Protective effect of metformin in CD1 mice placed on a high carbohydrate–high fat diet

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A B S T R A C T

A high carbohydrate–high fat (HC–HF) diet-associated with hyperinsulinemia has been previously reported to induce accelerated growth of prostate cancer in a xenograft model. High energy supply and insulin/insulin growth factor-1 axis are two of the mechanisms proposed. We hypothesize that metformin may have a protective effect against prostate cancer progression by affecting metabolisms associated with high energy intake. In the present study, animals were randomized into five groups, receiving a HC–HF diet with 50, 100, or 250 mg/kg body weight (mg/kg) metformin in drinking water, a standard diet or HC–HF diet alone. Animals on the HC–HF diet developed obesity and insulin resistance. They had significantly higher body weight, fasting blood glucose at an upper level of normal range, higher insulin secretion and utilization, and fatty degeneration of the liver. Metformin at the doses employed significantly reduced food and water consumption; however, only a dose of 250 mg/kg showed a significant reduction in body weight gain and suppression of gluconeogenesis as well remarkably reduced insulin secretion. There was no observed metformin-related hepato-toxicity in any of the groups. In summary, metformin at various doses exhibits protective effects on the metabolic disorder caused by the HC–HF diet with the most effective protection at a dose of 250 mg/kg. These effects may explain its translational role relating to its anti-neoplastic potential.

1. Introduction

High consumption of dietary carbohydrate and fat has been shown to have a direct impact on the promotion, progression and mortality of some solid tumors, including prostate cancer (PCa) [1–3]. Epidemiological and laboratory evidence suggests that high levels of serum insulin and IGF-1 [4,5] as well as the additional energy substrates [6–8] provided to tumor cells are possible mechanisms. We have previously reported that a HC–HF diet induces hyperinsulinemia, stimulates the growth of xenograft tumors, and conditions the serum conferring an increased mitogenic potential *in vitro* [9]. A low-fat diet reduces the incidences of prostate cancer development in a genetically predisposed animal model [10].

Metformin, a biguanide, is used as a first line anti-diabetic drug and also prescribed to patients with insulin resistant status such as polycystic ovary syndrome. Emerging studies have shown that metformin exhibits some anti-neoplastic activities both *in vitro* [11,12] and *in vivo* [11,13–15] in several tumors. It may be a candidate medication for intervention in cancer patients who are obese and hyperinsulinemic since it may improve their metabolic status and inhibit tumor growth [16,17].

The present study was designed to optimize the dose–response of metformin in CD1 animals placed on a HC–HF diet for a relative long period of treatment. We sought to investigate the role of metformin in the intervention of PCa progression in a xenograft model under the influence of a HC–HF diet.

2. Materials and methods

2.1. Animals and diets

Twenty-five 7-week-old male CD1 mice (Charles River Laboratories, Canada) were randomly and evenly divided into five groups, which were a standard diet group (Std Diet), a HC–HF diet group

Abbreviations: HC–HF, high carbohydrate–high fat; PCa, prostate cancer; IGF-1, insulin-like growth factor-1; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; H&E, hematoxylin and eosin.

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(HC–HF Diet), and three groups receiving the HC–HF diet and a respective dose of 50, 100, or 250 mg/kg of metformin (Met-50, Met-100 and Met-250). The standard diet consisted of 50.0% carbohydrate, 18.8% protein, 6.0% fat, which provided 3.3 kcal/g calories (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories Inc., Madison, WI, USA). The HC–HF diet was the same one as reported [9]. All procedures performed were in compliance with the "Care and Use of Experimental Animals guidelines of the Canadian Council on Animal Care" and "Cancer Endpoint Guidelines". The animals consumed food and water *ad libitum* and were kept under standard maintenance at our institute's animal facility.

2.2. Administration of metformin and assessment of body weight and water/food consumption

Metformin (1,1-dimethylbiguanide hydrochloride, Spectrum Chemical Corp., New Brunswick, NJ, USA) was administered in drinking water. Its concentration was adjusted weekly based on the average water consumption and body weight. It was replenished daily and the actual amount of water consumed was recorded daily. Animals were observed for metformin-related toxicity.

