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RESEARCH PAPER

Metformin and male reproduction: effects on Sertoli cell metabolism

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BACKGROUND AND PURPOSE

Metformin is commonly used to treat type 2 diabetes (T2D). While new clinical applications have been ascribed to metformin, including treatment of anovulatory infertility, its effects on male reproduction have not been investigated. The Sertoli cell (SC) is crucial for germ cell development, exerting metabolic control of spermatogenesis, therefore, we investigated the effects of metformin on SC metabolism.

EXPERIMENTAL APPROACH

Rat SCs were cultured in the absence and presence of metformin (5, 50 and 500 μM). mRNA and protein levels of glucose transporters (GLUT1 and GLUT3), phosphofructokinase 1 (PFK 1), lactate dehydrogenase (LDH) and monocarboxylate transporter 4 (MCT4) were determined by quantitative PCR and Western blot respectively. LDH activity was assessed and metabolite production/consumption determined by ^{1}H-NMR.

KEY RESULTS

Metformin (50 μM) decreased mRNA and protein levels of GLUT1, GLUT3, MCT4 and PFK 1 but did not affect LDH mRNA or protein levels. However, although glucose consumption was maintained in metformin-treated cells, LDH activity, lactate and alanine production were increased, indicating an enhanced glycolytic flux. No metabolic cytotoxicity was detected in SCs exposed to supra-pharmacological concentration of metformin.

CONCLUSIONS AND IMPLICATIONS

Our results indicate that metformin: (i) decreases mRNA and protein levels of glycolysis-related transporters in SCs but increases their activity; and (ii) stimulates alanine production, which induces antioxidant activity and maintains the NADH/NAD^{+} equilibrium. The increased lactate in metformin-treated SCs provides nutritional support and has an anti-apoptotic effect in developing germ cells. Thus, metformin can be considered as a suitable antidiabetic drug for male patients of reproductive age with T2D.

Abbreviations

DAB, 3,3′ diaminobenzidine hydrochloride; DM, diabetes mellitus; Ham's F12, Ham's nutrient mixture F12; dNTP, deoxynucleotide triphosphates; GLUT, glucose transporter; IgG-AP, alkaline phosphatase-linked igG; ITS, insulin–transferrin–sodium selenite; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; M-MLV RT, Moloney murine leukaemia virus reverse transcriptase; PCOS, polycystic ovary syndrome; PFK, phosphofructokinase; qPCR, quantitative real-time PCR; SC, Sertoli cell; T2D, type 2 diabetes mellitus

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Introduction

Metformin has been used clinically for over 50 years and is recognized as the first-line drug of choice for the treatment of patients with type 2 diabetes (T2D). In addition, it is the seventh most frequently prescribed generic drug in the USA (2011). Metformin is an oral biguanide insulin-sensitizing drug that is known to block liver gluconeogenesis, increase skeletal muscle uptake of glucose (Martineau, 2012) and reduce the absorption of glucose in the intestinal mucosa (Sakar *et al.*, 2010). Several other functions, rather than those directly related to its antihyperglycaemic effect, have been reported. For instance, metformin can inhibit the proliferation of various cancer cells both *in vivo* and *in vitro* (Wu *et al.*, 2010; Kato *et al.*, 2012), and, treatment with metformin for at least a year may reduce the incidence of breast cancer in diabetic women (Landman *et al.*, 2010).

Metformin has also been used to treat anovulatory infertility in patients with polycystic ovary syndrome (PCOS) (Palomba *et al.*, 2006). Metformin seems to increase the likelihood of ovulation and can, in combination with clomifene, increase the frequency of ovulation and pregnancy in PCOS women (Creanga *et al.*, 2008). However, intrauterine exposure to metformin is associated with elevated levels of sex hormone-binding globulin in newborns (Carlsen and Vanky, 2010) and boys have been shown to have elevated levels of both oestrogens and androgens when compared with girls (Carlsen and Vanky, 2010). More recently it has been found that metformin decreases testosterone production *in vitro* and reduces testicular size and the population of Sertoli cells (SCs) *in vivo* (Tartarin *et al.*, 2012), although this was only observed during fetal development. This reduction in SC population was due to a decrease in cell proliferation rather than cell death (Tartarin *et al.*, 2012), indicating that metformin alters the physiological functions of SCs. Thus, it is important to investigate the molecular mechanisms involved in the effects of metformin on SCs.

