Endurance training activates AMP-activated protein kinase, increases expression of uncoupling protein 2 and reduces insulin secretion from rat pancreatic islets

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Abstract

Endurance exercise is known to enhance peripheral insulin sensitivity and reduce insulin secretion. However, it is unknown whether the latter effect is due to the reduction in plasma substrate availability or alterations in β-cell secretory machinery. Here, we tested the hypothesis that endurance exercise reduces insulin secretion by altering the intracellular energy-sensitive AMP-activated kinase (AMPK) signaling pathway. Male Wistar rats were submitted to endurance protocol training one, three, or five times per week, over 8 weeks. After that, pancreatic islets were isolated, and glucose-induced insulin secretion (GIIS), glucose transporter 2 (GLUT2) protein content, total and phosphorylated calmodulin kinase kinase (CaMKII), and AMPK levels as well as peroxisome proliferator-activated receptor-γ coactivator-1-α (PGC-1α) and uncoupling protein 2 (UCP2) content were measured. After 8 weeks, chronic endurance exercise reduced GIIS in a dose–response manner proportionally to weekly exercise frequency. Contrariwise, increases in GLUT2 protein content, CaMKII and AMPK phosphorylation levels were observed. These alterations were accompanied by an increase in UCP2 content, probably mediated by an enhancement in PGC-1α protein expression. In conclusion, chronic endurance exercise induces adaptations in β-cells leading to a reduction in GIIS, probably by activating the AMPK signaling pathway.

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Introduction

Exercise is known to be a powerful strategy to improve blood glucose control in lean, obese, and diabetic individuals helping to prevent and control insulin resistance and diabetes development (Colberg 2007). It is widely accepted that exercise training facilitates this effect by increasing skeletal muscle expression and/or activity of key proteins involved in the insulin signaling, such as glucose transporter 4 (GLUT4), insulin receptor, and insulin receptor substrates 1/2, AMP-activated kinase (AMPK), protein kinase B/AKT substrate 160, as well as mitochondrial oxidative capacity and lipid oxidation and turnover (for review see: Zierath (2002) and Hawley & Lessard (2008)).

Despite the well-established effects of exercise upon skeletal muscle insulin action, ongoing evidence points toward its systemic effect in other tissues, which, in turn, are also involved in blood glucose homeostasis. In this sense, exercise increases liver insulin sensitivity (Hoene et al. 2009) reducing hepatic glucose production through the inhibition of FOXO/HNF-4α pathway and TBR3 expression in insulin-resistant and diabetic rodents (Lima et al. 2009, De Souza et al. 2010). It was also demonstrated that exercise increases hypothalamic insulin and leptin sensitivity (Flores et al. 2006) enhancing the AMPK/mammalian target of rapamycin pathway, leading to lowered appetite and consequently nutrient intake (Ropelle et al. 2008).

In response to the observed systemic insulin sensitivity enhancement, endurance exercise also induces insulinemia and insulin secretion reduction in rodents (Zawalich et al. 1982, de Souza et al. 2003, Scomparin et al. 2009) and in healthy and obese humans (Engdahl et al. 1995, Solomon et al. 2010). Although the mechanisms by which endurance exercise reduces pancreatic islets glucose-induced insulin secretion (GIIS) remain unclear, it was previously reported that trained rats exhibited reduced proinsulin and glucokinase mRNA content (Koranyi et al. 1991). Furthermore, endurance training was demonstrated to decrease GLUT2 content and total hexokinase plus glucokinase activity in rat pancreatic islets, reducing intracellular glucose flux, in addition to ATP availability (Ueda et al. 2003), which, in turn, would increase AMP-to-ATP ratio, favoring AMPK activation.
AMPK is an important intracellular metabolic sensor and regulates glucose homeostasis by acting upon insulin-sensitive tissues as well as pancreatic islet β-cells function (Rutter et al. 2003). In light of this, the AMPK pathway has been proposed to be an important intracellular target against diabetes development. It has been argued that type 2 diabetes treatment would be associated with insulin-sensitive tissues and AMPK activation, whereas in pancreatic islets, this pathway might be inhibited, raising insulin secretion and sensitivity (Viollet et al. 2009). It was recently reported that AMPK is involved with glucose sensing and insulin secretion, probably by increased expression of uncoupling protein 2 (UCP2) and granule docking control (Beall et al. 2010, Sun et al. 2010b). Indeed, it was also demonstrated that AMPK activation by metformin was able to reduce insulin secretion in human islets and MIN6 cells (Leclerc et al. 2004). On the other hand, tumor suppressor liver kinase B1 (LKB1) deletion, a well-known AMPK activator, enhanced mice pancreatic islets insulin secretion (Sun et al. 2010a).

