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Modulation of Cardiomyocyte and Hepatocyte Bioenergetics by Biguanides

Saeeda Almarzooqi[1*], Ali S. Alfazari[2], Alia Albawardi[1], Dhanya Saraswathiamma[1], Hidaya Mohammed Abdul-Kader[2], Sami Shaban[3] and Abdul-Kader Souid[4]

[1]*Department of Pathology, UAE University, Al-Ain, Abu Dhabi, United Arab Emirates*

[2]*Department of Medicine, UAE University, Al-Ain, Abu Dhabi, United Arab Emirates*

[3]*Department of Medical Education, UAE University, Al-Ain, Abu Dhabi, United Arab Emirates*

[4]*Department of Pediatrics, UAE University, Al-Ain, Abu Dhabi, United Arab Emirates*

[*]**Corresponding author:** Saeeda Almarzooqi, [1]Department of Pathology, UAE University, Al-Ain, Abu Dhabi, United Arab Emirates, Tel: +9-713-713-7471; Fax +9-713-767-2022; E-mail: saeeda.almarzooqi@uaeu.ac.ae

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Abstract

Biguanides (metformin, buformin and phenformin) have been developed for oral treatment of non-insulin-dependent diabetes mellitus. Metformin, the drug of choice in this class, controls blood glucose primarily by lowering hepatic gluconeogenesis (e.g., decreasing glucagon-mediated hepatic glucose output). Its mode-of-action, however, is more complex and may involve "refining" cellular bioenergetics (improving energy efficiency) in various cell types including myocytes. Buformin and phenformin presumably have similar mechanisms of action. The main purpose of this *in vitro* study was to assess the effects of these drugs on bioenergetics - cellular respiration (mitochondrial O_2 consumption) and ATP content - in tissue fragments from the heart muscle (cardiomyocytes) and liver (hepatocytes) of C57BL/6 mice. Cardiomyocyte respiration decreased by 10-26% in the presence of 100 μM metformin (p=0.093), buformin (p=0.028) or phenformin (p=0.015). Similar effects on cardiomyocyte respiration were noted with 1.0 mM drugs. Cardiomyocyte ATP, on the other hand, increased by 17-31% in the presence of 100 μM metformin (p=0.093), buformin (p=0.445) or phenformin (p=0.093). Hepatocyte respiration and ATP decreased by 11-26% and 8-25%, respectively in the presence of 1.0 mM drugs. Decreased respiration and ATP were also noted in hepatocytes exposed to 100 μM metformin for $1 \leq t \leq 6$ hours (13% and 5%, respectively). Thus, the effects of biguanides on cardiomyocyte bioenergetics differed from that on hepatocyte bioenergetics. These findings suggest that biguanides regulate cardiomyocyte energy conversion, favoring better fuel efficiency (↓respiration/↑ATP). The drug effects in hepatocyte are ↓respiration/↓ATP, favoring less fuel production (↓hepatic gluconeogenesis). Biguanide activities in various tissues may be coupled.

Keywords: Biguanides; Antidiabetic drugs; Metformin; Buformin; Phenformin; Cellular respiration; Cellular bioenergetics; Cellular ATP

Introduction

The oral antihyperglycemic drugs biguanides (guanyl-guanidines; H_2N-CNH-NH-CNH-NH_2; e.g., metformin, buformin and phenformin) are powerful regulators of cellular bioenergetics [1,2]. These important compounds refine critical energy conversion processes within the cell, resulting in less demand for glucose production via hepatic gluconeogenesis [3-5]. Their activity spectrum, however, is complex and deserves further studies. For example, metformin (best-in-class and mostly widely used) has been shown to decrease 3'-5'-cyclic adenosine monophosphate (cAMP, thus, suppressing hepatic glucagon signaling), stimulate liver and muscle 5'-adenosine monophosphate-activated protein kinase (AMPK, catalyzes the reaction: AMP+ATP→2ADP), and inhibit AMP deaminase (catalyzes the reaction: AMP→IMP+NH_3) [6-8]. Inhibition of AMP deaminase increases cellular AMP that activates AMPK, thus, surging mitochondrial ADP and oxidative phosphorylation [5,9]. Consistently, metformin has been recently shown to augment cellular bioenergetics in the L6 myogenic cell line (rat skeletal muscle myoblasts) [10]. High concentrations of biguanides, however, have been shown to inhibit complex I of the respiratory chain [11-14]. Figure 1 summarizes the effects of biguanides on hepatocyte bioenergetics characteristics. Similar changes are seen in cardiomyocyte.

