Review Article

Insulin release, peripheral insulin resistance and muscle function in protein malnutrition: a role of tricarboxylic acid cycle anaplerosis

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Pancrætic β-cells and skeletal muscle act in a synergetic way in the control of systemic glucose homeostasis. Several pyruvate-dependent and -independent shuttles enhance tricarboxylic acid cycle intermediate (TACI) anaplerosis and increase β-cell ATP:ADP ratio, triggering insulin exocytotic mechanisms. In addition, mitochondrial TACI cataplerosis gives rise to the so-called metabolic coupling factors, which are also related to insulin release. Periocrin insulin resistance seems to be related to skeletal muscle fatty acid (FA) accumulation and oxidation imbalance. In this sense, exercise has been shown to enhance skeletal muscle TACI anaplerosis, increasing FA oxidation and by this manner restores insulin sensitivity. Protein malnutrition reduces β-cell insulin synthesis, release and peripheral sensitivity. Despite little available data concerning mitochondrial metabolism under protein malnutrition, evidence points towards reduced β-cell and skeletal muscle mitochondrial capacity. The observed decrease in insulin synthesis and release may reflect reduced anaplerotic and cataplerotic capacity. Furthermore, insulin release is tightly coupled to ATP:ADP rise which in turn is related to TACI anaplerosis. The effect of protein malnutrition upon peripheral insulin resistance is time-dependent and directly related to FA oxidation capacity. In contrast to β-cells, TACI anaplerosis and cataplerosis pathways in skeletal muscle seem to control FA oxidation and regulate insulin resistance.

Mitochondrial metabolism: Skeletal muscle: Pancreatic islets

In pancreatic islet β-cells, ATP acts on ATP-dependent K+ channels leading to membrane depolarisation, opening of voltage-sensitive Ca2+ channels, increasing cytosolic Ca2+ concentration and stimulating insulin exocytosis. Under physiological conditions mitochondrial glucose metabolism provides most of the ATP required during insulin secretion. Glucose metabolism leads to the enhancement of net tricarboxylic acid cycle (TAC) intermediates (TACI) including citrate, malate, oxaloacetate, α-ketoglutarate and succinate, a process known as anaplerosis. The β-cell elevated TACI concentration has been associated with augmented oxidative energy production and insulin release. Despite the essential role of ATP, insulin release signalling by other metabolic coupling factors including malonyl-CoA, NADPH, leucine, glutamate and diacylglycerol has also been demonstrated, which suggests the existence of a complex process of insulin release regulation. Metabolic coupling factors are generally derived from TACI extramitochondrial metabolism after their exit from the mitochondria, a process known as cataplerosis. The TACI anaplerosis:cataplerosis ratio, therefore, plays a signalling role during insulin release in addition to contributing to enhanced ATP concentration.

Previous studies have demonstrated that pyruvate carboxylase (PC) activity and glucose-induced insulin secretion (GIIS) are strictly coupled, suggesting that an elevated anaplerosis–cataplerosis cycle is of substantial importance for insulin release. In 832/13 insulinoma cells treated with high concentrations of NEFA, GIIS is significantly reduced in association with a decreased mitochondrial anaplerosis–cataplerosis cycle.

Abbreviations: Akt or PKB, protein kinase B; CS, citrate synthase; FA, fatty acid; GDH, glutamate dehydrogenase; GIIS, glucose-induced insulin secretion; GPX, glutathione peroxidase; ICDe, cytosolic NADP-dependent isocitrate dehydrogenase; IMTG, intramuscular TAG; IRS, insulin receptor substrate; MEC, cytosolic malic enzyme; PC, pyruvate carboxylase; PI3, phosphatidylinositol 3,4,5-trisphosphate; ROS, reactive oxygen species; TAC, tricarboxylic acid cycle; TACI, tricarboxylic acid cycle intermediate.

