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4 **Chronic metformin associated cardioprotection against infarction: not just a glucose**  
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6 **lowering phenomenon**

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21 Running Title: Chronic metformin and cardioprotection in an aging, diabetic rat model.  
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4 PURPOSE

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6 Clinical and experimental investigations demonstrated that metformin, a widely used  
7 anti-diabetic drug, exhibits cardioprotective properties against myocardial infarction.  
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9 Interestingly, metformin was previously shown to increase the expression of PGC-1 $\alpha$  a  
10 key controller of energy metabolism in skeletal muscle, which is down-regulated in  
11 diabetic conditions. We hypothesized that chronic treatment with metformin could  
12 protect the aged, diabetic heart against ischemia-reperfusion injury (IRI) by up-regulating  
13 PGC-1 $\alpha$  and improving the impaired functionality of diabetic mitochondria.  
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23 METHODS

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25 Following 4 weeks of metformin (300mg/kg) administered in the drinking water, 12  
26 month-old diabetic Goto Kakizaki and non-diabetic Wistar rat hearts were assigned for  
27 infarct measurement following 35min ischemia and 60min reperfusion or for electron  
28 microscopy (EM) and Western blotting (WB) investigations.  
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35 RESULTS

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37 Metformin elicited a cardioprotective effect in both non-diabetic and diabetic hearts. In  
38 contrast with the diabetic non-treated hearts, the diabetic hearts treated with metformin  
39 showed more organized and elongated mitochondria and demonstrated a significant  
40 increase in phosphorylated AMPK and in PGC-1 $\alpha$  expression.  
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48 CONCLUSIONS

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50 In summary these results show for the first time that chronic metformin treatment  
51 augments myocardial resistance to ischemia-reperfusion injury, **by an alternative**  
52 **mechanism in addition to the lowering of blood glucose.** This consisted of a positive  
53 effect on mitochondrial structure possibly via a pathway involving AMPK activation and  
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PGC-1 $\alpha$ . Thus, metformin prescribed chronically to patients may lead to a basal state of cardioprotection thereby potentially limiting the occurrence of myocardial damage by cardiovascular events.

**KEYWORDS:**

Ischemia –reperfusion injury, Cardioprotection, PGC-1 $\alpha$ , Metformin.

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4 INTRODUCTION  
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7 Cardiovascular disease, with its main consequence myocardial infarction, is a leading  
8 cause of debilitation and death in Westernized societies. Diabetes mellitus is a major risk  
9 factor for ischemic heart disease (IHD) [1]. Patients with diabetes and IHD experience  
10 worse clinical outcomes [2], suggesting that the diabetic heart may be more susceptible to  
11 ischemia-reperfusion injury (IRI). Given these observations, novel cardioprotective  
12 strategies are required to try and eliminate the increased cardiovascular risk in diabetic  
13 patients. The hyperglycaemic profile and the associated microvascular consequences of  
14 diabetes have been suggested to play an important role in increasing patient's  
15 susceptibility to infarction [3].  
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28 Metformin, an oral anti-diabetic drug from the biguanide, insulin sensitizing class,  
29 has been suggested as a potential cardioprotective agent [4]. There are various  
30 mechanisms that have been suggested to play a role in metformin's glucose-lowering  
31 effect, including inhibition of complex I of the mitochondrial respiratory chain [5],  
32 decreasing hepatic glucose production [6], increasing glucose uptake [7] and stimulation  
33 of adenosine monophosphate-activated protein kinase (AMPK) [8]. However, the  
34 cardioprotective effect exerted by metformin could be independent of its hypoglycaemic  
35 actions [9].  
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48 AMPK, a serine-threonine kinase that can sense and restore energy  
49 homeostasis when a cell becomes deprived of energy, has been well documented as an  
50 important component in the pathway by which metformin produces its glucose lowering  
51 effects [8] however, more recent investigations have also implicated this kinase in the  
52 cardioprotective effect of this drug [10] . There have been numerous studies investigating  
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4 the action of metformin in an acute setting and it has been shown that metformin can  
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6 reduce infarct size in both non-diabetic and diabetic hearts [11]. In comparison, there has  
7  
8 been little experimental work investigating *chronic* effects of metformin, a scenario  
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10 reflecting patients on metformin treatment in the clinic. In addition, it has been  
11  
12 demonstrated that drugs may activate different pathways when given acutely *versus*  
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14 chronically. [12-14].  
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19 Metformin has a plethora of effects on endogenous factors with roles in cellular  
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21 survival; amongst them is Peroxisome Proliferator-Activated Receptor Gamma  
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23 Coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) [15], a tissue-specific transcriptional co-activator which plays a  
24  
25 key role in regulating energy metabolism [16]. PGC-1 $\alpha$  is predominantly expressed in  
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27 mitochondrial-rich tissues such as brown adipose tissue, skeletal muscle and the heart  
28  
29 [16]. As PGC-1 $\alpha$  plays a vital role in energy metabolism [16] and mitochondrial  
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31 bioenergetic responses [17], it is suggested that this coactivator can interact with  
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33 mitochondrial regulatory pathways [18] and possibly with factors involved in  
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35 mitochondrial dynamics such as mitofusion-2 (Mfn-2) [18].  
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41 Interestingly, the diabetic status has been associated with a reduced level of PGC-  
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43 1 $\alpha$  [19;20]; while metformin has been shown to increase the expression of PGC-1 $\alpha$  in  
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45 skeletal muscle possibly via AMPK activation [15]. Gundewar et al, 2009 showed that  
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47 chronic low dose (125 $\mu$ g/kg) metformin administered after irreversible LAD ligation in  
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49 normoglycaemic mice, exerts beneficial effects on cardiac function and survival in this  
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51 heart failure model via an increase in AMPK activation and an increase in PGC-1 $\alpha$   
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53 expression [21]. However, there is limited information regarding these effects in the  
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55 diabetic heart and their role in the protection against IRI. In addition to diabetes, age can  
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4 also contribute to the IRI damage [20;22]. Interestingly, Reznick et al 2007, compared  
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6 AMPK activity in young (3 months) and old (28 months) rats and demonstrated an age-  
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8 associated decrease in AMPK activity and mitochondrial biogenesis [23]. Age related  
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10 changes in metabolism and mitochondrial biogenesis, suggest PGC-1 $\alpha$  as a potential  
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12 target for anti-aging strategies [24;25].  
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16 The aim of the present study was to investigate in aged, rat heart, whether chronic  
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18 metformin treatment reduces myocardial infarct size through a mechanism independent  
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20 of lowering blood glucose. We hypothesized that chronic metformin treatment could  
21  
22 possibly activate AMPK and restore PGC-1 $\alpha$  expression. Furthermore, that this  
23  
24 restoration of PGC-1 $\alpha$  could lead to improved mitochondrial biogenesis in these hearts  
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26 and hence provide an enhanced capacity to be protected against an ischemic insult.  
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## 32 33 **METHODS**

### 34 35 **Animals**

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37 Male Goto-Kakizaki (GK) rats (a mild, non-obese diabetic model [26] obtained from  
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39 Taconic (Denmark), were kept in house until they reached 12 months of age; they  
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41 received humane care in accordance with the United Kingdom Animal (Scientific  
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43 Procedures) Act of 1986. Male Wistar rats (normoglycaemic) were obtained from Charles  
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45 River UK Limited (Margate, UK) and kept in house until they also reached 12 months of  
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47 age.  
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### 52 53 **Materials**