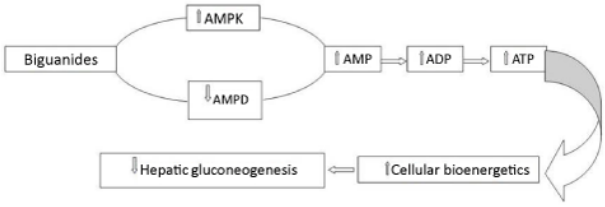

Figure 1: Summary of effects of Biguindes on cardiomyocytes and hepatocytes.

Abbreviations: AMPK: 5'-adenosine monophosphate-activated protein kinase; AMPD: AMP deaminase.

This study investigated the effects of these drugs on cellular mitochondrial O_2 consumption (cellular respiration) and ATP content in cardiomyocytes and hepatocytes from C57BL/6 mice [15-17]. The main purpose of these experiments was to investigate the mode-of-action of biguanides by measuring cardiomyocyte and hepatocyte bioenergetics in the presence of the drugs.

Methods

Reagents and solutions

Metformin (molecular weight, 165.6), buformin (molecular weight, 157.2), and phenformin (molecular weight, 241.7) were purchased from Toronto Research Chemicals (North York, Canada). All drugs used are of high purity as stated by manufacturer (98% purity). The biguanides were dissolved in H_2O immediately before use. Pd(II) complex of *meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin (Pd phosphor) was purchased from Porphyrin Products (Logan, UT). Pd phosphor solution (2.5 mg/mL=2 mM), NaCN (1.0 M) and glucose oxidase (10 mg/mL) were prepared and stored as previously described [7,8]. RPMI 1640 medium and remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Mice

C57BL/6 (9-10 weeks old) mice were housed at the animal facility in rooms maintained at 22°C, 60% humidity and 12-h light-dark cycles. The mice had *ad libitum* access to standard rodent chow and filtered water. The study received approval from the Animal Ethics Committee - United Arab Emirates University-College of Medicine and Health Sciences.

Tissue collection and processing

Urethane (25% w/v, 100 µL per 10 g) was used to anesthetize the mice. Heart muscle and liver fragments (20 to 30 mg each) were collected by 4-mm skin biopsy punches (Miltex GmbH, Germany) and immediately processed for measuring cellular respiration in the presence or absence of designated concentrations of the biguanides [16,17].

Moreover, liver fragments were incubated at 37°C in 50 mL in RPMI (gassed with 95% O_2: 5% CO_2) with and without 100 µM biguanide for up to 6 h. At designated time periods, samples were removed from the incubation solution and processed for measuring cellular respiration and ATP.

Cellular respiration

The phosphorescence O_2 analyzer was used to measure cellular O_2 consumption [16-19]. O_2 detection was with the aid of Pd phosphor with an absorption maximum of 625 nm and a phosphorescence emission maximum of 800 nm. Samples were exposed to 600 per min pulsed flashes with a peak output of 625 nm. The phosphorescence was filtered at 800 nm and detected by Hamamatsu photomultiplier tube. The phosphorescence decay rate ($1/\tau$) was single exponential; the values of $1/\tau$ were linear with dissolved O_2: $1/\tau=1/\tau^o + k_q[O_2]$, $1/\tau$=the phosphorescence decay rate in the presence of O_2, $1/\tau^o$=the phosphorescence decay rate in the absence of O_2, and k_q=the second-order O_2 quenching rate constant in $s^{-1} \bullet \mu M^{-1}$ [20]. A program was developed using Microsoft Visual Basic 6, Microsoft Access Database 2007, and Universal Library components (Universal Library for Measurements Computing Devices), which allowed direct reading from the PCI-DAS 4020/12 I/O Board (PCI-DAS 4020/12 I/O Board) [21].

O_2 measurements were performed at 37°C in glass vials that were sealed from air and contained a fragment of the heart or liver. The heart muscle was previously shown to be suitable for measuring cellular respiration and was used here as a representative of the muscles [21]. The respiratory substrates were endogenous metabolic fuels and glucose present in RPMI. O_2 concentration decreased linearly with time and this zero-order process was inhibited by cyanide (CN), confirming O_2 consumption occurred mainly in the mitochondrial respiratory chain (cellular respiration). The rate of respiration (k, in µM O_2 min^{-1}) was the negative of the slope $d[O_2]/dt$. The values of k were corrected by specimen weight (k_c) and expressed in µM O_2 min^{-1} mg^{-1}.

