrastructural morphology. Ultrastructural morphology was similar among groups regardless of ACF procedure or MET treatment, despite a marked increase in heart weights in both ACF groups. This can be explained by an increase in myocyte width and length [55]. An increase in both phosphorylated (pAMPK) and total (tAMPK) forms of AMP-activate protein kinase was observed with a preserved pAMPK/tAMPK ratio. On the level of acetyl-CoA carboxylase (ACC), which is a direct downstream target of AMPK, no differences in phosphorylated form of ACC (pACC), total ACC (tACC) nor in the pACC/tACC ratio occurred. This observation suggests a modest upstream AMPK activation due to ACF procedure, but the absence of a significant functional consequence at the level of the downstream target of AMPK. Insufficient AMPK activation could potentially explain the lack of MET benefit in our model. AMPK catalytic subunits are highly conserved, so inter-species structural differences are unlikely to explain the inability of MET to activate AMPK in our protocol. An absence of AMPK activation in our ACF-induced HF model could be explained by an unaltered myocardial ATP level in this model. Model-specific differences thus seem to provide an acceptable explanation. The cardioprotective effect of metformin can be at least partially ascribed to the attenuation of cardiac fibrosis [52], but compared to pressure overload, volume overload is not associated with increased myocardial fibrosis [22,23]. Compared to the study...
of Gundewar [44], we did not find any increase in AMPK activity or decrease in oxygen consumption rate or respiratory control index. It appears that in contrast to pressure overload, volume overload does not sufficiently alter resting mitochondrial function [23] and thus it may lack the substrate for metformin action. Finally, no insulin resistance was observed in our volume-overload HF model, so the lack of insulin resistance might also implicate a missing substrate for MET action. Despite all these specifics of the model, we should be aware that HF is a non-uniform syndrome and it should be studied in subsets. Volume overload is a clinically important condition and its most common form (mitral insufficiency) often complicates other heart diseases and independently increases mortality [56].

**Metabolic abnormalities in ACF HF model**

ACF-induced HF model showed several specific features. Despite gene expression analysis showed an extensive downregulation of β-oxidation pathway and several respiratory chain components in ACF, ATP-generating capacity of mitochondria in surplus of oxygen and substrates was preserved. This might be explained by a redundancy in enzyme activities and longer half-life [4,57]. Myocardial ATP content, presented for the first time in this model, was also normal. This is in agreement with the study from Marcil et al. [58] who showed normal myocardial oxidative capacity in compensated ACF-induced HF (15th week), but marked sensitivity of the heart to hypoxia, indicating preserved ATP levels at rest, but attenuated energetic reserve during increased stress. Low myocardial triglyceride content in ACF hearts, also reported for the first time, is probably related to limited reesterification of triglycerides due to low availability of NADPH [59] and this abnormality was unchanged by MET therapy. Although a number of works described connections between elevated levels of FFAs [16] and insulin resistance [15,60] in HF, muscle insulin sensitivity, measured as insulin-induced increment of glycogen synthesis [36,41], was preserved in ACF, making perhaps this model less prone to benefits of MET. Interestingly, fasting and postprandial insulinaemia were actually lower in ACF than in control animals. Pancreatic hypoperfusion or negative effects of chronically elevated FFAs on pancreatic β-cell secretory function might be responsible for this phenomenon [61].