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55 Metformin (Sigma, UK) was dissolved in water to give final concentration of 300mg/kg  
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57 per treated rat as described further. Antibodies for phospho- $\alpha$ -AMPK (Thr 172), total  $\alpha$ -  
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4 AMPK, phospho-Akt (Ser 473) and total Akt were obtained from Cell Signaling (Hitchin,  
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6 Kent). Antibodies for PGC-1 $\alpha$  and mitofusion-2 (Mfn-2) from Abcam, UK. Qproteome  
7  
8 nuclear protein kit used for nuclear fractionation of proteins was purchased from Qiagen.  
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10 All other reagents were of standard analytical grade. HbA1c, a measure of glycated  
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12 haemoglobin, was tested using an A1C now+ test kit (Bayer, UK). Overnight fasting  
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14 blood glucose was measured using Accu-chek system (Roche).  
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## 21 **Treatments**

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23 Animals were singly housed and randomly assigned to receive either 300mg/kg/day  
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25 metformin sweetened with a non-caloric sweetener in drinking water, or sweetener alone  
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27 in drinking water for 4 weeks. The metformin was sweetened to mask its metallic taste  
28  
29 [27] otherwise animals tended to have a reduced water intake. A pilot study was  
30  
31 performed on 6 animals, whereby the drinking rate was monitored for 7 days in order to  
32  
33 estimate the water consumption. Throughout the treatment the drinking rate was  
34  
35 monitored every 2 days and then the amount of powdered metformin was adjusted to  
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37 ensure each rat received a dose of 300mg/kg/day. The animals were randomly selected  
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39 for IRI followed by infarct measurements or for Western blot analysis and electron  
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41 microcopy studies.  
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48 There were 4 experimental groups: Wistar and Goto Kakizaki control groups  
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50 which received only the sweetener in the drinking water and Wistar and Goto Kakizaki  
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52 treated groups which received metformin added with the sweetener in the drinking water.  
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## 57 **Langendorff perfused heart studies**

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4 For determination of infarct size, GK and Wistar rats were anesthetized with sodium  
5 pentobarbital (55 mg/kg I.P.) and heparin (300 IU). The hearts were rapidly excised and  
6  
7 quickly mounted onto a Langendorff constant pressure perfusion system (ADInstruments,  
8  
9 Chalgrove, UK) as described by Bell et al, 2011 [28]. The hearts were perfused via the  
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11 aorta with modified Krebs-Henseleit bicarbonate buffer (KH) (mM: NaCl 118.5,  
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13 NaHCO<sub>3</sub> 25.0, KCl 4.8, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 1.7 and glucose 11.0), gassed  
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15 with 95% O<sub>2</sub>/5% CO<sub>2</sub> and heated to 37°C to maintain a pH of 7.4. Regional ischemia was  
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17 induced for 35min by occluding the left anterior descending coronary artery (LAD),  
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19 followed by 60min reperfusion [29]induced by the release of the occluding suture. Then  
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21 the suture was permanently tied around LAD, and 0.25% Evans blue dye was infused  
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23 through the aorta to delineate the risk zone, with the myocardium not at risk staining  
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25 blue. Infarct size was measured by incubating the heart sliced transversely in  
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27 triphenyltetrazolium chloride solution (TTC; 1% in phosphate buffer). TTC reacts with  
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29 intracellular dehydrogenases forming a red precipitate in the living cells leaving the dead  
30  
31 tissue off white. The slices were then transferred to 10% formalin overnight and then  
32  
33 scanned into the computer for analysis. Image J was used to calculate the volume of  
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35 infarction within the risk zone (I/R %).

### 46 47 **Measuring the heart function**

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49 A latex, fluid filled balloon was inserted into the left ventricle through an incision in the  
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51 left atrial appendage and inflated to a pressure of 8-10mmHg. Perfusion pressure was set  
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53 at 70mmHg, left ventricular end developed pressure (LVEDP) and heart rates (HR) were  
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55 permanently monitored and the rate pressure product (RPP) calculated. Coronary flow  
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4 was also collected at regular intervals throughout the investigation. A thermo probe  
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6 inserted into the pulmonary artery continuously monitored temperature.  
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### 10 11 **Electron microscopy and Western blot sample collection**

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13 In these investigations, three groups of animals, all 12 months of age, were used: a)  
14 control GK rats receiving only the sweetener; b) treated GK rats which received  
15 metformin and c) non-treated Wistar rats (normoglycaemic controls), (n=4 in each  
16 group), The animals were anesthetized with sodium pentobarbital (55 mg/kg I.P.) and  
17 heparin (300 IU). Hearts were harvested and samples from the left ventricle were  
18 collected in the electron microscopy fixative buffer, or snap frozen in liquid nitrogen for  
19 Western blot analysis.  
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### 33 **Electron microscopy analysis**

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35 To analyze mitochondrial appearance the heart samples were blindly coded and 4  
36 ultrathin sections were viewed with a Joel 1010 transition electron microscope (Joel Ltd,  
37 Warwickshire, UK.). At least 6 electron micrographs of each section were viewed and  
38 photos of the interfibrillar mitochondria taken and analyzed. Subsequently the codes were  
39 revealed and the photos investigated for patterns amongst groups.  
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### 50 **Western blot analysis**

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52 The tissue was homogenized in PBS buffer (pH to 7.4) containing Calbiochem protease  
53 inhibitor cocktail III EDTA free (1:1000), Sigma phosphatase inhibitor cocktail III  
54 (1:100) and EDTA 1mM. Protein content was then determined using bicinchoninic acid  
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4 (BCA) protein assay reagent (Sigma, UK) and protein levels corrected accordingly to  
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6 ensure equal protein loading. Equal volumes of Laemmli lysis buffer (Sigma, UK)  
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8 containing 5%  $\beta$ -mercaptoethanol were added and the samples were denatured by heating  
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10 to 80-90°C for 10 minutes. Sodium-dodecylsulphate (SDS) polyacrylamide gel  
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12 electrophoresis (PAGE) was used to analyze the protein expression and activation of our  
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14 proteins of interest using the Mini Protean III system (BioRad, UK) and incubation with  
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16 appropriate primary antibodies. The membranes were then probed with a horseradish  
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18 peroxidase (HRP) linked secondary antibody, either anti-mouse or anti-rabbit (1:1000)  
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20 dependent on the primary source. Protein bands were visualized by enhanced  
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22 chemiluminescence (ECL, Amersham, UK) and quantified by densitometry using Image  
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24 J software.  
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31 For detection of PGC-1 $\alpha$  in different sub cellular compartments, nuclear and  
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33 cytoplasmic extracts from GK and metformin treated GK rat hearts were prepared using a  
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35 nuclear/cytosol fractionation kit (Qiagen-Qproteome nuclear protein kit) according to the  
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37 manufacturer's instructions. Equal amounts of nuclear or cytoplasmic protein were  
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39 subjected to western blotting of PGC-1 $\alpha$ , and the fractions verified using nuclear TATA  
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41 binding protein and cytosolic  $\alpha$ -tubulin.  
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### 48 **Statistical Analysis**

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50 Data were analysed by either one-way ANOVA followed by Bonferroni's multiple  
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52 comparison test, students t-test or repeated measures ANOVA using GraphPad Prism 5.0  
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54 (GraphPad Software Inc, San Diego, California, USA). The choice of test is noted in the  
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4 figure legend. A p-value <0.05 was considered to be statistically significant. Values are  
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6 presented as means  $\pm$  standard error mean (SEM).  
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## 10 11 **RESULTS**