Cellular ATP

The tissue was homogenized in ice-cold 2% trichloroacetic acid (prepared immediately before use) and the supernatant was stored at -20°C. The supernatant was neutralized with 100 mM Tris-acetate, 2 mM ethylenediaminetetraacetic acid (pH 7.75) immediately before ATP determination by the Enliten ATP Assay System (Bioluminescence Detection Kit and Glomax Luminometer, Promega, Madison, WI) as previously described [16,17].

Statistical analysis

Data were analyzed on SPSS statistical package (version 19), using the nonparametric (2 independent samples) Mann-Whitney test.

Results

Cardiomyocyte respiration and ATP

Cardiomyocyte respiration was measured immediately after specimen collection in the presence and absence of 100 µM or 1.0 mM biguanides (the drugs were present in the O_2 measuring vials; drug exposure was ~40 to 60 min). Representative runs are shown in Figure 2A. The rate of respiration (k_c in µM O_2 min^{-1} mg^{-1}, n=6 mice for each condition, mean ± SD) without addition was 0.35 ± 0.04, with 100 µM metformin was 0.31 ± 0.03 (10% lower, p=0.093), with 100 µM buformin was 0.26 ± 0.02 (26% lower, p=0.004), and with 100 µM phenformin was 0.26 ± 0.04 (26% lower, p=0.015), Figure 2B. Cardiomyocyte ATP was measured after 60 min exposure to 100 µM biguanide (n=6-7 mice for each condition). Cellular ATP (pmol mg^{-1}) without addition was 12.2 ± 1.3, with metformin was 14.3 ± 2.5 (17% higher, p=0.093), with buformin was 15.3±5.0 (25% higher, p=0.445), and with phenformin was 16.0 ± 3.2 (31% higher, p=0.093), Figure 2C.

Cardiomyocyte respiration was then measured in the presence of 1.0 mM drugs (Figure. 2D). The rate of respiration (k_c in µM O_2 min^{-1} mg^{-1}) without addition was 0.37 ± 0.06 (n=14 mice), with 1.0 mM metformin was 0.36 ± 0.05 (2% lower, n=14 mice, p=0.874), with 1.0 mM buformin was 0.31 ± 0.03 (16% lower, n=8, p=0.035), and with 1.0 mM phenformin was 0.27 ± 0.02 (28% lower, n=8, p=0.001). Thus, the drugs lowered cardiomyocyte respiration and increased cardiomyocyte ATP.

Hepatocyte respiration and ATP

Hepatocyte respiration was measured immediately after specimen collection in the presence and absence of 1.0 mM biguanides (the drugs were present in the O_2 measuring vials). Representative runs are shown in Figure 3A. The rate of respiration (kc, µM O_2 min^{-1} mg^{-1}, n=7 mice per condition) without addition was 0.47 ± 0.05 (n=68 runs), with metformin was 0.42 ± 0.06 (11% lower, p=0.165), with buformin was 0.40 ± 0.07 (14% lower, p=0.053), and with phenformin was 0.35 ± 0.08 (26% lower, p=0.017), Figure 3B. Hepatocyte ATP was measured

after 60 min exposure to 1.0 mM biguanide (n=7 mice per condition). Cellular ATP (pmol mg^{-1}) without addition was 9.3 ± 3.3, with metformin was 8.4 ± 3.0 (10% lower, p=0.535), with buformin was 7.0 ± 3.4 (25% lower, p=0.456), and with phenformin was 8.6 ± 4.2 (8% lower, p=0.620), (Figure 3C). Thus, the drug had a mild decrease in the hepatocyte respiration, although mild, it was statistically significant in case of phenformin.