Animals were placed on the standard or HC–HF diet with or without metformin for 12 weeks. The diets were stored at 4 °C. The amount of food consumed by the animals was recorded daily. Body weight was measured weekly and the percent body weight gain was determined for each animal based on its corresponding baseline value.

2.3. Assessment of blood glucose level

Blood glucose levels were ascertained biweekly by a blood glucose meter (The FreeStyle Mini™ System, Abbott Diabetes Care Inc., Alameda, CA, USA) from saphenous vein bleeding. At the time of sacrifice they were determined from samples obtained by direct heart puncture. The animals were fasting for 6 h before the blood glucose was measured [18].

2.4. Measurement of C-peptide, insulin and IGF-1 in serum

At the time of sacrifice serum samples were collected through blood directly withdrawn from heart puncture following 6-h fasting and stored at −80 °C. Serum C-peptide was analyzed by a C-Peptide (mouse) EIA kit (Cat#: 48-CPEPM-011; ALPCO Diagnostics, Salem, NH, USA); insulin by a rat insulin ELISA kit (Cat#: 90060; Crystal Chem Inc., Downers Grove, IL, USA); and IGF-1 by a mouse/rat ELISA kit (Cat#: DSL-10-29200; Diagnostic Systems laboratories Inc. of Beckman Coulter, Webster, TX, USA). The samples were analyzed in duplicate in each assay except that IGF-1 was measured in group-pooled serum due to insufficient sample in each individual mouse.

2.5. Tissue preparation and histopathology

At necropsy, a part of the liver and the entire pancreas were harvested, fixed in PBS-buffered formalin, and processed for standard H&E staining. The pathology was assessed by a pathologist at our centre.

The surface area of pancreatic islet (islet) was measured using software provided by Leica IM1000 Imaging Manager (Richmond Hill, ON, Canada). The practical procedures were as follows. The image of each pancreas section was carefully taken under microscope at 50× magnificence without overlapping the fields. The surface area of each islet was measured with the tool bar. The software directly showed the surface area in square micrometers with the adjustment of magnification. The cut-off value was 500 μm^2. The number of islets from each pancreas section was determined by the number of islets measured in surface areas. The surface area of each pancreas section was measured in a similar way as islets were but at 25× magnification and in square millimeters.

2.6. Statistical analysis

Descriptive statistics were calculated for all variables of interest. Continuous measures were summarized using means and standard deviations whereas categorical measures were summarized using counts and percentages. Analysis of variance (ANOVA) models were used to run comparisons on variables of interest between the five groups (Std Diet, HC–HF Diet, Met-50, Met-100, Met-250). Tukey's tests were carried out to assess for pairwise group differences. All analyses were carried out using SAS Version 9.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. The HC–HF diet developed obesity and insulin resistance

Animals placed on the HC–HF diet with or without metformin for a period of 12 weeks appeared to be obese with a greasy coating except for those receiving metformin at a dose of 250 mg/kg. This high energy diet induced a metabolic status of insulin resistance (see details in the following sections).