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Peripheral tissues with insulin resistance are expected to exhibit an impaired mitochondrial oxidative capacity followed by an elevated intracellular lipid content\(^{(10\text{--}13)}\). Kelley et al. \(^{(10)}\) examined the respiratory quotient in diabetic, obese and lean subjects. It was observed that in contrast to lean subjects, diabetic and obese patients were unable to switch from lipid to carbohydrate metabolism after 8 h following an insulin-stimulated glucose loading. Paradoxically, diabetic and obese individuals preferentially oxidised carbohydrate, demonstrating a poor capacity for lipid oxidation. These findings are in agreement with the proposition that insulin-resistant subjects exhibit impaired mitochondrial capacity and reduced fatty acid (FA) \(\beta\)-oxidation\(^{(11,14)}\). In addition, insulin-resistant subjects are expected to express elevated uncoupling protein-3 content, demonstrating a low mitochondrial capacity\(^{(15,16)}\). In contrast, endurance-trained subjects exhibit elevated substrate consumption at rest and a high anaplerotic--cataplerotic capacity\(^{(17)}\). Chronic and acute exercise has also been shown to reduce insulin resistance\(^{(18\text{--}20)}\). Under this condition, TACI anaplerosis is postulated to be required for adequate skeletal muscle contraction\(^{(21)}\). Conversely, absence of an adequate TACI concentration leads to reduced muscle contraction tolerance, probably by a reduced mitochondrial oxidative flux\(^{(22,23)}\).

Intra-uterine and early postnatal protein malnutrition results in decreased insulin release\(^{(24\text{--}26)}\) and pancreatic \(\beta\)-cell altered glucose metabolism\(^{(27,28)}\). Our group has provided evidence that protein restriction reduces insulin secretion stimulated by glucose, amino acids, K\(^+\) and other secretagogues\(^{(29\text{--}32)}\). This decreased capacity is related to alterations in gene expression, including mitogen-activated protein kinases, voltage-gated K\(^+\) channel and glucose transporters\(^{(31\text{--}33)}\). Adult offspring of pregnancy and early postnatal malnutrition develop peripheral insulin resistance\(^{(27,28,34)}\), followed by reduced muscle contraction capacity\(^{(35)}\), alterations that are closely related to type 2 diabetes development. Thus, there seems to be a narrow concert governed by mitochondrial function between \(\beta\)-cell insulin release and peripheral tissue metabolism.

Protein malnutrition has been reported to induce similar effects to obesity. Mitochondrial metabolism in pancreatic \(\beta\)-cells as well as peripheral tissues under this situation has been poorly investigated. We propose that protein malnutrition induces an imbalanced anaplerotic:cataplerotic ratio due to the reduced amino acid pool. So, the withdrawal of TACI would increase intracellular amino acid availability for protein synthesis, reducing TACI flux and consequently decreasing insulin release and sensitivity. The purpose of the present review is to report evidence of anaplerotic mechanisms involved with the regulation of insulin secretion, peripheral resistance and muscle contraction metabolism under ordinary situations and under protein malnutrition, suggesting future directions for research in this field.

**Anaplerosis and insulin release**

An increased blood glucose concentration stimulates pancreatic \(\beta\)-cells to secrete insulin but the molecular aspects concerning GIIS are not completely understood. Recent findings have proposed an oscillatory pattern of downstream events, leading to an increase of cytoplasmic Ca\(^{2+}\) levels, insulin granule docking and fusion with the plasma membrane, enhanced cyclic AMP production, amplified Ca signalling and activation of secretory mechanisms by Ca\(^{2+}\) ions\(^{(2,36\text{--}38)}\). Several mechanisms have been proposed involving the K\(_{ATP}\) and Ca\(^{2+}\)-dependent pathway, the K\(_{ATP}\)-independent and Ca\(^{2+}\)-dependent pathway, and possibly a K\(^{+}\)- and Ca\(^{2+}\) independent pathway\(^{(39\text{--}43)}\). However, little is known about the metabolic regulation associated with these pathways.

