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Effects of metformin on insulin resistance and metabolic disorders in tumor-bearing rats with advanced cachexia

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ABSTRACT

Metformin (MET) is widely used in the correction of insulin (INS) resistance and metabolic abnormalities in type 2 diabetes. However, its effect on INS resistance and metabolic disorders associated with cancer cachexia is not established. We investigated the MET effects, isolated or associated with INS, on INS resistance and metabolic changes induced by Walker-256 tumor in rats with advanced cachexia. MET (500 mg.kg^{-1} , oral) and MET + INS (NPH, 1.0 IU.kg^{-1} , sc) were administered during 12 days, starting on the day of tumor cell inoculation. Tumor-bearing rats showed adipose and muscle mass wasting, weight loss, anorexia, decreased Akt phosphorylation in retroperitoneal and mesenteric adipose tissue, peripheral INS resistance, hypoinsulinemia, reduced INS content and secretion from pancreatic islets and also inhibition of glycolysis, gluconeogenesis and glycogenolysis in liver. MET and MET + INS treatments did not prevent these changes. It can be concluded that treatments with MET and MET + INS did not prevent the adipose and muscle mass wasting and weight loss of tumor-bearing rats possibly by not improving INS resistance. Therefore, MET, used for the treatment of INS resistance in type 2 diabetes, is not effective in improving INS resistance in the advanced stage of cancer cachexia, evidencing that the drug does not have the same beneficial effect in these two diseases.

Key words: Cancer, Hypercatabolism, Hypoinsulinemia, Metabolic changes, Insulin resistance.

1 INTRODUCTION

Cancer cachexia is a complex syndrome characterized by marked weight loss, anorexia, inflammation and changes in the metabolism of carbohydrates, lipids and proteins, being an important cause of mortality, since there is no effective treatment (Evans et al. 2008; Tisdale 2010; Vaughan et al. 2013; Porporato 2016).

The weight loss in cancer cachexia is a result of muscle and fat wasting due to hypercatabolism of proteins and lipids (Argilés et al. 2005; Evans et al. 2008; Tisdale 2010; Petruzzelli and Wagner 2016). The intense proteolysis and lipolysis is promoted by cachexia mediators, such as proteolysis inducing factor (PIF), lipid mobilizing factor (LMF) and cytokines such as tumor necrosis factor α (TNF α) and interleukins 1 (IL1) and 6 (IL6) (Mantovani et al. 2000; Argilés et al. 2005; Gordon et al. 2005; Patel and Patel 2016).

However, it is possible that insulin (INS) resistance present in cancer-bearing (Lundholm et al. 1978; Copeland et al. 1987; Honors and Kinzig 2012) also contributes to the establishment of cachexia, since INS has potent anabolic and anticatabolic effects on protein and lipid (Wilcox 2005; Chevalier and Farsijani 2014). Few studies have investigated the effects of INS sensitivities on muscle and fat wasting and other metabolic changes associated with cancer cachexia (Ropelle et al. 2007; Asp et al. 2010).

Metformin (MET) is an INS sensitizer used worldwide for the treatment of type 2 diabetes. Its INS sensitizing effect is mainly attributed to the stimulation of adenosine monophosphate activated kinase protein (AMPK) (Zhou et al. 2001; Hadad et al. 2013). Once activated, AMPK stimulates the INS signaling pathway proteins, such as protein kinase 3 (PI3K) and protein kinase B (Akt or PKB) (Kurth-Kraczek et al. 1999; Jakobsen et al. 2001). MET-activated AMPK also stimulates fatty acid oxidation and thereby reduces lipid (free fatty acids) interference on INS sensitivity in patients with type 2 diabetes (Zhou et al. 2001; Hawley et al. 2010; Andújar-Plata et al. 2012). Free fatty acids impair INS signaling by decreasing the proteins phosphorylation of INS signaling pathway, such as the Akt (Delarue and Magnan 2007).

Although the MET effects as an INS sensitizer in type 2 diabetes are well understood, its effects on INS resistance, as well as on several metabolic abnormalities and cancer-associated cachexia have been poorly investigated.

Considering that Walker-256 tumor-bearing rats exhibit INS resistance and hypoinsulinemia, which may contribute to the metabolic disorders and cachexia in these animals (Fernandes et al. 1990; De Souza et al. 2015a), the aim of this study was to evaluate the MET effects, isolated or associated with INS, on INS signaling pathway proteins (Akt), INS resistance and metabolic disorders in Walker-256 tumor-bearing rats with advanced cachexia.