It is well established that exercise training activates AMPK in several insulin-sensitive tissues (Zhang et al. 2009); however, the effect of endurance training upon pancreatic islets AMPK has not been addressed. Thus, the aims of the present study were to test the hypothesis that the reduction in insulin secretion by endurance exercise is related to the activation of the AMPK–UCP2 pathway, and if these adaptations depend on exercise frequency.

Materials and Methods

Ethical approval
All the experiments described herein were approved by the State University of Campinas Committee for Ethics in Animal Experimentation and performed according to the ‘Principles of laboratory animal care’, which are in agreement with those stated by Drummond (2009).

Antibodies, chemicals, and buffers
All reagents used were from analytical grade. Antibodies against GLUT2 (sc-7580) and UCP2 (sc-6526) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against peroxisome proliferator-activated receptor-γ coactivator-1-α (PGC-1α) (#2178), AMPK (#2532), and pAMPK (#2535) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against calmodulin kinase kinase (CaMKII) (ab22609) and pCaMKII (ab5683) were obtained from Abcam (Cambridge, MA, USA). Nitrocellulose paper (Hybond ECL, 0.45 μm) were obtained from Amersham Biosciences. Human recombinant insulin (Biohulin N) was obtained from Biobra’s (Mogi das Cruzes, Brazil). Reagents for SDS-PAGE and immunoblotting were obtained from Bio–Rad. HEPES, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X–100, Tween 20, glycerol, collagenase (type V), and BSA (fraction V) were obtained from Sigma–Aldrich. Chemiluminescence kit was obtained from Pierce (Rockford, IL, USA).

Experimental rats
Male Wistar rats from the University of Campinas Animal Breeding Center were used in the experiments. The rats were maintained on 12 h light:12 h darkness photocycle (lights on at 0600 h) in a room at 22 ± 2 ºC and maintained on a diet of Purina Laboratory Chow and water made available ad libitum. Three rats were maintained in each cage. At 6 weeks of age, the animals were randomly assigned into four groups (n = 5 for each group): sedentary control (CTL), exercising once a week, exercising three times a week, and exercising five times a week over 8 weeks. Initially, the rats were tested for glucose and insulin tolerance. The rats were then acclimatized to treadmill running with daily sessions from 5 to 10 min (5 m/min at 0º inclination) for 2 weeks. Following baseline experiments and treadmill acclimatization, the rats started their treadmill exercise (8 weeks of age). Rats were weighed before, during, and after the training programme.

Endurance exercise training protocol
The CTL group remained sedentary, allowed only regular movements inside the cages. The other groups were exercised 1 (TRE1x), 3 (TRE3x), or 5 (TRE5x) times per week over a period of 8 weeks. Rats ran initially for 5 min at 5 m/min, reaching 60 min at 30 m/min in the last training week. Training speed and duration were adjusted every week, as described in Fig. 1. Rats were continually monitored during their training sessions. If a rat refused to run it was removed from the training session, examined for possible injuries, and removed from the treadmill for that day. If the rat continued to refuse to run in the following session, it was removed from the training programme.

Intraperitoneal glucose tolerance test
The intraperitoneal glucose tolerance test (ipGTT) was done at baseline and at the end of the 8th week of training. Rats rested for at least 24 h before the ipGTT. Food was withdrawn 12 h before the experiment, and free access to water was...
allowed. Rats were weighted and had their tail tip cut for measurement of blood glucose and insulin levels (time = 0). A solution of glucose 50% (w:v) was administered by i.p. injection at a dose of 2 g/kg body weight, and blood samples were collected at 15, 30, 60, and 120 min to glucose and insulin determination. The area under the curve (AUC) was calculated from values for each rat. For plasma insulin measurement ~100 μl of total blood was collected in heparinized tubes and quantified by RIA.