Improved TACI anaplerosis--cataplerosis flux is needed to enhance intracellular ATP content\(^{(44,45)}\). Although the mechanism involved remains unknown, a strong correlation has been documented between PC activity and GIIS\(^{(44,45)}\). This enzyme, located in the mitochondrial matrix, catalyses the ATP-dependent carboxylation of pyruvate to form oxaloacetate\(^{(46)}\). PC is highly expressed in the liver and kidney, and participates with phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase of gluconeogenesis\(^{(44,47,48)}\). The lack of phosphoenolpyruvate carboxykinase activity and relatively low lipogenic capacity suggest an important role of PC in \(\beta\)-cells during oxidative energy metabolism. A proportion of about 40–50\% pyruvate enters \(\beta\)-cells during mitochondrial metabolism through PC reactions at stimulating glucose concentrations, a very high flux for a non-gluconeogenic tissue. The anaplerosis of glucose carbons, therefore, highly correlates with GIIS by \(\beta\)-cells\(^{(4,49)}\). In INS cells (from the insulinoma cell line), anaplerosis was reported to be increased followed by a high basal insulin secretion after exposure to glucose. However, at a low glucose concentration, PC expression was demonstrated to be markedly decreased\(^{(8,49)}\). Farfari et al. \(^{(50)}\) demonstrated that phenylacetic acid, a PC inhibitor, reduced GIIS in INS cells and pancreatic islets, an effect that was associated with reduced citrate accumulation. Similarly, Fransson et al. \(^{(5)}\), examining the phenylacetic acid-induced effect on PC during insulin release in rat islets, demonstrated that anaplerosis via PC is required for an appropriated rise in the ATP:ADP ratio and insulin secretion. However, caution must be taken from the above studies once phenylacetic acid specificity is limited toward PC\(^{(44)}\). Very recently, Hasan et al. \(^{(51)}\), testing the hypothesis that anaplerosis via PC is important for GIIS in cell lines, verified that reduced expression of this enzyme using transfection of short hairpin RNA was associated with reduced PC activity and insulin release in response to glucose and other secretagogues. Although the mechanism remains to be elucidated, the influx of carbon intermediates into the TAC is critical for appropriate ATP generation in \(\beta\)-cells\(^{(45)}\).

The proposed mechanisms have been extensively studied and supported by different metabolic pathways in which pyruvate is metabolised and/or recycled\(^{(44,49,50,52,53)}\). As in most mammalian tissues, pyruvate may follow two different routes, feeding the TAC with acetyl-CoA via pyruvate dehydrogenase and oxaloacetate via PC in pancreatic \(\beta\)-cells\(^{(45)}\).

Pyruvate dehydrogenase contributes with the TAC by the production of acetyl-CoA. In this reaction, one carbon from pyruvate is lost as CO\(_2\) and two are converted in the acetyl-CoA molecule, which will condensate with oxaloacetate to form citrate by citrate synthase (CS)\(^{(52)}\). The oxaloacetate molecule is regenerated with the TACI, remaining constant at the expense of acetyl-CoA. Although much attention has been given to PC, the flux of pyruvate decarboxylation through pyruvate dehydrogenase in \(\beta\)-cells is estimated to be
similar to that observed during pyruvate carboxylation by PC\(^{(53)}\). Sustained glucose supply raises the intracellular citrate content in \(\beta\)-cells, suggesting an association between PC and pyruvate dehydrogenase during the anaplerosis process\(^{(48)}\). However, the high \(K_m\) of the CS for acetyl-CoA compared with oxaloacetate suggests that the flux of oxaloacetate from pyruvate via PC is greatly favoured, increasing the content of intermediates in the second span of TAC\(^{(54)}\). Moreover, acetyl-CoA is a well known allosteric activator of PC, favouring the anaplerosis process and consequently the rate of intracellular ATP production\(^{(55)}\). However, the elevated TAC content exerts an inhibitory effect on most of the regulatory sites from the TAC\(^{(4,55,56)}\), which will further allow the TAC export from the mitochondria to the cytosol. Once accumulated, TAC not only provide ATP through the mitochondrial electron transport chain but also are exported (cataplerosis) to the cytosol\(^{(4)}\). In this latter process, carbon derived from pyruvate carboxylation exits from the mitochondria to the cytosol, primarily as malate\(^{(4,53)}\). In the cytosol, malate is converted to pyruvate in a NADPH generation reaction catalysed by cytosolic malic enzyme (MEc), which can be transported back to the mitochondria\(^{(4)}\). This cycle, also known as the pyruvate–malate shuttle, occurs inside the mitochondrial matrix and exerts a critical role in \(\beta\)-cells during both the anaplerosis and cataplerosis processes by recycling pyruvate and NADPH production, a potent metabolic coupling factor\(^{(52,53)}\) (Fig. 1). Evidence of pyruvate–malate shuttle activity was recently demonstrated during GHS in INS cells.