The SC population determines the efficiency of spermatogenesis (Petersen and Soder, 2006), as indicated by a strong correlation between the number of germ cells and sperm production. Moreover, each SC can only support a limited number of germ cells (Weber *et al.*, 1983). These cells are responsible for the maintenance of the ionic concentration of the tubular fluid (Oliveira *et al.*, 2009a,b; Rato *et al.*, 2010) and also for providing the nutritional and physical support for the developing germ cells (Rato *et al.*, 2012b). SCs take up glucose from the interstitial fluid via glucose transporters (GLUTs), particularly GLUT1 and GLUT3 (Galardo *et al.*, 2008; Alves *et al.*, 2013d), and convert it into lactate via LDH (Alves *et al.*, 2013d). The lactate produced is then exported by specific monocarboxylate transporters (MCTs) (Rato *et al.*, 2012a). The developing germ cells need this exogenous lactate for energy production, and lactate supply can regulate spermatocyte survival and metabolic activity (Jutte *et al.*, 1982). SCs have recently been demonstrated to secrete acetate (Alves *et al.*, 2012b), which is essential for the maintenance of a high rate of lipid synthesis by the developing germ cells (Alves *et al.*, 2012b). This metabolic cooperation between SCs and germ cells is crucial for normal spermatogenesis and is sensitive not only to hormonal levels (Galardo *et al.*, 2008; Oliveira *et al.*, 2012; Alves *et al.*, 2013d), but also to endocrine disruptors (Alves *et al.*, 2013c), suggesting that SC metabolism is a good indicator of possible deleterious effects of pharmacological agents on male reproductive function. Although new functions have been ascribed to metformin in women without diabetes, there is a lack of studies investigating the effect of metformin on male reproduction. Hence, we hypothesized that exposure to metformin can alter SC glucose metabolism, which is crucial for spermatogenesis. To test our hypothesis, we determined the effects of sub-pharmacological (5 μM), pharmacological (50 μM) and supra-pharmacological (500 μM) concentrations of metformin on the function of rat SCs *in vitro*. This is the first report to focus on the effects of metformin on SC metabolism, revealing new perspectives into the effects of this pharmacological agent on male reproduction.

Methods

Chemicals

D_2O (99.9%) was purchased from Cambridge Isotope Laboratories Inc. (Cambridge, MA, USA). Taq DNA polymerase was purchased from Fermentas Life Sciences (Ontario, Canada). Random primers, polyclonal antibodies and Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) were purchased from Invitrogen (Carlsbad, CA, USA), deoxynucleotide triphosphates (dNTPs) were purchased from GE Healthcare (Buckinghamshire, UK) and FBS was obtained from Biochrom AG (Berlin, Germany). Maxima SYBR Green/Fluorescein qPCR Master Mix was purchased from Thermo Scientific (San Jose, CA, USA). Metformin (1,1-dimethylbiguanide hydrochloride), HBSS, DMEM Ham's nutrient mixture F12 (DMEM: Ham's F12), EDTA, soybean trypsin inhibitor, DNase, collagenase type I, BSA, ExtrAvidin-Peroxidase Staining Kit, 3,3′ diaminobenzidine hydrochloride (DAB), trypsin–EDTA, insulin–transferrin–sodium selenite supplement (ITS supplement), TRI reagent and other chemicals were all purchased from Sigma–Aldrich (St. Louis, MO, USA).

Animals

Ten 20-day old male Wistar rats (*Rattus norvegicus*) were housed under a 12 h light–12 h darkness cycle and constant room temperature (20 ± 2°C) in our accredited animal facilities. Animals were maintained with food and water *ad libitum*. Accommodation, maintenance and animal handling were performed in accordance with the 'Guide for the Care and Use of Laboratory Animals'; available in the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and the rules for the care and handling of laboratory animals (Directive 2010/63/EU).

SC culture

Animals were killed by cervical dislocation, the testes were immediately excised in aseptic conditions and washed twice in a 50 mL conical tube with 20 mL of ice-cold HBSS containing 10 000 $U \cdot mL^{-1}$ of penicillin, 10 $mg \cdot mL^{-1}$ streptomycin and 25 $\mu g \cdot mL^{-1}$ amphotericin B (pH 7.4). SCs were isolated and cultured (in phenol-red free media) using a previously

described method by Rato *et al.* (2012a). Briefly, tissue from decapsulated testes was placed in a Petri dish with glycine medium [HBSS plus 1 M glycine, 2 mM EDTA, 0.002% (w v^{-1}) soybean trypsin inhibitor; pH 7.2] and the tubular pellet was digested with collagenase type I and DNAse in HBSS for 15 and 20 min, respectively, at room temperature. The SCs suspension was collected by centrifugation (300 x *g* for 3 min), washed in HBSS and resuspended in Sertoli culture medium, which consisted of a 1:1 mixture of DMEM:Ham's F12 (pH 7.2–7.4) supplemented with 15 mM HEPES, 50 U·mL^{-1} penicillin and 50 mg·mL^{-1} streptomycin sulfate, 0.5 mg·mL^{-1} fungizone, 50 µg·mL^{-1} gentamicin and 10% heat-inactivated FBS. In order to disaggregate large Sertoli clusters, the cellular suspension was then forced through a 20 G needle. The cellular suspension was then placed on culture flasks (Cell+; Sarstedt, Germany), and incubated at 33°C in an atmosphere of 5% CO_2, 95% O_2 in Sertoli culture medium. The purity of the cultured SCs was assessed by the immunoperoxidase detection of a specific marker, Vimentin (Dako, Glostrup, Denmark, M072501), using standard methods (Rato *et al.*, 2012a; Alves *et al.*, 2013c; Simoes *et al.*, 2013). Briefly, SCs were grown in culture plates, incubated overnight at 4°C with primary polyclonal antibody and labelled with streptavidin–biotin method using an ExtrAvidin-Peroxidase Staining Kit. SCs obtain a brown colour after reacting with diaminobenzidine. The nucleus was then stained with haematoxylin. Negative-control incubations were always executed using PBS instead of primary antibody. Stained cultures were then examined through a microscope and selected only if cell contaminants were below 5%. We used 20-day-old rats because SCs cease to divide at the 15th post-natal day and have a fully established blood–testis barrier (Orth, 1982; Li *et al.*, 2009). Thus, SCs from 20-day-old rats are well known for being fully differentiated and functionally similar to those isolated from adult rats (Li *et al.*, 2009). Moreover, primary cultures of SCs obtained from 20-day-old rats are less contaminated with other testicular cell types, remaining responsive not only to hormonal treatments, but also to environmental pollutants and several substances (Monsees *et al.*, 2000; Vigier *et al.*, 2004; Rato *et al.*, 2012a; Alves *et al.*, 2013c).