In conclusion, the presented study shows that long-term MET therapy in rats with HF due to volume-overload decreases circulating FFA, decreases myocardial glucose oxidation and increases myocardial palmitate oxidation, but these effects have neutral impact on cardiac performance and survival in HF. Recently reported cardioprotective effects of MET may not be universal to all forms of HF and may require AMPK activation or ATP depletion. Prolonged exposure of a large group of severely symptomatic HF animals to high-dose MET led to no apparent increase in mortality, which provides robust data to the toxicology of MET [40] and supports its safe use in HF patients with diabetes.
Funding
This work was supported by the EU Operational Program Prague – Competitiveness; project “CEVKOON” [CZ.2.16/3.1.00/22126 to L.C.]; institutional support of the Institute for Clinical and Experimental Medicine [MZO 00023001 to VM., J.B. and L.C.]; Center for Cardiovascular Research [MSMT-1MO510 to VM., J.B. and L.C.]; grants from Ministry of Heath [MZO-00023001 to V.M. and L.C., IGA MZCR 10300-3 to J.P., NS10497-3/2009 to V.M.]; Ministry of Education [MSMT-1MO510 to V.M., J.B. and L.C., VZ 0021620806 to D.S., 1M6837805002 to H.S., M.K., J.H. and J.K., 0021620806 to J.P., LC06044 to J.P., SVV-2010-254260507 to J.P.]; the Grant agency [305/09/1390 to V.M.]; and the Academy of sciences [AV0Z50520514 to H.S. and M.K., AV0Z50110509 to D.S., J.H. and J.K., AV0Z50200510 to O.B.] of the Czech Republic.

Acknowledgements
Acknowledgement to Ms. Jarmila Svatunkova for assistance with unbiased point counting.

Conflict of interest:
None declared.
Figure legends

Figure 1. Serum levels of glucose, free fatty acids (FFA) and insulin. A. Serum glucose levels during oral glucose tolerance test (oGTT). B. Area under the curve (AUC) of serum glucose during oGTT. C. Serum insulin level at the beginning of oGTT. D. Serum insulin level in the 60th minute of oGTT. E. Serum FFA at the beginning of oGTT. F. Serum FFA in the 60th minute of oGTT. White: controls with placebo (SH+PL), Hatched white: controls with MET (SH+MET), Grey: aorto-caval fistula with placebo (ACF+PL), Hatched grey: aorto-caval fistula with MET (ACF+MET). Results are expressed as means ± S.E.M.

Figure 2. Content and metabolism of lipids and glucose. A. Triglycerides in the serum, B. Triglycerides in the heart, C. Triglycerides in the liver, D. Glycogen in the heart, E. 14C-glucose oxidation in the heart, F. 14C-palmitate oxidation in the heart. White: controls with placebo (SH+PL), Hatched white: controls with MET (SH+MET), Grey: aorto-caval fistula with placebo (ACF+PL), Hatched grey: aorto-caval fistula with MET (ACF+MET). Results are expressed as means ± S.E.M.

Figure 3. Diaphragm glycogen synthesis. A. Diaphragm glycogen synthesis under basal conditions, B. Diaphragm glycogen synthesis; increase in glycogen synthesis due to insulin stimulation. White: controls with placebo (SH+PL), Hatched white: controls with MET (SH+MET), Grey: aorto-caval fistula with placebo (ACF+PL), Hatched grey: aorto-caval fistula with MET (ACF+MET). Results are expressed as means ± S.E.M.

Figure 4. Heart AMPK activation. A. Total content of AMP-activated protein kinase (tAMPK), B. Phosphorylated AMP-activated protein kinase (pAMPK), C. Ratio between phosphorylated and total AMP-activated protein kinase (pAMPK/tAMPK), D. Total content of acetyl-CoA carboxylase (tACC), E. Phosphorylated acetyl-CoA carboxylase (pACC), F. Ratio between phosphorylated and total acetyl-CoA carboxylase (pACC/tACC). White: controls with placebo (SH+PL), Hatched white: controls with MET (SH+MET), Grey: aorto-caval fistula with placebo (ACF+PL), Hatched grey: aorto-caval fistula with MET (ACF+MET). Results are expressed as means ± S.E.M.

Figure 5. Mitochondrial function. A. ATP content, B. Mitochondrial respiratory control index (with glutamate, without/with oligomycin), C. Maximal mitochondrial oxidation rate (with malate, palmitoyl carnitine, succinate, glutamate and ADP), D. Cytochrome c oxidase activity E. Citrate synthase activity. White: controls with placebo (SH+PL), Hatched white: controls with MET (SH+MET), Grey: aorto-caval fistula with placebo (ACF+PL), Hatched grey: aorto-caval fistula with MET (ACF+MET). Results are expressed as means ± S.E.M.