Fig. 1. Anaplerosis and cataplerosis pathways during insulin secretion in \(\beta\)-cells. The pyruvate cycling is the main cytosolic site of NADPH production. NADPH is one of the most important antioxidants in \(\beta\)-cells. This anaplerotic process includes the pyruvate–malate shuttle, pyruvate–citrate cycle and pyruvate–isocitrate–\(\alpha\)-ketoglutarate cycle. NADPHox, NADPH–oxidase enzymic complex; SOD, superoxide dismutase; \(O_2^-\), superoxide anion; GSSG, oxidised glutathione; GPX, glutathione peroxidase; GSH, glutathione; GR, glutathione reductase; cME, cytosolic malic enzyme; glucose 6-P, glucose 6-phosphate; MDH, malate dehydrogenase; CPT, carnitine palmitoyltransferase; mME, mitochondrial malic enzyme; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; ETC, electron transport chain; Pi, inorganic phosphate.
favouring rapid TAC expansion\(^{(44,52,53)}\). Similarly to malate, citrate after exiting the mitochondria is converted by ATP–citrate lyase to oxaloacetate and acetyl-CoA\(^{(44,53)}\). Oxaloacetate in a sequence of two reactions is reduced to malate by malate dehydrogenase, which will further be converted to pyruvate by MEc. In this reaction, NADPH is used as cofactor favouring the rise of cytosolic NADPH:NADP\(^+\) and pyruvate transport back to the mitochondria. This cycle is defined as the pyruvate–citrate cycle\(^{(44,50,53)}\) (Fig. 1). Recent studies have demonstrated that oscillatory ATP production is strongly associated with cytosolic citrate content\(^{(56)}\). This relationship suggests that citrate might be by itself coordinating the activities of TAC and anaerobic glycolysis. Inside the mitochondrial matrix, citrate has been described as an important inhibitor of CS\(^{(55)}\). This effect will favour its mitochondrial accumulation and consequently its exit toward the cytosol. Once accumulated into the cytosol, citrate might inhibit glycolysis via allosteric modulation of phosphofructokinase\(^{(55,57)}\). This mechanism, associated with elevated cytosolic NADPH: NADP\(^+\), could explain, at least in part, the oscillatory pattern of insulin secretion in \(\beta\)-cells.

An increased NADPH:NADP\(^+\) inhibits the pentose phosphate pathway. Although this route is described as exhibiting low activity in \(\beta\)-cells, inhibition of the pentose phosphate pathway might exert a metabolic-saving effect, driving the glucose-6-phosphate to glycolysis\(^{(50)}\). On the other hand, acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase, a well-known inhibitor of FA metabolism\(^{(58,59)}\). In a tissue with elevated anaplerosis capacity, the cytosolic NADH:NAD\(^+\) ratio is reduced, allowing sustained glycolaldehyde 3-phosphate dehydrogenase activity and glycolytic flux, leading to an increase in the ATP:ADP ratio\(^{(4,55)}\). In \(\beta\)-cells, glucose oxidation provides a higher mitochondrial electrochemical gradient (\(\Delta\psi\)) and ATP:ADP ratio than FA\(^{(60)}\), whereas FA oxidation seems to induce mitochondrial uncoupling and reduced ATP synthesis\(^{(61)}\). There is strong evidence linking PC activity and the pyruvate–citrate cycle with insulin secretion\(^{(5,58,53,62)}\). PC inhibition resulted in decreased GIIS, which was positively correlated with citrate concentrations\(^{(50,51)}\). Furthermore, impairment of mitochondria citrate metabolism by either inhibition of mitochondrial di- and tricarboxylate carriers or deletion of citrate lyase and MEc genes leads to reduced GIIS, with no change in glucose oxidation, probably by a reduction in malonyl-CoA and NADPH synthesis\(^{(53)}\).