Experimental groups

SCs were allowed to grow until they reach 90–95% confluence, and then washed thoroughly and the medium replaced by serum and phenol-red free media (DMEM : F12, 1:1, with ITS supplement, pH 7.4). SCs were either untreated or treated with metformin. The concentrations chosen were 5 µM, a pharmacological concentration of 50 µM (Pentikäinen *et al.*, 1979; Sarabia *et al.*, 1992; Robert *et al.*, 2003) and 500 µM. After a 50 h incubation period, the cells were detached with a trypsin–EDTA solution and collected using standard methods. A viability test was executed on cells of the different experimental groups using the Trypan Blue Exclusion Test. Viability averaged 85–95%, always with values higher than 85%. At the end of the treatment, the total number of cells per flask was determined with a Neubauer chamber, extracellular media were collected for ^{1}H-NMR analysis and the cells were collected for RNA or protein extraction as well as LDH activity.

RNA extraction and cDNA synthesis

Extraction of total RNA (RNAt) of cells was performed using the E.Z.N.A. Total RNA Kit (Omega bio-tek, Norcross, GA, USA) following the manufacturer's instructions. RNA concentration and absorbance ratios (A260/A280) were determined by spectrophotometry (NanophotometerTM, Implen, Germany). The RNAt obtained for each sample was reverse transcribed in a mixture containing 0.5 mM of each dNTP, 250 ng of random hexamer primers, 1 µg of RNAt and sterile H_2O up to a final volume of 13.5 µL. The mixture was initially incubated for 5 min at 65°C. Then, 200 U of M-MLV RT and 2 µL of reaction buffer were added and the samples were incubated sequentially at 25°C for 10 min, 37°C for 50 min and 70°C for 15 min.

Quantitative real-time PCR (qPCR)

qPCR was performed to analyse the expressions of phosphofructokinase 1 (PFK 1), MCT4, GLUT1, GLUT3 and LDH mRNA. Specific primers were designed for the amplification of the target and housekeeping transcripts (Table 1). qPCR was carried out in an iQ5 system (Bio-Rad, Hercules, CA, USA) and efficiency of the amplification was determined for all primer sets using serial dilutions of cDNA. qPCR conditions and reagent concentrations were optimized and specificity of the amplicons was determined by melting curves. qPCR amplifications used 1 µg of synthesized cDNA in a 20 µL reaction sample containing: 10 µL Maxima SYBR Green/Fluorescein qPCR Master Mix and 0.3 µM of sense and antisense primers for each gene. Amplification conditions comprised an initial denaturation step of 5 min at 95°C, followed by 35 cycles of (i) denaturation, 95°C for 10 s, (ii) annealing, a specific temperature for each set of primers for 30 s (Table 1); and (iii) extension, 72°C for 10 s. β-2-microglobulin transcript levels were used to normalize the mRNA expression of PFK 1, MCT4, GLUT1, GLUT3 and LDH. The fold variation of the expression of target genes was calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001).

Western blot

Western blot procedure was performed as previously described by Alves and collaborators (2011). The membranes were incubated overnight at 4°C with rabbit anti-GLUT1 (1:300, Millipore, Temecula, CA, USA, CBL242), or rabbit anti-GLUT3 (1:500, Abcam, Cambridge, MA, USA ab41525), or rabbit anti-PFK 1 (1:400, Santa Cruz Biotechnology Heidelberg, Germany, Sc 67028), or rabbit anti-MCT4 (1:1000, Santa Cruz Biotechnology, Sc 50329) or rabbit anti-LDH (1:10 000, Abcam, ab52488). Mouse anti-tubulin was used as a protein loading control (1:5000, Sigma, Roedermark, Germany, T 9026). The immune-reactive proteins were detected separately with goat anti-rabbit alkaline phosphatase-linked igG (IgG-AP; 1:5000, Santa Cruz Biotechnology, Sc 2007) or goat anti-mouse IgG-AP (1:5000, Santa Cruz Biotechnology, Sc 2008). Membranes were reacted with an enhanced chemifluorescence detection system (GE Healthcare, Weßling, Germany) and read with the Bio-Rad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). The Quantity One Software (Bio-Rad) was used to obtain band densities following standard procedures. The band density measured was divided by the

Table 1

Oligonucleotides and cycling conditions for qPCR amplification of GLUT3 and GLUT1, PFK, LDH, MCT4 and β-2-microglobulin