Figure 6. Survival analysis. Open triangles: controls with placebo (SH+PL), Open circles: controls with MET (SH+MET), Filled triangles: aorto-caval fistula with placebo (ACF+PL), Filled circles: aorto-caval fistula with MET (ACF+MET)
References


metabolism adaptation to chronic hypoxia in right and left ventricles. 

30 Pecina, P., Capkova, M., Chowdhury, S. K., Drahota, Z., Dubot, A., Vojtiskova, A., 
Functional alteration of cytochrome c oxidase by SURF1 mutations in Leigh 


32 Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, 
B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., 
Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A. J., Sawitzki, G., Smith, 
software development for computational biology and bioinformatics. Genome 
Biol. 5, R80.

33 Strnad, H., Lacina, L., Kolar, M., Cada, Z., Vlcek, C., Dvorankova, B., Betka, J., Plzak, 
(2010) Head and neck squamous cancer stromal fibroblasts produce growth 
Biol. 133, 201-211.


impaired response of non-obese hereditary hypertriglyceridemic rats to glucose 
load is associated with low glucose storage in energy reserves. Exp. Clin. 

Captopril enhanced insulin-stimulated glycogen synthesis in skeletal muscle but 
not fatty acid synthesis in adipose tissue of hereditary hypertriglyceridemic rats. 
Metabolism 52, 1406-1412.

of myocardial GLUT 4 and increased glucose uptake through activation of 

38 Sugimoto, K., Kazdova, L., Qi, N. R., Hyakukoku, M., Kren, V., Simakova, M., Zidek, 
oxidation in skeletal muscle through a peroxisome proliferator-activated 
receptor-gamma dependent pathway. J. Hypertens. 26, 1209-1215.

39 Glucophage SPC. 
http://www.accessdata.fda.gov/drugsatfda_docs/label/2008/020357s031,021202s 
016lbl.pdf.


41 Kraegen, E. W., Jenkins, A. B., Storlien, L. H. and Chisholm, D. J. (1990) Tracer 
studies of in vivo insulin action and glucose metabolism in individual peripheral 

Asakura, M., Kim, J., Minamino, T., Takashima, S., Sanada, S., Sugimachi, M., 
progression of heart failure in dogs: role of AMP-activated protein kinase. 
Circulation 119, 2568-2577.


Table 1, Morphometric characteristics

<table>
<thead>
<tr>
<th></th>
<th>SH+PL</th>
<th>SH+MET</th>
<th>ACF+PL</th>
<th>ACF+MET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>592 ± 20.9</td>
<td>603 ± 11.9</td>
<td>586 ± 23.4</td>
<td>579 ± 22.0</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.63 ± 0.08</td>
<td>1.74 ± 0.04</td>
<td>3.10 ± 0.16</td>
<td>* 3.23 ± 0.21</td>
</tr>
<tr>
<td>HW/BW, g/100g</td>
<td>2.80 ± 0.12</td>
<td>2.90 ± 0.12</td>
<td>5.29 ± 0.18</td>
<td>* 5.59 ± 0.33</td>
</tr>
<tr>
<td>Lung weight/BW, g/100g</td>
<td>3.30 ± 0.16</td>
<td>3.69 ± 0.53</td>
<td>4.23 ± 0.19</td>
<td>* 4.48 ± 0.29</td>
</tr>
<tr>
<td>Liver weight/BW, g/100g</td>
<td>28.7 ± 0.93</td>
<td>28.3 ± 0.99</td>
<td>27.7 ± 1.01</td>
<td>27.0 ± 1.04</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>43.2 ± 0.30</td>
<td>42.3 ± 0.41</td>
<td>43.1 ± 0.43</td>
<td>43.1 ± 0.42</td>
</tr>
<tr>
<td>Epididymal fat body/BW, g/100g</td>
<td>1.63 ± 0.16</td>
<td>1.46 ± 0.14</td>
<td>1.22 ± 0.06</td>
<td>* 1.30 ± 0.11</td>
</tr>
<tr>
<td>Perirenal fat body/BW, g/100g</td>
<td>1.52 ± 0.17</td>
<td>1.41 ± 0.24</td>
<td>1.05 ± 0.08</td>
<td>* 1.25 ± 0.11</td>
</tr>
<tr>
<td>Subcutaneous fat body/BW, g/100g</td>
<td>0.26 ± 0.04</td>
<td>0.28 ± 0.02</td>
<td>0.26 ± 0.12</td>
<td>0.25 ± 0.09</td>
</tr>
</tbody>
</table>