2. MATERIALS AND METHODS

2.1 Drugs and chemicals

Metformin (Glifage®) was purchased from Merck and Co. (Darmstad, Germany) and NPH INS (Humulin®) and regular INS (Humalog®) from Eli Lilly (Rio de Janeiro, Brazil). Primary and secondary antibodies were acquired from Cell Signaling Technology® (Danvers, USA), Abcam® (Cambridge, USA), Santa Cruz Biotechnology® (Dallas, USA) or Jackson ImmunoResearch Laboratories, Inc. (West Grove, USA). Protease inhibitors were acquired from AMRESCO® LLC (Solon, USA). Radioactive INS was purchased from PerkinElmer (Massachusetts, USA). The perfusion fluid salts and other chemicals were obtained from Sigma Chemical Co. (St Louis), Merck and Co. (Darmstadt, Germany), Reagen (Rio de Janeiro, Brazil) or Laborclin (Pinhais, Brazil).

2.2 Animals and Walker-256 tumor inoculation

The experimental protocols were approved by the Ethics Committee for Animal Use of the State University of Londrina (CEUA/Uel, register number 09161) and followed the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council, Washington, DC, National Academy Press, no. 85-23, revised 1996).

Male Wistar rats (220-230 g), kept in collective boxes, $23 \pm 2^{\circ}\text{C}$, light/dark cycle of 12 h, with free access to water and standard rodent chow (Nuvilab, CR-1 Nuvital®, Colombo, Brazil) were used in all experiments. Walker-256 carcinosarcoma cells were maintained as previously described (Cassolla et al. 2012). For tumor inoculation, 8×10^7 tumor cells were suspended in phosphate buffered saline (PBS: 16.5 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and inoculated subcutaneously in the right flank of rats (tumor-bearing rats). Healthy rats were injected with PBS in the same place.

2.3 Treatment protocol

Walker-256 tumor-bearing rats were treated with MET (500 mg.kg^{-1} , oral gavage), alone or in association with INS (NPH, 1.0 UI.kg^{-1} , sc), once a day (17:00 pm) for 12 days, since the day of tumor cells inoculation. Doses were based on previous study (Ropelle et al. 2007; Takada et al. 2008). MET was daily dissolved in water and INS was diluted in saline (0.9% NaCl). Control rats (tumor-bearing and healthy) received vehicles.

The experiments were performed on day 12 after tumor cells inoculation, 3 h after the last treatment, in fed rats. The experiments for evaluation of glycolysis and gluconeogenesis were performed in rats fasted for 24 hours.

2.4 Assessment of cachexia-anorexia parameters and tumor growth

Food intake was measured (day 11) by the difference between the amount of feed supplied and the remainder after 24 h. On day 12, rats were weighed, anesthetized with thiopental (50 mg.kg^{-1} , ip) and laparotomized to collect of blood samples from the inferior vena cava to evaluate the concentrations of plasma INS by radioimmunoassay (Yalow and Berson 1960). Thereafter, the rats were euthanatized and retroperitoneal and mesenteric adipose tissue and gastrocnemius and extensor digitorum longus (EDL) muscle were also carefully removed, weighed, quickly frozen in liquid nitrogen and stored at -80°C for protein analysis. The pancreas

also was removed for analysis of islet INS secretion and content. The tumor was dissected and weighed for assessment of tumor growth. The change in body mass was measured by the difference between the final body mass (day 12), discounted the tumor mass, and initial body mass (day 1).

2.5 Western blotting to assess total Akt and p-Akt

The retroperitoneal and mesenteric adipose tissues and the gastrocnemius muscle, stored at -80°C , were homogenized in buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , 1 mM EDTA, 5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 10 mM NaF, Triton X100 1%, glycerol 10%, 0.5 mM Na_3VO_4 , 20 mM Tris, pH 7.8) containing 0.2 mM of protease inhibitors cocktails (PIC) and phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged (15294 g, 40 min, 4°C) and the total proteins of the supernatant were quantified (Bradford 1976). Equal amounts of total protein (80 μg) diluted in Laemmli buffer, were applied to 10% polyacrylamide gel, submitted to electrophoresis (SDS-PAGE) and transference to nitrocellulose membrane. The membranes were incubated with primary antibody (1:1000) anti-Akt (Cell Signaling®, catalog n° 9272), anti-phospho-Akt^{Ser473} (Cell Signaling®, catalog n° 9271) or anti- γ -tubulin (Sigma®, catalog n° T5326) overnight at 4°C , followed by incubation with secondary antibody conjugate to peroxidase (1:5000) and chemiluminescence detection with peroxidase substrate (ECL) in Amersham Image® photodocumentator. The density of the blots was analyzed in Image J software (National Institutes of Health, USA) and expressed in arbitrary units (AU) after normalization by constitutive protein γ -tubulin.