Gene	Primer sequence (5′-3′)	AT (°C)	Amplicon size (bp)	C
GLUT1	Sense: TCCATTCTCCGTTTCACAGC	55	145	40
	Antisense: CCGGTGTTATAGCCGAACTG			
GLUT3	Sense: GCTCTGGTCGTTATGTGTGG	62	171	35
	Antisense: TCAACCGACTCCGCTATCTT			
PFK 1	Sense: TGATTGGCTGTTCATCCCTG	50	150	35
	Antisense: GAGATAGGCTTTCCATGCCG			
LDH	Sense: CGTCGTCCCCCATCGTGCAC	60	345	35
	Antisense: GGGCCCCCGCGGTGATAATG			
MCT4	Sense: ACACTTAGGAGACAACAC	47	132	37
	Antisense: GGCAATATAGGAGACTGG			
β-2-microglobulin	Sense: ATGAGTATGCCTGCCGTGTG	58	92	30
	Antisense:CAAACCTCCATGATGCTGCTTAC			

AT, annealing temperature; C, number of cycles during exponential phase of amplification.

respective tubulin band density and then normalized as percentage of the respective control.

LDH activity assay

LDH activity was determined using a commercial assay kit (Promega, Madison, WI, USA) and following the manufacturer's instructions. Briefly, the LDH enzymatic activity was calculated by measuring the shift in the absorbance (492 nm) of protein samples that resulted from the conversion of a tetrazolium salt into a red formazan product. The amount of formazan formed is directly proportional to the activity of LDH in the samples. The method was calibrated with the LDH positive control included in the assay kit. The activities measured were calculated using the molar absorptivity of formazan and expressed in nmol·min^{-1}·mg^{-1} protein.

NMR spectroscopy

^{1}H-NMR spectroscopy was used to determine metabolite variation in the extracellular media of cultured SCs. ^{1}H-NMR spectra of the extracellular media were obtained at 14.1 T, 25°C, using a Bruker Avance 600 MHz spectrometer equipped with a 5 mm QXI probe with a z-gradient (Bruker Biospin, Karlsruhe, Germany) using standard methods (Alves *et al.*, 2011; 2013c). Sodium fumarate (final concentration of 2 mM) was used as reference (singlet, 6.50 p.p.m.) to quantify the metabolites in solution (multiplet, p.p.m.): lactate (doublet, 1.33); alanine (doublet, 1.45); acetate (singlet, 1.9) and H1-α glucose (doublet, 5.22). The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis programme (Acorn NMR, Inc., Fremont, CA, USA).

Statistical analysis

The statistical significance among the experimental groups was assessed by two-way ANOVA, followed by Bonferroni's *post* test. All experimental data are shown as mean ± SEM ($n = 5$ for each condition). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

Results

A pharmacological concentration of metformin modulates GLUT1, GLUT3 and PFK 1 levels in cultured rat SCs

SCs exposed to 5 and 50 μM metformin significantly decreased the mRNA levels of GLUT1 and GLUT3 compared to the control groups (Figure 1A and C). The highest metformin concentration used (500 μM) significantly increased GLUT1 and GLUT3 mRNA compared to the control group. GLUT1 and GLUT3 protein levels were decreased compared to controls when cells were exposed to 50 μM metformin (Figure 1B and D). Metformin 500 μM slightly but not significantly increased GLUT1 and GLUT3 protein levels compared to the control group (Figure 1B and D).

With regard to PFK 1, the lowest doses of metformin (5 and 50 μM) significantly decreased PFK 1 mRNA and protein expression when compared with the control group (Figure 1E and F). However, 500 μM metformin increased PFK 1 mRNA expression (Figure 1E) but decreased its protein expression compared to the control group (Figure 1F).

SCs exposed to a pharmacological concentration of metformin have increased LDH activity and decreased mRNA and protein levels of MCT4

Metformin did not alter LDH mRNA (Figure 2A) or protein levels (Figure 2B) in SCs. However, all the metformin concentrations (5, 50 and 500 μM) significantly increased LDH activity in the SCs (Figure 3). MCT4 mRNA levels were significantly decreased in SCs exposed to all concentrations of