**Intraperitoneal insulin tolerance test**

The intraperitoneal insulin tolerance test (ipITT) was done at baseline and at the end of the 8th week of training, 5 days after the ipGTT. Rats rested for at least 24 h before the ipITT. Fed rats were weighted and had their tail tip cut for blood glucose levels measurement (time = 0). Following this they received 2 U/kg body weight of human recombinant insulin by i.p. injection, and blood samples were collected at 5, 10, 15, 20, 25, and 30 min for serum glucose determination. The constant rate for glucose disappearance (K\text{dis}) was calculated using the formula 0.693/t_{1/2}. Glucose t_{1/2} was calculated from the slope of the least-squares analysis of plasma glucose concentrations during the linear decay phase.

**Pancreatic islets isolation**

Rats were anesthetized in a sealed CO\text{2} chamber and killed by decapitation. Trained rats were killed at least 24 h after their last training session. Pancreatic islets were then isolated by collagenase digestion as described previously (Bordin et al. 1995). Briefly, after killing, the pancreas was inflated with 10 ml Hank’s solution containing collagenase 0.8 mg/ml, and then removed from the rat and kept at 37 °C for 23 min. Islets were then collected manually one-by-one under a microscope.

**Static insulin secretion studies**

Groups of four freshly isolated islets were initially preincubated for 45 min at 37 °C in Krebs-Ringer bicarbonate buffer with the following composition (in mmol/l): NaCl, 115; KCl, 5; CaCl\text{2}, 2.56; MgCl\text{2}, 1; NaHCO\text{3}, 24; and glucose, 5.6; supplemented with BSA (0.3% w:v) and equilibrated with a mixture of 95% O\text{2}:5% CO\text{2}, pH 7.4. The solution was then replaced, and islets were incubated for 1 h under the experimental conditions (2.8 or 16.7 mmol/l glucose). At the end of the incubation time, the insulin content of the medium was determined by RIA (Scott et al. 1981).

**Western blot analysis**

After isolation, groups of 800–1000 freshly isolated islets from each experimental group were homogenized by sonication (3 times, 15 s pulses each) (Sonics & Materials, Newtown, CT, USA) in a freshly prepared ice-cold anti-protease buffer (1% Triton X-100, 100 mmol/l Tris, pH 7.4, 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium vanadate, 2 mmol/l phenylmethylsulfonyl fluoride, and 0.1 mg aprotinin). Insoluble material was removed by centrifugation for 30 min at 15 300 g in a 70.Ti rotor (Beckman, Fullerton, CA, USA) at 4 °C. The protein concentration of the supernatants was determined by the Bradford dye binding method (Bradford 1976). Thirty (30) micrograms of the total protein extracts from pancreatic islets were separated by SDS-PAGE. Proteins were then transferred from gel to nitrocellulose membrane. Membranes were blocked in 5% nonfat dried milk in 139 mM NaCl, 2.7 mM KH\text{2}PO\text{4}, 9.9 mM Na\text{2}HPO\text{4}, and 0.1% Tween 20 for 2 h at room temperature and then incubated overnight at 4 °C with specific antibodies. After incubation with the specific secondary antibody, immune complexes were detected using the enhanced SuperSignal West Pico Chemiluminescent Substrate (Pierce), as described by the manufacturer, and the visualization was performed by exposure of the membranes to X-ray films. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image Software – Scion Corporation, Frederick, MD, USA).

**Statistical analysis**

Mean values ± S.E.M. obtained from all experiments were compared using ANOVA. Post hoc Tukey test was employed when required. The level of significance was set at P<0.05. Significant differences symbols are detailed in figure captions.

<table>
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<tr>
<th>Table 1</th>
<th>Body weight throughout endurance training period. Results are mean±S.E.M. (n=4)</th>
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<td>Before training</td>
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<td>CTL</td>
<td>193.4±11.9</td>
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<td>TRE1x</td>
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<td>TRE3x</td>
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<td>TRE5x</td>
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*P<0.001 compared to CTL and TRE1x after 8 weeks of training.
Results

Endurance exercise reduced body weight and improved systemic glucose tolerance and insulin sensitivity

After 8 weeks of the endurance training programme, rats from groups TRE3x and TRE5x showed lower body weight compared to CTL and TRE1x (Table 1). Although body composition was not investigated, the observed difference might rely on a reduced fat storage in TRE3x and TRE5x.