### 12 13 **Metformin treatment and glucose level**

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15 As metformin has insulin-sensitizing effects and therefore the ability to lower blood  
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17 glucose, the diabetic status of the GK and Wistar rats was monitored throughout the 4  
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19 week study. Blood glucose, haemoglobin A1C (HbA1c) and body mass were measured  
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21 prior to the study, then at 2 weeks and at 4 weeks. Metformin reduced blood glucose and  
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23 HbA1c by 33.4% and 23.8% ( $P<0.005$ ), respectively following 4 weeks of treatment in  
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25 GK rats. However, Metformin did not reduce blood glucose in normoglycaemic Wistar  
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27 rats ( $P=ns$ ). As expected, as a side effect of metformin treatment, GK rats receiving the  
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29 drug showed a 7.4% ( $P<0.005$ ) decrease in body weight. In the control groups no  
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31 significant changes were recorded for these parameters. Data is shown in *tables 1* and *2*.  
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33 Mean plasma insulin levels were not assessed in this study, however in accordance with  
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35 Portha et al, 1991, the glycaemic status we report is consistent with impaired insulin  
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37 secretion and resistance previously reported in the GK model of type 2 diabetes [30].  
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### 48 49 **Chronic metformin treatment decreases infarct size in both the diabetic and non-** 50 51 **diabetic heart**

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53 Metformin given chronically for 4 weeks at 300mg/kg/day significantly reduced infarct  
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55 size in diabetic rat hearts by 65.4% compared to non-treated GK rats (I/R %;  $16.6\% \pm 2.0$   
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57 *vs*  $47.8 \pm 2.0$   $P<0.0005$ ). Data were expressed as infarct to risk volume percentage  
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4 (Figure 1a). There was no difference regarding the area at risk (AAR) between the  
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6 groups ( $56.2\% \pm 3.5$  vs  $58.1\% \pm 1.6$ ). The functional parameters were also monitored  
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8 throughout the experimental protocol. It appears that metformin treatment had no  
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10 significant effect on either RPP or coronary flow (Figure 1b and c) in this model. This  
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12 treatment also significantly reduced infarct size in normoglycaemic Wistar rat hearts (I/R  
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14 %;  $40.8 \pm 4.5$  vs  $58.9 \pm 3.5$  P<0.05) (Figure 2a), however the protection was blunted  
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16 compared to the effect seen in the GK rat. Metformin treatment in Wistar rat hearts also  
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18 had no significant effect on either RPP or coronary flow (Figure 2b and c) in this model.  
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### 26 **Mitochondrial appearance is improved after chronic metformin treatment in the** 27 **diabetic heart** 28 29

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31 To investigate the hypothesis that chronic metformin treatment is associated with  
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33 improved mitochondrial structure and function, in the first instance we used electron  
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35 microscopy to visualise the shape, structure and number of mitochondria in the hearts of  
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37 the GK rats treated or not with metformin for 4 weeks. The mitochondria in the untreated  
38  
39 hyperglycaemic GK hearts appeared unorganised and more spherical in shape (Figure  
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41 3a). Interestingly, following metformin treatment, the mitochondria appear more  
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43 organised and elongated (Figure 3b), resembling those in the Wistar normoglycaemic  
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45 heart (Figure 3c).  
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### 53 **AMPK but not Akt is activated in the diabetic heart following chronic metformin** 54 **treatment** 55 56 57 58 59 60 61 62 63 64 65

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4 To assess the effect of metformin treatment in diabetic rat hearts we measured the  
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6 baseline levels of AMPK and Akt phosphorylation following 4 weeks treatment. The  
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8 reduction in infarct size was mirrored by a significant increase in the phosphorylation of  
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10 AMPK at baseline, prior to ischemia ( $1.4 \pm 0.1$  vs  $0.9 \pm 0.1$  a.u.,  $P < 0.05$ ) with no change  
11  
12 noted in total AMPK protein levels (*Figure 4a*). In contrast, phosphorylated Akt was  
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14 unchanged following 4 weeks of metformin treatment ( $3.9 \pm 0.4$  vs  $4.0 \pm 0.2$  a.u.  $P = ns$ )  
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19 (*Figure 4b*).  
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24 **An increase in PGC-1 $\alpha$  and Mfn 2 levels are seen in the diabetic heart following**  
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26 **metformin treatment.**  
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29 It has been documented that PGC-1 $\alpha$ 's expression is down regulated in type 2 diabetes  
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31 [19;20] and that metformin can increase the expression of PGC-1 $\alpha$  in skeletal muscle  
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33 [15]. Indeed, we saw a down regulation of PGC-1 $\alpha$  protein in the diabetic rat hearts in  
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35 comparison to the non-diabetic hearts ( $0.38 \pm 0.1$  vs  $0.54 \pm 0.1$ ,  $P < 0.0005$ ), however  
36  
37 following metformin treatment, the expression of PGC-1 $\alpha$  was significantly restored  
38  
39 ( $0.38 \pm 0.1$  vs  $0.47 \pm 0.1$   $P < 0.005$ ) (*Figure 5a*). Of note, our Western blot analysis of  
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41 PGC-1 $\alpha$  revealed two protein bands at 90 and 113kDa similar to that in the study by  
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43 Aquilano et al, 2010 [31]. They explained the double banding by potential splicing or by  
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45 a post translational change such as acetylation or phosphorylation of the protein and  
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47 performed extensive investigations to convincingly demonstrate that both bands are in  
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49 fact PGC-1 $\alpha$  [31].  
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55 We have also investigated if metformin treatment and the subsequent up  
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57 regulation of PGC-1 $\alpha$  affected the protein expression levels of Mfn-2. Diabetic status had  
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4 no significant effect on the levels of Mfn-2 at baseline ( $2.6 \pm 0.2$ , Wistar vs  $2.3 \pm 0.2$ , GK  
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7 *a.u.*  $P=NS$ ); however, metformin treatment significantly increased the expression of Mfn-  
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9 2 in diabetic hearts to a level greater than that seen in the normoglycaemic Wistar hearts  
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11 ( $2.6 \pm 0.2$  vs  $3.3 \pm 0.1$  *a.u.* ,  $P < 0.005$ ) (*Figure 5b*).

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14 PGC-1 $\alpha$  is a dynamic molecule that is known to migrate between different  
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16 subcellular compartments of the cell to exert different effects [32]; therefore we sought to  
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18 investigate if metformin treatment had an impact on this process. Following subcellular  
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20 fractionation we found that the nuclear PGC-1 $\alpha$  expression decreased following  
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22 metformin treatment compared to untreated GK hearts (*Figure 6*). Surprisingly, the  
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24 cytosolic fraction showed no difference in PGC-1 $\alpha$  expression. Moreover, the cytosolic  
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26 fractions appear on the blots as a single band compared to the double band seen in the  
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28 total cell extract (*Figure 5*) and in the nuclear fraction (*Figure 6*).

## 32 33 34 35 36 **Discussion**

37  
38 Metformin has been demonstrated to be cardioprotective in different experimental models  
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40 of ischemia-reperfusion injury. By using a model which corroborates 2 co-morbidities  
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42 (diabetes and age) the data presented in this paper enhance the information regarding this  
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44 protection. Investigating the effect of chronic metformin treatment in 12 month old  
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46 diabetic and non-diabetic rats, we have demonstrated that this drug widely prescribed to  
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48 type II diabetic patients reduced infarct size in both diabetic and non-diabetic hearts,  
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50 albeit to a lesser extent in the latter group. This suggests that the cardioprotection elicited  
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52 by metformin in the diabetic heart is not entirely glucose dependent. The cardioprotection  
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54 was associated with an improvement in mitochondrial arrangement and appearance,  
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4 AMPK phosphorylation and increased myocardial expression of PGC-1 $\alpha$  and Mfn-2 in  
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7 the diabetic heart.

