Doxorubicin caused severe hyperglycaemia and insulin resistance, mediated by inhibition in AMPk signalling in skeletal muscle

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Abstract

Background Cancer is considered the second leading cause of death in the world, and for the treatment of this disease, pharmacological intervention strategies are frequently based on chemotherapy. Doxorubicin (DOX) is one of the most widely used chemotherapeutic agents in clinical practice for treating a number of solid tumours. The treatment with DOX mimics some effects of cancer cachexia, such as anorexia, asthenia, decreases in fat and skeletal muscle mass and fatigue. We observed that treatment with DOX increased the systemic insulin resistance and caused a massive increase in glucose levels in serum. Skeletal muscle is a major tissue responsible for glucose uptake, and the positive role of AMPk protein (AMP-activated protein kinase) in GLUT-4 (Glucose Transporter type 4) translocation, is well established. With this, our aim was to assess the insulin sensitivity after treatment with DOX and involvement of AMPk signalling in skeletal muscle in this process.

Methods We used Wistar rats which received a single dose of doxorubicin (DOX group) or saline (CT group) intraperitoneally at a dose of 15 mg/kg b.w. The expression of proteins involved in insulin sensitivity, glucose uptake, inflammation, and activity of electron transport chain was assessed in extensor digitorum longus muscle, as well as the histological evaluation. In vitro assays were performed in L6 myocytes to assess glucose uptake after treatment with DOX. Agonist of AMPk [5-aminoimidazole-4-carboxamide (AICAR)] and the antioxidant n-acetyl cysteine were used in L6 cells to evaluate its effect on glucose uptake and cell viability.

Results The animals showed a significant insulin resistance, hyperglycaemia, and hyperinsulinemia. A decrease in the expression of AMKP and GLUT-4 was observed in the extensor digitorum longus muscle. Also in L6 cells, DOX leads to a decrease in glucose uptake, which is reversed with AICAR.

Conclusions DOX leads to conditions similar to cachexia, with severe glucose intolerance both in vivo and in vitro. The decrease of AMPk activity of the protein is modulated negatively with DOX, and treatment with agonist of AMPk (AICAR) has proved to be a possible therapeutic target, which is able to recover glucose sensitivity in skeletal muscle.

Keywords Doxorubicin; Skeletal muscle; AMPk; Glucose intolerance; Hyperglycaemia; Chemotherapy; Anthracycline

Introduction

Doxorubicin (DOX) is a chemotherapeutic, in the family of anthracyclines, developed in the 1960s.¹ It is an antineoplastic antibiotic that is still widely used in the treatment of a variety of malignancies, effective in acute leukaemia, non-Hodgkin lymphomas, breast cancer, Hodgkin’s disease, and sarcomas.² Doxorubicin exerts antitumor activity by inhibiting DNA Topoisomerase II, thus disrupting DNA replication.³ Moreover, DOX induces the generation of reactive oxygen species
(ROS) leading to DNA damage and apoptosis, by stimulation of p53-DNA binding, which initiates the caspase signalling, and DNA cross-linking.4,5

Despite the great antitumoural efficiency of DOX, the use of this drug in therapy is limited, whereas since the 1970s, its cardiotoxicity was demonstrated in DOX treatment; therefore, this limits its use in cancer treatment.6 Although the deleterious effects of DOX on the heart muscle are the main targets of investigation discussed in the literature, skeletal muscle is also affected. DOX treatment causes severe fatigue7 and muscle weaknesses that reflect a poor quality of life.8 This tissue has important biological functions, and various metabolic disorders are related to changes in metabolism.

It has been shown that the decrease in insulin sensitivity in skeletal muscle may be secondary to a number of pharmacological therapies.9,10 In humans, skeletal muscle accounts for approximately 50–60% of body weight. In insulin-stimulated conditions, skeletal muscle is responsible for about 75% of uptake in circulating glucose.11 Insulin resistance favours muscle wasting, a process called sarcopenia.12,13 Sarcopenia is part of the physiological ageing process, but it is also present in several morbidities, such as diabetes, cancer, and kidney disease, which affect the quality and life expectancy of individuals.14,15 Clinically, patients usually report severe fatigue during DOX treatment.16 Previous studies have shown that DOX is able to generate muscle dysfunction, leading to lost performance and the physical appearance of debilitating fatigue.17–19 Worsening in the parameters of maximum strength, maximum relaxation and fatigue were evident and were probably caused by changes in sarcoplasmic calcium metabolism.20,21

However, few studies have been proposed to investigate a possible association between the use of DOX and insulin resistance, as well as the role of skeletal muscle in the process.