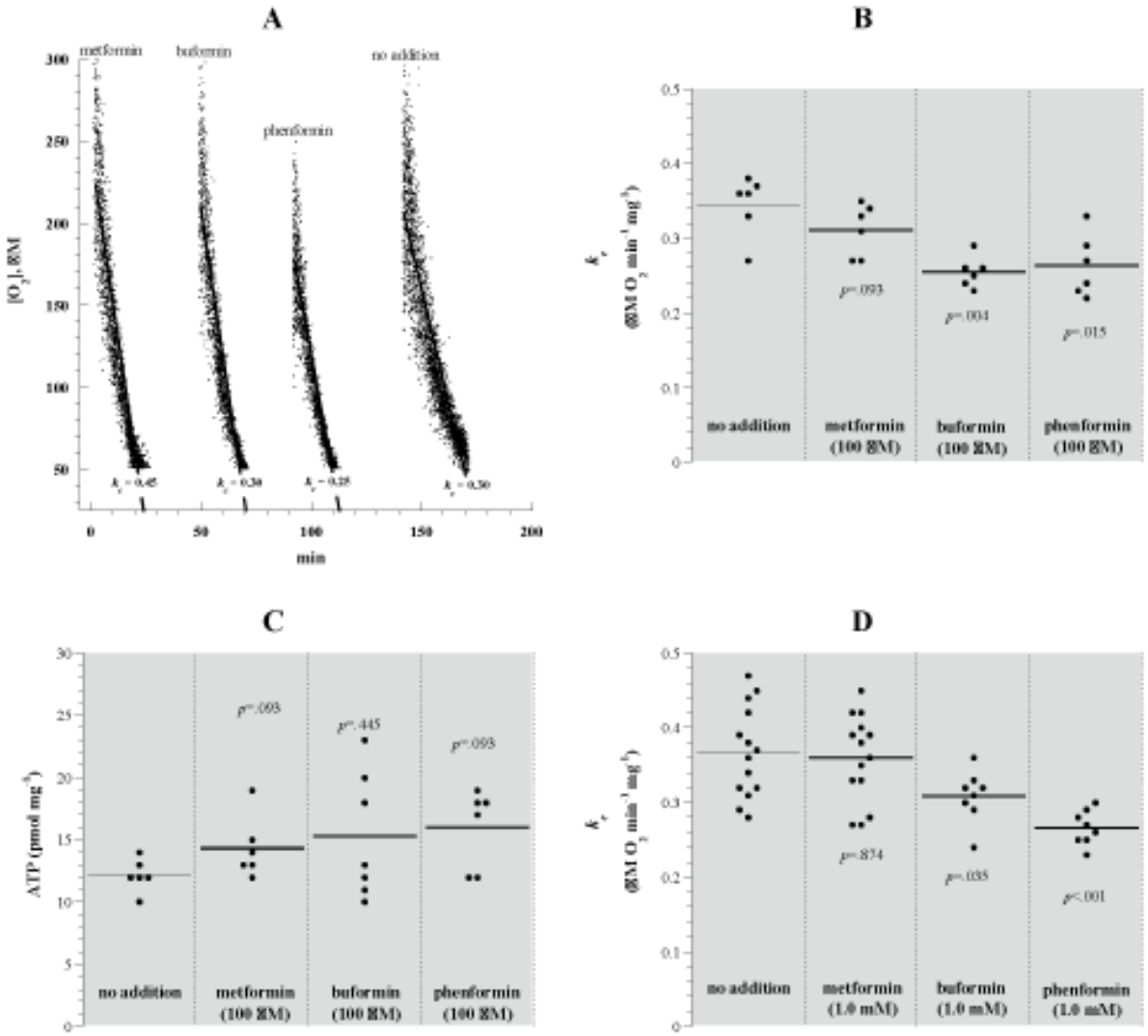

Figure 2: Effects of the biguanides on cardiomyocyte bioenergetics. Heart specimens were collected as described in Methods and immediately placed in the O_2 vial for measuring cellular respiration at 37°C in the presence and absence of 100 µM or 1.0 mM biguanides (Panels A, B and D). For ATP determination, collected heart specimens were incubated at 37°C in RPMI (gassed with 95% O_2: 5% CO_2) for 60 min in with and without 100 µM biguanides (Panel C). Panel A: Representative O_2 runs (a typical experiment involving 4 mice) in the presence of 1.0 mM metformin, buformin, phenformin or no addition. The lines are linear fits. The rate of respiration (k, µM O_2 min^{-1}) was the negative of the slope of [O_2] vs. t. The values of k_c (µM O_2 min^{-1} mg^{-1}) are shown at the bottom of each run. Panel B: Summary of the values of k_c (6 separate experiments involving 24 mice) with 100 µM metformin, buformin, phenformin or no addition. The lines are mean. Panel C: Summary of cellular ATP with 100 µM metformin, buformin, phenformin or no addition (six separate experiments involving 25 mice). The lines are mean. Panel D: Summary of the values of k_c (8 separate experiments involving 32 mice) with 1.0 mM metformin, buformin, phenformin or no addition. The lines are mean.