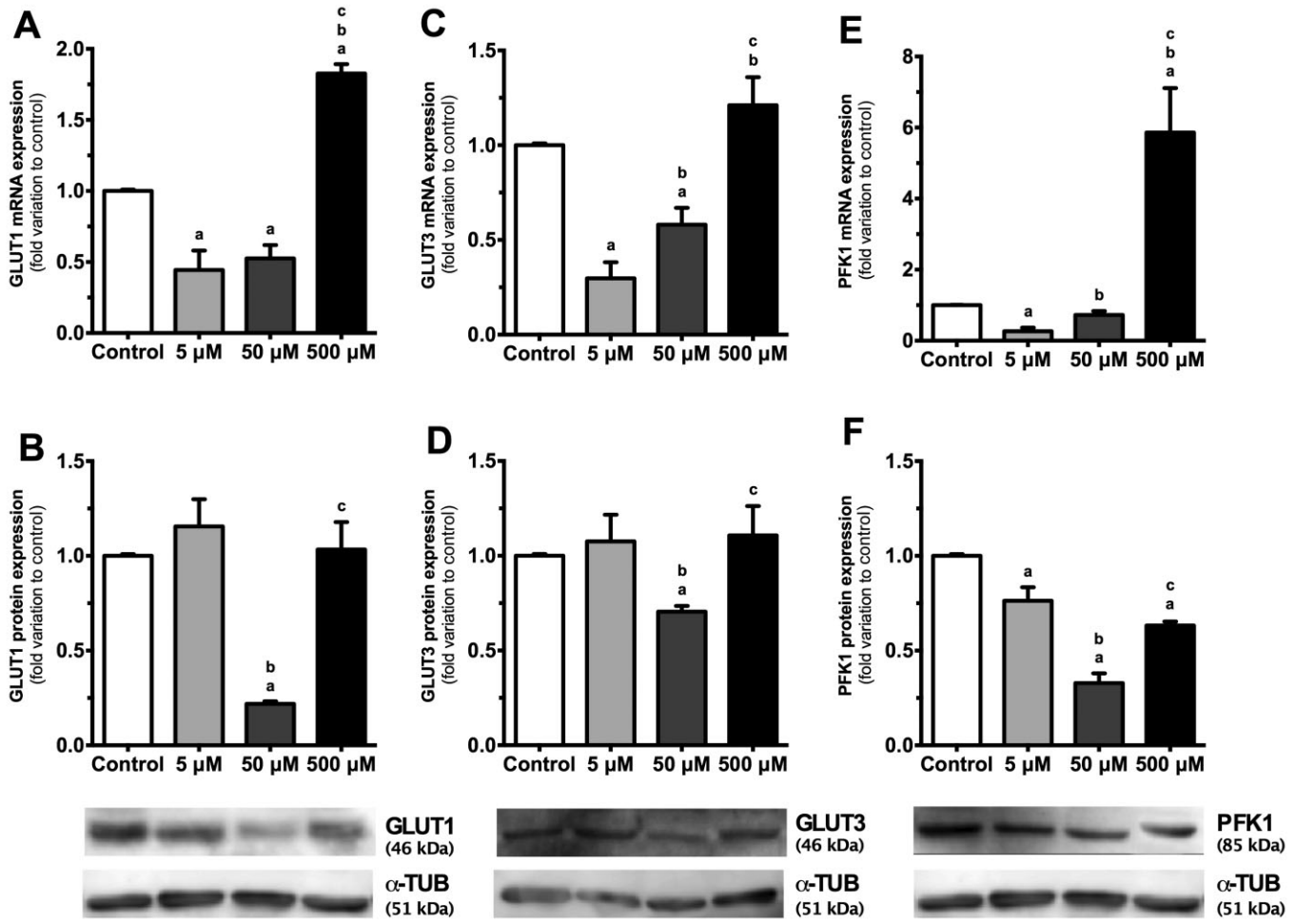

Figure 1

Effect of metformin (5, 50 and 500 μM) on GLUT1, GLUT3, PFK 1 mRNA (panels A, C and E) and protein levels (panels B, D and F). The figure shows pooled data of independent experiments, indicating the fold variation in mRNA and protein levels found in rat SCs cultured in the presence of metformin when compared with the control condition. Representative blots are also presented. Results are expressed as mean ± SEM (n = 5 for each condition). Significantly different results (P < 0.05) are indicated as: a – relative to control; b – relative to 5 μM; c – relative to 50 μM.

metformin (Figure 2C). Interestingly, MCT4 protein levels were only significantly decreased in cells exposed to the pharmacological dose of metformin (50 μM) (Figure 2D).

A pharmacological concentration of metformin increases lactate and alanine production by cultured SCs

Glucose consumption by cultured SCs was only significantly decreased when the cells were exposed to the lowest concentration of metformin (Figure 4A). Nevertheless, lactate production was not altered in cells exposed to this concentration of metformin, whereas 50 and 500 μM metformin significantly stimulated lactate production in these cells (Figure 4B).

Lactate is produced from pyruvate that is the intermediary compound in the lactate/alanine pathway. Our results show that alanine production was stimulated in cells exposed to 5, 50 and 500 μM metformin (Figure 3C). This is very interesting because the lactate/alanine ratio reflects the NADH/NAD^+ equilibrium (Alves *et al.*, 2012a) and thus, the cellular redox state. Our results showed that the increased lactate production in SCs induced by metformin was followed by a significant increase in alanine production, indicating that the lactate/alanine ratio is maintained in these metformin-treated cells, except in those exposed to 5 μM, where this ratio was significantly decreased. In contrast, acetate production was only increased in cells exposed to 5 μM metformin (Figure 4D).