The importance of the anaplerotic PC–pyruvate–citrate cycle pathway was reinforced when \(\beta\)-cells were incubated in the presence of weak insulin secretagogues including acetate, \(\beta\)-hydroxybutyrate, monomethyl succinate and lactate. When incubated together, these metabolites increased acetyl-CoA, oxaloacetate and consequently citrate concentrations, that were able to enhance insulin release by 10- to 20-fold, almost the same effect observed for GIIS\(^{(7)}\). However, a strong correlation between GIIS and a non-PC-derived anaplerosis pathway in insulinoma cell lines was observed, suggesting that other pathways, probably aspartate aminotransferase, act in combination with PC in TAC anaplerosis-induced GIIS, enhancing oxaloacetate levels through aspartate and \(\alpha\)-ketoglutarate consumption, respectively\(^{(63)}\). MacDonald\(\textit{et al.}\)\(^{(6)}\) demonstrated that incorporation of carbon from pyruvate into lipids was not lowered in the citrate lyase-deficient INS-L cell line, suggesting that citrate is not the only catalytic carbon carrier from the mitochondria to cytosol. Further studies are, therefore, needed to clarify other anaplerotic and cataplerotic pathways and substrates that regulate GIIS.

Alternatively, pyruvate cycling during TAC expansion can also occur via pyruvate, isocitrate and \(\alpha\)-ketoglutarate\(^{(44)}\). Unlike the pyruvate–malate shuttle, oxaloacetate formed by PC is converted to citrate and isocitrate, which then leave the mitochondria forming oxaloacetate and acetyl-CoA through citrate lyase or \(\alpha\)-ketoglutarate through cytosolic NADPH-dependent isocitrate dehydrogenase (ICDc). The pyruvate cycling can take place via conversion of oxaloacetate to malate and further to pyruvate by malate dehydrogenase and MEc, respectively\(^{(44,45,53)}\). In addition, \(\alpha\)-ketoglutarate can re-enter the mitochondria forming malate by following the TAC reactions, which will be converted to pyruvate via MEc or mitochondrial malic enzyme with elevated NADPH production (Fig. 1). Ronnebaum\(\textit{et al.}\)\(^{(64)}\), examining the role of ICDc in control of GIIS in \(\beta\)-cells using ICDc iRNA, observed that suppression of ICDc attenuated the glucose-induced increment in pyruvate cycling and intracellular NADPH content. These findings, therefore, suggest that the pyruvate cycling pathway involving ICDc plays an important role in the control of GIIS.

In addition to the pyruvate cycling shuttle enzymes, the role of glutamate dehydrogenase (GDH) during anaplerosis-induced insulin release has recently been reviewed\(^{(60)}\). Carobbio and colleagues\(^{(66)}\), using GDH knockout rats, showed a near 37 % reduction in GIIS. Studies using leucine, a GDH-positive allosteric modulator, as an insulin secretagogue, showed enhanced insulin release\(^{(52,67)}\). To date, it is not known if GDH acts as an anaplerotic enzyme producing \(\alpha\)-ketoglutarate or has a cataplerotic function generating glutamate at the expense of \(\alpha\)-ketoglutarate during insulin release. Accumulated evidence has shown the link between GDH and GIIS\(^{(64)}\). In addition to the possible anaplerotic and cataplerotic role during insulin release, GDH produces NADPH, another important metabolic coupling factor\(^{(4)}\).