AMPk (AMP-activated protein kinase) is a protein with serine-threonine kinase residues,22 which acts as a key sensor of cellular energy levels. The activity of this protein appears to play a pivotal role in increasing glucose uptake in skeletal muscle. In 2005, Tokarska-Schlattner et al.23 showed that this disruption in AMPk signalling negatively affected the energetic metabolism in cardiomiocytes of the animals treated with DOX. AMPk activation by 5-Aminoimidazole-4-carboxamide (AICAR) riboside or genetic approach reduced DOX cardiotoxicity in culture cells.24

The explanation of this condition is extremely important, because insulin resistance alters the pharmacokinetics of the drug, increases cardiotoxicity, and increases oxidative stress.25,26 Therefore, the skeletal muscle has an important role in glucose and insulin homeostasis. Accordingly, the aim of this study was to evaluate insulin sensitivity after treatment with DOX and involvement of AMPk signalling in skeletal muscle in this process.

Methods

Animals

The Experimental Research Committee of the University of São Paulo approved all procedures for the care of the animals used in this study. A total of 26 male Wistar rats approximately 14 weeks of age were used. They were housed four per cage in an animal room under a 12 h light-dark cycle at 22 ± 1°C and 60 ± 5% humidity and received a chow diet and water ad libitum. The experiments were carried out after a one-week acclimation period. Rats were randomly divided into two groups: (i) saline control (CT) (n = 13) and (ii) DOX group (DOX) (n = 13). After the acclimation period, the DOX-treated group received 15 mg/kg, i.p., DOX chloride (Eurofarma Laboratory, Campinas, Brazil); DOX chloride received an equal volume of saline. Food intake and body weight were assessed daily.

The animals performed 6 h of fasting previous to euthanasia, by decapitation, 72 h after the DOX treatment. Following euthanasia, extensor digitorius longus (EDL) and retroperitoneal adipose tissue were removed, weighted, snap frozen in liquid nitrogen, and stored at −80°C. The epididymal adipose tissue and liver were only weighed. Whole blood was drawn, centrifuged at 3000 g for 15 min at 4°C. Serum was removed and kept frozen at −80°C for later determination.

Serum analysed

Fasting blood glucose, uric acid, and aspartate transaminase was assessed using Labtest© kits. Serum insulin, adiponectin, testosterone, and corticosterone were quantified using enzyme-linked immunosorbent assay (ELISA). For insulin the kit was obtained from Millipore Corp. Bedford, MA, USA, for adiponectin the kit was obtained from R&D Systems, Minneapolis, MN, USA, and for corticosterone and testosterone from Assay Designs, Inc., Ann Arbor, MI, USA. Serum free fatty acid (FFA) levels were analysed in rats using the NEFA-kit-U (Wako Chemical GmbH, Neuss, Germany). Homeostatic model assessment of insulin resistance was used to evaluate insulin resistance. The index was determined by calculating: fasting serum insulin (μU/mL) × fasting plasma glucose (mmol l–1)/22.5.

Histology analysis

The EDL muscle was cut in cryostat sections (10 μm thick) at −25 ° temperature. The sections were incubated with hematoxylin and eosin for the analysis of cross-sectional area of the fibre (aspartate transaminase). The morphometric analysis was analysed under a microscope (Nikon Eclipse E600, Fukuoka, Japan) equipped with a digital video camera.
coupled to software to analyse the images (Metamorph, Universal Corporation, Downingtown, USA). The scanned images were analysed using Image-Pro Plus (Media Cybernetics, Silver Spring, MD) software in a double-blind manner. For analysis of cross-sectional area of the fibre, approximately 1000 EDL muscle fibres were analysed per group. Four to five EDL fields per animal were analysed in groups studied.

**Enzymatic assays**

The EDL muscle was homogenized in SETH buffer, pH 7.4 (250 Mm sucrose, 2 mM EDTA, 10 mM Trizma base, and 50 IU/mL heparin). The enzymatic activity of citrate synthase, malate dehydrogenase, and mitochondrial complexes 1 and 3 were performed as described in27.