Discussion

Diabetes mellitus (DM) is a major public health threat and the number of diabetic individuals is rapidly increasing. Among which, there is an alarming number of children and adolescents (Silink, 2002). Glycaemic control can affect male reproductive function and recent reports highlight the

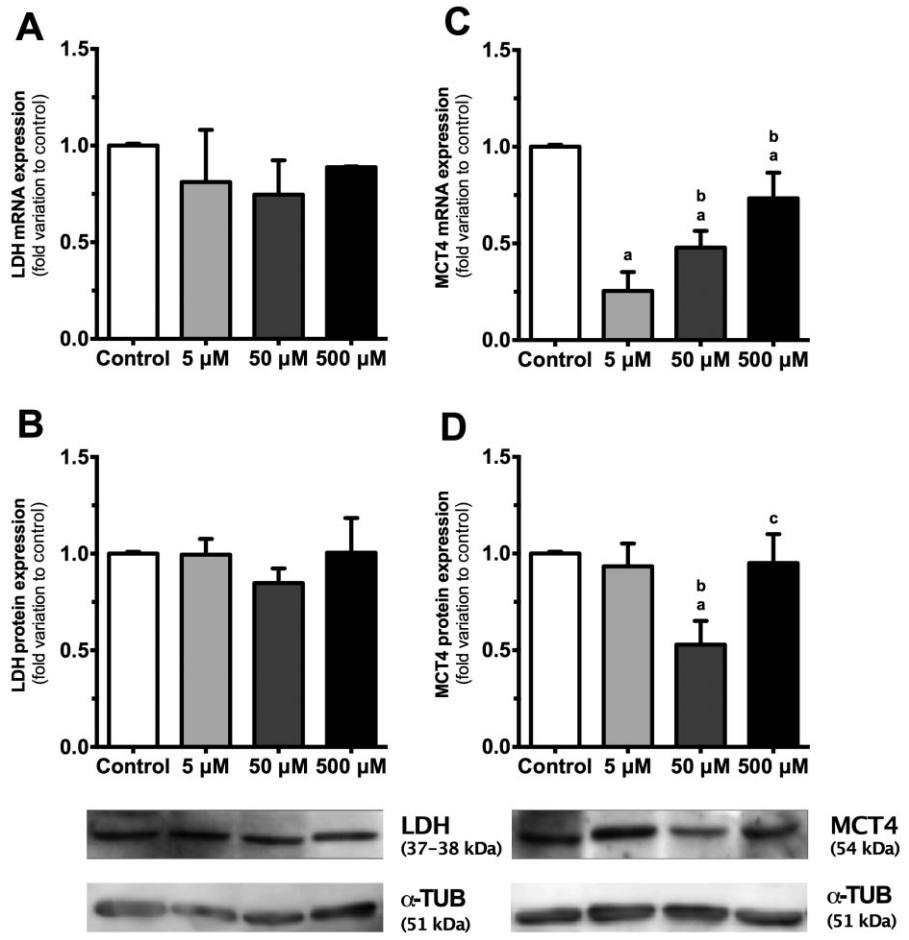

Figure 2

Effect of metformin (5, 50 and 500 μM) on LDH and MCT4 mRNA (panels A and C) and protein levels (panels B and D). The figure shows pooled data of independent experiments, indicating the fold variation in mRNA and protein levels found in rat SCs cultured in the presence of metformin when compared with the control condition. Representative blots are also presented. Results are expressed as mean ± SEM (n = 5 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control; b – relative to 5 μM; c – relative to 50 μM.

relevance of DM to male fertility (Alves *et al.*, 2013a,b). While new clinical applications have already been reported for the anti-diabetic drug metformin, such as improving female reproductive function, the effects of metformin on male reproduction remain largely unknown. Thus, as SCs are the somatic component of the seminiferous epithelium and its metabolic function is known to exert a tight control over spermatogenesis, we investigated the effects of metformin on SC metabolism.

Glucose uptake by SCs is not stimulated by metformin. In fact, exposure to the lowest concentration even decreased glucose consumption by SCs. Nevertheless, both GLUT1 and GLUT3 mRNA levels were significantly decreased after exposure to 5 and 50 μM metformin. It has been reported that metformin can change the functional properties of insulin- and glucose-sensitive transporters (Wiernsperger and Bailey, 1999). The *in vivo* administration of supra-therapeutic concentrations of metformin to insulin-resistant fatty rats increased GLUTs-mediated transport without changing the total number of glucose transporters (Matthaei *et al.*, 1993), indicating that metformin can increase the functional activity of glucose transporters. Therefore, as our results showed that glucose consumption is not decreased by exposure to 5 and 50 μM metformin, the decreased levels of glucose transporters suggests that in SC, these concentrations of metformin increase the functional activity of these transporters. Others have also reported an increase in glucose transport by metformin in skeletal muscle, vascular smooth muscle cells and erythrocytes (Sasson *et al.*, 1996; Thomas *et al.*, 1998). In SC, glucose transport mechanisms are sensitive to hormonal treatment (Oliveira *et al.*, 2011; Rato *et al.*, 2012a) and it has a high metabolic plasticity to ensure an adequate lactate concentration in the microenvironment where germ cells develop. Thus, although there was a significant decrease in both GLUT1 and GLUT3 levels in SCs exposed to 5 and 50 μM metformin, these cells may maintain glucose consumption as in control conditions by increasing glucose transport activity, as detected in other cell types (Sasson *et al.*, 1996; Thomas *et al.*, 1998). It is noteworthy that the GLUT1 and GLUT3 protein levels were only found to be decreased in SCs exposed