**Anaplerosis, intracellular redox status and insulin release in \(\beta\)-cells**

Interestingly, most alterations in anaplerotic- and cataplerotic-related mechanisms are involved with NADPH production (Fig. 2). As first reported by MacDonald\(^{(52)}\), the pyruvate cycling may be the main cytosolic site of NADPH production with much higher capacity than the pentose phosphate pathway in \(\beta\)-cells. In this sense, the cataplerosis of malate and citrate would increase NADPH production. The \(\beta\)-cells’ needs for this reducing agent are still not known. However, NADPH is involved in FA synthesis and cellular redox modulation. As \(\beta\)-cells exhibit a relatively low rate of FA synthesis\(^{(68)}\), the main NADPH function could be attributed to the regulation of intracellular redox status. Also, one would speculate that the low pentose phosphate pathway activity must be of relevance for \(\beta\)-cells, as this pathway is highly associated with lipid synthesis in different tissues. However, a high intracellular lipid content might be potentially toxic for \(\beta\)-cells, in which an elevated anaplerosis and cataplerosis process is required during GIIS.
Redox alteration of β-cells to a more reduced status is needed for proper insulin secretion. The NADPH production during anaplerosis provides a substrate for glutathione and thioredoxin systems, which in turn will favour the intracellular redox status. GSH, glutathione; GSSG, oxidised glutathione; SOD, superoxide dismutase; O$_2^-$, superoxide anion; OH, hydroxyl radical; GPX, glutathione peroxidase; GR, glutathione reductase; CAT, catalase; S-S, thiol and disulfide (–S–S–).

Fig. 2. The β-cell antioxidant system. The NADPH production during anaplerosis provides a substrate for glutathione and thioredoxin systems, which in turn will favour the intracellular redox status. GSH, glutathione; GSSG, oxidised glutathione; SOD, superoxide dismutase; O$_2^-$, superoxide anion; OH, hydroxyl radical; GPX, glutathione peroxidase; GR, glutathione reductase; CAT, catalase; S-S, thiol and disulfide (–S–S–).

Protein malnutrition, β-cell molecular alterations and insulin release

Protein-deficient diets lead to impaired insulin secretion in response to oral or intravenous glucose infusion and to other secretagogues. Under such conditions, the expression of pancreatic and duodenal homeobox-1, a transcription factor that plays a role in the maintenance of β-cell homeostasis, is markedly reduced in association with reduced pancreatic islet area and insulin release. Expression of signalling proteins, such as protein kinase A and protein kinase C, is also reduced during protein malnutrition. Several genes involved in insulin production and secretion mechanisms also have their expression altered. Recently, it was demonstrated that Ca$^{2+}$ uptake and insulin mRNA content were also reduced in undernourished rats, leading to reduced insulin release in response to glucose. These outcomes might in part be attributed to the reduced expression of both constitutive and inducible NO synthase isozymes under protein malnutrition. NO has been demonstrated to protect against endoplasmic reticulum stress and has a stimulating effect upon insulin gene transcription. NO is synthesised by NO synthase, which also uses NADPH as a substrate. The redox imbalance leading to β-cell oxidative stress has been implicated in several dysfunctions including low insulin release, cell proliferation and death, which are directly related to type 2 diabetes development.

Robertson & Harmon described the importance of GPX for the control of β-cell redox status. This enzyme acts in combination with glutathione reductase. Under low NADPH content, GPX has its ROS detoxification capacity markedly compromised. NO has also been proposed to have a protective effect upon the endoplasmic reticulum under ROS-induced stress. In addition, NO has been reported to stimulate insulin gene transcription. NO synthesis, which also uses NADPH as a substrate. The redox imbalance leading to β-cell oxidative stress has been implicated in several dysfunctions including low insulin release, cell proliferation and death, which are directly related to type 2 diabetes development.

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Protein malnutrition, anaplerosis and insulin release

Concerning metabolic aspects of protein malnutrition-induced reduction in insulin release, Sener et al. (93) have shown that low-protein-fed rats have reduced glucose oxidation as demonstrated by a decreased metabolite flux through the glycerol phosphate shuttle in β-cells from malnourished rats, probably by a low mitochondrial FAD-linked glycero phosphate dehydrogenase activity (94). In addition, at high glucose concentration, low-protein-fed rats showed an elevated glycolytic flux. However, leucine transamination to α-ketoisocaproic acid and further production of α-ketoglutarate was significantly reduced, indicating a poor anaplerotic capacity (93).