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9 To our knowledge, this is the first time that (i) PGC-1 $\alpha$  protein expression has  
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11 been shown to be down-regulated in aged, diabetic rat myocardial tissue (ii) that in the  
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13 aged, diabetic rat heart metformin chronically restores PGC-1 $\alpha$  expression and that (iii)  
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15 Mfn-2 could potentially play a role in metformin induced protection in this set up.  
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17 Although we do not show any direct evidence, it is possible that metformin through  
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19 phosphorylation of AMPK can up-regulate and provide an increased platform for  
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21 activation of PGC-1 $\alpha$  via either phosphorylation or deacetylation [33]. This increase in  
22  
23 activity may then act downstream on the mitofusion protein Mfn-2 which contributes to  
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25 improved mitochondrial biogenesis and renders the diabetic heart less prone to damage  
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27 by IRI.  
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34 **Plasma levels of metformin were not measured in this study; the dose given**  
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36 **of 300mg/kg/day may appear high compared to the dose of 20mg/kg/day given to**  
37  
38 **patients in the clinical setting. However, investigations have shown that even when**  
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40 **given as 50mg/kg/day, the plasma level of metformin in mouse blood was almost**  
41  
42 **undetectable after 24 hours [34]. There have been numerous data suggesting that**  
43  
44 **300mg/kg/day in rodent studies is required to mirror the effect of 50 $\mu$ M of**  
45  
46 **metformin in human plasma [35;36]. On the contrary, other studies have suggested**  
47  
48 **this dose to be approximately 3-5 times greater than daily dose recommended in**  
49  
50 **humans [37]. However, it has be shown that, for metformin to exert its**  
51  
52 **hypoglycaemic actions in diabetic rats, relatively high doses are required to reach**  
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54 **plasma concentrations similar to those found in humans[38;39].**  
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4           There has been a great interest in the molecular mechanisms of metformin's  
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6 cardioprotection following the release of clinical data suggesting that diabetic patients  
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8 receiving metformin had less cardiovascular complications than patients on other  
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10 treatments [40]. However, the molecular mechanisms underlying this protection seem to  
11  
12 be complex and not fully understood yet. The majority of experimental studies have been  
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14 performed in an acute setting, either administering metformin for a short period before  
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16 an ischemic insult [10] or following ischemia [41] in a post-conditioning fashion.  
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18 However, if a long term treatment can potentially lower the incidence of cardiovascular  
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20 events, prevention is often preferable to cure. Therefore investigations into the  
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22 mechanisms underlying why long term treatment with metformin proves beneficial is  
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24 essential. The majority of experimental evidence in the acute setting thus far shows a  
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26 strong involvement via AMPK activation [42;43]. Our study as well shows that chronic  
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28 metformin treatment causes long term activation of AMPK by phosphorylation. This is  
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30 supported by a clinical study by Musi et al, 2002 [44] demonstrating that AMPK is still  
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32 activated in skeletal muscle following 10 weeks of oral metformin treatment in type II  
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34 diabetic patients [44]. In contrast, Haunton, 2011, could not find an increase in the  
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36 phosphorylation of AMPK [45] after only 2 weeks of treatment in normoglycaemic rat  
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38 heart, using the same treatment. Interestingly, at a higher dose of metformin an increase  
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40 in AMPK phosphorylation is seen [45], suggesting that perhaps the cardioprotective  
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42 benefits of metformin are time and/or dose-dependent [46]. **When interpreting our**  
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44 **data, it is important to have in mind that the *in vivo* administration of a drug such**  
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46 **as metformin will lead to a plethora of downstream effects that will promote cell**  
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4 **survival in both the heart and other body systems. However our research is focused**  
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6 **on investigating the pathway involving AMPK and PGC-1 $\alpha$  in the heart [47].**  
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10 | In addition, some authors support the hypothesis of an AMPK-independent effect  
11 of metformin [48]. This variability in results may be caused by the use of different  
12 experimental models such as normoglycaemic, genetically modified or chemically  
13 induced diabetic animals. In this respect, we consider the old Goto Kakizaki rat model  
14 used in this study, to more closely mirror a non-obese middle aged diabetic patient.  
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21 Previous studies have shown that acute metformin treatment given at the time of  
22 reperfusion can activate the RISK pathway kinase Akt via phosphorylation [41]. This  
23 activation led to a PI3K-dependent inhibition of the mitochondrial permeability transition  
24 pore (mPTP) and therefore reduced infarct size in this model of IRI [41]. However,  
25 chronic treatments may not manifest the same outcomes as acute drug administration due  
26 to inhibitory feedback regulation mechanisms [12]. This may explain why we did not find  
27 an increased level of Akt phosphorylation following chronic metformin treatment.  
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38 Firstly, we observed for the first time an increase of the PGC-1 $\alpha$  in the diabetic  
39 heart following chronic metformin treatment. Previously the same effect was noted in  
40 skeletal muscle [15]. PGC-1 $\alpha$  is extensively documented as a key regulator of energy  
41 metabolism [16], its expression is finely regulated to meet the energy demands of cellular  
42 systems [49]. There are numerous mechanisms that can control the expression levels of  
43 PGC-1 $\alpha$ ; however, these are dependent on the tissue and the circumstance. Interestingly,  
44 research investigating the regulation of PGC-1 $\alpha$  in skeletal muscle showed that increasing  
45 the levels of the energy sensor AMPK by pharmacological activation, either by 5-  
46 aminoimidazole-4-carboxamide-1- $\beta$ -d-ribofuranoside (AICAR) [50] or metformin [15]  
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4 caused an enhanced activation of mitochondrial biogenesis via increasing PGC-1 $\alpha$   
5 expression. Yet, the mechanisms by which increasing AMPK activity can lead to a  
6 subsequent increase in PGC-1 $\alpha$  expression are still under investigation. Our study  
7 replicates this scenario: following administration of metformin for 4 weeks we recorded  
8 an increase in activation of AMPK and a subsequent increase in the expression levels of  
9 PGC-1 $\alpha$  in the heart, an effect similar to that previously seen in skeletal muscle.  
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19 Up regulation of PGC-1 $\alpha$  expression can provide a platform for post translational  
20 modifications to occur. A dynamic shift in localisation can take place in response to a  
21 change in the energy status of the cell, with the main pool localised in the cytoplasm  
22 rapidly modified by phosphorylation, de/acetylation, ubiquination, methylation or  
23 GlcNacylation [49]. Subsequently PGC-1 $\alpha$  can be both activated and transported into a  
24 different cellular location, for example into the nucleus to encode target genes, or remains  
25 in an inactive form in the cytoplasm, all depending on the cellular demands [32].  
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36 **We sought to assess the effect metformin had on the subcellular location of**  
37 **PGC-1 $\alpha$ . In the whole cell, PGC-1 $\alpha$ 's expression was decreased in diabetes and**  
38 **recovered in metformin treated hearts. Interestingly, following subcellular**  
39 **fractionation, the cytoplasmic fraction displayed no difference in PGC-1 $\alpha$**   
40 **expression following metformin treatment but unexpectedly PGC-1 $\alpha$  expression was**  
41 **decreased in the nuclear extract of the same hearts.**  
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50 **This was somewhat surprising as the literature suggests that PGC-1 $\alpha$  is**  
51 **active when transported into the nucleus [32]. A possible explanation could be that**  
52 **the PGC-1 $\alpha$  double banding seen in the nuclear and whole cell homogenates could**  
53 **represent a post-translational process as suggested by other authors [31]. Based on**  
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4 **this** the results are intriguing and difficult to interpret, but for sure they indicate  
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6 trafficking of PGC-1 $\alpha$  between the cell compartments following metformin treatment.  
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9 However, a thorough investigation is needed in order to assess its functional state in these  
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11 compartments by measuring the acetylation and phosphorylation of the protein.  
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13 Intriguingly, very recent evidence suggests that PGC-1 $\alpha$  can also reside directly in the  
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15 mitochondria [31] and on activation potentially stimulates target genes directly within the  
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17 mitochondria, thereby strengthening a direct link to mitochondrial biogenesis.  
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19 Interestingly, AMPK phosphorylation following metformin administration was shown to  
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21 improve mitochondrial biogenesis by inducing PGC-1 $\alpha$  transcription in skeletal muscle  
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23 [15], or by direct activation of PGC-1 $\alpha$  by phosphorylation [51] suggesting a dual  
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25 effector system following metformin treatment.  
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31           There has been a recent emergence in evidence that proteins that are crucial in  
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33 mitochondrial dynamics namely Mfn-2, can also regulate mitochondrial metabolism [18].  
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35 Mfn-2 has pleiotropic roles within the cellular system, and has emerged as a key target of  
36  
37 the PGC-1 $\alpha$  regulatory pathway [52]. Soriano et al, 2006 showed that overexpression of  
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39 PGC-1 $\alpha$  in cultured muscle cells induced both Mfn-2 mRNA and protein expression  
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41 above the levels of other genes typically enhanced by PGC-1 $\alpha$ . They also found that  
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43 PGC-1 $\alpha$  stimulates the expression of Mfn-2 through its transcriptional properties [53].  
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45 Taking these findings alongside data showing that type 2 diabetes patients have a  
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47 decreased expression of Mfn-2 in skeletal muscle [54] we hypothesised that if metformin  
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49 administration can increase PGC-1 $\alpha$  expression and improve mitochondrial organisation  
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51 and appearance, Mfn-2 could be a link in the puzzle. However, we could not see a  
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4 significant reduction in Mfn-2 levels in the diabetic heart, yet interestingly; following  
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6 metformin treatment Mfn-2 expression is greatly enhanced.  
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9 **Nevertheless there are limitations to this study. The chronic administration**  
10 **of metformin was performed *in vivo* for 4 weeks followed by infarct size**  
11 **determination conducted in *ex vivo* isolated heart preparations. Therefore our data**  
12 **revealed a direct effect of metformin on the cardiac tissue, without the influence of**  
13 **the whole body system which may occur in an *in vivo* model of ischemia reperfusion**  
14 **injury. Our molecular investigations were limited to whole heart tissue; however,**  
15 **the effect of metformin on the regulation of PGC-1 $\alpha$  needs to be investigated in**  
16 **further studies using cellular and molecular models.**  
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### 31 **Conclusions**