Glucose tolerance and insulin sensitivity were not different among groups before the beginning of the training programme (data not shown). After the end of endurance training, TRE3x and TRE5x rats displayed progressive higher blood glucose clearance demonstrated by a reduced ipGTT (Fig. 2A) and AUC 17 and 26% lower in TR3x and TR5x respectively than the control group ($P<0.001$; Fig. 2B). Plasma insulin during ipGTT was significantly decreased (18.7 and 28%) in TRE3x and TRE5x respectively compared to the control group ($P<0.001$; Fig. 2C). The decrease observed in TRE5x was significantly different from TRE3x ($P<0.05$). Higher insulin sensitivity was also demonstrated by $K_\text{itt}$ values, which showed an up-regulating tendency already in TRE3x and was significantly ($P<0.0001$) higher in TRE5x (Fig. 2D).

Endurance exercise reduces pancreatic islets GIIS

The GIIS in isolated islets was significantly reduced at basal (glucose 2.8 mM) and stimulatory glucose concentration (16.7 mM) in TRE3x and TRE5x. GIIS decrease was also related to training frequency. In TRE3x, GIIS decreased by 17 and 32%, whereas in TRE5x, the reduction was of 40 and 44% at 2.8 and 16.7 mM glucose respectively compared with the control group ($P<0.001$; Fig. 3A and B).

Effect of endurance exercise upon several islet parameters

As illustrated in Fig. 4, chronic endurance training progressively increased GLUT2 content in a dose-dependent fashion. The expression of this protein was two-, three-, and fourfold higher in the TRE1x, TRE2x, and TRE3x groups respectively compared with the CTL group ($P<0.001$; Fig. 4A). A significant increase in CaMKII content (Fig. 4B) and phosphorylation (Fig. 4C) was observed in islets from the TRE5x group ($P<0.001$). As a consequence, the ratio between pAMPK/AMPK was also significantly higher in islets from the TRE3x and TRE5x groups ($P<0.0001$; Fig. 4D). PGC-1α was increased according to exercise frequency attaining 1.8- and 2.6-fold in TRE3x and TRE5x respectively compared with CTL islets ($P<0.001$; Fig. 4E). Finally, UCP2 also significantly increased by 1.5- and 2.3-fold in TRE3x and TRE5x islets respectively compared with CTL islets ($P<0.001$; Fig. 4F).

Figure 2 Effect of endurance training on whole body glucose tolerance and insulin sensitivity. After rats were submitted to an endurance training protocol one (TRE1x), three (TRE3x), or five (TRE5x) times per week, the observed elevated blood glucose attenuated during ipGTT in TRE3x and TRE5x (A), an effect that was demonstrated by reduced area under the curve (B). Endurance training improved insulin sensitivity in TRE3x and TRE5x shown by reduced blood insulin content (C) and elevated $K_\text{itt}$ values (D). All values are expressed as means ± S.E.M. ($n=5$, *$P<0.0001$ versus CTL; *$P<0.05$ versus TRE3x).
Discussion

Endurance exercise has been described to increase insulin sensitivity, reducing insulin secretion, which suggests crosstalk between insulin-sensitive tissues and pancreatic islets. Information concerning the effect of exercise on the improvement of insulin sensitivity is abundant in the literature; however, it is rare regarding insulin secretion modulation.

In agreement with literature data, our endurance-trained groups were more glucose tolerant due to enhanced insulin sensitivity, as shown by the ipGTT and $K_m$ data. The observed increase upon whole body insulin sensitivity might be related to higher insulin signal transduction, mainly in skeletal muscle and liver. Exercise training-induced increase in skeletal muscle and liver insulin signaling pathway results in higher blood glucose uptake (Zierath 2002, Hawley & Lessard 2008, Hoene et al. 2009, Ropelle et al. 2009). In addition, endurance exercise also improves skeletal muscle and liver glycogen synthesis capacity by increased glycogen synthase activity (Abernethy et al. 1990, Garrido et al. 1996), enhancing blood glucose disposal, allowing faster glycemia reduction. Thus, faster training-induced blood glucose clearance by insulin-sensitive tissues, together with the low GLUT2 and glucokinase affinity, due to their elevated $K_m$ (Iynedjian 1993, Schuit 1997), might account for impaired GIIS signaling in pancreatic β-cells, reducing blood insulin content observed during ipGTT. Therefore, these data provide some evidence for a mechanism by which the crosstalk between insulin-sensitive tissues and pancreatic islets might occur.

