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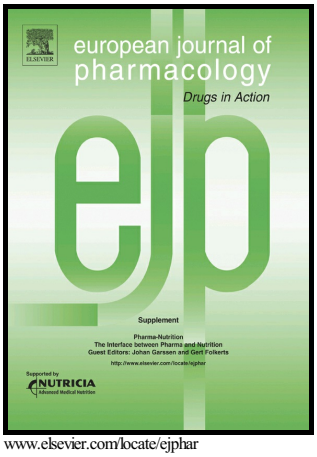

www.elsevier.com/locate/ejphar

PII: S0014-2999(18)30136-5
DOI: https://doi.org/10.1016/j.ejphar.2018.02.050
Reference: EJP71703

To appear in: *European Journal of Pharmacology*

Received date: 31 August 2017
Revised date: 27 February 2018
Accepted date: 28 February 2018

Cite this article as: Massa Oi, Daniel Donner, Jason Peart, Belinda Beck, Lauren Wendt, John P. Headrick and Eugene F. du Toit, Pravastatin improves risk factors but not ischaemic tolerance in obese rats, *European Journal of Pharmacology,* https://doi.org/10.1016/j.ejphar.2018.02.050

Pravastatin improves risk factors but not ischaemic tolerance in obese rats.

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Abstract

Statins are effective in management of dyslipidaemia, and a cornerstone of CVD prevention strategies. However, the impacts of their pleiotropic effects on other cardiovascular risk factors and myocardial responses to infarction are not well characterised. We hypothesised that pravastatin treatment in obesity improves lipid profiles, insulin-resistance and myocardial resistance to ischaemia/reperfusion (I/R) injury. Wistar rats were fed a control (C) chow or high carbohydrate and fat diet (HCFD) for 16 weeks with vehicle or pravastatin (prava 7.5 mg/kg/day) treatment for 8 weeks. At 16 weeks HOMAs were performed, blood samples collected and hearts excised for Langendorff perfusions/biochemical analyses. Anti-oxidant activity and proteins regulating mitochondrial fission/fusion and apoptosis were assessed. The HCFD increased body weight (736±15 vs. 655±12 g for C; P<0.001), serum triglycerides (2.91±0.52 vs. 1.64±0.26 mmol/L for C; P<0.001) and insulin-resistance (HOMA- 6.9±0.8 vs. 4.2±0.5 for C; P<0.05) while prava prevented diet induced changes and paradoxically increased lipid peroxidation. The HCFD increased infarct size (34.1±3.1% vs. 18.8±3.0% of AAR for C; P<0.05), which was unchanged by prava in C and HCFD animals. The HCFD decreased cardiac TxR activity and mitochondrial MFN-1 and increased mitochondrial DRP-1 (reducing MFN-1:DRP-1 ratio) and Bax expression, with the latter changes prevented by prava. While unaltered by diet, cytosolic levels of Bax and caspase-3 were reduced by prava in C and HCFD hearts (without changes in cleaved caspase-3). We conclude that obesity, hyper-triglyceridemia and impaired glycemic control in HCFD rats are countered by prava. Despite improved risk factors, prava did not reduce myocardial infarct size, potentially reflecting its complex pleiotropic impacts on cardiac GPX activity and MFN-1, DRP-1, caspase-3 and Bcl-2 proteins.

Keywords: Statins; Ischaemia-reperfusion; Myocardial Infarction; Obesity; Reperfusion Injury; Mitochondrial dynamics.

1. Introduction

The pronounced increase in global incidence of obesity has led to a high prevalence of its co-morbidities, which are all major cardiovascular disease (CVD) risk factors. Patients with 3 or more obesity comorbidities (dyslipidaemia, hypertension, insulin-resistance/diabetes) have the metabolic syndrome (MetS) (Eckel et al., 2010), which greatly increases risk of developing coronary artery disease (Kannel et al., 1961), decreases myocardial tolerance to I/R (Bouhidel et al., 2008; Du Toit et al., 2008; Clark et al., 2011; Wensley et al., 2013) and increases the risk of cardiac death 2-4 fold (Lakka et al., 2002). In this growing population statin therapy is the cornerstone for management of dyslipidaemia and reduction of cardiovascular risk (Gu et al., 2014; O'Keeffe, Nazareth and Petersen, 2016). Although statins are highly effective for the maintenance of normal lipid levels, they also exert complex pleiotropic effects independent of lipid lowering actions (Zhou and Liao, 2009; Allen and Mamotte, 2017). As the single most prescribed cardiovascular drug (Stewart, 2017), it is important to delineate these pleiotropic actions and how chronic statins may modify disease processes in those that ultimately do suffer an ischaemic insult.

We recently showed that myocardial tolerance to I/R in obesity is dependent on the presence or absence of insulin-resistance (Donner et al., 2013). Data suggest that isolated obesity and dyslipidaemia may actually protect the heart whereas obesity with insulin-resistance adversely impacts ischaemic tolerance in a rodent model of MetS (Donner et al., 2013). Cardioprotection with obesity has also been recently reported by others (Salie et al., 2014; Webster et al. 2017). Prolonged untreated dyslipidaemia and elevated circulating non-esterified fatty acids associated with obesity are implicated in the aetiology of insulin-resistance and diabetes (Lopaschuk et al., 2007; Chess and Stanley, 2008). However, the efficacy of lipid lowering drugs for the prevention/attenuation of insulin-resistance is

controversial, with studies suggesting statins can decrease (Bellia et al., 2012; Sato et al., 2012), have no effect (Panz et al., 2012) or improve (Fraloub et al., 2012; Guo et al., 2012) insulin sensitivity in insulin-resistant/diabetic animals and patients. Several studies suggest pravastatin may also improve insulin-resistance in hypercholesterolaemic patients (Koh et al., 2013) and rats (Bełtowski et al., 2011). Improved insulin sensitivity may in turn impact cardiac mitochondrial turnover (Gonzalez-Franquesa and Patti, 2015; Montgomery and Turner. 2015) and apoptosis (Li et al., 2017), with insulin-resistance strongly associated with mitochondrial dysfunction (Dumas et al., 2009; Pagel-Langenickel et al., 2010; Gonzalez-Franquesa and Patti, 2015; Montgomery and Turner. 2015). Statin dependent changes in insulin resistance may therefore influence I/R injury during and after a myocardial infarct.

We hypothesise that pravastatin improves cardiometabolic risk factors (adiposity, lipid profiles, insulin sensitivity) and intrinsic myocardial resistance to I/R injury. In this study we assess the effects of pravastatin treatment in lean and obese rats on: 1) morphometry and body composition; 2) cardiometabolic risk factors; 3) myocardial I/R tolerance; 4) myocardial antioxidant enzyme activity; and 5) myocardial expression of proteins regulating apoptosis and mitochondrial fission and fusion.

2. Materials and methods

2.1. Animal model

All experiments were conducted in accordance with the NHMRC's *Australian code for the care and use of animals for scientific purposes*. This project was approved by the Griffith University Animal Ethics committee (MSC/01/14/AEC). Rats were housed in the Griffith University Animal Unit with a 12/12 h light/dark cycle and temperature and humidity set at 21°C and 40% respectively. Sixty male Wistar rats (8 week old) were randomly assigned to

one of four groups: a control diet fed group (C); a high fat diet (HCFD) fed group; a control diet plus pravastatin (Prava) treated (C+Prava) group; and a HCFD plus pravastatin treated (HCFD+Prava) group. Animals were on the respective diets for 8 weeks before vehicle or pravastatin treatment was commenced for 8 weeks. Pravastatin (7.5 mg/kg/day) or vehicle was administered daily via oral gavage. Rats were weighed and pravastatin dosages adjusted weekly to compensate for body weight changes during the 8 week treatment period. Standard rat chow contained 70% carbohydrates, 23% proteins and 7% fat; while the HCFD contained 70% carbohydrates, 13% proteins and 17% fat. All animals were allowed *ad libitum* access to fresh food and water.

Ten rats from each group were randomly assigned to *ex vivo* Langendorff perfusion experiments in which cardiac function and myocardial infarct size was assessed. The remaining 5 animals from each group were used for blood and myocardial tissue collection for blood chemistry, ELISA analysis, and myocardial enzyme activity (spectrophotometry) and protein expression (Western blot) analyses.

2.2. Body weight, body composition and glucose tolerance and lipid profile analyses

2.2.1. Body weight: Animals were weighed weekly and dual-energy X-ray absorptiometry (DXA) scans and glucose tolerance tests (GTTs) were performed at 8 and 16 weeks to assess effects of diet and pravastatin on body composition and insulin sensitivity.

2.2.2. DXA scans: Animals were anaesthetised with an i.p. injection of 50 mg/kg ketamine (Ketamil, Troy Laboratories, Australia) and 3 mg/kg xylazine (Ilium xylazil-20, Troy Labrotories, Australia). DXA scans (XR-36 Quickscan densitometer, Norland Medical

Systems, Inc., USA) were performed at 8 and 16 weeks, with scans performed at a high resolution (1.5 x 1.5 mm, speed of 6 mm/s) in "small animal mode".

2.2.3. GTTs and lipid profiles: The GTTs were performed 3 days following DXA scanning to allow complete recovery from associated anaesthesia. Lipid profiles were determined using a blood-lipid analyser (L.D.X Cholestech Analyser, Alere™, Australia). Animals were fasted for 6 h with only access to water, anaesthetised with iso-fluourene (5%), and glucose was administered via i.p. injection at a dose of 2 g/kg/body weight. Blood glucose was monitored at 30 min intervals for 2 h (L.D.X Cholestech Analyser, Alere™, Australia).

2.3. Heart perfusions for assessing myocardial mechanical function and infarct responses

Rats were anesthetised (60 mg/kg sodium pentobarbital i.p.) and hearts removed and perfused in Langendorff mode as described in detail previously (Du Toit et al., 2005; Du Toit et al., 2008; Maarman et al., 2012). Hearts were excised and transferred to 4^{o}C perfusion fluid, the aorta was cannulated and coronary vessels perfused with modified Krebs- Henseleit buffer containing (in mM): 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 11 D-glucose, and 0.5 EDTA at a pressure of 100 cm H_2O. Perfusion fluid was saturated with 95% O_2-5% CO_2 at 37°C giving a pH of 7.4. A fluid-filled polyvinyl chloride balloon was inserted into the left ventricle and connected to a P23XL pressure transducer (Viggo-Spectramed, Oxnard, CA) for measurement of ventricular pressure. Balloon volume was adjusted to achieve an end-diastolic pressure of 4-6 mmHg. Functional data were recorded at 1 KHz on a 4-channel MacLab system (ADInstruments, Castle Hill, Australia) connected to an Apple iMac computer. Peak systolic, diastolic and developed pressures, heart rate, and rates of contraction or relaxation over time (dP/d*t*) were documented. Coronary flow was monitored via volumetric analysis of coronary effluent.