3.2. Metformin significantly altered food and water consumption

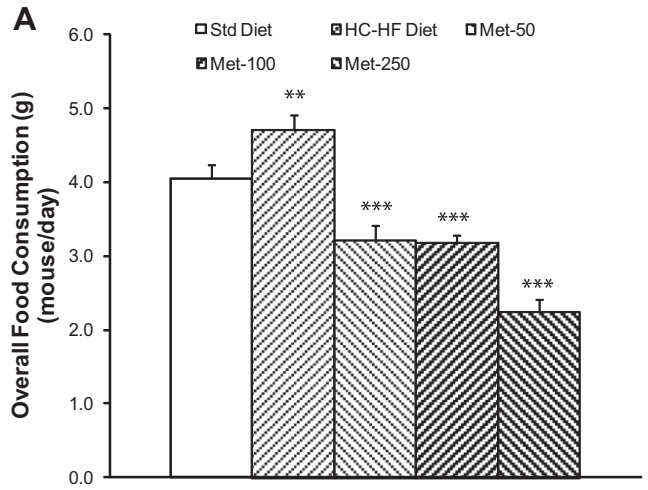
Compared to those on the standard diet, animals on the HC–HF diet consumed significantly higher amount of food and water (Fig. 1A and B). Administration of metformin caused a significant reduction in food and water consumption (Fig. 1A and B). There were no recognizable signs of metformin-related toxicity with any of the doses. Though metformin reduced food consumption, thereby impacting energy intake, this reduced food consumption was still sufficient to provide excess calories with the exception of metformin at 250 mg/kg. Energy intake of each group calculated in calories (per mouse per day in average as kcal) was as follows: Std Diet, 3.3 kcal/g × 4.2 g = 13.86 kcal; HC–HF Diet, 4.76 kcal/g × 5.2 g = 24.752 kcal; Met-50 and -100, 4.76 kcal/g × 3.2 g = 15.232 kcal; and Met-250, 4.76 kcal/g × 2.3 g = 10.948 kcal. The ratio of energy supply that was provided by carbohydrate and protein vs. that provided by fat was 83/17 in the standard diet and 55/45 in the HC–HF diet.

3.3. Metformin at 250 mg/kg caused a significant reduction in body weight gain

Due to the small differences in animals' body weights at the commencement of the study, we used body weight gain relative to the baseline in order to make comparisons (Fig. 2). Compared to those on the standard diet, animals on the HC–HF diet developed significantly higher percent body weight gain. Metformin at 50 and 100 mg/kg enhanced the body weight gain several weeks post administration; however, at 250 mg/kg it gradually brought body weight gain under control. The weight gain appeared cyclical under the influence of the HC–HF diet; and metformin lengthened or shortened these cycles in a dose-dependent manner.

3.4. Metformin at 250 mg/kg caused a significant suppression of gluconeogenesis

Blood glucose levels monitored using saphenous vein samples remained relatively stable within and across groups throughout the experimental period (data not shown). However, the average fasting blood glucose in the animals on the HC–HF diet gradually reached the upper level of normal range (Fig. 3A). Blood (fasting)



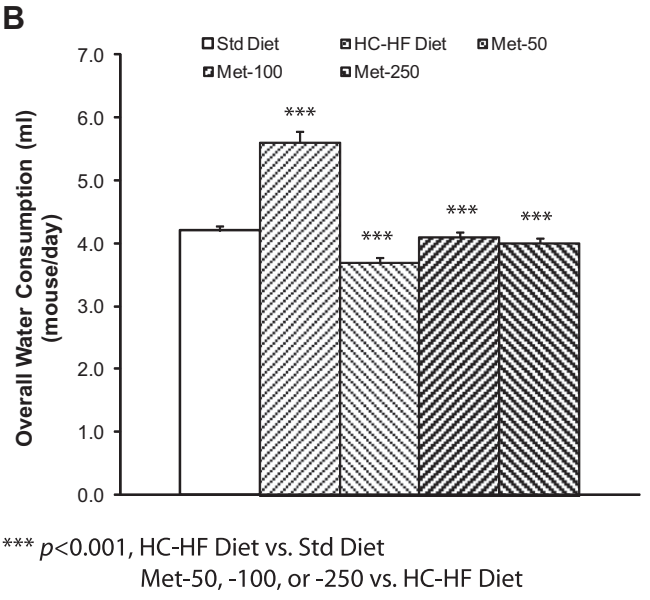


Fig. 1. Effect of metformin on food (A) and water (B) consumption. Animals on the HC–HF diet consumed significantly more food and water. Metformin at various doses significantly reduced the food and water consumption. The values are Mean ± SEM, $n = 5$ or 4/group.