**Intraperitoneal tolerance test**

Forty-eight hours after starting treatment, some of the animals were subjected to the insulin tolerance test (ITT). After 6 h fasting, insulin (2 IU/kg) was administered by intraperitoneal injection, and blood samples were collected from the tail at 0, 5, 10, 15, 20, 25, and 30 min for measurement of serum glucose.

**Quantitative real-time polymerase chain reaction**

Total RNA from the EDL muscle was extracted with Trizol reagent (Invitrogen Life Technologies)28 and reverse transcribed to cDNA using the High-Capacity cDNA kit (Applied Biosystems). Gene expression was evaluated by real-time PCR using a Rotor Gene (Qiagen) and SYBR Green as fluorescent dye. Primer sequences are shown in Table S1. Quantification of gene expression was carried,29 with RPL-19 gene as an internal control. Primer sequences are shown in Table S1.

**Protein analysis by western blotting**

The EDL muscle and L6 myocytes were lysed in extraction buffer containing protease and phosphatase inhibitors. The extracts were then centrifuged at 12 000 rpm at 4°C for 40 min to remove insoluble material. Protein determination in the supernatants was performed by the Bradford dye method using the Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA, USA). GLUT-4 (glucose transporter 4), GAPDH, and β-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) diluted in blocking buffer added with 1% bovine serum album (BSA) and then washed for 1 h in a blocking buffer without BSA. The blots were subsequently incubated with peroxidase-conjugated secondary antibody for 1 h.

By evaluation of protein loading, membranes were stripped and reblotted with GAPDH or β-tubulin antibody, as appropriate. Specific bands were detected by chemiluminescence, and visualization/capture was performed by exposure of the membranes to RX films. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software-Scion Corporation, Frederick, MD, USA).

**L6 cell culture**

The cells were maintained in Dulbecco’s modified Eagle’s medium at 37°C in humidified atmosphere containing 5% CO2 and supplemented with 10% fetal bovine serum and 2% antibiotic solution. To differentiate, cells were allowed to reach confluence, and the medium was changed to medium containing 2% fetal bovine serum for 7 days, with medium changes every 2nd day. The serum was removed for 6 h for the determination of glucose uptake ability.

**MTT assay**

For the assessment of cell viability, MTT assay was performed. 2X10⁴ cells per well in 96-well plates were cultivated and were differentiated after 24 h. Cells were treated with different concentrations of DOX (0 to 1 μM) for 48 h, and after this period, we performed the cell viability assay. The cells’ medium was replaced with 200 μL fresh medium/well containing 0.125 mg/mL MTT and cultivated for another 3 h darkened in the cells’ incubator. The supernatant was removed (until reaching a final volume of 25 μL), and 100 μL isopropanol/HCl (11 M) were added per well. The absorbance at 595 nm was measured. Two wells per plate without cells served as a blank. The effect of the DOX on cell viability was relativized by DMSO group.

**Glucose uptake assay**

Glucose uptake in L6 cells was measured using the 2-deoxy-[C14]-D-glucose. The 5.9 x 10⁴ cells per well in 24-well plates were seeded and were differentiated after 24 h, with a confluence of approximately 80%. Differentiated L6 cells were treated with 100 nMol of DOX or DMSO for 48 h. To evaluate the chronic effect of AICAR, this drug was added together to start treatment with DOX (2 mMol/48 h).

For the glucose uptake assay the cells remained fasting (serum-free medium) for 6 h. After this, cells were washed in dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose membranes were incubated overnight at 4°C with antibodies against Akt/PKB, and phospho Akt S473, AMPk α, phospho AMPkα T172, and IR (insulin receptor) were obtained from Cell Signaling Technology® (Danvers, MA, USA). GLUT-4 (glucose transporter 4), GAPDH, and β-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) diluted in blocking buffer added with 1% bovine serum album (BSA) and then washed for 1 h in a blocking buffer without BSA. The blots were subsequently incubated with peroxidase-conjugated secondary antibody for 1 h.