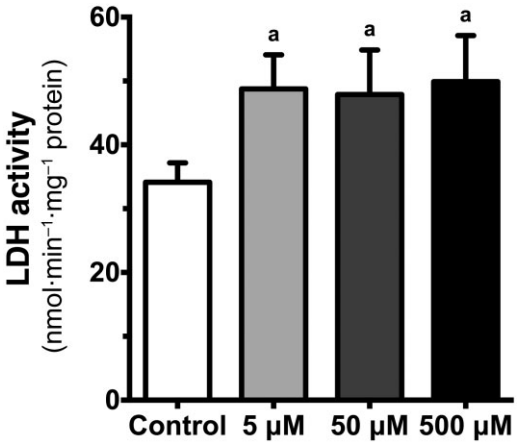

Figure 3

Effect of metformin (5, 50 and 500 µM) on LDH activity in cultured SCs. The figure shows pooled data of independent experiments, indicating LDH activity in nmol·min^{-1}·mg^{-1} protein. Results are expressed as mean ± SEM (n = 5 for each condition). Significantly different results (P < 0.05) are indicated as: a – relative to control.

to the pharmacological concentration of metformin, further indicating that the functional activity of GLUTs is essential to maintain the glucose consumption in this scenario.

Once glucose enters the SC, it follows the glycolytic pathway. It is well known that PFK is a rate-limiting control point in glycolysis. In SCs exposed to 5 and 50 µM metformin, the PFK 1 mRNA and protein levels were decreased. Whereas in SCs exposed to a supra-pharmacological concentration of metformin the mRNA levels of PFK 1 were increased and only the protein levels were decreased. Overall, this would suggest that glycolysis could be inhibited. However, no differences were found in the LDH levels between metformin-treated and untreated cells, whereas LDH activity was significantly increased in cells exposed to all metformin concentrations. LDH activity is often associated with a stimulation of the glycolytic flux. These results provide evidence that metformin, again, stimulates enzymatic activities of glycolysis-related enzymes without changing its protein expression. In fact, SC can modulate its glycolytic flux without changing extracellular glucose consumption to maintain lactate production at high rates (Riera *et al.*, 2009; Oliveira *et al.*, 2011; 2012; Rato *et al.*, 2012a; Alves *et al.*, 2013c). Our results also confirm this unusual property of SCs.

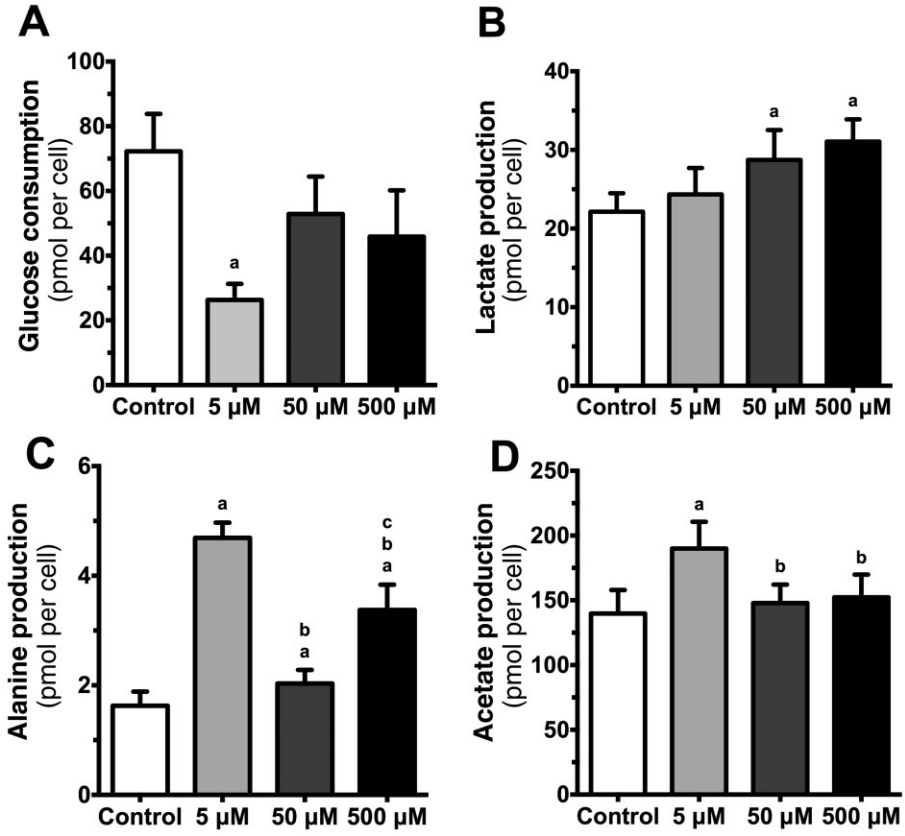

Figure 4

Effect of metformin (5, 50 and 500 µM) on extracellular metabolites. The figure shows pooled data of independent experiments, indicating glucose consumption (panel A) and the production of lactate (panel B), alanine (panel C) and acetate (panel D) in cultured SCs. Results are expressed as mean ± SEM (n = 5 for each condition). Significantly different results (P < 0.05) are indicated as: a – relative to control; b – relative to 5 µM; c – relative to 50 µM.