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33 In co-morbidities such as old age and diabetes, cardiomyocytes' mitochondrial  
34 respiration and metabolism are thought to be affected [55]. Our results suggest that  
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36 chronic metformin treatment can be cardioprotective by improving mitochondrial  
37  
38 biogenesis in the diabetic heart. This could be achieved via a pathway involving  
39  
40 AMPK/PGC-1 $\alpha$ /Mfn-2; a pathway that certainly warrants further investigation.  
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11 researched data and assisted in experimental work. D.J.H. contributed to discussion.  
12  
13 D.M.Y contributed to discussion and reviewed & edited the manuscript. M.M.M  
14 researched data, contributed to discussion, reviewed, and edited the manuscript.  
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### 32 33 **Conflict of interest**

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35 None declared.  
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4 **Tables**  
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7 **Table 1**

	Goto Kakizaki - Control			Goto Kakizaki -Metformin		
Measurement	Initial	2weeks	4weeks	Initial	2weeks	4weeks
<i>Body mass (g)</i>	439.7 ± 8.2	436.4 ± 8.5	432.4 ± 7.9	461.4 ± 8.4	431.9 ± 6.7*	427.1 ± 7.1**
<i>Heart mass(g)</i>	-	-	2.77 ± 0.07	-	-	2.66 ± 0.08
<i>Blood Glucose (mmol/L)</i>	12.4 ± 0.8	10.5 ± 0.6	11.8 ± 0.9	15.5 ± 1.6	9.7 ± 0.4	10.3 ± 0.5**
<i>HbA1c (mmol/mol)</i>	67.6 ± 3.3	-	61.4 ± 3.7	58.2 ± 5.2	-	44.3 ± 3.4*

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22 Table 1: **Summary of parameters measured before, during and after administration of water +non-caloric sweetener or 300mg/kg/day metformin in water + non-caloric sweetener.** Body mass, blood glucose and HbA1c were measured initially, at 2 weeks and at 4 weeks prior to excision of the heart for experimentation. Heart mass was measured following isolated Langendorff heart preparation. Data is represented as mean ± S.E.M, n≥6. \*P<0.05 *versus* initial reading within group. \*\*P<0.005 *versus* initial reading within group. Repeated measures ANOVA was used to determine statistical significance.  
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33 **Table 2**

	Wistar - Control			Wistar -Metformin		
Measurement	Initial	2weeks	4weeks	Initial	2weeks	4weeks
<i>Body mass (g)</i>	573.1 ± 14.1	550.3 ± 14.3	544.5 ± 13.9	553.6 ± 26.1	543.3 ± 23.7	549.1 ± 29.1
<i>Heart mass(g)</i>	-	-	1.85 ± 0.06	-	-	1.90 ± 0.08
<i>Blood Glucose (mmol/L)</i>	7.5 ± 0.2	7.0 ± 0.4	6.8 ± 0.9	7.6 ± 0.4	7.1 ± 0.3	7.5 ± 0.5
<i>HbA1c (mmol/mol)</i>	26.6 ± 0.6	-	25.7 ± 1.6	27.9 ± 1.2	-	24.3 ± 0.7