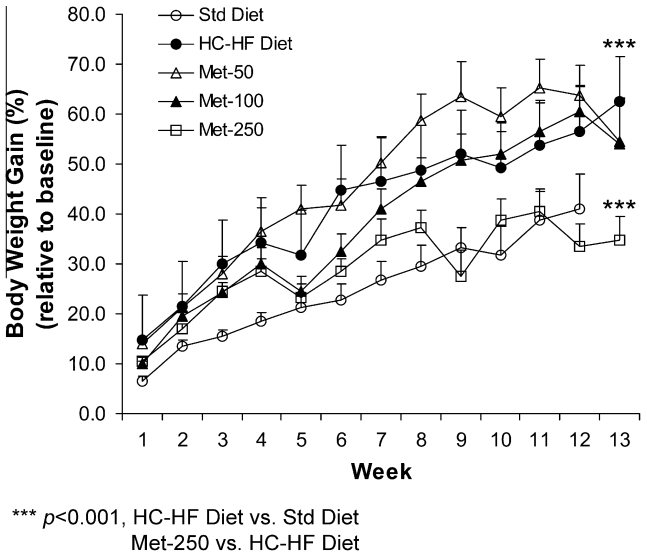


Fig. 2. Effect of the HC–HF diet and metformin on body weight. The HC–HF diet resulted in a significantly higher percent body weight gain (relative to the baseline). While at 50 and 100 mg/kg metformin stimulated body weight gain 5 weeks post treatment, at 250 mg/kg caused a significant reduction. The Mean ± SEM ($n = 5$ or 4/group) is shown.

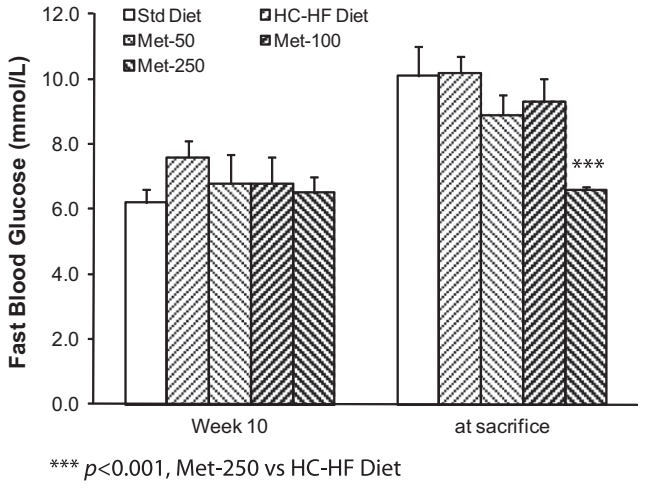


Fig. 3A. Influence of the HC–HF diet in blood glucose and inhibition of gluconeogenesis by metformin. Fasting blood glucose was influenced by the HC–HF diet as shown at Week 10. It reached to the upper level of normal range. Metformin at various doses inhibited gluconeogenesis as shown by the levels of blood glucose at sacrifice but at 250 mg/kg significantly inhibited the process. The values are Mean ± SEM, $n = 5$ or 4/group.

drawn by direct heart puncture reflects the status of gluconeogenesis by liver cells since there is little glucose consumed by the tissues. Only metformin at a dose of 250 mg/kg resulted in significant suppression of gluconeogenesis at sacrifice with no statistical differences found at week 10 (Fig. 3A).

3.5. The stimulation of insulin secretion by the HC–HF diet, the status of serum insulin, and the utilization of insulin

The HC–HF diet caused stimulation in insulin secretion as shown by the levels of fasting serum C-peptide (Fig. 3B). Interestingly, while metformin at 50 and 100 mg/kg was associated with more insulin secretion compared to that by the HC–HF diet alone, the 250 mg/kg dose inhibited the HC–HF diet-associated stimulation of insulin secretion to some extent (Fig. 3B). The serum insulin status of each group was of interest as well. The actual levels in these groups seemed to be affected by the HC–HF diet alone and the combination of the HC–HF diet and metformin treatment. Since neither insulin secretion nor actual level of insulin could precisely reflect the role of insulin in the metabolism of the HC–HF diet and metformin's effect on such metabolism, we looked into the matter by introducing insulin utilization.