In addition to lactate, SCs are also responsible for generating essential cofactors and other metabolites such as acetate (Alves *et al*., 2012b). Our results show that acetate production was not affected by metformin, except when cells were exposed to the lowest concentration. Although several studies have reported a relationship between acetate and glucose metabolism, supporting a possible conversion between acetate and glucose metabolism through Acetyl-CoA, our results show that a pharmacological concentration of metformin only induces important changes in glucose metabolism without altering acetate production. However, SCs exposed to the lowest metformin dose show decreased glucose consumption and increased acetate production without an effect on lactate production. This suggests a role for the endogenous substrates and deserves special attention because it has been reported that SCs possess glycogen and glycogen phosphorylase activity (Leiderman and Mancini, 1969; Slaughter and Means, 1983).

Several studies have demonstrated that metformin has a marked effect in oxidative stress (Bonnefont-Rousselot *et al*., 2003; Esteghamati *et al*., 2013). Indeed, SCs treated with metformin 50 and 500 μM had increased lactate production and overall glycolytic flux. The cellular glucose sensing machinery and lactate metabolism are closely related to the production of reactive oxygen species (Bonnefont-Rousselot *et al*., 2003; Rato *et al*., 2013). Our results suggest that the increased lactate production induced by exposure to the highest metformin concentrations is followed by alanine production leading to the maintenance of the lactate/alanine ratio. This ratio is an index of cellular redox state as the conversion of pyruvate or its conversion into alanine is coupled to the re-oxidation of NADH to NAD^+ (O'Donnell *et al*., 2004). Thus, our results suggest that stimulation of alanine production, at least in rat cultured SCs, is a mechanism by which the exposure to a pharmacological concentration of metformin (50 μM) exerts its antioxidant activity. Interestingly, even when cells were exposed to 5 μM metformin, alanine production was stimulated and thus the lactate/alanine ratio is significantly decreased, indicating lower redox state levels in these cells. This provides further evidence that stimulation of alanine production may be a mechanism by which metformin acts to exert its antioxidant activity.

The results presented herein are an important step in the evaluation of the effects of metformin on male reproduction. We have previously reported that deregulation of insulin is detrimental to the male reproductive system as it modulates SC metabolism (Oliveira *et al*., 2012; Alves *et al*., 2012b). One of the major effects associated with insulin deregulation is inhibition of lactate production. Thus, the results presented herein suggest that a pharmacological concentration of metformin (50 μM) may counteract this undesirable effect. Moreover, SC can only support a limited number of germ cells and even in physiological conditions, a significant number of germ cells die by apoptosis. In fact, treatment with intratesticular lactate infusion was reported to improve spermatogenesis *in vivo* (Courtens and Ploen, 1999). Moreover, germ cell apoptosis in the human testis was also found to be inhibited in a dose-dependent way by lactate (Erkkila *et al*., 2002). Therefore, our results suggest that treatment with a pharmacological concentration of metformin (50 μM) may improve the male reproductive potential of diabetic male individuals through inhibition of germ cell apoptosis. Finally, it should be noted that although we used a supra-pharmacological concentration of metformin (500 μM), SCs did not present any cytotoxic metabolic behaviour and the mechanisms of metformin's action at this dose were very similar to the overall action of a pharmacological dose, with the exception of the mRNA levels of the GLUTs and PFK 1. This was expected, as supra-therapeutic concentrations of metformin induce acidosis by stimulating the glycolytic flux. Nevertheless, SCs are well known as lactate-producing cells and, thus, they maintain a high glycolytic flux even in physiological conditions (Martins *et al*., 2013).

Recently, it was reported that metformin may interfere with normal testicular physiological processes leading to spermatogenic failure (Adaramoye *et al*., 2013). Those authors evaluated the toxic effects of this drug in healthy adult male rats, which were administered metformin, p.o., for three consecutive weeks. They observed marked histological alterations (such as necrosis and seminiferous tubules degeneration and atrophy), concomitant with a decrease in sperm counts and motility. These are indeed important results regarding the toxicity of metformin, which are apparently contradictory to the results presented here. Nevertheless, there is a striking feature of the study by Adaramoye *et al*. (2013) that might explain these differences. Apart from the experimental approach used, the results obtained using the *in vivo* model were obtained from healthy animals with apparently normal levels of blood glucose. In these animals, all tissues and cells were exposed to an oral antidiabetic while having available lower levels of glucose, which may explain the reproductive deleterious results observed. Indeed, our *in vitro* approach was done in hyperglycaemic conditions because most cell culture media (as the one used in our studies) contain glucose concentrations well above plasma glucose physiological levels in healthy individuals, within the range of plasma values observed in the diabetic condition.

Metformin is a drug involved in metabolic homeostasis and SC metabolism has been shown to be essential for spermatogenesis (Alves *et al*., 2013d). Although the results presented herein were obtained using primary cultures of rat SCs, it is not expected that human SC metabolism is significantly different (Oliveira *et al*., 2011; Rato *et al*., 2012a). Overall, our results suggest that metformin may improve the male reproductive potential and it is, therefore, a suitable anti-diabetic agent for young adults and adolescents with T2D, as it will not severely compromise their reproductive function. Further experiments are needed to clarify the effects of metformin on the metabolic cooperation between SC and germ cells and on male reproductive health in general.

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Conflict of interest

None declared.

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