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48 Table 2: **Summary of parameters measured before, during and after administration of water +non-caloric sweetener or 300mg/kg/day metformin in water + non-caloric sweetener.** Body mass, blood glucose and HbA1c were measured initially, at 2 weeks and at 4 weeks prior to excision of the heart for experimentation. Heart mass was measured following isolated Langendorff heart preparation. Data is represented as mean ± S.E.M, n≥6. Repeated measures ANOVA was used to determine statistical significance.  
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4 **Figure legends**  
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7 **Figure 1: The effect of chronic metformin on infarct size and cardiac function in**  
8 **the GK rat** a. Infarct size is expressed as the infarcted volume within the area at risk  
9 of the left ventricle (% I/R) in hearts either treated with metformin for 4 weeks or  
10 control hearts. Chronic metformin significantly reduced the infarct size compared to  
11 the control group. Data is shown as mean  $\pm$  S.E.M,  $n \geq 6$ . \*\*\*  $P < 0.0001$  *versus* control;  
12 b. Rate pressure product (RPP) was calculated as HR x LVDP; c. Coronary flow, no  
13 significant differences were seen following metformin treatment. White circles= non-  
14 treated Goto Kakizaki, Black squares = metformin-treated Goto Kakizaki. Student's t-  
15 test was used for statistical analysis.  
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19 **Figure 2: The effect of chronic metformin on infarct size and cardiac function in**  
20 **the Wistar rat** a. Infarct size is expressed as the infarcted volume within the area at  
21 risk of the left ventricle (% I/R) in hearts either treated with metformin for 4 weeks or  
22 control hearts. Chronic metformin significantly reduced the infarct size compared to  
23 the control group. Data is shown as mean  $\pm$  S.E.M,  $n \geq 6$ . \*  $P < 0.05$  *versus* control; b.  
24 Rate pressure product (RPP) was calculated as HR x LVDP; c. Coronary flow, no  
25 significant differences were seen following metformin treatment. White circles= non-  
26 treated Wistar, Black squares = metformin-treated Wistar. Student's t-test was used  
27 for statistical analysis.  
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32 **Figure 3: Chronic metformin treatment leads to mitochondrial appearance in**  
33 **the diabetic heart to become more like that of Wistar hearts.** a. Non treated 12  
34 month old Goto Kakizaki diabetic heart. b. Metformin treated (4 weeks  
35 300mg/kg/day) Goto Kakizaki diabetic heart. c. 12 month old Wistar  
36 normoglycaemic heart. (Representative electron micrograph photos)  
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40 **Figure 4: The effect of chronic metformin treatment on AMPK and Akt level**  
41 **and phosphorylation.** a. Metformin increases AMPK phosphorylation but has no  
42 effect on AMPK's total levels in Goto Kakizaki (GK) rat hearts. Data is shown as  
43 mean  $\pm$  S.E.M,  $n = 4$  per group. \* $P < 0.05$  \*\* $P < 0.005$  *versus* non-treated GK. b. Akt  
44 total level and activation are unaffected following 4 weeks metformin treatment. Data  
45 is shown as mean  $\pm$  S.E.M,  $n = 4$  per group. One-way ANOVA followed by  
46 Bonferroni's multiple comparison test were used to determine statistical significance.  
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50 **Figure 5: Chronic metformin treatment increases PGC-1 $\alpha$  and Mfn-2**  
51 **expression.** a. PGC-1 $\alpha$  protein expression is decreased in myocardial tissue of type II  
52 diabetic rats compared to non-diabetic Wistar rat hearts. Following chronic  
53 metformin treatment PGC-1 $\alpha$  expression is increased. Data is shown as mean  $\pm$   
54 S.E.M,  $n = 4$  per group. \* $P < 0.05$  \*\*\* $P < 0.0005$  *versus* non treated GK. Densitometric  
55 results express the sum of two representative bands of PGC-1 $\alpha$  protein. b. Mfn-2  
56 expression did not significantly differ between diabetic and non-diabetic heart tissue.  
57 Chronic metformin treatment increased the expression of Mfn-2. Data is shown as  
58 mean  $\pm$  S.E.M,  $n = 4$  per group. \* $P < 0.05$  \*\* $P < 0.005$  *versus* treated GK. One-way  
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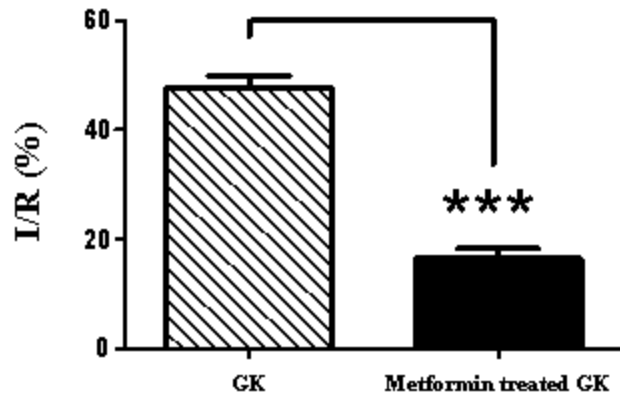
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4 ANOVA followed by Bonferroni's multiple comparison test were used to determine  
5 statistical significance.  
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8 **Figure 6: Chronic metformin treatment leads to decreased nuclear accumulation**  
9 **of PGC-1 $\alpha$ .** Following metformin treatment, PGC-1 $\alpha$  expression in the nuclear  
10 fragment decreased compared to that of the non-treated Goto Kakizaki. Data is shown  
11 as mean  $\pm$  S.E.M, n=4 per group, \*\*P<0.005. PGC-1 $\alpha$  expression is calculated by  
12 measuring both bands and corrected to the nuclear loading control TATA. Student's  
13 t-test was used for statistical analysis.  
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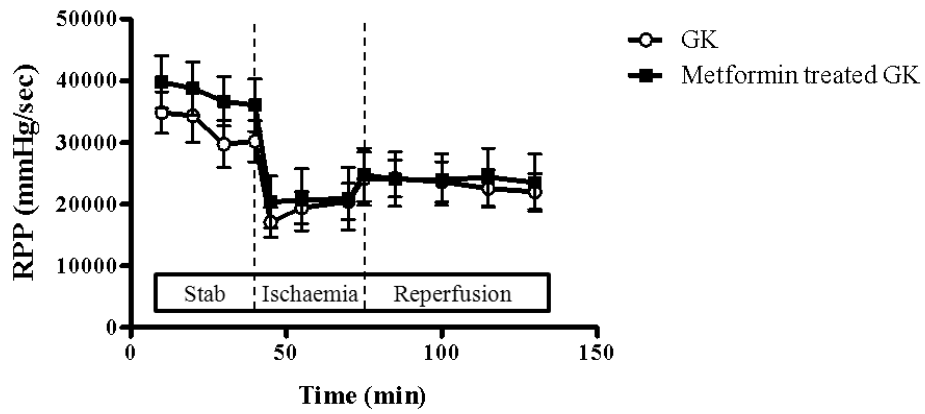
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6 **Figures**

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8 **Figure 1**

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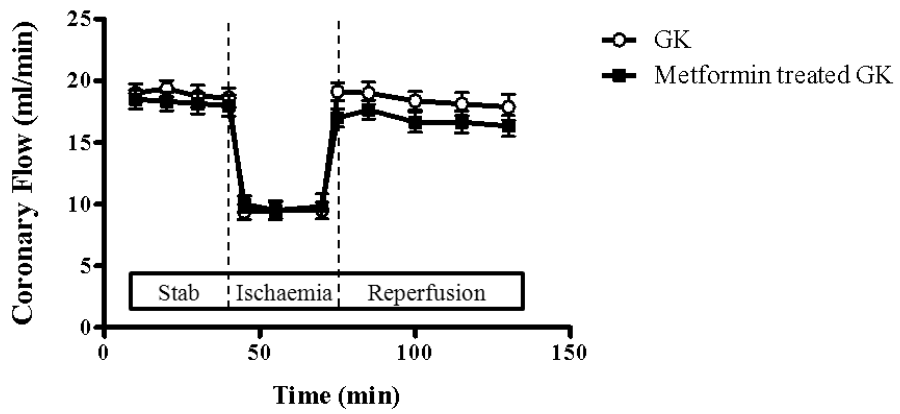
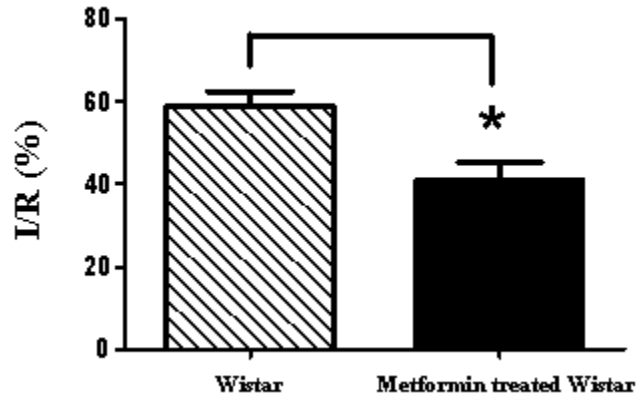
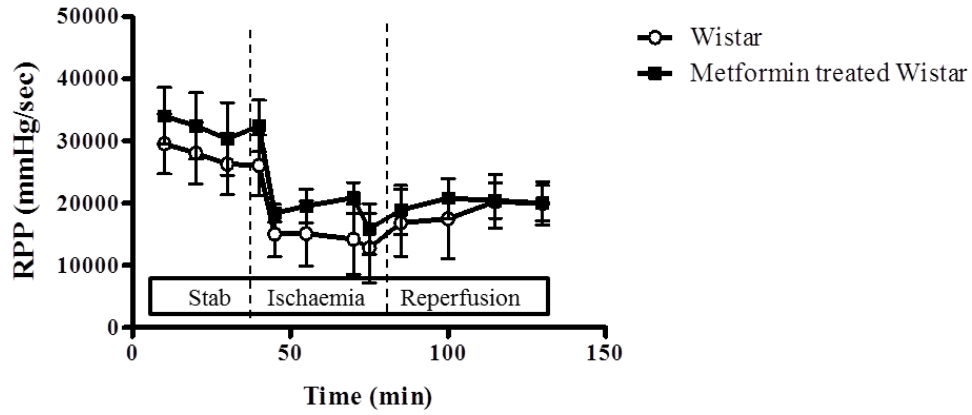