However, when pancreatic islets were isolated, removing the interaction with other tissues, and incubated with glucose, it was observed that endurance-trained rats islets secreted almost 50% less insulin in response to basal and stimulatory glucose concentrations than CTL islets. Our results are in line with Zawalich et al. (1982) who reported a reduction in GIIS of about 40–50% in islets from endurance-trained rats. Taken together, these evidences suggest that, despite the well-known peripheral insulin-sensitizing effect of endurance exercise, it also induces alterations upon pancreatic β-cell function.

Reduced GIIS in isolated islets from exercise-trained rats was previously attributed to decreased β-cell glucose uptake and metabolism. Lower glucokinase concentration and expression of proinsulin mRNA was demonstrated. In addition, glucokinase activity and glucose utilization were reduced in endurance-trained rats, which, in turn, decreased islet ATP synthesis (Koranyi et al. 1991). Ueda et al. (2003) also observed lower levels of GLUT2 protein content in trained rats. Although all these alterations could be triggered by the exercise-induced reduction upon blood glucose availability, the lowered β-cell glucose metabolism could reduce ATP synthesis, leading to activation of intracellular signaling pathways, such as AMPK. This information prompted us to investigate this possible effect.

In contrast to the report of Ueda et al. (2003), our results showed increased GLUT2 content in trained rat islets. We speculate that the observed increase might reflect a tendency to counteract the higher rates of blood glucose clearance in trained animals. However, at the moment, we do not have a clear explanation for these discrepancies; we can only suggest that they may be the consequence of different training protocols.

On the other hand, our data demonstrated that endurance training increased β-cell pAMPK/AMPK ratio, activating this pathway, according to exercise frequency. As stated above, exercise-induced β-cell AMPK activation might be related to AMP-to-ATP ratio increase, resulting from a possible decreased glucose metabolism. Despite the unexpected GLUT2 increase, we cannot exclude a reduction in glucose metabolism in our experimental groups, as we did not measure any parameter to investigate this effect. Thus, it is possible that, as observed in previous reports, glucokinase activity might be reduced, lowering glycolytic flux.

In addition, similar to a previous report (Ueda et al. 2003), our results showed that exercise training increased total and phosphorylated CaMKII. It is well established that AMPK is a substrate for CaMKII (Viollet et al. 2009). Thus, the higher levels of CaMKII phosphorylation also explain the increased levels of AMPK activation in islets from trained rats. Moreover, these results suggest that endurance exercise increases, somehow, pancreatic islet CaMKII gene expression as well as calcium sensitivity, as this specific kinase phosphorylation is mediated by cytosolic calcium increase (Viollet et al. 2009).

β-cell AMPK activation was demonstrated to reduce GIIS by several mechanisms. Activated AMPK pathway blocks secretory granules transit (Tsuboi et al. 2003, Sun et al. 2010b). Furthermore, AMPK activation increases UCP2 content in several tissues such as liver, skeletal muscle, and hypothalamus (Pedersen et al. 2001, Foretz et al. 2005).
Andrews et al. 2008). In addition, recent studies provided evidence for effects of the AMPK signaling pathway on the expression control of UCP2 in the MIN6 insulinoma cell line and mice pancreatic islets, an effect that seems to be mediated by PGC-1α (Beall et al. 2010, Wang et al. 2010). In agreement, our data provided evidence that endurance exercise enhances AMPK activation as well as UCP2 expression in isolated pancreatic islets, probably by enhancing PGC-1α content. Therefore, our findings support the idea that endurance exercise reduces GHS, at least in part, by AMPK activation. This activation seems to be related to increased CaMKII phosphorylation and positive AMP allosteric modulation.

However, in spite of increased AMPK activation, one cannot exclude other factors that could also be involved with exercise-induced lowered GIIS. During exercise, a well-known result is the blood catecholamine (i.e. adrenaline, noradrenaline) rise. Catecholamines reduce insulin secretion mainly by adrenergic receptors α (2A) and α (2C)-G protein-mediated decreases in cAMP (Peterhoff et al. 2003). Thus, it is possible that exercise training could enhance β-cell responsiveness to catecholamines, and consequently reduce GHS.