The impaired insulin release in malnourished rats might be, therefore, related to the lower mitochondrial oxidative and anaplerotic capacity (Fig. 3). The reduced β-cell anaplerosis in malnutrition is supported by findings that insulin synthesis is also regulated by succinate and/or succinyl-CoA cataplerosis (95,96). This finding is in accordance with reduced β-cell insulin mRNA levels in undernourished rats (90,92). Moreover, malonyl-CoA content, another insulin release signalling molecule, has been shown to be reduced under protein malnutrition (90). Malonyl-CoA is produced through mitochondrial citrate cataplerosis, and one of its actions is to limit FA oxidation, stimulating GIIS (4). There seems to be a pyruvate–citrate cycle impairment under protein malnutrition, since intracellular malonyl-CoA content is very low. Thus, PC expression might be altered under this situation and the reasons are still unknown. Aspartate aminotransferase is identified as another main anaplerotic pathway under physiological conditions (63). However, it has not been investigated in protein restriction conditions. Under physiological conditions, glutamate is described as a non-essential anaplerotic substrate (63) but, under protein malnutrition, this amino acid may exert a role during anaplerosis. The hypothesis for a main glutamate anaplerotic route is based on the higher activity of glutamate–pyruvate transaminase shown in undernourished rats (94). In this condition, an increased insulin release is observed in response to leucine stimulation, a positive GDH allosteric modulator (32). One possible reason could be an alternative pathway for pyruvate, α-ketoglutarate and NADPH synthesis. In agreement, we have recently demonstrated that GDH protein expression is reduced in rats submitted to protein undernourishment. However, leucine supplementation restored GDH expression as well as GIIS to control levels (97), probably by enhanced α-ketoglutarate and NADPH production.

Reusens et al. (98), examining the effect of a low-protein diet in pregnant rats, demonstrated that the gene expression of most TAC proteins was substantially up-regulated, an effect that was accompanied by a reduced expression of superoxide dismutase and heat shock protein-1, -1a and -1b. However, cytochrome c oxidase activity and ATP production were markedly reduced. Preliminary data from our laboratory provided evidence for a reduction of catalease activity, but GPX activity remained unchanged (APG Cappelli, CC Zoppi, A Trevisan, TM Batista, PMR da Silva and EM Carneiro, unpublished results). It seems reasonable, therefore, that during protein deficiency anaplerosis might be compromised, leading to reduced NADH:NAD+ and NADPH:NADP+ ratios and insulin synthesis and release. Further studies are needed to investigate the expression and content of anaplerotic enzymes as well as their substrate oscillations during insulin release events in undernourished β-cells.

Anaplerosis and peripheral insulin resistance

Skeletal muscle operates in a coordinated way with pancreatic islets in the control of glucose levels (13). The binding of insulin to its receptor induces insulin receptor tyrosine kinase activity. Tyrosine phosphorylation of insulin receptor substrate (IRS)-1 results in activation of the p85 regulatory subunit of phosphatidylinositol 3,4,5-trisphosphate (PI3) kinase and activates the p110 catalytic subunit, which increases phosphoinositides such as PI3. This leads to activation of phosphoinositide-dependent protein kinase and downstream PKB (Akt) and/or atypical protein kinase C (99). Phosphorylation of Akt substrate 160 (AS160), which has a GTPase-activating domain (Rab4), facilitates translocation of GLUT-4 to the sarcolemma, favouring glucose uptake (100).

Intramuscular TAG (IMTG) accumulation leads to an increased concentration of FA metabolites including...
diacylglycerol, fatty acyl-CoA and ceramides that in turn activate serine kinase leading to IRS-1 phosphorylation of serine residues, and consequently inhibition of insulin downstream events(101). In addition, higher levels of IMTG may increase ROS production and induce inflammation(100,102).