The insulin utilization was estimated by the percent exhaustion of insulin secreted (Fig. 3C). The trend suggested that the high energy diet demanded more insulin than the standard diet. The insulin utilization was significantly greater in HC–HF Diet and Met-250 vs. Std Diet (Fig. 3C). There was no alteration in the levels of IGF-1 that was estimated in the pooled serum samples (data not shown).

3.6. Effects of the HC–HF diet and metformin on pancreatic islets

The average number of islets per pancreas section from animals on the HC–HF diet was 167% greater than that found on the standard diet. A significant increase was found in groups with

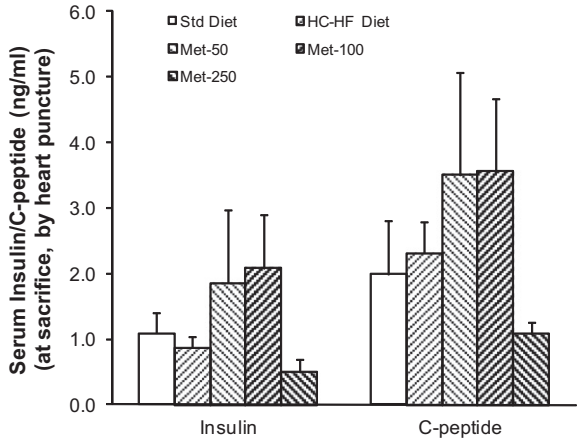


Fig. 3B. Effect of the HC–HF diet and metformin on insulin secretion and serum insulin. The HC–HF diet stimulated more insulin secretion as demonstrated by higher fasting serum C-peptide level. Metformin at 50 and 100 mg/kg dramatically induced more insulin secretion while at 250 mg/kg remarkably inhibited its secretion. Actual fasting serum insulin was used to estimate insulin utilization. The values are Mean ± SEM, $n = 5$ or 4/group.

metformin treatment at 100 and 250 mg/kg as compared to the standard diet (Table 1). To dissect the mechanism as to how the effect was carried out, the number of islets were grouped by arbitrarily classified ranges of sizes (from $\leqslant$5000 to >40,000 μm^2). The largest range included all islets larger than 40,000 μm^2 for the convenience of graphing and comparison (Fig. 4). This analysis revealed that the effects of the HC–HF diet on islets seemed to be dynamic as more islets appeared and more became larger in size. In terms of the number of islets, there was a marginal statistical significance between Std Diet and HC–HF Diet/Met-50, and a statistically significant difference between Std Diet and Met-100/Met-250 in $\leqslant$5000 μm^2 range. A much greater increase in the number of islets was observed than in the actual size of the islets. Therefore, the HC–HF diet primarily increased islets by number.

Based on two pieces of information that we obtained in this study, we could state that metformin had a minor and secondary effect on islet's size. Firstly, the dynamic pattern of the HC–HF diet's effect was still retained in metformin-treated groups. Secondly, metformin mainly affected larger islets since we noted that the number of islets in the largest range (40,000 μm^2) was expanded (Fig. 4), probably resulting in a slight increase in the average size (Table 1). At a dose of 250 mg/kg of metformin, we observed that only a few islets were enlarged beyond 30,000 μm^2, with more newly formed islets staying within less than 5000 μm^2, probably accounting for the greatly reduced islet size (Table 1 and Fig. 4).