After balloon placement, hearts were immersed in a water-jacketed chamber maintained at 37°C. Perfusion fluid temperature was monitored using a thermistor probe (Model 52II, Fluke Corporation, Everett, Western Australia) in the coronary sinus. Hearts were excluded from study after a 15-min stabilization period if they failed to meet any of the following functional criteria: *i*) coronary flow >20 ml/min, *ii*) unstable (fluctuating) contractile function, *iii*) left ventricular systolic pressure <90 mmHg, or *iv*) significant cardiac arrhythmias.

After an initial 15 min normoxic stabilization period, hearts were paced at 300 beats/min (Grass S9 stimulator, Quincy, MA), normalizing rate to allow comparison of rate-dependent measures of contractile function (systolic and developed ventricular pressures) and inotropic state (dP/d*t*). Baseline measures were made after 10 and 20 min. The LAD was occluded for 40 min to induce regional ischaemia followed by 60 min of reperfusion achieved by ligature release. At the end of reperfusion the ligature was retied, the hearts stained with Evan's Blue dye and frozen overnight. Hearts were then sectioned and stained with TTC (Clark et al., 2011), to delineate viable and necrotic myocardium. Tissue areas (viable tissue, tissue at risk and necrotic tissue) were quantified by volumetric planimetry using a flat-bed scanner for image capture and computer software for image analysis (UTHSCSA Image Tool, V3). Infarct size is expressed as % of area at risk (AAR).

2.4. Blood biochemical analyses

Animals set aside for blood biochemical analyses and myocardial tissue collection were fasted for 6 h before anaesthesia, blood collection and heart excision. Epididymal, peritoneal and retro-peritoneal fat was removed and weighed.

For serum analysis, blood samples were placed in serum separation tubes (BD Vacutainer

tubes), centrifuged and serum stored at -80 °C for later analysis. Blood triglycerides, high density lipoprotein (HDL), cholesterol and glucose levels were determined in fresh whole-blood using a Blood-Lipid and Glucose Analyser (L.D.X. Cholestech Analyser, Alere™, Australia).

Levels of insulin (ALPCO Immunoassays™, Salem) and TBARS (R&D systems, Minneapolis, MN, USA.) were determined in 96-well enzyme-linked immunosorbant assay's (ELISAs) according to manufacturer instructions.

Coronary effluent LDH activity was determined spectrophotometrically, as described previously (Peart and Headrick, 2003). To assess insulin resistance in all animals the homeostasis model assessment (HOMA) index was measured. Fasting blood glucose and insulin levels were used to determine the HOMA index using the standard formula: [fasting insulin (μIU/ml) x fasting glucose (mmol/l)] / 22.5.

2.5. Myocardial enzyme activity assays

Glutathione peroxidase (GPx) activity was measured spectrophotometrically using a method described by Flohe and Gunzler (1984) and adapted to work in a 96-well microplate. Thioredoxin reductase (TrxR) activity was quantified using a Thioredoxin Reductase Colorimetric Assay Kit (Cayman Chemicals, Ann Arbor, Michigan, USA).

2.6. Western immunoblot analyses

2.6.1. Tissue extraction. Hearts were sectioned and homogenised in a glass dounce with 1.0 ml of ice-cold isolation buffer containing protease and phosphatase inhibitors (70 mM sucrose, 190 mM mannitol, 20 mM HEPES and 0.2 mM EDTA, 1 mM PMSF, 10 μM

leupeptin, 3 mM benzamidine, 5 μM pepstatin A, 1 mM NaO). Whole homogenate samples were removed and stored in lysis buffer for later analysis. Tissue homogenates underwent centrifugation at 600g (rcf) for 10 min at 4°C. The supernatant was removed and the nuclear pellet washed in isolation buffer containing protease and phosphatase inhibitors and spun at 600g for 10 min before being re-suspended in lysis buffer and stored. The supernatant containing mitochondria, cytosol and plasma membrane was centrifuged at 10,000g for 30 min. The mitochondria enriched pellet was washed in isolation buffer at 600g for 10 min and re-suspended in lysis buffer, with this and the non-mitochondrial fraction stored at -80°C until analysis.

Protein concentrations were determined using a BCA assay in a 96-well microplate (Pierce BCA protein assay kit), with absorbance measured at 540 nm (Tecan infinite M200 Pro, Mannedorf, Switzerland). Aliquots of 20 μg protein were prepared with appropriate volumes of Kinexus buffer and protease inhibitors (20 mM Mops, 2 mM EGTA; 5 mM EDTA; 30 mM sodium fluoride; 40 mM β-glycerophosphate; 20 mM sodium tetrapyrophosphate) and stored at -80°C until analysis.

2.6.2. Electrophoresis: Myocardial or cell fractions containing equal quantities of protein were loaded onto hand-cast 10% acrylamide gels and separated at 150 V for 80 min. Transfer of proteins was achieved using a polyvinylidene difluoride fluorescent (PVDF) membrane at a 350 mA current for ~2 h, and blocked with Odyssey fish serum for a further 2 h at room temperature. Transferred proteins were incubated with primary antibody (dilution 1:750-1:1000) for 15-18 h at 4°C with gentle rocking. The PVDF membrane was washed in TBS for 5 min and again in TBST (4 cycles of 5 min) before incubation with secondary antibody at room temperature in the dark. Membranes were subsequently visualised on a Licor Odyssey

Infrared Imaging System (Millennium Science, Mulgrave, Australia) with protein densitometry normalised to an internal standard and loading control.

Primary antibodies that were used included: Mfn-1, Mfn-2, OPA-1, DRP-1 and Bax (Abcam, Cambridge, MA), and Bcl-2 and total and cleaved caspase-3 (Cell Signalling Technology Inc., Danvers, MA, USA).

2.7. Statistical analyses

Unless otherwise stated, all results are expressed as means ± S.E.M. Differences between two or more groups were tested via 1- or 2-way ANOVA, with a Newman-Keuls post-hoc test applied when significant effects were detected. Significant differences were accepted for $P<0.05$.

3. Results

3.1. Effects of diet and pravastatin on body weight, body composition and cardiometabolic risk factors

The HCFD significantly increased both body mass (655±12g for C vs. 736±15g for HCFD, $P<0.001$) and fat mass (153±21g for C vs. 310±10g for HCFD, $P<0.0001$), with prava preventing the body mass change in HCFD animals (Table 1 and Fig. 1). The HCFD increased serum triglycerides and HOMA-IR (Table 2 and Fig. 2), and prava treatment prevented these increases. Blood glucose levels were unchanged and similar in all 4 groups. Plasma TBARS, reflecting systemic lipid peroxidation, were unaltered by the HCFD, and selectively increased by prava treatment in HCFD animals (Fig. 3).

3.2. Effects of diet and pravastatin on myocardial function and reperfusion LDH release and infarct size

Neither diet nor pravastatin treatment influenced myocardial basal or post-ischaemic mechanical function. Post-ischaemic myocardial LDH release was similar for all groups (data not presented), however infarct size was increased in HCFD animals. Despite improving myocardial cardiometabolic risk factors (triglyceride levels and HOMA-IR) in HCFD animals, prava had no effects on myocardial infarction: infarct size was similar in the prava treated control and HCFD fed animals (Fig. 4).

3.3. Effects of diet and pravastatin on myocardial GPX and TrxR activities

The HCFD had no effect on myocardial glutathione peroxidase activity but decreased thioredoxin reductase activity compared to the control diet fed animals (Fig. 5A and 5B). Prava reduced cardiac glutathione peroxidase activity in HCFD animals (Fig. 5A) but had no effect on thioredoxin reductase activity.

3.4. Effects of diet and pravastatin on myocardial expression of proteins regulating mitochondrial fission/fusion and apoptosis

The HCFD decreased mitochondrial MFN-1 and increased mitochondrial DRP-1 expression without effecting MFN-2, OPA-1 or cytosolic DRP-1 levels (Fig. 6A-E). Prava decreased mitochondrial MFN-1 expression in control diet animals without modifying MFN-2 or OPA-1 (Fig. 6A, Fig. 6B and Fig. 6C). However, prava was without effect on these proteins in HCFD rats. Mitochondrial DRP-1 expression was insensitive to prava in control animals while levels in HCFD fed rats were reduced by treatment (Fig. 6D). Prava had no effect on cytosolic DRP-1 expression in either the control or HCFD fed animals (Fig. 6E). Overall, the

HCFD promotes pro-fusion changes (decreased ratio of MFN1:DRP1) (Fig. 6F) that are countered by prava treatment.

In terms of apoptotic proteins, while there was a tendency to increased mitochondrial Bax, the HCFD had no significant effects on cardiac Bax, Bcl2, the Bax:Bcl2 ratio or caspase-3 levels (Fig. 7A-E). Nonetheless, prava treatment significantly decreased mitochondrial Bax expression in HCFD animals (Fig. 7A), and cytosolic Bax (Fig. 7B) and caspase-3 levels (Fig. 7E) in both control and HCFD animals. Prava treatment had no significant effect on cleaved caspase-3 expression in either control or HCFD animals (Fig. 7F). The mitochondrial Bax:Bcl-2 ratio was unaltered by diet or prava (Fig. 7D). Thus, HCFD does not appear to induce pro-apoptotic protein changes, while prava generally reduces pro-apoptotic proteins in hearts from both lean and obese groups.

4. Discussion

In this study we demonstrate that a HCFD increases body weight, serum triglycerides and insulin-resistance, and worsens myocardial infarct tolerance without influencing baseline cardiac function. This effect is associated with reduced myocardial TrxR (not GPX) activity, and reduced mitochondrial MFN-1 vs. increased DRP-1 expression, without consistent changes in apoptosis proteins. While prava treatment prevented key risk factor changes (increased weight, adiposity, triglycerides, HOMA-IR) in HCFD animals, the drug had no effect on myocardial infarct size. This may reflect complex pleiotropic effects of prava on anti-oxidant, mitochondrial fusion:fusion and apoptosis proteins: prava decreased GPX activity in HCFD hearts, reduced mitochondrial MFN1 levels in control but not HCFD hearts while selectively countering the HCFD-dependent increase in mitochondrial DRP-1, and

reduced cytosolic Bax and caspase-3 levels in both groups while reducing mitochondrial Bax specifically in HCFD hearts.