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PBS, glucose-free medium (Hepes, 140 mmol NaCl, 20 mmol Hepes-Na pH 7.4, 5 mmol KCl, 2.5 mmol MgSO4, and 1 mmol CaCl2) was added for 30 min, with or without insulin (100 nmol) or AICAR (2 mmol/1 h—only for the acute treatment). After this, Hepes medium containing 2-deoxy-[C14]-D-glucose (1 mCi/mL) was added for 30 min. Reactions were terminated by washing twice in ice-cold NaCl (0.9%), cells were digested (50 mmol NaOH), and part of the sample was used for total protein quantification by Bradford, and in part transferred to a scintillation vial with liquid scintillant. The radioactivity was measured by beta counter.

**TNF-α, IL-10, IL-6, and adiponectin protein level determination**

Frozen tissues (0.1–0.3 g) were homogenized in radio immunoprecipitation assay buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylendiamine tetra acetic acid at pH 7.4) containing 10 μg/mL of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri). Homogenates were centrifuged at 12 000 g for 10 min at 4°C, the supernatant was saved, and protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, California) with bovine serum albumin for 10 min at 4°C, the supernatant was saved, and protein concentration was determined using the Bradford assay with bovine serum albumin, and part of the sample was used for total protein quantification by Bradford, and in part transferred to a scintillation vial with liquid scintillant. The radioactivity was measured by beta counter.

**Statistical analysis**

The statistical analysis was performed using the GraphPad Prism statistics software package version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). The data are expressed as the means ± SD. Implementation of the Kolmogorov–Smirnov test revealed that the results of experiments were distributed normally. The data were analysed using a Student’s t-test for comparison between two groups. For comparison of assays in cell culture, the ANOVA one-way with Tukey post-test test, or ANOVA two-way test with Bonferroni post-test were used. A value of $P < 0.05$ was considered statistically significant.

**Results**

**Doxorubicin chemotherapy leads to severe anorexia and sarcopenia-like morbidity**

Treatment with DOX causes a marked decrease in body weight and relevant anorectic condition (Table 1). Seventy-two hours after the initial application of chemotherapy, a severe drop in the weight of epididymal adipose pad (Table 1) and EDL skeletal muscle (Figure 1A) was observed. No change in the weight of the liver, adrenal, and retroperitoneal adipose tissue (Table 2S) was observed. The high levels of aspartate aminotransferase (AST) and uric acid confirm the deleterious effects of chemotherapy with DOX on hepatic and renal function (Table 2S).

**Acute treatment with doxorubicin increases systemic insulin resistance**

Doxorubicin groups showed substantial increased levels of insulin, glucose, and FFA (Table 1) in serum, after 72 h of DOX treatment (15 mg/kg). Meanwhile, the protein expression of adiponectin in serum and retroperitoneal adipose tissue was decreased (data in press).

**Table 1** Doxorubicin leads to a severe loss of body weight and anorexia with a disruption in systemic metabolism

<table>
<thead>
<tr>
<th></th>
<th>CT</th>
<th>DOX</th>
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<tbody>
<tr>
<td>Delta body weight (g)</td>
<td>12.78 ± 1.26</td>
<td>30.05 ± 1.46 ***</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>27.10 ± 2.30</td>
<td>4.58 ± 0.70 ***</td>
</tr>
<tr>
<td>Epidymal adipose tissue (g)</td>
<td>5.99 ± 0.27</td>
<td>4.00 ± 0.32 **</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>143.2 ± 2.78</td>
<td>310.7 ± 11.13 ***</td>
</tr>
<tr>
<td>NEFA (mEq/L)</td>
<td>0.62 ± 0.02</td>
<td>0.72 ± 0.03 *</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>1.68 ± 0.12</td>
<td>2.46 ± 0.31 *</td>
</tr>
</tbody>
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Values represent the means and ± SD of the data obtained from analysis of 8–10 animals per group.

* $P < 0.05$.

**Table 1** Doxorubicin leads to a severe loss of body weight and anorexia with a disruption in systemic metabolism

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$**P < 0.01$.

$***P < 0.001$ vs. CT.
However, protein levels of insulin receptor (Figure 2E and 2F) and gene and protein expression of AKT protein downstream of PI3-K were not altered (Figure 2D, 2E, and 2F) in the EDL muscle after treatment. Nevertheless, other proteins involved in the insulin pathway showed decreased gene expression, such as IRS-1, GSK3-b (Figure 2D) in skeletal muscle.