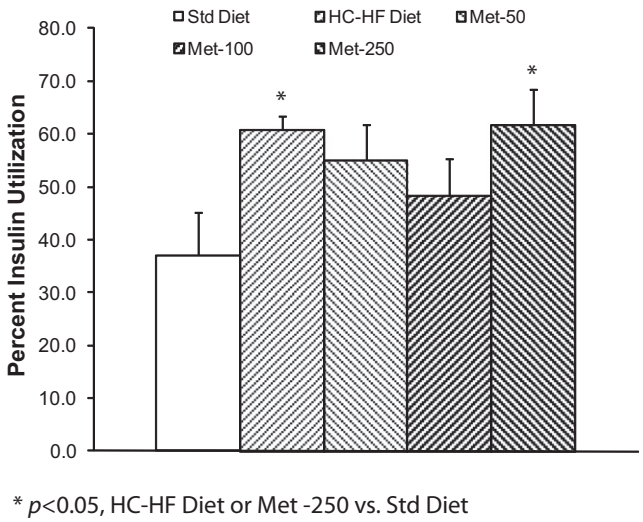


Fig. 3C. Effect of the HC–HF diet and metformin on insulin utilization. Insulin utilization was estimated by percent exhaustion of insulin secreted [(C-peptide – insulin)/C-peptide]. The HC–HF diet and metformin-treated groups utilized higher percent of insulin secreted on the whole with significance between some groups. Values were Mean ± SEM, $n = 5$ or 4/group.

3.7. The HC–HF diet was associated with the development of fatty degeneration in the liver

The HC–HF diet resulted in accumulation of microvesicular fat droplets in the cytoplasm of hepatocytes (Supplementary Fig. 1). Cells around central veins were mostly affected. In some cells, the accumulation of fat droplets was so profound that the cells were doubled or even tripled in size. Metformin at doses of 50 and 100 mg/kg did not abrogate the HC–HF diet-associated fatty changes; rather there were more hepatocytes affected and more fat droplets accumulated in the cytoplasm. Surprisingly, animals that received metformin at 250 mg/kg displayed either a relatively normal liver pathology (2/5) or very mild fatty degeneration (3/5). There was no metformin-related toxicity observed in the liver tissue at any given doses.

4. Discussion

The present study was conducted in normal male CD1 mice placed on a HC–HF (high energy) diet *ad libitum* for continuous 12 weeks with and without the administration of metformin at varying doses. The results showed that feeding the animals with the HC–HF diet induced obesity and insulin resistance. This was manifested with higher body weight (Fig. 2), stimulation of insulin secretion (Fig. 3B) and higher percent insulin utilization (Fig. 3C) but more glucose left in blood (Fig. 3A), and development of liver fatty degeneration (Supplementary Fig. 1).

The outcome of our study was different from those conducted with the standard diet. The lack of statistical significance between the groups receiving a standard diet and HC–HF diet as well between the groups of the HC–HF diet and metformin administration could be attributed to small numbers of animals in each group as well as internal variations.

The integrated data from our study demonstrated that once the energy intake provided by the HC–HF diet was inhibited by metformin, several of the unfavorable consequences associated with the HC–HF diet were lessened or corrected to a certain degree.

Table 1
Overview of surface area and number of pancreatic islets per pancreas section (Mean ± SEM).

	Std Diet ($n = 5$)	HC–HF diet ($n = 4$)	Met-50 ($n = 4$)	Met-100 ($n = 5$)	Met-250 ($n = 5$)
Surface area of pancreatic islets (μm^2)[#]	15,504 ± 1850	18,848 ± 2321	22,707 ± 2491	25,601 ± 3658	8882 ± 954
Number of islets/mm^2 pancreas section (percent of Std Diet)	0.48 ± 0.04 (100)	0.80 ± 0.13 (167)	0.82 ± 0.08 (171)	0.82 ± 0.14[*] (171)	0.91 ± 0.14[*] (190)

[#] No statistical significance found, HC–HF diet vs. Std Diet; Met-50, Met-100 or Met-250 vs. HC–HF diet.
[*] $p < 0.05$, Met-100 or Met-250 vs. Std Diet.