2.6 Assessment of INS secretion and content in pancreatic islets

Pancreatic islets were isolated by pancreas digestion with collagenase as previously described (Lacy and Kostianovsky 1967). Groups of 5 islets were pre-incubated at 37°C during 30 min in Krebs-Henseleit (KH) with 0.2% bovine serum albumin (BSA) and 5.6 mmol.L^{-1}

glucose. Thereafter, islets were incubated at 37°C during 1 h in KH with 0.2% BSA in different concentrations of glucose (5.6, 11.1 or 16.7 mM). For total INS content, islets in each well were disrupted in acid ethanol solution (1.4% chloridric acid and 74% ethanol) and sonicated (3 pulses of 5 seconds). At the end of the experiments, the medium was collected and INS was measured by radioimmunoassay (Yalow and Berson 1960).

2.7 Insulin tolerance test (ITT)

For the ITT, regular INS (0.25 UI.kg^{-1}) was injected into inferior vena cava in rats anesthetized with thiopental (50 mg.kg^{-1} ; ip). Blood samples for evaluation of glycemia (Bergmeyer and Bernt 1974) were collected from inferior vena cava at 0 (basal), 5, 10 and 15 min after INS injection. The constant of plasma glucose disappearance (KITT), an indicator of INS peripheral response, was calculated as previously described (Seraphim et al. 2001; De Souza et al. 2015a).

2.8 Liver perfusion to assess glycolysis, glycogenolysis and gluconeogenesis

For *in situ* liver perfusion the rats were anesthetized with thiopental (50 mg.kg^{-1} ; ip). The portal and inferior cava vein were cannulated as previously described (De Moraes et al. 2012; Da Rocha et al. 2013; De Souza et al. 2015b; Bassoli et al. 2015). The perfusion liquid, Krebs-Henseleit buffer (KH: NaCl 115 mM, NaHCO_3 25 mM, KCl 5.8 mM, 1.2 mM Na_2SO_4 , 1.18 mM MgCl_2 , 1.2 mM NaH_2PO_4 and 2.5 mM CaCl_2), pH 7.4 at 37 °C and saturated $\text{O}_2\text{:CO}_2$ (95:5%) was introduced into portal vein (4 ml.min^{-1} per gram of liver) and the liquid effluent of the liver was collected from inferior cava vein at 2 min intervals to assess the production of glucose (Bergmeyer and Bernt 1974), lactate (Gutmann and Wahlefeld 1974) and pyruvate (Czok and Lamprecht 1974).

For assessment of glycolysis and gluconeogenesis livers of 24 hours fasted rats (to deplete liver glycogen) were perfused with KH for 10 minutes followed by perfusion for 30 minutes with

KH + 20 mM glucose (glycolysis evaluation) or KH + 2.5 mM alanine (gluconeogenesis evaluation). For the assessment of glycogenolysis livers of fed rats were perfused for 30 minutes with KH. Glycolysis was calculated as the sum of pyruvate plus lactate production (lactate + pyruvate) and glycogenolysis as the sum of glucose production plus the half-sum of lactate and pyruvate production [glucose + $\frac{1}{2}$ (lactate + pyruvate)] (Miksza et al. 2013; Da Rocha et al. 2014; De Souza et al. 2015b).

2.9 Statistical analysis

Data were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Brown Forsythe test) and appropriate statistical test (One-Way ANOVA followed by Tukey) were employed. Statistical analysis were carried out with the program GraphPad Prism 6.0, at a significance level of 5% ($p < 0.05$). Data were expressed as mean $\pm$ standard error of the mean (SEM).

3 RESULTS

The p-Akt:total Akt ratio of retroperitoneal (Figure 1A) and mesenteric (Figure 1B) adipose tissue, but not the gastrocnemius muscle (Figure 1C), was lower in tumor-bearing rats compared to healthy rats. MET and MET + INS treatments had no effect on p-Akt:total Akt of these tissues.