The activity of AMP-activated protein kinase protein is decreased with treatment with doxorubicin

GLUT4 and AMPk α (pT172) were decreased in mRNA and protein levels (Figure 2D, 2E, and 2F). Our in vitro data corroborate with the decrease of GLUT4 expression and phosphorylation of AMPK α observed in the EDL muscle (Figure 2G and 2H). After 48 h, the L6 myocyte culture treated with DOX presents a decreased phosphorylation of AMPK α in the residue T172, without changing its overall content. The GLUT-4 expression was also reduced in the treatment. These data suggest that although the insulin signalling cascade has not been completely disrupted, important proteins with effective participation in glucose uptake are less expressed.

Treatment with doxorubicin decreases glucose uptake in L6 myocytes and this effect is reversed by chronic activation of AMP-activated protein kinase

Our results in isolated myocytes confirm our hypothesis that metabolic chaos generated by this drug on carbohydrate metabolism is due in part to impaired glucose uptake in both the stimulated and basal state (Figure 3A).

Accordingly, myocytes were treated with DOX and AICAR acutely (1 h) or chronically (48 h). As previously demonstrated, in vitro treatment with DOX decreased the phosphorylation ofAMPK. The acute intervention (1 h) with AICAR is effective in phosphorylation of this protein in control and treated group with DOX compared with the group treated only with DOX. We did not find an increase in the phosphorylation of AMPK α after chronic treatment (48 h) with AICAR (Figure 3B, 3C, and 3D).

Intervention with AICAR recovers glucose uptake in myocytes treated with DOX. By 48 h, the reestablishment of glucose uptake was complete (Figure 3E). Despite the fact that treatment with AICAR for 1 h increased uptake, it was not enough to be statistically significant compared with the group treated with DOX.

Cell viability was increased by n-acetyl cysteine treatment, but the glucose uptake was not restored by n-acetyl cysteine

The deleterious role of oxidative stress in chemotherapy treatment, especially with DOX has been well established in cardiac and skeletal muscle tissue. Indeed, our results suggest a potential increase in production of ERO’s, because treated animals had increased activity of mitochondrial complex 1 and a decrease in activity of mitochondrial Complex 3 (Figure 4A).
Figure 2  Doxorubicin leads to impaired systemic insulin sensitivity. (A) Homeostatic model assessment of insulin resistance. (B) Curve of the insulin tolerance test C. Kitt. (D) Gene expression involved in glucose metabolism. (E/F) Protein expression involved in glucose metabolism in extensor digitorum longus muscle. (G/H) AMPk and GLUT-4 protein expression in culture L6 myocytes treated with doxorubicin. The groups were compared by Test T. P < 0.05 was considered statistically significant. * P < 0.5, ** P < 0.01, *** P < 0.001. n = 3–8.
To assess whether oxidative stress could be involved in the decrease in cell viability we treated L6 myocytes with DOX for 48 h at a concentration of 1 mmol in the presence or absence of NAC (20 mmol/high concentration and 2 mmol/low concentration). As shown in Figure S2A, the concentration of DOX 1 mmol leads to a decrease in cell viability, whereas the chronic treatment with high concentrations of NAC (20 mmol) reverses this effect (Figure S2B). Low concentrations of NAC were not able to reverse the cell viability.

We investigated whether treatment with the antioxidant NAC reverses glucose uptake in the myocytes treated with this chemotherapeutic agent. Our data show that the treatment with NAC was unable to reverse the reduction in glucose uptake resulting from treatment with DOX (Figure S2C).

**Doxorubicin-induced decreased the proinflammatory cytokines protein content**

Pro-inflammatory cytokine levels (IL-6/TNF-α) were not elevated compared with the control group (Figure S3A), including decreased levels of antiinflammatory cytokine IL-10. Still, the content of IL-6 released into the medium of myocytes treated with DOX in the presence of LPS is decreased compared with the control group treated with LPS (Figure S3B).

The pathway of inflammasome also seems to be not involved in this process, because the expression of IL-1β, as well as proteins involved in the maturation of this protein, was not modulated by treatment (Figure S3C).
Figure 4 Mitochondrial complex 1 and 3 activity. The groups were compared by Test T. P < 0.05 was considered statistically significant. * P < 0.5. n = 4–6.