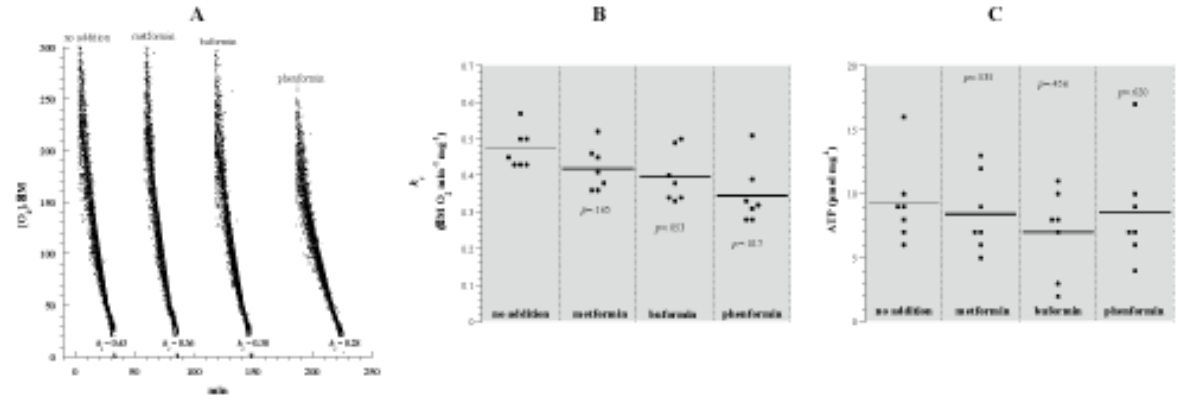

Figure 3: Effects of the biguanides (1.0 mM) and hepatocyte bioenergetics. Liver specimens were collected as described in Methods and immediately placed in the O_2 vial for measuring cellular respiration at 37°C in the presence and absence of 1.0 mM biguanides (Panels A-B). For ATP determination, collected liver specimens were incubated at 37°C in RPMI (gassed with 95% O2: 5% CO_2) for 60 min in with and without 1.0 mM biguanides (Panel C). Panel A: Representative O_2 runs (a typical experiment involving 4 mice) in the presence of 1.0 mM metformin, buformin, phenformin or no addition. The lines are linear fits. The rate of respiration (k, μM O_2 min^{-1}) was the negative of the slope of [O_2] vs. t. The values of k_c (μM O2 min^{-1} mg^{-1}) are shown at the bottom of each run. Panel B: Summary of the values of k_c (seven separate experiments involving 28 mice) with 1.0 mM metformin, buformin, phenformin or no addition. The lines are mean. Panel C: Summary of cellular ATP with 1.0 mM metformin, buformin, phenformin or no addition (seven separate experiments involving 28 mice). The lines are mean.

Hepatocyte respiration and ATP were then determined after *in vitro* incubation at 37°C in RPMI (gasses with 5% CO_2: 95% O_2) with and without 100 μM metformin. At designated time periods, samples were rinsed with RMPI and processed for O_2 and ATP measurements. Drug exposure was 60 to 300 min. Representative O_2 runs are shown in Figure 4A. The rate of respiration (k_c in μM O_2 min-1 mg-1, $t \leq 6$ h; 6 separate experiments, 6 mice, 22 O_2 runs for each condition) without addition was 0.23 ± 0.10 and with metformin was 0.20 ± 0.08 (13% lower; p=0.317), Figure 4B. Hepatocyte ATP was determined after 3 and 6 h of drug exposure (100 μM). The results of four separate experiments are summarized in Figure 4C. Cellular ATP (pmol mg^{-1}) without addition was 22.2 ± 15.2 (n=9) and with metformin was 21.1 ± 8.8 (5% lower, n=12, p=0.554). Thus, consistent with the results in Figure 2, metformin lowered hepatocyte respiration and ATP.