21st week after ACF. N= 6-8 /group. Data are mean ± SE. HW: Heart weight, BW: Body weight, SH: sham-operated, MET: MET-treated, PL: placebo, ACF: aorto-caval fistula group, * significantly different (p<0.05) than sham.
Table 2, Hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>SH+PL</th>
<th>SH+MET</th>
<th>ACF+PL</th>
<th>ACF+MET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal LV pressure, mmHg</td>
<td>129 ± 7.11</td>
<td>139 ± 8.33</td>
<td>120 ± 3.96</td>
<td>128 ± 6.94</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>6.7 ± 0.84</td>
<td>6.8 ± 0.45</td>
<td>12.1 ± 0.66</td>
<td>* 11.4 ± 1.35</td>
</tr>
<tr>
<td>Systolic duration, as % of cycle duration</td>
<td>46.7 ± 4.79</td>
<td>49.5 ± 1.77</td>
<td>59.8 ± 1.95</td>
<td>* 55.3 ± 1.21</td>
</tr>
<tr>
<td>Heart rate, s⁻¹</td>
<td>345 ± 13.3</td>
<td>386 ± 10.3</td>
<td>360 ± 10.8</td>
<td>323 ± 13.3</td>
</tr>
<tr>
<td>dP/dt max, mmHg/s</td>
<td>11 417 ± 965</td>
<td>12 730 ± 834</td>
<td>11 504 ± 733</td>
<td>11 655 ± 1 030</td>
</tr>
<tr>
<td>dP/dt min, mmHg/s</td>
<td>-13 314 ± 1593</td>
<td>-12 520 ± 4 192</td>
<td>-11 056 ± 2 540</td>
<td>-11 508 ± 1 977</td>
</tr>
<tr>
<td>LV relaxation constant tau, s</td>
<td>0.015 ± 0.0013</td>
<td>0.012 ± 0.0013</td>
<td>0.034 ± 0.0188</td>
<td>0.039 ± 0.0203</td>
</tr>
</tbody>
</table>

21st week after ACF. N= 6-8/group. Data are mean ± SE. LV: left ventricle, SH: sham-operated, MET: MET- treated, PL: placebo, ACF: aorto-caval fistula group, * significantly different (p<0.05) than sham.
Table 3, Echocardiography