Discussion

Our main results suggest that treatment with DOX caused hyperglycaemia and insulin resistance mediated by inhibition in AMPk, moreover, the treatment leads to atrophy, weight loss, and anorexia mimicking similar conditions to cachexia. In addition, we observed that rats treated with DOX showed hyperglycaemia, insulin resistance (kiTT), increases in FFA, and corticosterone serum concentration. To our knowledge, this is the first study to demonstrate an association between treatment with DOX and insulin resistance in skeletal muscle metabolism.

Chemotherapy can alter insulin sensitivity in clinical practice.30,31 The treatment with DOX causes the deterioration in glucose metabolism both in vivo and in vitro. Recently, Arunachalam and colleagues32 review that DOX may mimic type 2 diabetes, whereas the adipose tissue metabolism was negatively affected. We showed that skeletal muscle plays a significant role in this condition, with dramatically reduced GLUT-4 protein and mRNA expression in the animal model, and DOX treatment decreased the glucose uptake in the basal and stimulated condition in L6 myocytes.

Insulin is a key hormone in glucose uptake in skeletal muscle. The activation of proteins involved in signalling of this hormone is essential for the translocation of GLUT4 from intracellular vesicles to the sarcolemma. Dysfunction of proteins in this pathway causes a strong insulin resistance.33,34 Our data showed a decrease in insulin sensitivity, whereas in the kiTT test, the DOX group showed decreases in the kiTT constant. However, in molecular levels our results suggest that the signalling cascade of insulin is not completely disrupted, although important proteins with effective participation in glucose uptake are less expressed. The protein levels of insulin receptors and gene and protein expression of AKT protein downstream of Pl-3-kinase were not affected in the EDL muscle after treatment. Nevertheless, other proteins involved in glucose metabolism had decreased expression such IRS-1 and GS3kb mRNA expression, and GLUT-4 mRNA and protein expression. Even with a partially competent signalling of insulin, the need for this glucose transporter is essential as demonstrated by studies using a knockout model for GLUT4.35

Several hypotheses were reported to trigger the development of glucose intolerance. It is well established that DOX increases oxidative stress and mitochondrial dysfunction36,37 including in skeletal muscle,38,39 which both mechanisms are important to lead the insulin resistance. DOX leads to increased ROS through different mechanisms. The interaction of this drug with iron III, the perturbation of regulation of nitric oxide (NO), and the redox cycling of this drug, which takes place by reoxidation of radical DOX-semiquinone to DOX, leads to the formation of ROS.40–42

Although our data suggest that treatment with DOX alters the activity of mitochondrial complexes, leading to a proton gradient that is favourable to the formation of ROS with increases in activity of the Complex I and inhibition of Complex III, which are important sites where there is formation of ROS.43,44 Many studies showed the strong association between oxidative stress and a decrease in glucose uptake in skeletal muscle.45–48 But the treatment with the antioxidant n-acetylcysteine (NAC) was not able to reverse the decrease in glucose uptake induced by DOX in L6 culture. However, when cells were treated with concentrations of DOX capable of decreasing cell viability, antioxidant treatment (high concentrations of NAC) was able to reverse this effect. Probably, the absence of effect in restored the glucose uptake after NAC treatment occurs because the oxidative stress leads to impair in glucose uptake mediated by insulin response.46 and the contribution of this pathway in glucose intolerance is less effective after the DOX treatment. Whereas, the main pathway disrupted by DOX is the AMPK signalling in skeletal muscle. The AMPK is the other major regulator of glucose transport, and it leads to GLUT-4 for the sarcolemma independently of insulin.

Additionally, AMPK regulates the gene expression of GLUT-4 to the activation and translocation of transcription factors into the nucleus to bind to the promoter of the GLUT4 gene region, as the MEF2A (myocytes enhancer factor) and GEF (GLUT4 enhancer factor).49,50

Our data demonstrated a large inhibition of GLUT-4 protein expression in EDL in rats treated with DOX. Together with this, the AMPk mRNA expression and phospho AMPk α thr172 follow the decreases in the GLUT-4 protein. Because of this we hypothesized that AMPk signalling was disrupted in skeletal muscle in the DOX group. We evaluated the treatment with an agonist of AMPk, AICAR, that was able to completely reverse glucose uptake stimulated by insulin in L6 myocytes.