Figure 2

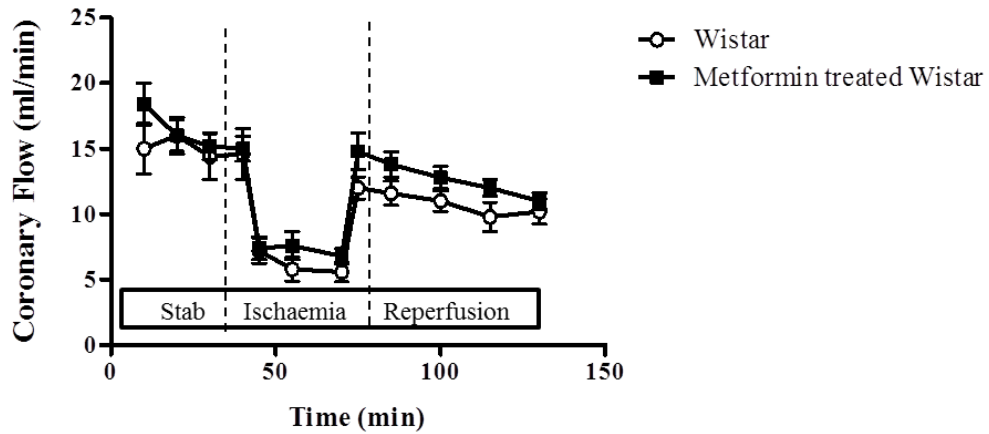
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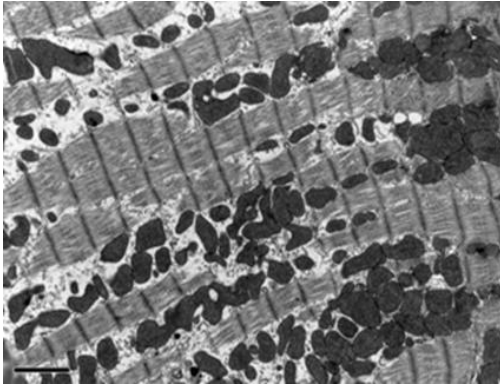
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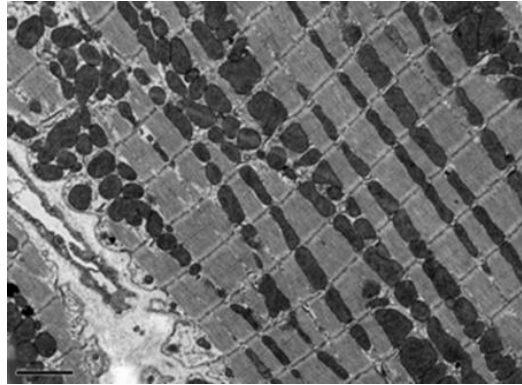
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**Figure 3**

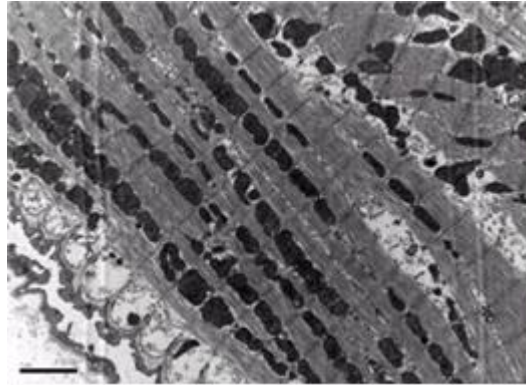
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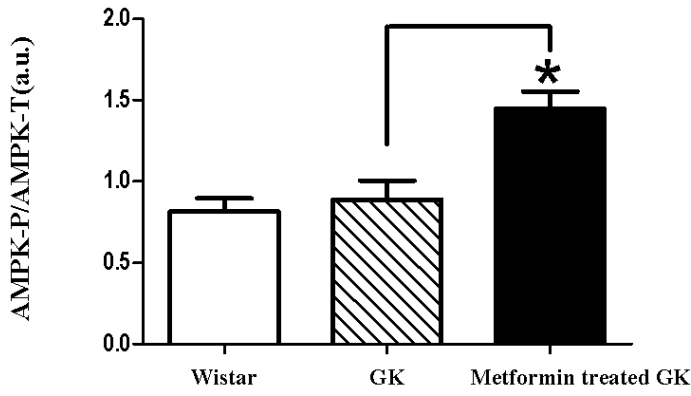
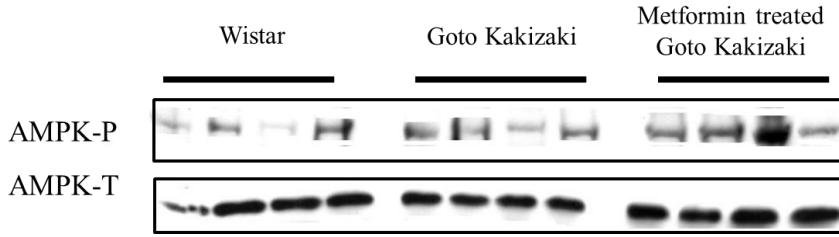
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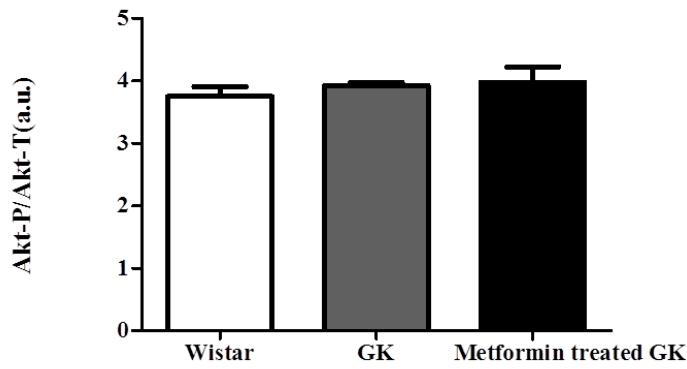
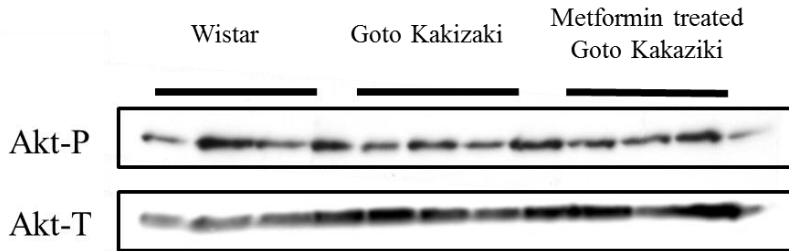