<table>
<thead>
<tr>
<th></th>
<th>SH+PL</th>
<th>SH+MET</th>
<th>ACF+PL</th>
<th>ACF+MET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, s⁻¹</td>
<td>425 ± 12.3</td>
<td>432 ± 12.7</td>
<td>370 ± 27.4</td>
<td>375 ± 18.2</td>
</tr>
<tr>
<td>LV diastolic diameter, mm</td>
<td>6.08 ± 0.40</td>
<td>6.70 ± 0.09</td>
<td>10.20 ± 0.48</td>
<td>* 9.75 ± 0.40</td>
</tr>
<tr>
<td>LV systolic diameter, mm</td>
<td>1.95 ± 0.42</td>
<td>2.63 ± 0.19</td>
<td>5.47 ± 0.42</td>
<td>* 4.62 ± 0.39</td>
</tr>
<tr>
<td>LV fractional shortening, %</td>
<td>69.2 ± 5.00</td>
<td>60.8 ± 2.78</td>
<td>46.7 ± 2.46</td>
<td>* 53.0 ± 2.58</td>
</tr>
<tr>
<td>LV anterior wall thickness in diastole, mm</td>
<td>2.30 ± 0.08</td>
<td>2.28 ± 0.07</td>
<td>2.33 ± 0.09</td>
<td>2.25 ± 0.09</td>
</tr>
<tr>
<td>LV posterior wall thickness in diastole, mm</td>
<td>2.33 ± 0.07</td>
<td>2.32 ± 0.09</td>
<td>2.31 ± 0.09</td>
<td>2.23 ± 0.12</td>
</tr>
<tr>
<td>RV diastolic diameter, mm</td>
<td>2.85 ± 0.18</td>
<td>3.34 ± 0.30</td>
<td>5.07 ± 0.29</td>
<td>* 4.96 ± 0.52</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>93.0 ± 2.36</td>
<td>88.6 ± 1.72</td>
<td>75.2 ± 2.66</td>
<td>* 81.4 ± 2.56</td>
</tr>
<tr>
<td>Stroke volume, μl</td>
<td>174 ± 21.6</td>
<td>205 ± 7.07</td>
<td>447 ± 44.6</td>
<td>* 436 ± 35.3</td>
</tr>
<tr>
<td>Cardiac output, ml.min⁻¹</td>
<td>73.57 ± 8.6</td>
<td>88.80 ± 4.7</td>
<td>159.85 ± 10.8</td>
<td>* 160.41 ± 9.2</td>
</tr>
</tbody>
</table>

21st week after ACF. N= 6-8/group. Data are mean ± SE. LV: left ventricle, RV: right ventricle, SH: sham-operated, MET: MET- treated, PL: placebo, ACF: aorto-caval fistula group, * significantly different (p<0.05) than sham.
Figure 1

A. Glucose

B. Serum glucose AUC

C. Insulin at the oGTT baseline

D. Insulin in the 60th minute of oGTT

E. Free fatty acids at the oGTT baseline

F. Free fatty acids in the 60th minute of oGTT

* p<0.05 ACF vs. SHAM
# p<0.05 PLAC vs. MET
Figure 2

A. Serum triglycerides

B. Heart triglyceride content

C. Liver triglyceride content

D. Heart glycogen content

E. Glucose oxidation in the heart

F. Palmitate oxidation in the heart

n. s.

* p<0.05 SHAM vs. ACF

* p<0.05 ACF vs. SHAM

* p<0.05 PLAC vs. MET

& p<0.01 ACF+MET vs. ACF+PL

© 2011 The Authors Journal compilation © 2011 Portland Press Limited
Figure 3

A) Diaphragm glycogen synthesis (basal conditions)

B) Diaphragm glycogen synthesis (insulin induced increase)

* p<0.05 ACF vs. SHAM

n. s.
Figure 4

A. Total AMP-activated protein kinase (TAMPK)

B. Phospho AMP-activated protein kinase (pAMPK)

C. pAMPK/TAMPK

D. Total acetyl-CoA carboxylase (tACC)

E. Phospho acetyl-CoA carboxylase (pACC)

F. pACC/tACC

* p<0.05 ACF vs. SHAM

n.s. = not significant
Figure 5

**A** Myocardial ATP

**B** Mitochondrial respiratory control index (without/with oligomycin)

**C** Maximal mitochondrial oxidation rate (Mal+ palmitoylcarnitine + Suc+ Glut+ ADP)

**D** Cytochrome c oxidase activity

**E** Citrate synthase activity
Figure 6

**Survival**

![Survival Graph](image)

- SH+PL
- SH+MET
- ACF+PL
- ACF+MET

Percent survival

$p = 0.39$

weeks

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.

© 2011 The Authors Journal compilation © 2011 Portland Press Limited