4.1. Effects of the HCFD and pravastatin on morphometric and cardiometabolic risk factors

4.1.1. Body weight and body composition: Effects of a HCFD on body weight and composition in rodents are well characterized (Arkari et al., 2008; Wensley et al, 2013; Salie et al., 2014), confirmed here with significantly increased body and body fat mass. The increased body weight was primarily due to increased fat mass, with DXA scans revealing significantly increased fat mass and marginally decreased lean mass in HCFD animals. Effects of statins on body weight remain controversial, with studies reporting either increased (Kang et al, 2009), unaltered (Yamada et al., 2017; Sun et al., 2017) or decreased (Araki et al., 2008; Fraulob et al., 2012; Zhang et al., 2017; Cui et al., 2017) weight with statin therapy. Here prava treatment did attenuate the HCFD-induced increase in body weight. This corroborates observations of Dalaklioglu and co-workers (2013) who documented slight body weight reduction with prava treatment in rats. To our knowledge no other animal studies have reported on the body weight effects of chronic prava treatment. While physical activity was not measured, changes are unlikely to contribute to (indeed may act to counter) this weight loss effect given evdence of statin-dependent reductions in physcial activity and exercise capacity in both humans and rodents (Meador and Huey, 2010; Bouitbir et al., 2011; Lee et al., 2014).

4.1.2. Blood lipids and insulin sensitivity: Dietary obesity was accompanied by anticipated increases in circulating triglycerides and HOMA-IR values. These data corroborate our previous findings (Du, Toit et al 2008; Wensley et al 2013) and those of others (Araki et al., 2008; Lalli et al., 2008). Pravastatin treatment decreased triglyceride levels in HCFD obese but not control lean rats. This triglyceride lowering effect is consistent with reported efficacy

of prava in decreasing triglycerides in obese (Araki et al., 2008) and hypercholesterolaemic mice (Lorza-Gil et al., 2016), and in clinical trials in dyslipidaemic patients (Barakat et al., 2013 Schweitzer et al 2002; Baker et al., 2010).

Dyslipidaemia is implicated in the aetiology of insulin-resistance and diabetes (Eckel, et al., 2010 Yoon et al., 2016, Chrysant, 2017). Management is thus predicted to improve insulin sensitivity and prevent or delay onset of frank diabetes. Paradoxically, emerging evidence suggests lipid lowering statins may actually exacerbate insulin-resistance and accelerate onset of diabetes (Preiss et al. 2012; Muscogiuri et al., 2014; Bellia et al., 2012; Sato et al., 2012). Nonetheless, data regarding effects of statins on insulin sensitivity are controversial. One study found that while atorvastatin increased HOMA-IR and fasting insulin in rats, pravastatin had no effect on these parameters (Beltowski et al., 2011). Two other animal studies demonstrated that lovastatin improved insulin sensitivity in high-fat fed rats (Lalli et al., 2008) and rosuvastatin had similar effects in high-fat fed mice (Fraulob et al., 2012).

Several clinical trials have shown prava can improve lipid profiles and insulin sensitivity. Pravastatin improved lipid profiles and insulin sensitivity in dyslipidaemic, insulin insensitive patients (Sugiyama et al., 2007; Koh et al., 2013) while pravastatin/valsartan combination therapy had additive effects and improved these parameters above values observed with monotherapy (Koh et al., 2013).

4.2. Effects of the HCFD and pravastatin on myocardial infarct size

Ourselves (Du Toit et al., 2008; Wensley et al., 2013, Donner et al., 2016) and others (Huisamen et al., 2011) present evidence that diet-induced obesity with insulin-resistance decreases myocardial tolerance to ischaemia. This is confirmed here, with infarct size increased in obese, insulin-resistant rats when compared with control littermates (Fig. 4). Chronic prava treatment, however, had no effect on infarct size in lean or obese HCFD

animals, despite other benefits. This agrees with an early study of the effects of chronic statin therapy on post ischaemic functional outcomes in pigs, which found no benefit with 3 weeks of pre-ischaemic treatment with either simvastatin, atorvastatin or pravastatin (Rendig et al., 2003). In contrast, several studies exploring the effects of acute or short-term (minutes to days) statin treatment support reductions in myocardial I/R injury. A 3 day atorvastatin treatment regime or bolus injection 15 min before ischaemia reportedly decreases myocardial infarct size in rats (Ye et al., 2006; Ye et al 2008), and simvastatin administered 20 min prior to ischaemia decreases infarct size in rabbits (Bao et al., 2009) and mice (Andres et al., 2014). The acuteness of benefit in these studies further highlights the pleiotropic basis of cardiac protection. Data from clinical studies are less consistent, with a study by Post *et al.* (2012) suggesting that 1 week of atorvastatin treatment prior to percutaneous coronary intervention had no impact on infarct size, while another study reported decreased troponin I levels in unstable angina patients receiving long-term high-dose statin therapy (Gordin et al., 2012). Chronic statin therapy is associated with a reduction in major adverse cardiac events in myocardial infarct patients (Piao et al., 2017), consistent with its risk reduction effects, however treatment also improved post-infarct mechanical function, suggesting potentially direct myocardial effects (Auscher et al., 2017).

4.3. Effects of the HCFD and pravastatin on anti-oxidant enzyme activities

The effects of obesity on anti-oxidant enzyme activity are well characterized (Utus and Picklo, 2011; Panday et al., 2015). Here we found there was a marginal decline in myocardial GPX activity and significant decrease in thioredoxin reductase activity in HCFD fed animals. A reduction in thioredoxin reductase activity has been recently reported in HCFD fed rats (Shrivastava et al., 2013). However, effects of statins on oxidative stress and anti-oxidant enzyme activity are more controversial. Here, prava decreased myocardial GPX activity in HCFD animals, consistent with reduced serum GPX activity in coronary artery

disease patients treated with statins (Palazhy et al. 2015). However, an earlier study found no effect of prava on plasma GPX activity in hypercholesterolaemic patients (Chen et al., 1997). These observations are challenged by a study showing that rosuvastatin increases GPX activity in obese rats fed a high fat diet (Ansari et al., 2012).

The reduction in GPX activity in the HCHF+Prava animals here was also accompanied by an increase in serum TBARS. Since TBARS are a measure of free radical induced lipid peroxidation there appears a clear link between reduced anti-oxidant enzyme activity and increased lipid peroxidation in obese prava treated animals. Increased oxidative stress, in turn, is a well established driver of apoptosis (Abdel-Hamidand Firgany, 2015; Manickam et al., 2017). In the current study this reduction in GPX activity in the HCHF+Prava group may contribute to increased apoptosis.

4.4. Effects of the HCFD and pravastatin on mitochondrial fission/fusion and apoptosis proteins

4.4.1. Effects of the HCFD: Shifts in autophagy and apoptosis on transition from obesity to the metabolic syndrome have been characterised (Li et al., 2012), whereas effects of dietary obesity on mitochondrial fission and fusion are less well researched. We demonstrate that a HCFD that induces significant obesity and insulin-resistance decreases pro-fusion MFN-1 and increases pro-fission DRP-1 in cardiac mitochondria without modifying MFN-2 or OPA-1 levels. These differential changes in mitochondrial DRP-1 *vs.* MFN1 are predicted to favour fission, in turn promoting post-ischaemic cell death (Calo et al., 2013). This effect is thought to involve shifts in Bcl2 proteins (Aouacheria et al., 2017), and the HCFD did increase mitochondrial Bax levels, although Bcl-2, the Bax:Bcl-2 ratio and cytosolic levels of total and cleaved caspase-3 were unchanged. These observations contrast reports of increased

apoptosis and pro-apoptotic proteins in porcine (Li et al., 2012) and rodent (Ballal et al., 2010) models of obesity and metabolic syndrome.

The effects of HCFD and obesity on mitochondrial dynamics and the proteins regulating this process are controversial. Decreased mitochondrial levels of pro-fusion MFN-1 and increased levels of pro-fission DRP-1 in HCFD hearts here are consistent with the findings of Jheng and co-workers who report palmitate-induced increases in DRP-1 expression, mitochondrial fragmentation and insulin-resistance in C2Cl2 muscle cells (Jheng et al., 2012), together with increased skeletal muscle DRP-1 and fission-dependent insulin-resistance in *ob/ob* mice (Jheng et al., 2012). Other work shows that in utero and post-weaning exposure of rats to an obesogenic HCFD reduces Mfn1/2 and OPA-1 mRNA levels in muscle and liver (Borengasser et al., 2014), and that repression of Mfn-2 promotes mitochondrial fragmentation and inhibits glucose oxidation and respiration in humans (Bach et al., 2003). Obesity has been shown to reduce Mfn-2 expression, potentially contributing to disrupted mitochondrial dynamics (Bach et al., 2003).

4.4.2. Effects of Pravastatin: The effects of statins on myocyte structure and function are not well defined, though a range of pleiotropic cardiovascular actions have been revealed in recent years (Oesterle et al., 2017). Here prava improved body weight, circulating lipids and insulin sensitivity without influencing myocardial infarct size or LDH release. This may reflect complex pleiotropic effects of the drug in myocardium. Pravastatin decreased mitochondrial MFN1 in control but not HCFD hearts, while decreasing DRP-1 expression in HCFD but not control hearts without modifying MFN2 or OPA-1 expression (ie. decreased MFN1:DRP-1 ratio in control diet *vs*. increased ratio in HCFD hearts). Chronic repression of DRP-1 expression may promotes fission and mitophagy, promoting mitochondrial depletion and cardiomyopathy (Song et al., 2015). In contrast, acute repression of DRP-1 during an I/R event may be protective (Jin et al., 2018). While cardiac function was unaltered after 8 week

prava treatment, we cannot exclude the possibility that prava promoted fission and mitophagy in our study. The absence of an improvement in infarct size with prava, despite repression of DRP-1 expression, suggest that chronic prava (with potential mitochondrial depletion) may counter possible cardioprotective effects of DRP-1 repression during I/R.

Changes in apoptotic proteins were similarly complex: prava selectively decreased mitochondrial levels of pro-apoptotic Bax and anti-apoptotic Bcl2 in HCFD but not control hearts, and repressed cytosolic levels of Bax and total (but not cleaved) caspase-3 in both groups. These changes in pro- and anti-apoptotic proteins are difficult to interpret in terms of predicting effects on cell death, with little evidence of an overt pro- or anti-apoptotic effect of prava at the regulatory protein level. Shifts in Bax and Bcl2 are balanced such that the Bax:Bcl2 ratio is unaltered, and reduced total caspase 3 without a change in cleaved caspase-3 levels suggests prava may decrease apoptotic 'potential' (ie. the caspase-3 pool available for cleavage) without directly influencing execution of caspase-3 dependent apoptosis. Potential effects on cell death are additionally complicated by reduced GPX expression with prava in HCFD hearts, and differential changes in fusion:fission proteins, which may influence the balance of cell survival vs. death. Interestingly, early studies of the cardioprotective effects of statins suggest that acute simvastatin may limit I/R injury via up-regulating mitophagy (Andres et al., 2014). Other work suggests that DRP-1 mediates protective mitophagy during metabolic insult such as I/R (Ikeda et al., 2015). Nonetheless, considerable attention has focused on DRP-1 inhibition in cardioprotection (Mukherjee et al., 2015), despite important questions and potential limitations regarding such a strategy (Dong et al., 2016; Rosdah et al., 2016).