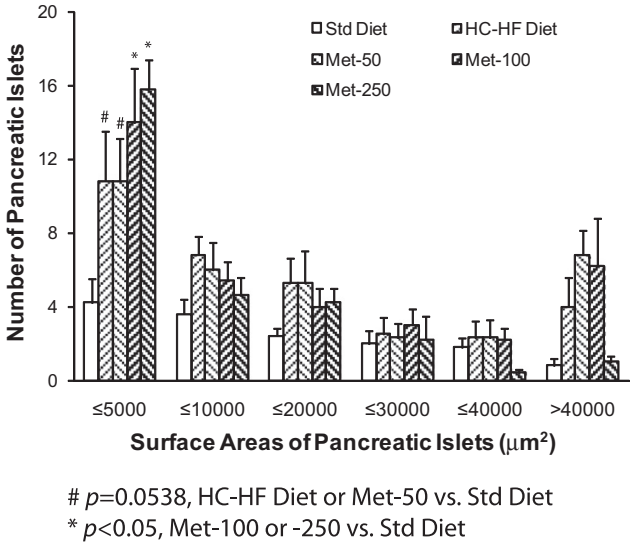


Fig. 4. Effects of the HC–HF diet and metformin on islets illustrated by number of islets in various size ranges. Marginally significantly or significantly more numbers of islets clustered in the range of surface areas $\leqslant 5000\ \mu m^2$. Metformin at 50 and 100 mg/kg had a minor effect on islet' size while at 250 mg/kg seemed to protect islets from being enlarged. The values are Mean ± SEM, $n = 5$ or 4/group. The data could be comprehended more clearly together with Table 1.

Metformin's capacity to significantly reduced food consumption may be its fundamental impact. The reduced ingestion could be due to the metabolic effects produced by metformin [19] or in part due to the impact on the gastrointestinal stress [20] or both. Nonetheless, at a sufficient dose it was capable of reducing energy intake to the level comparable to that provided by the standard diet (Fig. 1A). The difference was that 17% of the energy supply was contributed from fat in the standard diet while 45% was in the HC–HF diet.

It has been reported that β-cells can be desensitized after chronic exposure to high concentration of glucose or fatty acids [21]. Metformin has been demonstrated to restore β-cells' sensation in the presence of high levels of blood glucose or fatty acids so as to enable them to secret more insulin [21]. Our data provided similar evidence *in vivo*. Insulin secretion was not dramatically increased by the HC–HF diet though energy intake was relatively high (24.752 kcal with the HC–HF vs. 13.86 kcal with the standard diet). This was probably due to the fact that β-cells were chronically exposed to high level of fatty acids and/or blood glucose provided by the HC–HF diet [22]. The administration of metformin at 50 and 100 mg/kg may successfully restored insulin secretion to meet the metabolic needs (Fig. 3B). Metformin is also reported to sensitize peripheral tissues to react to insulin [20]. It seemed plausible that a combination of these two effects might have produced the outcome observed in our study, namely the excess energy provided by the HC–HF diet was transformed by metformin into body weight and this in turn transferred into the hepatocytes as fat droplets. Thus, metformin at lower doses such as 50 and 100 mg/kg might paradoxically improve the systemic metabolism.

Our data also illustrated that metformin at a dose of 250 mg/kg significantly inhibited gluconeogenesis (Fig. 3A). Under such inhibition, the blood glucose remains similar to that of the standard diet (Fig. 3A), which likely implied that the alternative energy supply came more from fatty acids rather than from glucose. Since insulin secretion was remarkably inhibited by metformin (Fig. 3B) and more insulin was needed to metabolize alternatively supplied energy, additional amount of secreted insulin was utilized (Fig. 3C). Taken together, sufficient doses of metformin might be required to exhibit the most effective protection to excess energy supply.

The unique finding in our study was that there seemed to be some pancreatic islets reserved for metabolic needs. Feeding the animals with the HC–HF diet might increase or enlarge islets through hyperplasia and/or hypertrophy (Table 1 and Fig. 4). Given that the entire mouse pancreas resembles a piece of paper-thin fatty tissue, we speculate that the surface areas of islets would represent the sizes of islets dispersed in the entire pancreas and that the number of islets in one section of pancreas would proportionally represent the actual number of islets in the pancreas. In order to limit sampling bias, the surface area of each pancreas section was measured and taken as the reference and the results observed was fairly consistent (Table 1).

5. Conclusion

Our study has demonstrated that feeding the animals with a HC–HF diet induces obesity and insulin resistances and the simultaneous administration of metformin influences both the systemic and cellular metabolisms. Metformin, at sufficient doses, is able to offer protection from the unfavorable metabolic consequences of the HC–HF diet.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.05.152.

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