Other important regulator of AMPk pathway and glucose uptake is the adiponectin, an adipokine produced in adipose tissue and responsible by a cross link between it and other tissues, because its circulating concentration positively
correlated with an insulin sensitivity. The adiponectin expression in adipose tissue and the circulating levels was decreased (data not shown) in the DOX group and may be explained, at least in part, by the decrease in AMPk activity and mRNA expression, whereas adiponectin is a positive modulator of AMPk activity.\textsuperscript{51} Beyond the adiponectin, the Interleukin-6 increased the AMPk activity in myocytes.\textsuperscript{52,53} In our results, the DOX treatment inhibits the IL-6 released in L6 myocytes after LPS stimulation, although this phenomenon did not occur in vivo.

Our results showed that DOX treatment lead animals to develop a cachexia-like syndrome. Cachexia is characterized as a multifactorial syndrome, in which there is involuntary weight loss.\textsuperscript{54} In agreement with other supporting studies, the use of some chemotherapy drugs appears to contribute to the development of symptoms of cachexia, contributing to an unfavourable metabolic state.\textsuperscript{19,55,56} The DOX also caused increases in glucose intolerance, induced extreme atrophy, weight, and adipose tissue mass loss, anorexia, and a catabolic environment, as demonstrated by an increase in corticosterone/testosterone ratio, with a decrease in myocyte area.

The literature showed that inflammatory mediators are a possible mediator of glucose intolerance, sarcopenia, and anorexia. However, our results do not support this hypothesis in DOX treatment. Although TNF-\textalpha was decreased in the DOX group without changes in the protein content of the cytokine receptors assessed, there was no change in gene expression of proteins involved in the inflammassome pathway in muscle.

In conclusion, our results show that treatment with DOX leads to conditions similar to cachexia, with a severe glucose intolerance both in vivo and in vitro. The decrease of AMPk activity of the protein is modulated negatively with treatment and has proved to be a possible therapeutic target, which is able to recover glucose sensitivity in skeletal muscle, and can lead to improvements in quality of life in chemotherapy patients.

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The authors certify that they comply with the ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2015. Please enter here a numbered reference: 57 See below reference list no. 57.

Conflict of interest

Edson Alves de Lima Junior, Alex Shimura Yamashita, Gustavo, Pimentel, Luís Gustavo de Sousa, Ronaldo Vagner T. dos Santos, Cinara Gonçalves, Emílio Streck, Fábio Lira, and José Cesar Rosa Neto declare that they have no conflict of interests.

References


Online supplementary material

Supporting Information is available at Journal of Cachexia, Sarcopenia and Muscle online.

Table S1. Primer sequences of the genes studies for real-time PCR.

Table S2. Organs weight and hepatic and renal function.

Figure S1. Treatment with doxorubicin don’t change the activity of mitochondrial oxidative enzymes in EDL muscle. The groups were compared by Test T. p < 0.05 was considered statistically significant. n=3-5.

Figure S2. Cell viability assay and 2-deoxy-[C14]-D-glucose uptake in L6 cells. A. Differentiated L6 cells were treated for 48 hours with doxorubicin in different concentrations. The MTT assay was performed thereafter to assess cell viability; B. Treatment with high concentrations of NAC reverses the viability of doxorubicin-treated myocytes. The groups were compared using ANOVA one-way test with Tukey post-test to compare the groups. p < 0.05 was considered statistically significant. * p<0.5, ** p<0.01, ***p<0.005. n=7. C. 2-deoxy-[C14]-D-glucose uptake in L6 cells treated with NAC. This cells were subjected to treatment with doxorubicin, associated with insulin, and submitted or not to treatment with NAC. The results were relativized by control group without insulin stimulation. For the glucose uptake assay the groups were compared using ANOVA two-way test with Bonferroni post-test to compare the groups. p < 0.05 was considered statistically significant. * p<0.5, ** p<0.01, *** p<0.001. n=4-6.

Fig. S3. Expression of proteins involved in inflammation and cytokines in skeletal muscle and myocytes. A. Concentration of cytokines TNF-\textalpha, IL-6 and IL-10 in the EDL muscle; B. IL-6 content in the middle of L6 myocytes after 24 hours of treatment with doxorubicin (100 nmol) in the presence or absence of LPS (1 ug / ml). C. Caspase 1, NLRP1 gene expression, NLRP-3 gene expression, NOD-1, NOD-2 and TLR-4 gene expression. n=5-10. The groups were compared by Test T. p < 0.05 was considered statistically significant. * p<0.5, ** p<0.01, *** p<0.001. n = 4-7.