5. Conclusions

The current study indicates that despite improvements in key systemic risk factors (including body weight, insulin-resistance and triglyceride levels), prava treatment failed to limit the exaggeration of myocardial infarction observed in dietary obesity with insulin-resistance. This increase in infarction in HCFD is consistent with impaired anti-oxidant activity, increased mitochondrial Bax, and increased fission (DRP-1) vs. fusion (MFN1) protein expression. The lack of a cardioprotective effect of prava may reflect diverse pleiotropic effects of the statin, which induced opposing changes in proteins governing apoptosis, fusion and fission, while suppressing cardiac GPX activity. The basis and impacts of these distinct molecular changes, which highlight the complexities of statin effects in healthy and diseased hearts, warrant further study.

Acknowledgements and disclosures

This study was supported by funds from the Griffith University Research Grant scheme and the Griffith Health Institute. We wish to thank Owain Evans for his technical assistance with the TrxR and GPX activity assays.

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

Conceived and designed experiments: EFDT, DD.

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Figure legends

Fig. 1. Body mass distribution in animals from the four groups as determined using DXA scans. Closed bars represent lean mass and open bars represent fat mass.

n=10-15

*< P,0.05

Fig. 2. Homeostasis model assessment for insulin resistance (HOMA-IR) values for control and HCFD fed animals treated with vehicle or pravastatin.

n=6

*< P,0.05

Fig. 3. Thiobarbituric Acid Reactive Substances (TBARS) levels in serum from the four groups of animals.

n=8

*P<0.05

Fig. 4. Myocardial infarct size as a percentage of the area at risk for control, HCFD, C+Prava and HCFD+Prava animals.

n=8-10

* P<0.05

Fig. 5 A and 5B. A. Myocardial glutathione peroxidase activity in hearts from control and HCFD fed animals treated with vehicle or pravastatin. B. Myocardial thioredoxin reductase activity in hearts from control and HCFD fed animals treated with vehicle or pravastatin.

n=5

*P<0.05

Fig. 6A-F. Expression of proteins implicated in mitochondrial fission/fusion. **A.** Mitochondrial MFN-1 expression from control and HCFD fed animals treated with vehicle or pravastatin. **B.** Mitochondrial MFN-2 expression from control and HCFD fed animals treated with vehicle or pravastatin. **C.** Mitochondrial OPA-1 expression from control and HCFD fed animals treated with vehicle or pravastatin. **D.** Mitochondrial DRP-1 expression from control and HCFD fed animals treated with vehicle or pravastatin. E. Cytosolic DRP-1 expression

from control and HCFD fed animals treated with vehicle or pravastatin. F. Mitochondrial MFN-1:DRP-1 ratio in these hearts.

n=4-6

*P<0.05

Fig. 7 A-F. Expression of pro- and anti-apoptitic proteins in the hearts of animals from the control, HCFD, C+Prava, HCFD and HCFD+Prava groups. **A.** Mitochondrial Bax expression, **B.** cytosolic Bax expression, **C.** mitochondrial Bcl-2 expression, **D.** Mitochondrial Bax:Bcl-2 ratio, **E.** total cytosolic caspase-3 expression and F. Cytosolic cleaved caspase -3 expression.

n=4-6

*P<0.05

	Body Composition		
	Body Weight (g)	**Visceral Fat Weight (g)**	**(Visceral Fat/Body Weight)*100%**
Groups			
C	655±12	40.6±3.1	6.15±0.3
C+Prava	616±17	35.3±1.3	5.70±0.2
HCFD	736±15[b] (+ 12%)	63.9±3.5[c] (+57%)	8.64±0.4[b] (+40%)
HCFD+Prava	696±11 (+13%)*	58.8±3.5 (+66%)	8.42±0.4 (+48%)

Table 1. Body weight, visceral fat and visceral fat as a percentage of body weight for the four groups of animals after 16 weeks.

Value in brackets represent the % change for the HCHF group compared to control group, and the HCHF+Prava group compared to the C+Prava group.

n=11-18

[a] P<0.05 for HCFD vs. HCFD + Prava

[b] P<0.001 for C vs HCFD

[c] P<0.0001 for C vs. HCFD

Groups		**Serum Lipid Levels**	
	Total Cholesterol (mmol/L)	**HDL (mmol/L)**	**Triglycerides (mmol/L)**
C	> 2.59	0.78±0.07	1.64±0.26
C + Prava	> 2.59	0.98±0.18	1.36±0.165
HCFD	> 2.59	0.69±0.06	2.91±0.52[a]
HCFD + Prava	> 2.59	0.59±0.09	1.68±0.12[b]

Table 2. Serum lipid levels of the four groups of animals after 16 weeks.

n=5-7

[a] P<0.05 for C vs. HCFD

[b] P<0.05 for HCFD vs. HCFD+Prava

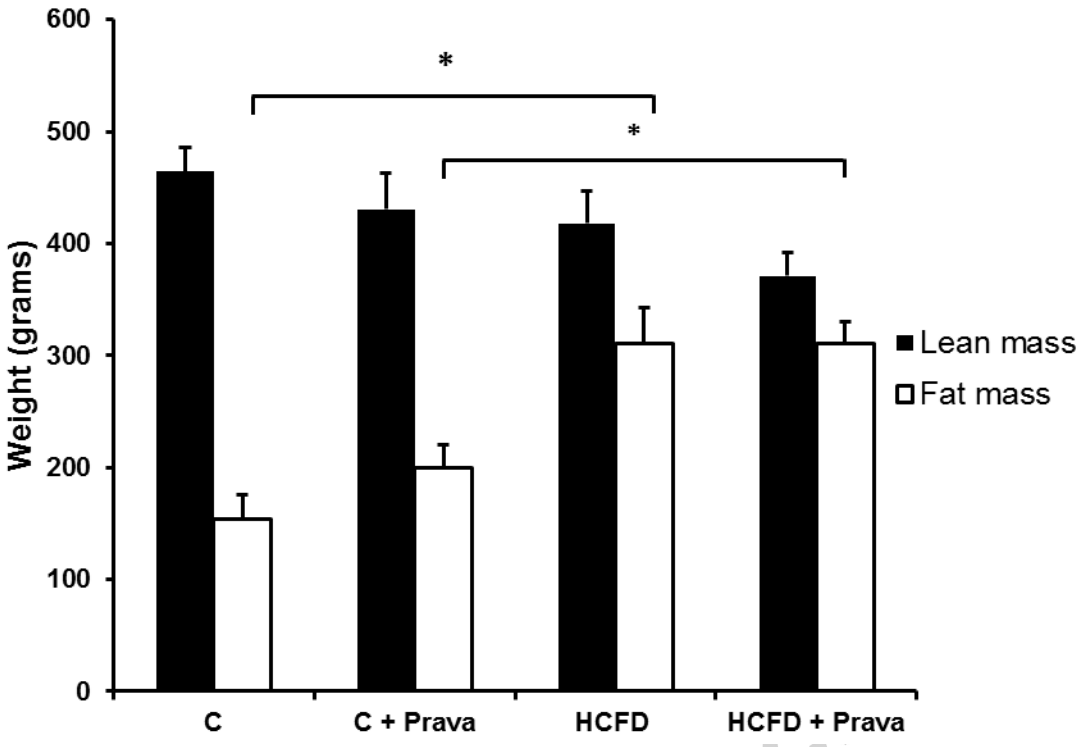


Fig 1.

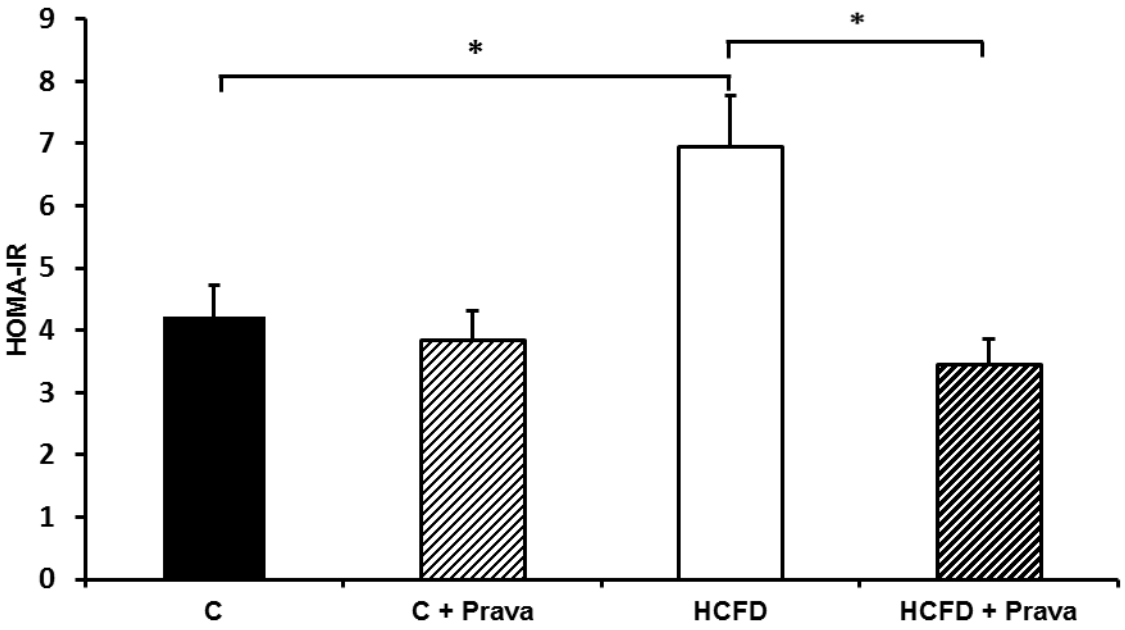
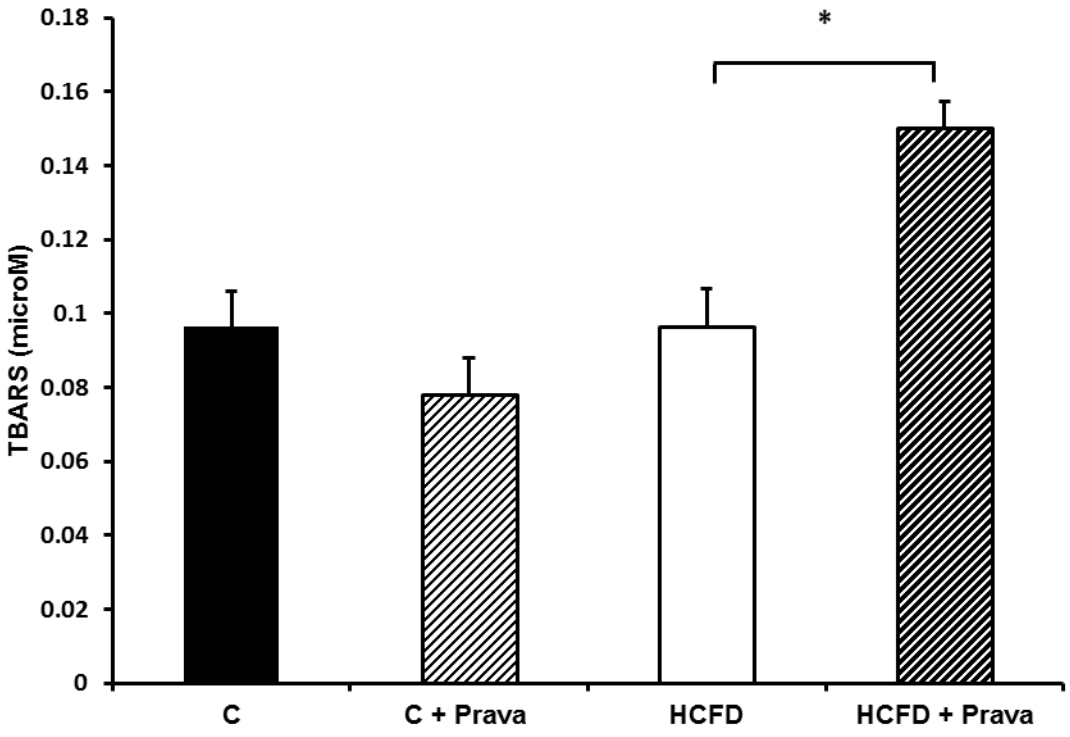