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4 **Figure 4**

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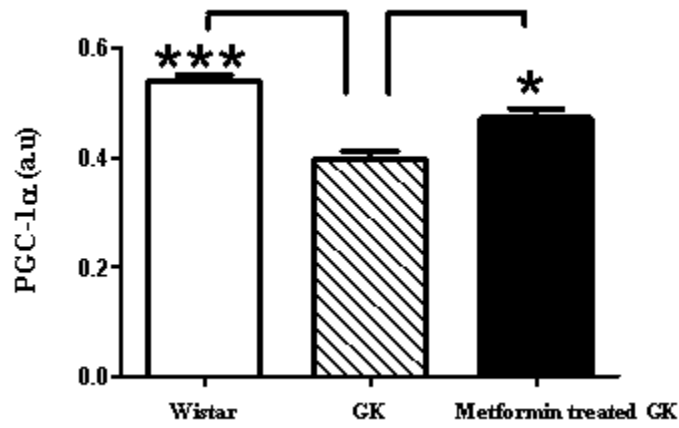
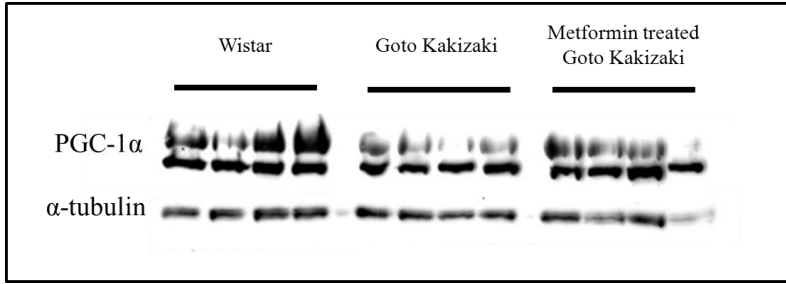


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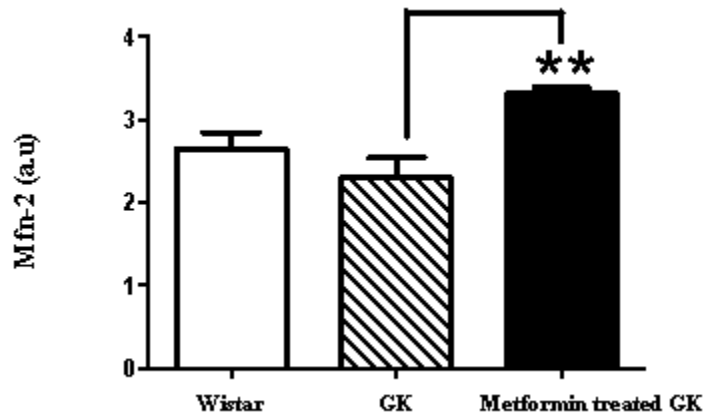
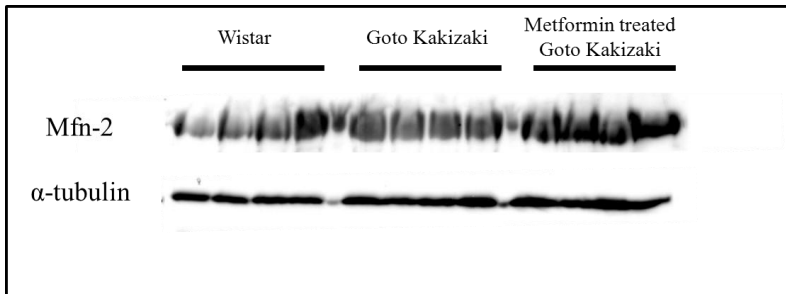


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4 **Figure 5**

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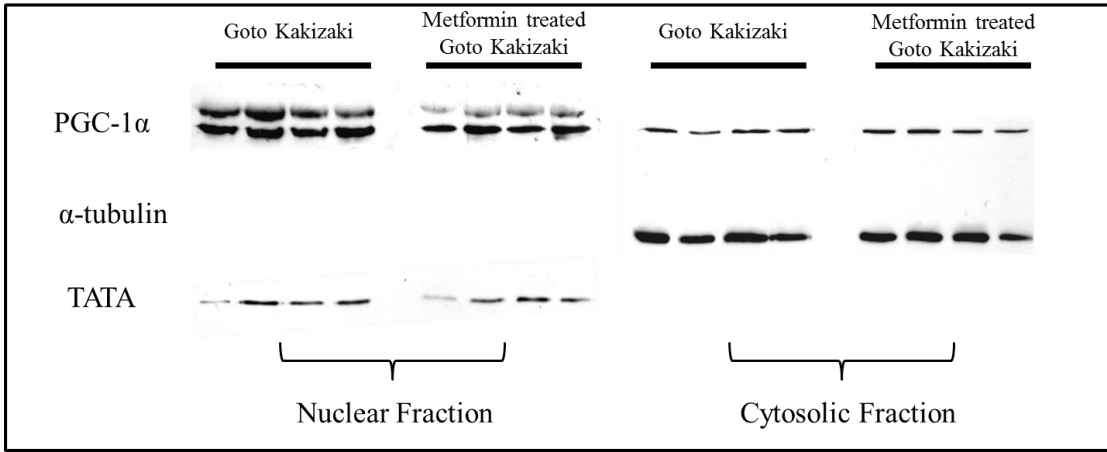


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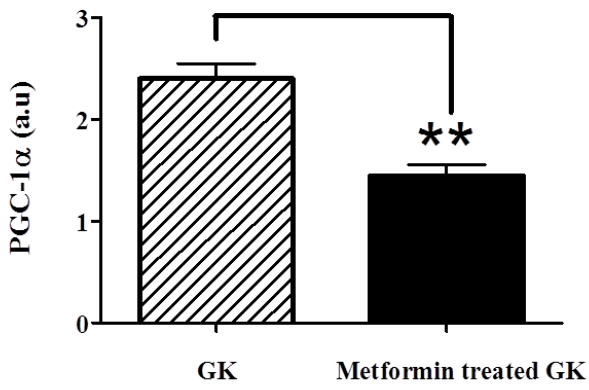


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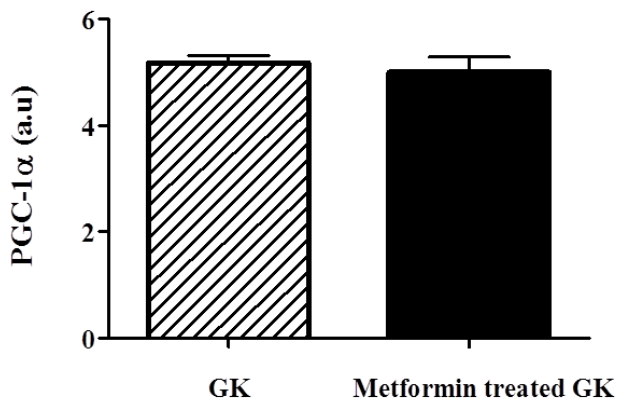
**Figure 6**



**Nuclear Fraction**



**Cytosolic Fraction**



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