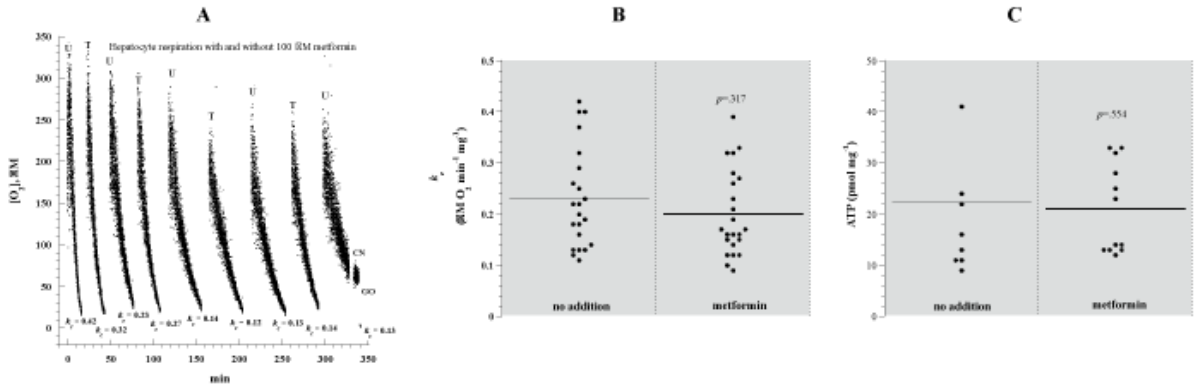

Figure 4: Effects of incubation with 100 μM metformin on hepatocyte bioenergetics. Liver specimens were incubated at 37°C in 50 mL RPMI (gassed with 95% O_2: 5% CO_2) with and without 100 μM metformin. At designated time periods (Panel A), samples were rinsed with RMPI and processed for O2 measurements. Representative O_2 runs are shown in Panel A. The lines are linear fits. The rate of respiration (k, μM O_2 min^{-1}) was the negative of the slope of [O_2] vs. t. The values of k_c (μM O_2 min^{-1} mg^{-1}) are shown at the bottom of each run (U, untreated; T, treated). Panel B: Summary of the values of k_c (6 separate experiments involving 6 mice; 22 untreated specimens and 23 treated specimens) in the presence of 100 μM metformin or no addition. The lines are mean. Panel C: Summary of cellular ATP (4 separate experiments involving 4 mice; 9 untreated specimens and 12 treated specimens) with 100 μM metformin for 3 and 6 h. The lines are mean.

Discussion

The American Diabetes Association recommends metformin as the first therapeutic line for type 2 diabetes mellitus and for prevention of diabetes in high risk individuals [22]. Buformin is available in Japan and Eastern Europe. Phenformin (10 times more potent than metformin) has been withdrawn from the market since 1978, due to lack of activity and reports of fatal lactic acidosis [23]. Buformin has a relatively limited use. Undoubtedly, improvements in our understanding of biguanides' molecular targets will result in follow-up compounds with better properties.

The biguanides have been proposed to target complex I of the respiratory chain [11]. In one study, high metformin dosing (30 mM) reduced the activity of complex I in skeletal muscle homogenates by about 15%. A 24-h exposure of muscles to 270 μM metformin reduced cellular respiration by 30% and increased lactate production by 80% [12]. Similarly, exposure of rat liver mitochondria to high metformin doses (≥ 10 mM) impaired oxidations in the respiratory chain and decreased the membrane potential [13]. The clinical significance of the high concentrations used in these studies remains to be determined since the human plasma concentrations of metformin and buformin rarely exceed 5 mg/L (about 30 μM) [24-27]. Higher (up to 2-fold) drug concentrations, however, accumulate in the liver [3]. mainly due to expression of the organic cation transporter 1 that facilitates metformin hepatocyte uptake [14]. The level of phenformin is much lower (150 μg/L or 0.6 μM) [28]. In this study, the biguanides were investigated at 100 μM and 1.0 mM (several fold higher than therapeutic levels) [24-28].

The bioavailability of biguanides is about 50%, and their binding to plasma proteins is negligible. Metformin and buformin are excreted in the urine as free drugs. Phenformin is metabolized in the liver and then excreted in the urine [29].