Fig 2.



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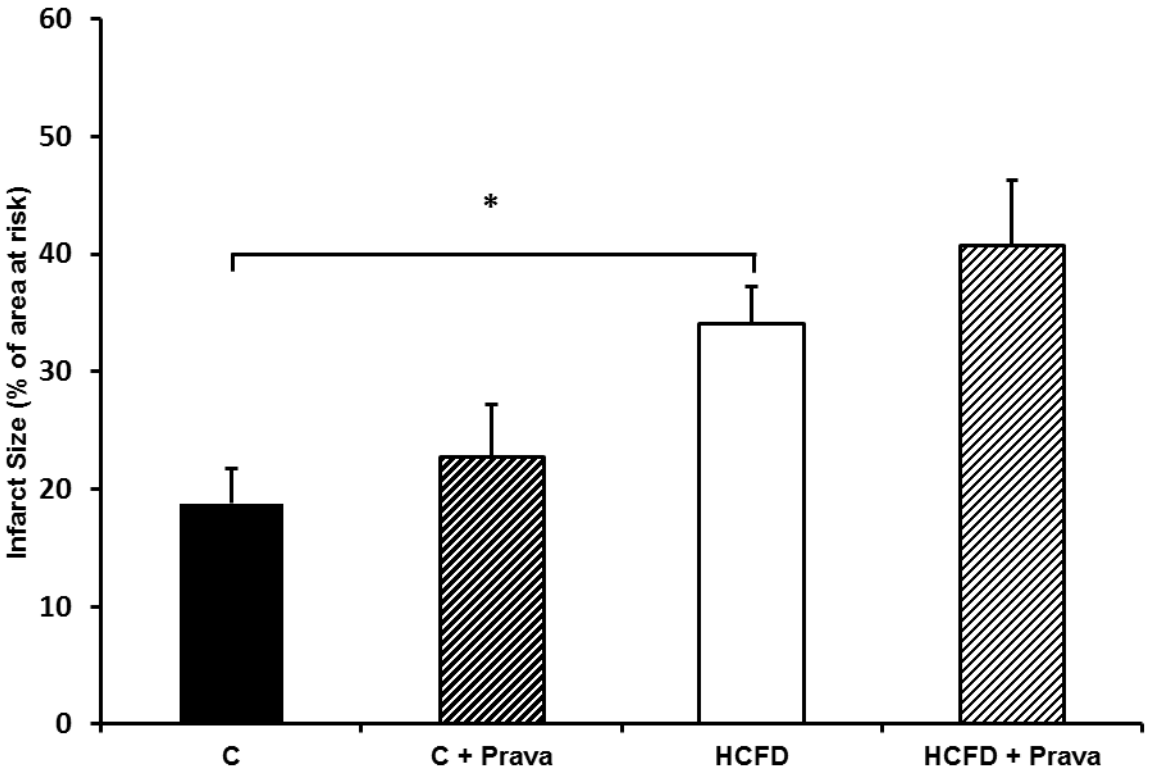

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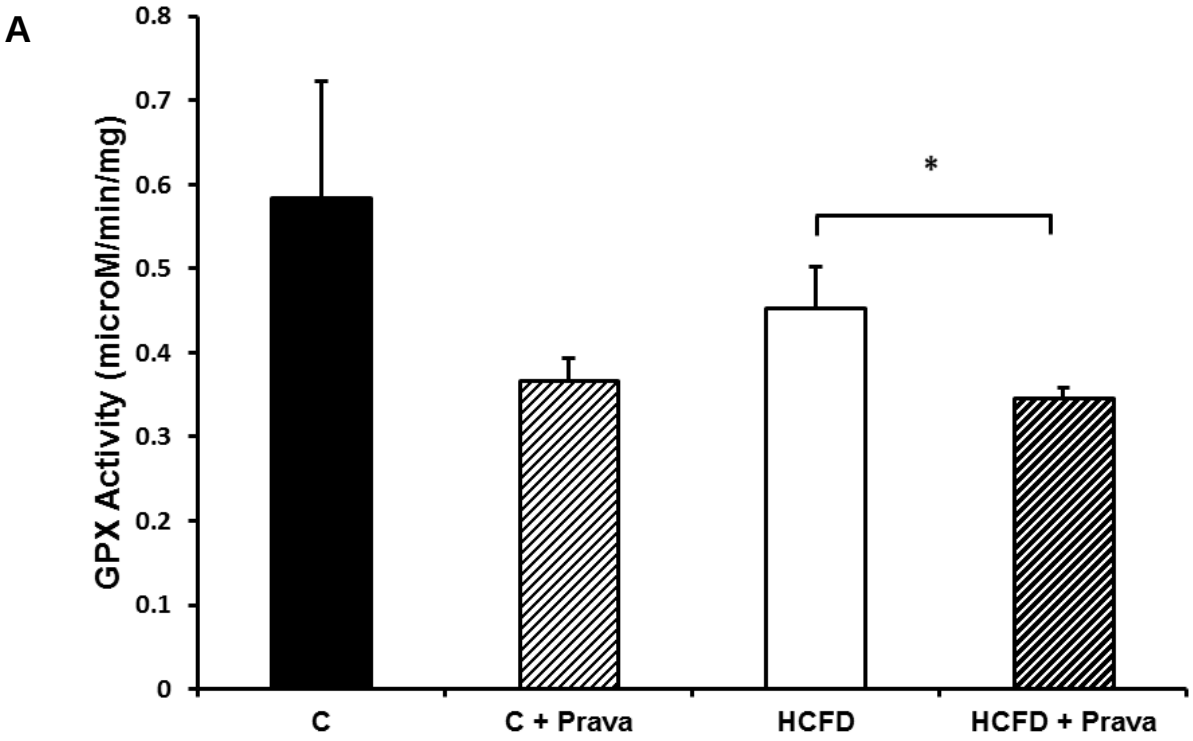

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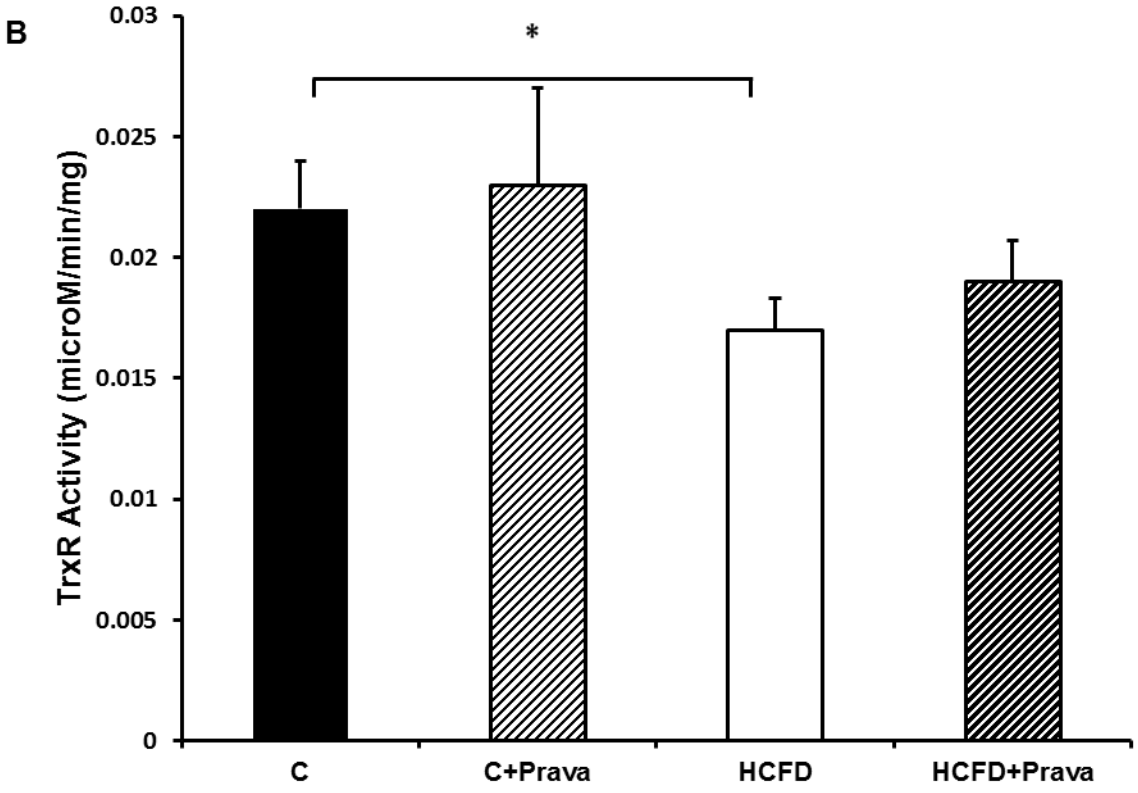