Exercise training and AMPK have been pointed out as an important strategy and target respectively against the development of obesity and diabetes. According to this proposal, treatment with AICAR prevented hyperinsulinemia and preserved β-cell mass in fat Zucker rats (Pold et al. 2005). In contrast, it was also demonstrated that AICAR activation of AMPK in isolated β-cells increased apoptosis (Kefas et al. 2003), whereas AMPK inhibition protected β-cell toxicity and apoptosis (Riboulet-Chavey et al. 2008). Based on our data and on the aforementioned evidence, one can assume that overall exercise-induced AMPK activation would protect β-cells by counteracting elevated blood nutrient glucolipotoxicity-induced damage as well as by reducing GIIS-associated stress, rather than enhancing intrinsic β-cell survival pathways. Moreover, exercise-induced decreased blood glucose could alter β-cell programming, favoring its function and survival. It was demonstrated that glucose activates the carbohydrate response element-binding protein (ChREBP; Davies et al. 2008), and ChREBP

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**Figure 4** Effect of endurance exercise upon several islet parameters. After rats were submitted to an endurance training protocol one (TRE1x), three (TRE3x), or five (TRE5x) times per week, several islet proteins involved with insulin secretion modulation content were quantified by western blotting. Representative bands and densitometry quantification demonstrated by vertical bars of GLUT2 (A). Total CaMKII (B) and phosphorylated CaMKII (pCaMKII) (C). Total and phosphorylated AMPK ratio (D). PGC-1α (E) and UCP2 (F). Values are normalized by β-actin, exception made to total to phosphorylated AMPK ratio. All values are expressed as means ± S.E.M. (n=5, *P<0.001 versus CTL; #P<0.001 versus TRE1x; *P<0.001 versus TRE3x).
inactivation was associated with increased β-cell protection (Da Silva Xavier et al. 2010); however, further investigations are needed to clarify the effect of exercise training upon β-cell survival.

In addition to the endurance exercise-induced enhanced AMPK–UCP2 signaling pathway, our results demonstrated that this effect is dependent on the exercise frequency, as observed by the graduated response seen in different experimental groups. Although exercise-induced whole body insulin sensitivity changes could influence nutrient control of pancreatic islet adaptations, the observed dose–response effect may also indicate skeletal muscle and pancreatic islet crosstalk cytokine modulation.

In light of this, interleukin 6 (IL6) appears to be a good candidate since it is produced by contracting skeletal muscle (Pedersen 2006) leading to a rise in the concentration of IL6 in blood, (GokHale et al. 2007, Wardyn et al. 2008). IL6 was previously demonstrated to be involved with skeletal muscle and hypothalamic insulin sensitivity (Flores et al. 2006, Stefanyk & Dyck 2010). In addition, IL6 activates AMPK in several tissues such as liver, skeletal muscle, and adipose tissues (Ruderman et al. 2006). Moreover, this statement is in agreement with previous study, which suggests a cytokine control of the crosstalk between skeletal muscle and pancreatic β-cells. In this study, it was reported that isolated islets incubated with IL6 reduced GHS, whereas insulin secretion induced by arginine was not altered, evidencing IL6 metabolic rather than membrane depolarization effects (Handschin et al. 2007). However, to date, literature still lacks direct evidence linking exercise-induced blood IL6 increase, β-cell AMPK activation, and GHS control. Therefore, further studies are needed to investigate this possible relationship.

In conclusion, our data provide evidence that endurance exercise modulates isolated pancreatic islet GHS by activating AMPK, increasing UCP2 protein content. Furthermore, exercise-induced alterations in insulin sensitivity and secretion are dose-dependent phenomena, reinforcing skeletal muscle and pancreatic islets direct crosstalk.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Author contribution statement**

V C C and C C Z were involved in the conception and design of the experiments, collection, analysis, and interpretation of data and article drafting. L F R was involved in the collection, analysis and interpretation of data, and drafting the article. L R S, E M C, and A C B were involved in the conception and design of the experiments and critically revising the article for important intellectual content.

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