It is generally accepted that metformin treats non-insulin-dependent diabetes mellitus by suppressing hepatic glucose output. This highly-regulated activity is complex and it best tested in the targeted tissue rather than cell lines. This study investigated the effects of biguanides on murine heart muscle and liver specimens, using preparations that allowed measuring cellular energy biomarkers (cellular respiration and ATP) [16-17]. The purpose of these experiments was to measure cardiomyocyte and hepatocyte bioenergetics in the presence of metformin and other biguanides.

The main finding in this study is that 100 μM metformin lowered cardiomyocyte respiration by 10% (Figure 1B) and increased cardiomyocyte ATP by 18% (Figure 2C). These two processes (↓respiration/↑ATP) may reflect a mildly improved cellular bioenergetics (metabolic energy conversion). It is worth noting that this mode-of-action is unique to this class of drugs and may be responsible for lowering the demand for hepatic gluconeogenesis. Consistently, metformin lowered hepatocyte respiration by 11-13% (Figure 3B and 4B) and decreased hepatocyte ATP by 5-10% (Figure 3C and 4C). A simple explanation of these two processes (↓respiration/↓ATP) is that metformin slightly decreases hepatocyte bioenergetics and, thus, lowing hepatic gluconeogenesis.

These data are consistent with the recent finding that metformin augments myoblast bioenergetics following energy interruption by uncouplers (dinitrophenol) or respiratory chain inhibitors (azide) [10]. The results also are consistent with the current understanding that metformin controls blood glucose by favorably modulating metabolic pathways without directly targeting oxidative phosphorylation [1].

The experiments reported here have important limitations. Heart muscle specimens were not suitable for several hours of *in vitro* incubation. Their respiration, thus, was measured immediately after tissue collection (Figure 2A-D); the drug exposure was limited to about one hour. Liver specimens, on the other hand, were relatively more stable, allowing *in vitro* incubation with and without the drugs for up to ~6 h (Figure 3A-D) [15-17]. It is worth noting, however, that without addition, hepatocyte ATP at 0 h (immediately after tissue collection) was 235 ± 72 pmol mg^{-1} and at 1 h was 39 ± 15 pmol mg^{-1}. These results confirm significant deterioration of hepatocyte bioenergetics *in vitro*.

Metformin has been shown to have beneficial effects on cardiomyocyte in Diabetic Mice. In the study by Xie et al. [30] they have shown an increased AMP-activated protein kinase in cardiac myocyte in OVE26 diabetic mice after treatment with metformin in comparison to control group. These increases lead to enhancement of cellular autophagy; a process responsible for the removal of damaged organelles and aggregated proteins thus preserving cardiac function [30].

Similar results have been demonstrated by in SHHF rats, an animal mode of insulin resistant diabetic rats, as activation of AMP-activated protein kinase leads to increased production of nitric oxide that plays a major rule in regulation of vascular tone and hence cardiac function. In addition, study by Cittadini et al. [31] reveals a number of beneficial cellular alterations within including reduction in lipid accumulation and decrease in tumor necrosis factor-α expression and myocyte apoptosis. These recently highlighted cellular changes have been documented in a number of clinical studies that proposed the protective rule of use of biguanides e.g. metformin on enhancing cardiac function and decreasing risk of diabetes associated cardiomyopathy [32,33].

Treatment with metformin increases the expression of transcription factors involved in gluconeogenesis and hence produce its diabetes therapeutic effects [34,35]. In a recent study by Takayama et.al, they have added a new insight on mechanism of action of metformin. They have demonstrated a reduction in selenoprotein P, a protein secreted by hepatocytes in insulin resistant type-2 diabetes, induced by AMP-activated kinase [36].

In conclusion, cardiomyocyte respiration is slightly decreased and ATP is slightly increased in the presence of metformin. The drug effects on hepatocytes are different. Hepatocyte respiration and ATP are both slightly decreased in the presence of metformin. The effects of buformin and phenformin are more prominent than metformin, pointing to the importance of investigating several biguanides in all targeted organs.

Author Contributions

Alia Albawardi, Ali Alfazari and Saeeda Almarzooqi designed the study, carried out the analysis, interpreted the data and drafted the manuscript. Sami Shaban programed the oxygen analyzer and data analysis. Dhanya Saraswathiamma, Hidaya Mohammed Abdul-Kader and Abdul-Kader Souid performed and analyzed the oxygen and ATP measurements. The authors read, edited and approved the final manuscript. The manuscript was written through contributions of all

authors. All authors have given approval to the final version of the manuscript.

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