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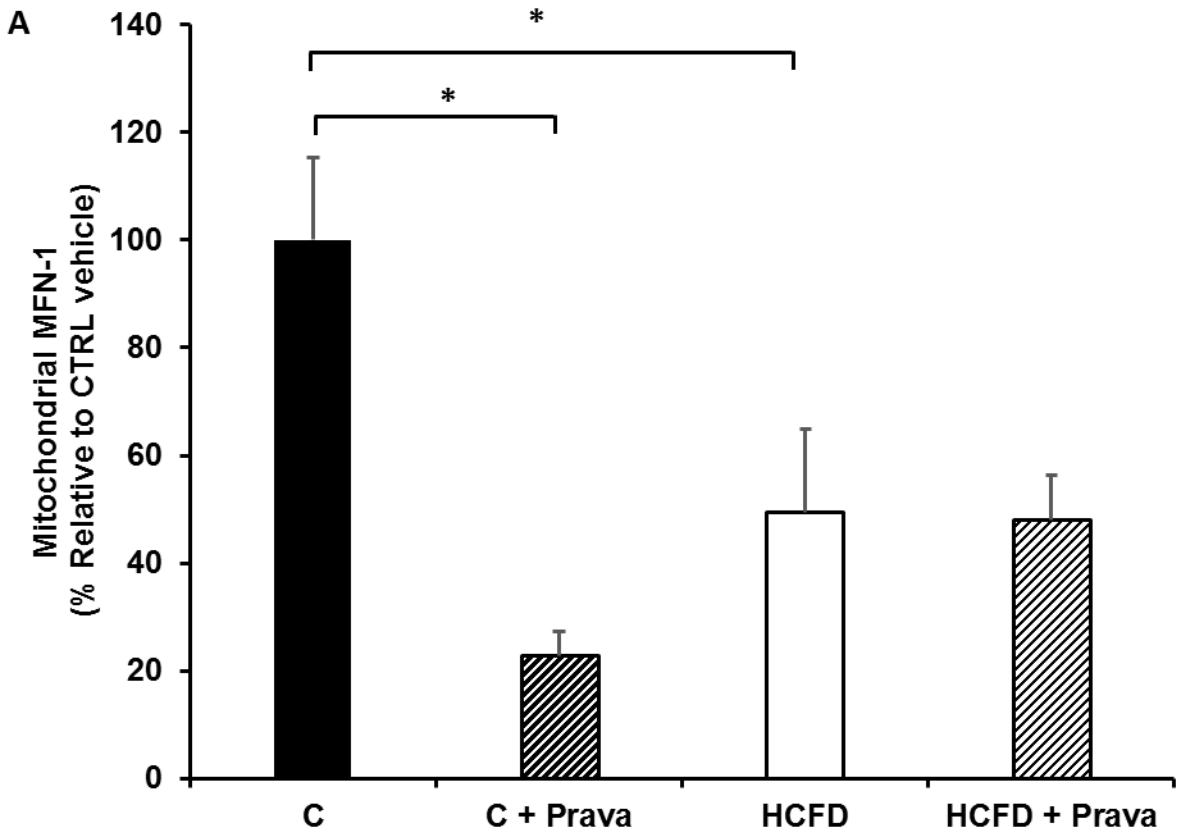

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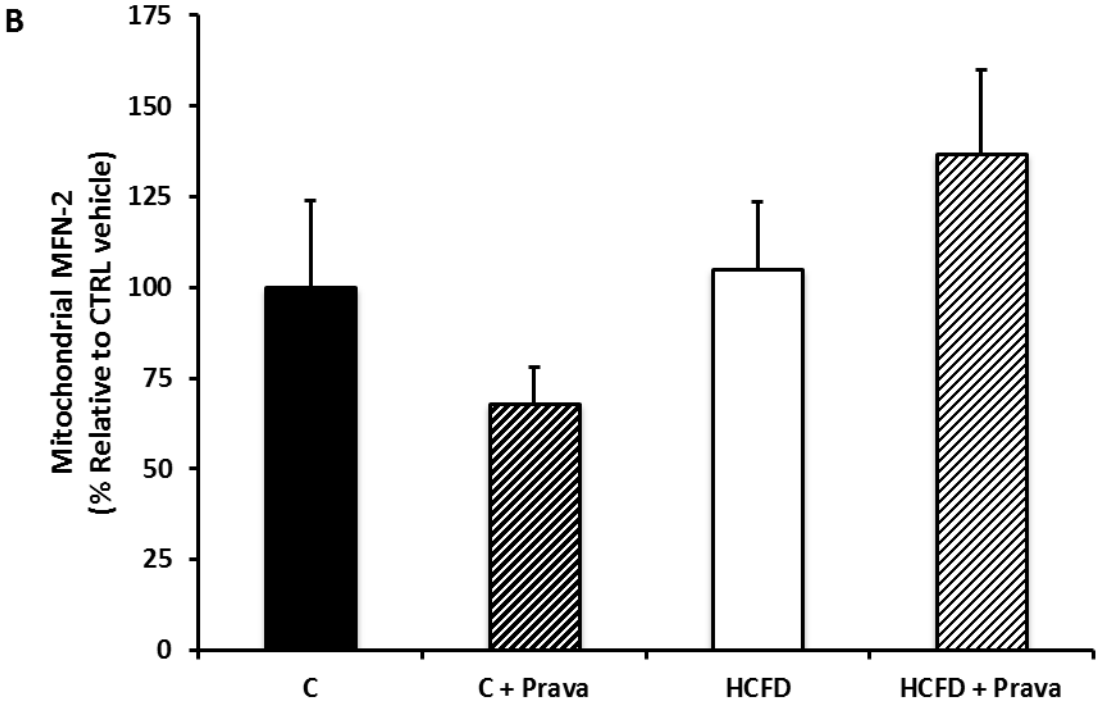

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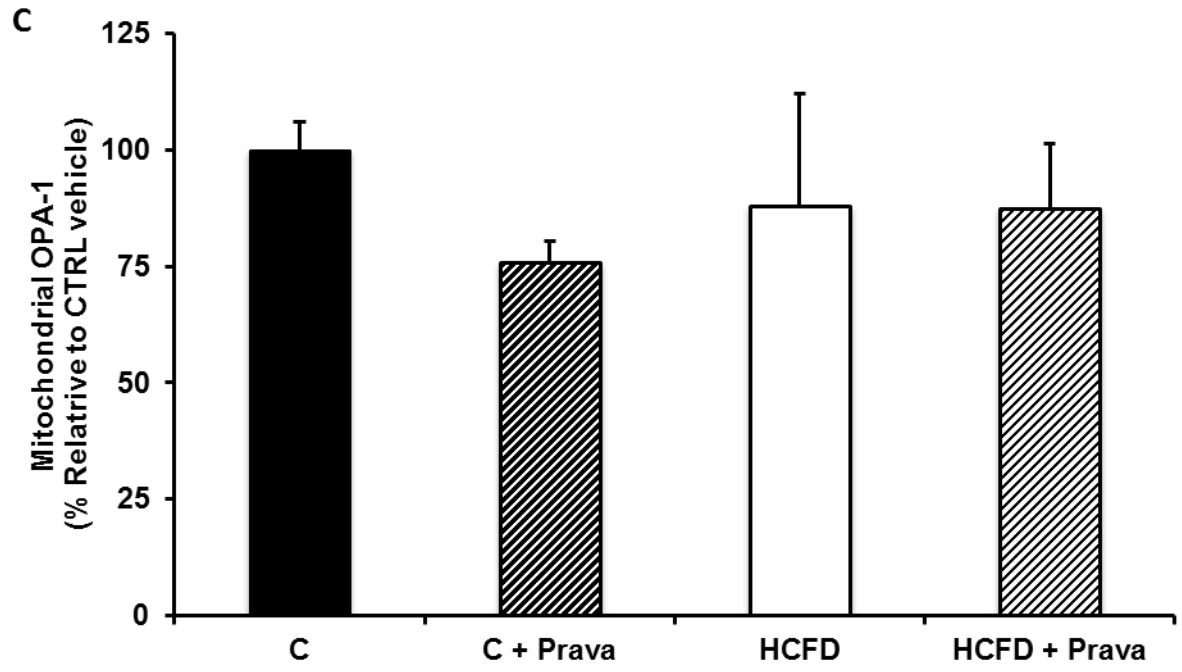

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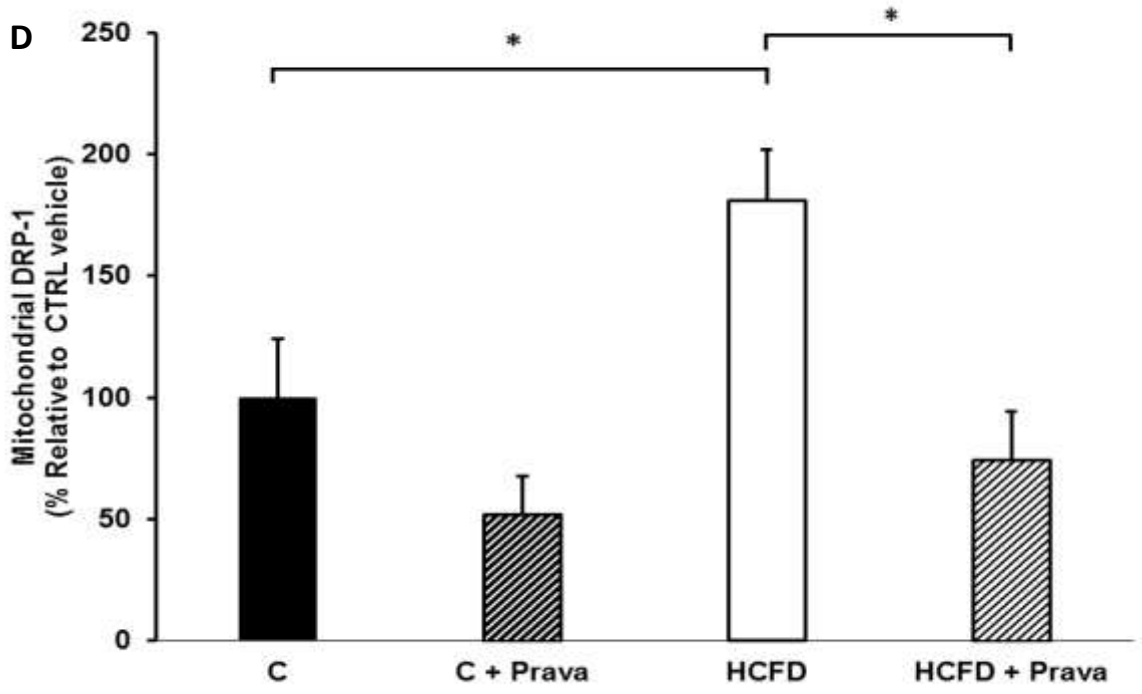