Tumor-bearing rats had reduced INS peripheral response compared to healthy rats (Figure 2A), as evidenced by lower kITT (Figure 2B). MET and MET + INS did not improve the INS resistance of tumor-bearing rats (Figure 2B).

Blood INS (Figure 3A), INS secretion from islets incubated with 16.7 mM glucose (Figure 3B) and INS intracellular content of islets (Figure 3C) were lower in tumor-bearing rats compared to healthy rats and were not improved by MET and MET + INS treatments (Figures 3A, 3B and 3C).

Tumor-bearing rats showed tumor mass about 25 g, which was associated with body mass loss, retroperitoneal and mesenteric adipose mass wasting, gastrocnemius and EDL muscle mass wasting and anorexia (Table 1). MET and MET + INS had no effect on tumor growth or parameters of cachexia and anorexia (Table 1).

Regarding the hepatic parameters, tumor-bearing rats showed reduced production of lactate (Figure 4A) and pyruvate (Figure 4B) and consequently of glycolysis (Figure 4C) from exogenous glucose, compared to healthy rats. MET and MET + INS had no effect on these parameters, as shown by AUCs (Figure 4D).

Tumor-bearing rats showed decreased hepatic glucose production (Figures 5A) and glycogenolysis (Figures 5B) compared to healthy rats. MET treatment did not change these parameters, but MET + INS increased glucose production and glycogenolysis in tumor-bearing rats compared to rats with tumor untreated, as evidenced by AUCs (Figures 5C and 5D).

Finally, tumor-bearing rats showed reduced production of glucose (Figure 6A), lactate (Figure 6B) and pyruvate (Figure 6C) compared to healthy rats. MET and MET + INS did not change glucose production but increased the lactate and pyruvate production of tumor-bearing rats, as shown by AUCs (Figure 6D).

4 DISCUSSION

Given that MET reduces INS resistance and improves metabolic abnormalities in type 2 diabetes, we decided to investigate whether these beneficial effects of MET also occur in Walker-256 tumor-bearing rats with advanced cachexia. We showed that treatment with MET or MET + INS did not improve INS resistance and the metabolic disorders induced by the Walker-256 tumor at this advanced stage of the cachectic syndrome.

Tumor-bearing rats showed reduced Akt phosphorylation in retroperitoneal and mesenteric adipose tissue but not in the gastrocnemius muscle (Figure 1). Gastrocnemius p-Akt was not reduced probably because of the compensatory overload of this muscle, caused by the

reduced capacity of support and locomotion of contralateral limb, where the tumor was located (Bodine et al. 2001).

Consistent with the reduction of p-Akt in adipose tissue, tumor-bearing rats showed reduced INS peripheral response (Figure 2), indicating INS resistance. INS resistance in cancer has been related to the production of cytokines (TNF α and IL6) and to excess of blood free fatty acids resulting from intense lipolysis (Yoshikawa et al. 1999). These factors interfere in the INS signal transduction, since they change the protein phosphorylation of INS signaling cascade as the Akt (Kanety et al. 1995; Boden 2002; Kim et al. 2004; Delarue and Magnan 2007). MET and MET + INS treatments did not improve the INS signal transduction in adipose tissue, as indicated by the decreased Akt phosphorylation (Figure 1), and nor the INS peripheral response (Figure 2).

Tumor-bearing rats also showed hypoinsulinemia (Figure 3A), confirming previous findings (Fernandes et al. 1990), which is probably consequence of decreased INS secretion (Figure 3B) and synthesis (Figure 3C) by pancreatic- β cells. Treatments with MET e MET + INS did not improve the secretory dysfunction and decreased INS synthesis of the islet and nor hypoinsulinemia (Figure 3). INS supplementation did not change the hypoinsulinemia of tumor-bearing rats, probably due to its metabolism. It is noteworthy that blood INS was evaluated 3 h after the last treatment with low INS dose.

Consistent with the lack of treatments effect on INS resistance and hypoinsulinemia, MET and MET + INS treatments did not prevent weight loss, and adipose (retroperitoneal and mesenteric) and muscle (gastrocnemius and EDL muscle) mass wasting (Table 1). Weight loss in cancer cachexia is attributed to intense muscle proteolysis and lipolysis, due to the action of factors such as PIF, LMF and cytokines (TNF α , IL1 and IL6), which activate proteolytic and lipolytic pathways, as well as reducing protein synthesis and adipogenesis (Mantovani et al. 2000; Gordon et al. 2005; Patel and Patel 2016).

Weight loss in patients and animals with cancer is also attributed to anorexia. Anorexia in cancer appears to be related to the hypothalamic effect of cytokines (TNF α and IL6) that stimulate

the release of anorexigenic factors, such as corticotrophin releasing factor (CRH), and reduce the release of orexigenic factors such as neuropeptide Y (NPY) (Martignoni 2003; Argilés et al. 2005; Patra and Arora 2012). In our study, treatment with MET + INS did not prevent tumor-induced anorexia (Table 1). MET and MET + INS treatments also did not change tumor growth (Table 1), corroborating with other studies (Ropelle et al. 2007).

Another parameter affected by the tumor and regulated by INS, which could be benefited by treatment with an INS sensitizer is the glucose metabolism in the liver. Tumor-bearing rats showed inhibition of glycolysis from exogenous glucose (Figure 4), corroborating our previous studies (Mikszta et al. 2013; De Souza et al. 2015b). These results are consistent with inhibition of glucokinase observed in rats with Walker-256 tumor (Vicentino et al. 2002). The glucokinase inhibition decreases glucose uptake by liver and may have contributed to the lower peripheral response to INS (KITT) in tumor-bearing rats (Figure 2). Although MET stimulates ATP-generating pathways, MET and MET + INS treatments did not prevent inhibition of glycolysis in tumor-bearing rats (Figure 4), probably due to inhibition of glucokinase (Vicentino et al. 2002).

Tumor-bearing rats also showed inhibition of hepatic glucose production and glycogenolysis (Figure 5), results that reflect the low glycogen content in liver, as observed in our studies (Cassolla et al. 2012). MET treatment did not change glucose production and glycogenolysis, but MET + INS treatment increased these parameters (Figure 5), probably because INS increased glycogen content and MET activated glycogenolysis (Salani et al. 2014).

In addition, tumor-bearing rats showed inhibition of hepatic gluconeogenesis from alanine (Figure 6), confirming our previous studies (Moreira et al. 2013; De Souza et al. 2015b). MET treatment did not change the reduced glucose production, but MET + INS treatment increased lactate and pyruvate production, which is reduced in tumor-bearing rats (Figure 6), an effect that may be due to the stimulatory action of MET on the catabolism of glucose (glycolysis) generated by gluconeogenesis (Salani et al. 2014).

It can be concluded that MET and MET + INS treatments did not prevent the adipose and muscle mass wasting and weight loss of the tumor-bearing rats possibly by not improving INS resistance. Therefore, MET, widely used for the treatment of INS resistance in type 2 diabetes, is not effective in improving INS resistance in the advanced stage of cancer cachexia, evidencing that the drug does not have the same beneficial effect in these two diseases.

Although some studies (Fonseca et al. 2011, Franco et al. 2014, Oliveira and Gomes-Marcondes 2016) show beneficial effects of MET treatment in Walker-256 tumor-bearing rats, it is noteworthy that the treatment protocols, the animal model (obese tumor-bearing rats), the stage of cachexia and/or age of the animals were different from those used in the present study.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1. Tumor mass, initial weight, final weight (excluding tumor weight), change of body mass, retroperitoneal and mesenteric fat mass, gastrocnemius and EDL muscle mass and food intake of healthy and Walker-256 tumor-bearing rats treated with metformin (Tumor + MET), metformin plus insulin (Tumor + MET + INS) or vehicles during 12 days.

Parameter	Healthy	Tumor	Tumor MET	Tumor MET+ INS
Tumor mass (g)	---	24,72±1,32 (21)	26,86±2,39 (17)	27,48±2,14 (17)
Initial weight (g)	219,7±3,24 (10)	219,6±2,36 (23)	222,4±2,61 (17)	224,0±2,67 (17)
Final weight (g)	261,4±3,99 (10)	213,2±4,51*** (23)	206,2±5,97*** (17)	208,0±5,66*** (17)
Change body mass (g)	41,7±2,59 (10)	-6,4±3,43*** (23)	-16,2±4,67*** (17)	-16,0±3,76*** (17)
Retroperitoneal fat (g%)	0,772±0,053 (9)	0,544±0,038* (21)	0,414±0,052*** (16)	0,371±0,039*** (17)
Mesenteric fat (g%)	0,463±0,024 (9)	0,334±0,021** (20)	0,324±0,036** (17)	0,282±0,027*** (17)
Gastrocnemius muscle (g%)	0,639±0,014 (15)	0,582±0,011* (18)	0,573±0,012** (17)	0,586±0,016* (17)
EDL muscle (g%)	0,049±0,002 (11)	0,044±0,001* (20)	0,042±0,002** (15)	0,044±0,001* (16)
Food intake (g%)	9,33±0,44 (12)	7,32±0,33* (18)	8,37±0,27 (8)	6,39±0,48** (8)

* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ vs healthy (One-Way ANOVA followed by Tukey). The values in parentheses indicate the number of animals.