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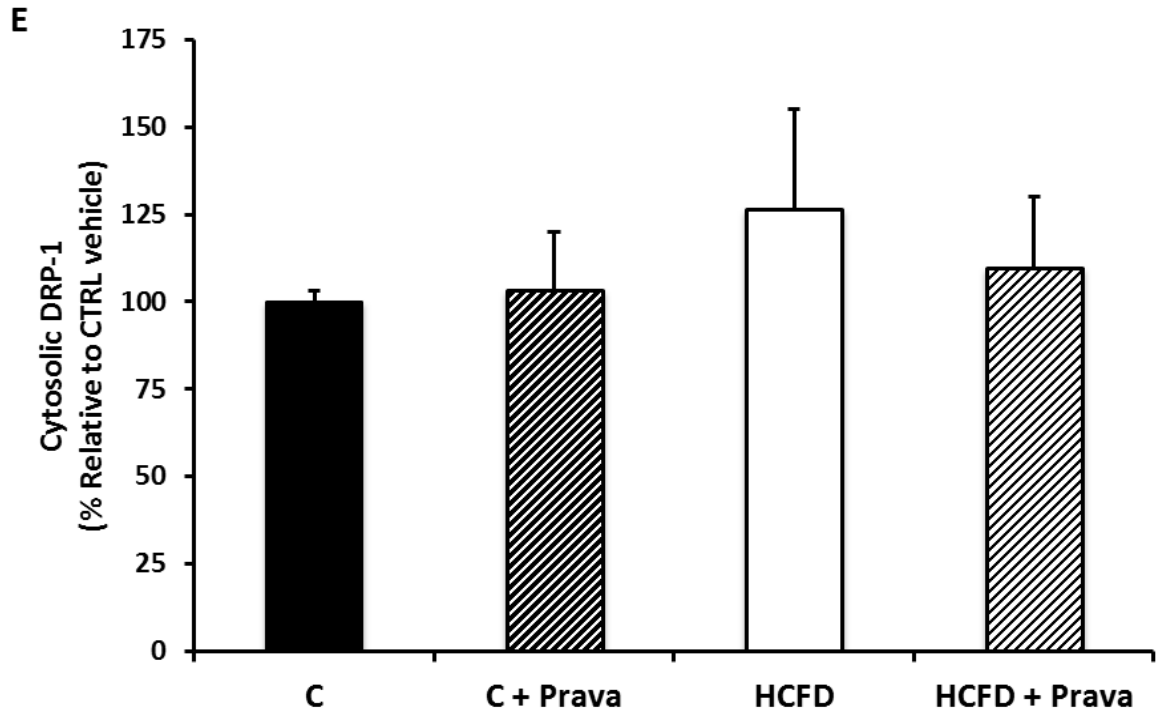

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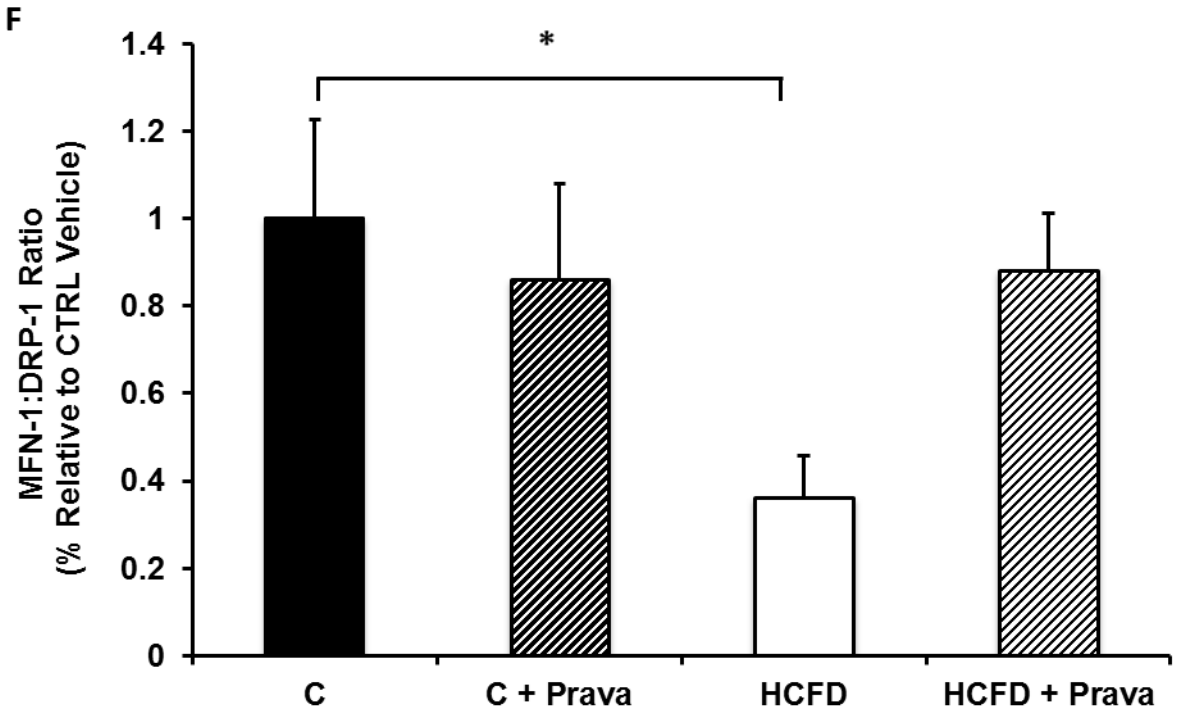

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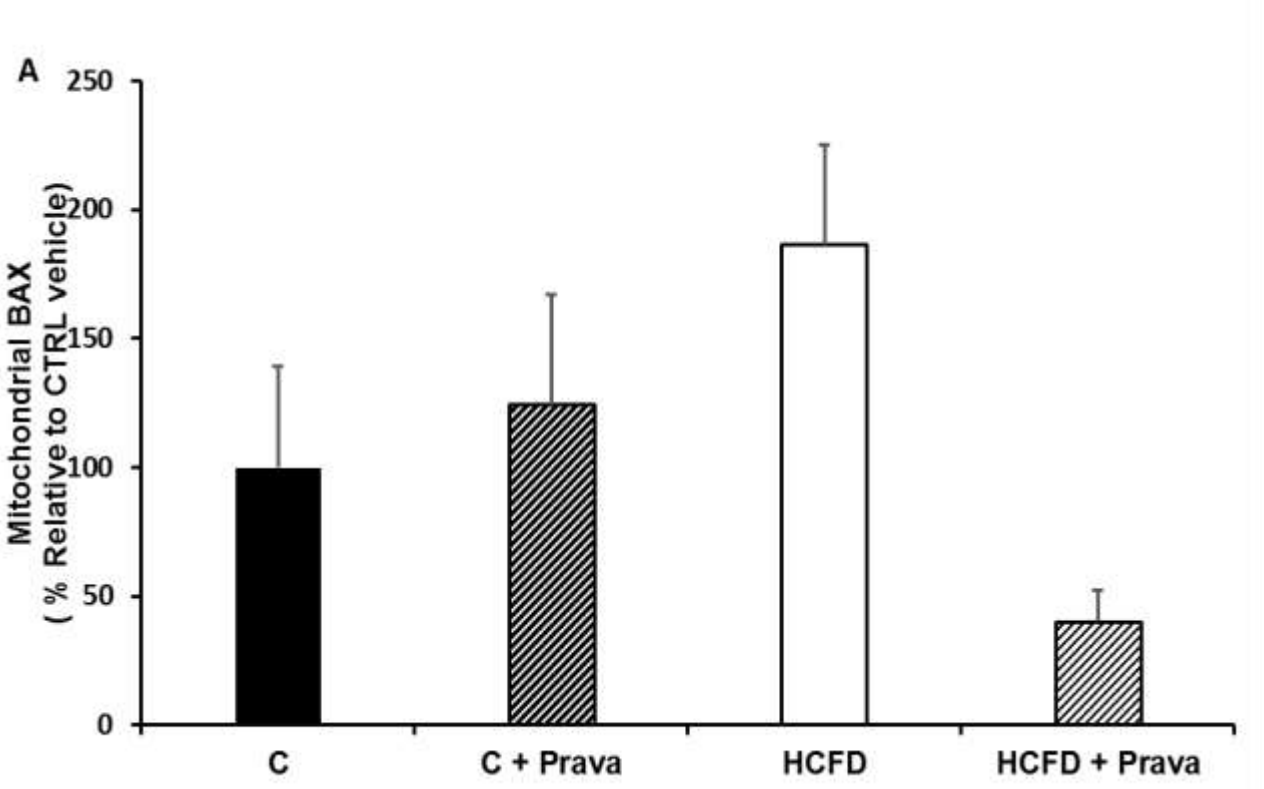

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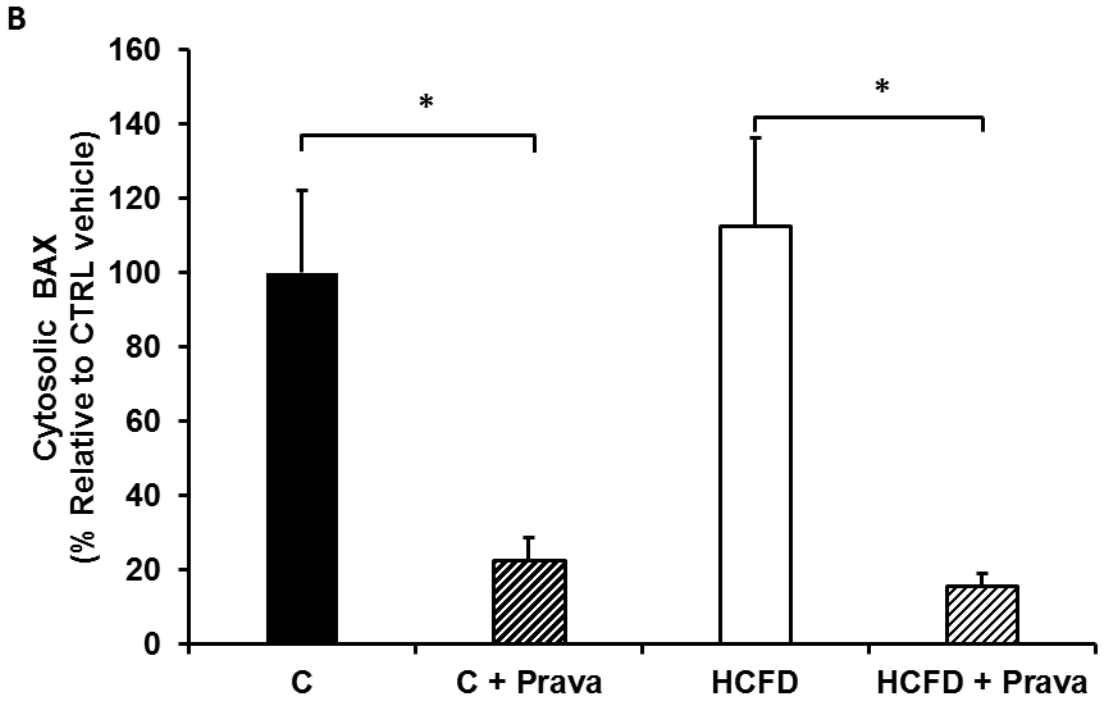