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FIGURE CAPTIONS

Figure 1. p-Akt^{Ser473}:total Akt in retroperitoneal fat (A), mesenteric fat (B) and gastrocnemius muscle (C) in healthy rats and Walker-256 tumor-bearing rats treated with metformin (Tumor + MET), metformin plus insulin (Tumor + MET + INS) or with vehicles for 12 days. The proteins were normalized by γ -tubulin. Data are mean $\pm$ SEM of 8-12 fed rats. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ vs healthy (One-Way ANOVA followed by Tukey).

Figure 2. Glycemia (A) and constant of serum glucose disappearance (KITT) (B) of healthy rats and Walker-256 tumor-bearing rats treated with metformin (Tumor + MET), metformin plus insulin (Tumor + MET + INS) or with vehicles for 12 days. Glycemia was evaluated at 0 (basal), 5, 10 and 15 minutes after the injection of regular insulin (0.25 UI.kg^{-1}). Data are mean $\pm$ SEM of 5–15 fed rats. The glycemia values within the same group were compared to basal (0 min) by repeated measures ANOVA followed by Tukey; *** $p < 0.01$. Differences between kITTs: * $p < 0.05$ and ** $p < 0.01$ versus healthy (One-way ANOVA followed by Tukey).

Figure 3. Plasma insulin (A), insulin secretion-stimulated by 5.6, 11.1 and 16.7 mM glucose (B) and the respective intracellular insulin content (C), after 1h of incubation of the islets, of healthy and Walker-256 tumor-bearing rats treated with metformin (Tumor + MET) or with metformin plus insulin (Tumor + MET + INS) or with vehicles for 12 days. Data are mean $\pm$ SEM of 4-8 fed rats. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs healthy (One-Way ANOVA followed by Tukey).

Figure 4. Production of lactate (A), production pyruvate (B) and glycolysis (C) from exogenous glucose, and areas under curves (AUCs) (D), in livers of healthy rats and Walker-256 tumor-bearing rats treated with metformin (Tumor + MET), metformin plus insulin (Tumor + MET + INS) or with vehicles for 12 days. Livers were perfused as described in Material and Methods. Glucose (20 mM) was infused between 10 and 40 min. Data are mean $\pm$ SEM of 5–6 rats fasted

24 hours. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ vs healthy (One-Way ANOVA followed by Tukey).

Figure 5. Production of glucose (A), glycogenolysis (B) and the means of glucose production (C) and glycogenolysis (D) in livers of healthy rats and Walker-256 tumor-bearing rats treated with metformin (Tumor + MET), metformin plus insulin (Tumor + MET + INS) or with vehicles for 12 days. Livers were perfused as described in Materials and Methods. Data are mean $\pm$ SEM of 3–7 fed rats. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ vs healthy; ### $p < 0.001$ vs tumor (One-Way ANOVA followed by Tukey).

Figure 6. Production of glucose (A), lactate (B) and pyruvate (C) from alanine, and areas under curves (D), in livers of healthy rats and Walker-256 tumor-bearing rats treated with metformin (Tumor + MET), metformin plus insulin (Tumor + MET + INS) or with vehicles for 12 days. Livers were perfused as described in Material and Methods. Alanine (2.5 mM) was infused between 10 and 40 min. Data are mean $\pm$ SEM of 5–6 rats fasted 24 hours. * $p < 0.05$; ** $p < 0.01$ vs healthy; # $p < 0.05$ and ### $p < 0.001$ vs tumor (One-Way ANOVA followed by Tukey).

Figure 1

32x17mm (300 x 300 DPI)

Figure 2

25x12mm (300 x 300 DPI)

Figure 3

42x124mm (300 x 300 DPI)

Figure 4

45x38mm (300 x 300 DPI)

Figure 5

48x50mm (300 x 300 DPI)

Figure 6

44x38mm (300 x 300 DPI)