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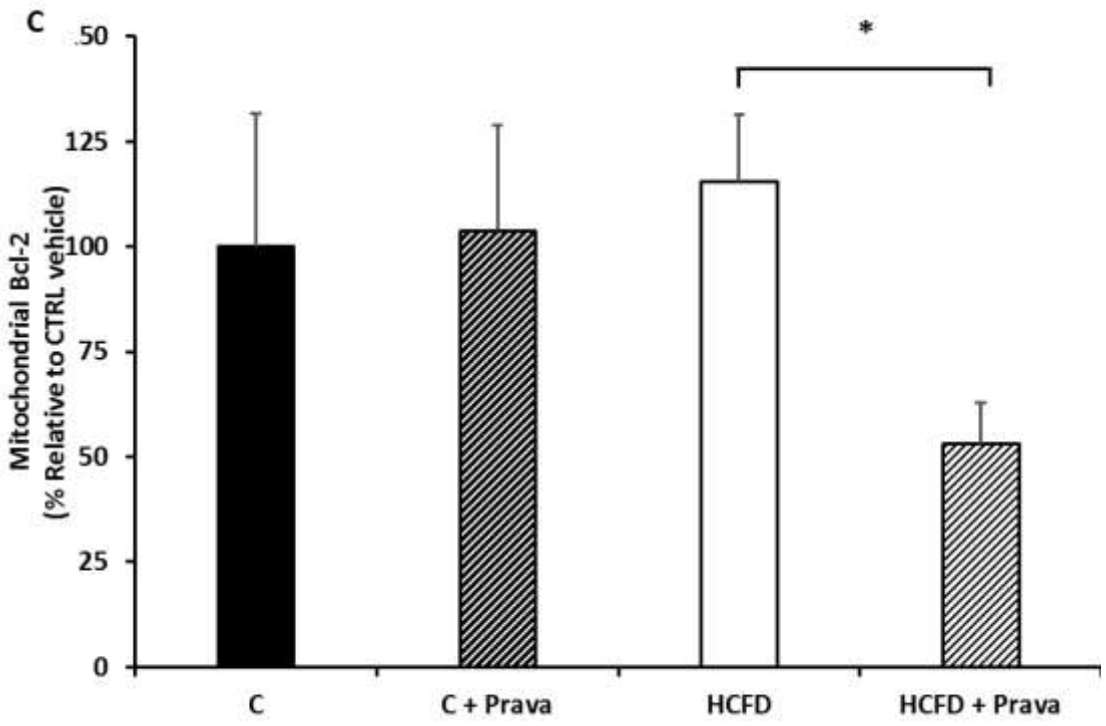

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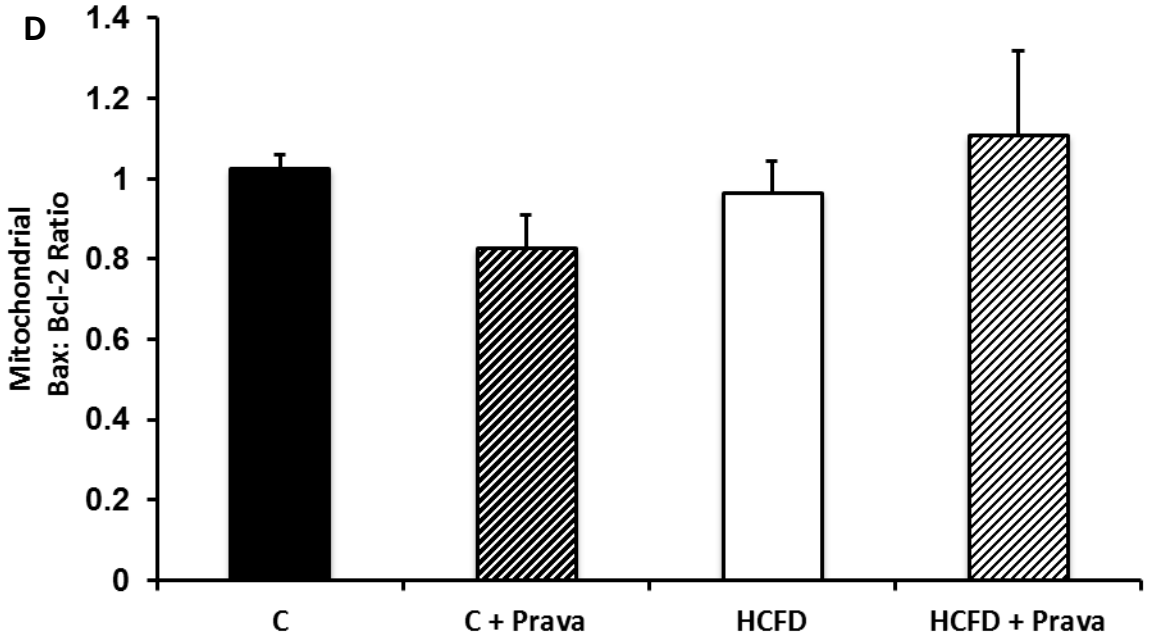

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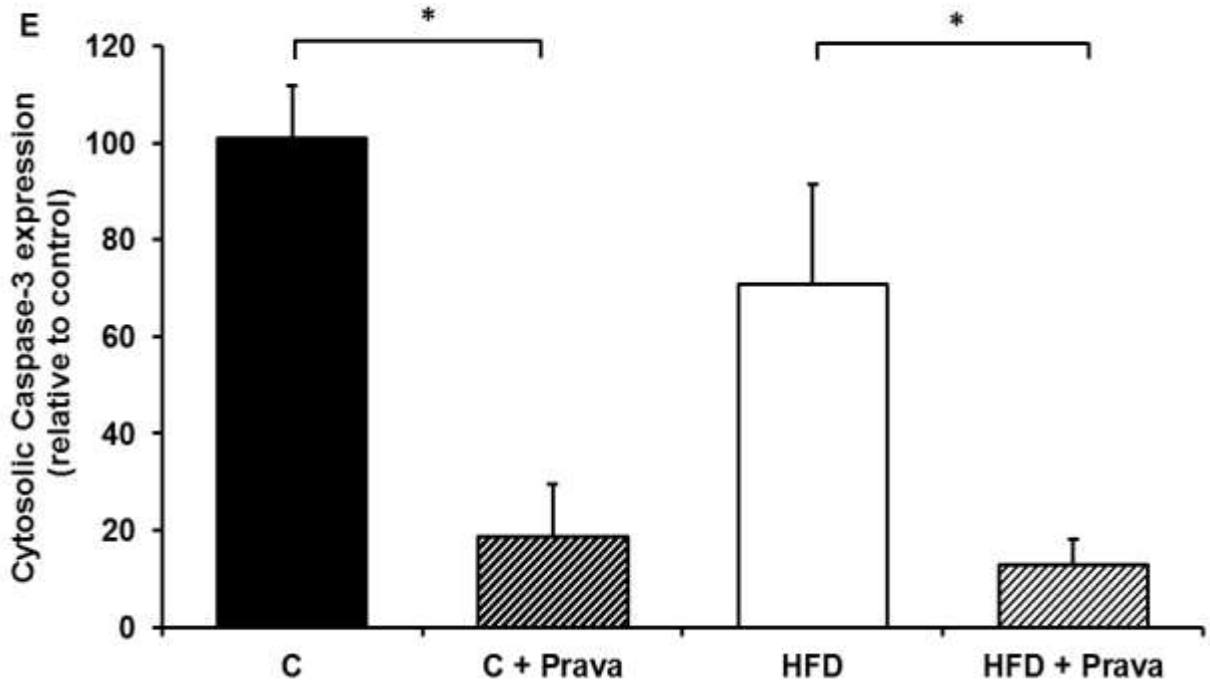

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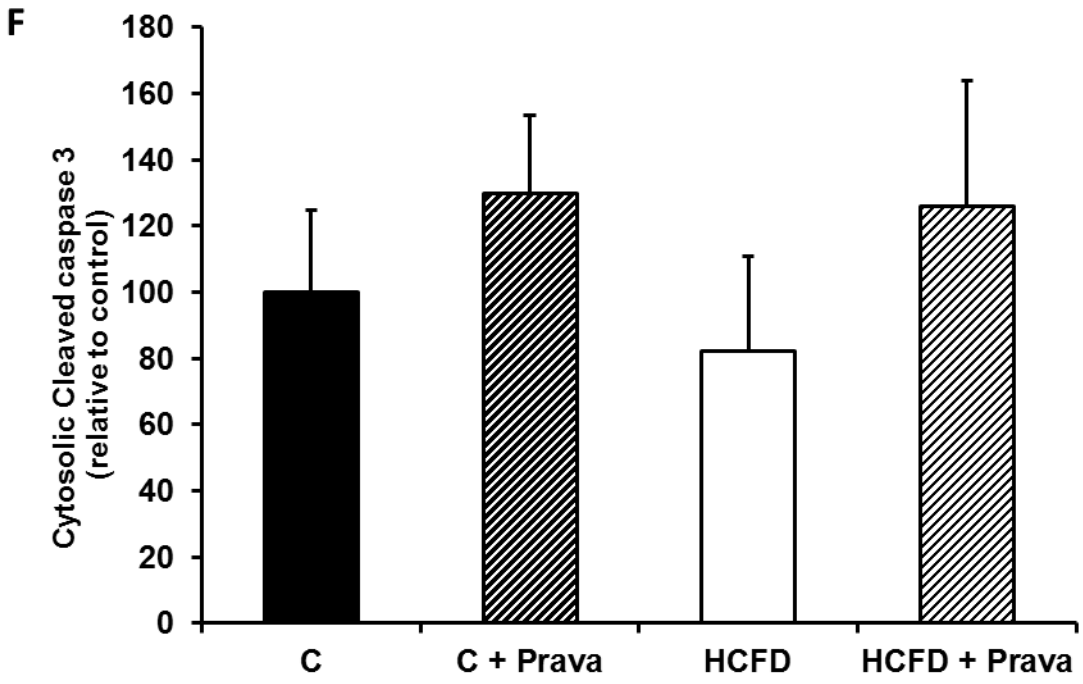

Fig 7.