Abnormal mitochondrial metabolism has been described in the insulin-resistant state(12,103–105). However, IMTG accumulation with no mitochondrial dysfunction has also been associated with the development of peripheral insulin resistance(106–109). Therefore, whether mitochondrial dysfunction is a cause or consequence of peripheral insulin resistance development is still unknown(113).

Interestingly, when sedentary obese insulin-resistant subjects were submitted to a moderate exercise training programme, insulin sensitivity was increased despite enhanced IMTG stores. However, this effect was associated with a reduction in diacylglycerol and ceramide content followed by an increased mitochondrial oxidative capacity(118). Similar results have been obtained in obese Zucker rats submitted to acute exercise. Despite an unchanged or increased concentration of intramuscular diacylglycerol and long-chain acyl-CoA, acute exercise improved insulin sensitivity, probably by the enhanced phosphorylation of AS160(25). Skeletal muscle contraction is a well-known stimulus to increase GLUT-4 translocation by an insulin-independent mechanism(119). Although the full mechanisms involved in contraction-induced GLUT-4 translocation are still unclear, sarcoplasmic Ca2+ efflux and the AS160 phosphorylation by the Akt–AMP-activated kinase pathway seem to play a pivotal role also in glucose uptake(111).

If mitochondrial dysfunction is not the main cause of insulin resistance, it can, at least in part, contribute to the development of this condition. A conciliatory hypothesis has been proposed that the link between mitochondrial metabolism and IMTG lipotoxicity-induced insulin resistance would be confined to the level of IMTG turnover inside muscle fibres. Thus, a mismatch between IMTG hydrolisis (lipolysis) and mitochondrial β-oxidation increases the intracellular lipid content with detrimental effects on insulin signalling and glucose metabolism. So, regular physical exercise plays a key role to protect against IMTG accumulation-induced insulin resistance(112).

The endproduct of FA β-oxidation is acetyl-CoA, and the further oxidation of this compound occurs inside the TAC. However, the first TAC reaction catalysed by CS is the condensation of acetyl-CoA with oxaloacetate, giving rise to citrate. Thus for a complete oxidation of FA molecules, a regular production of oxaloacetate is needed(113). Muoio & Koves(114) reviewed the molecular mechanism-induced mitochondrial metabolism dysfunction and proposed several mechanisms that may be involved in peripheral insulin resistance, including a reduced TAC flux. Indeed, type 2 diabetic patients have reduced TAC flux and consequently impaired complete FA oxidation(115). In addition, reduced ADP phosphorylation in mitochondria from type 2 diabetic patients might result from reduced TAC and electron transport chain flux(116).

One possible mechanism to explain the reduced TAC flux is an impaired anaplerotic capacity (Fig. 4). However, there are scarce data regarding anaplerotic pathways in insulin-resistant tissues. Befroy et al.(117) examined whether resting skeletal muscle metabolism is altered in endurance-trained compared with sedentary subjects. These authors reported that trained subjects exhibit elevated substrate consumption at rest, suggesting a high anaplerotic and cataplerotic capacity. In contrast, elevated uncoupling protein-3 content has been demonstrated in insulin-resistant subjects, indicating an elevated IMTG content and consequently poor anaplerosis and cataplerosis capacity(115,16,115).

Reduced TAC flux can also be impaired by oxidative stress as previously reported in peripheral insulin resistance, a metabolic state which stimulates ROS production(59,117–119). In fact, elevated concentrations of the superoxide anion might inhibit aconitase activity(120). Likewise, high levels of H2O2 reduce the activity of α-ketoglutarate dehydrogenase, one far from near-equilibrium regulatory enzyme of the TAC(121). In contrast to what is observed for β-cells, reduction in FA oxidation by malonyl-CoA is not desirable for skeletal muscle fibres. Under conditions of increased ROS production, aconitase inhibition may induce citrate accumulation and cataplerosis, resulting in decreased glycolytic flux and enhanced malonyl-CoA synthesis, leading to reduced FA oxidation. In agreement with this statement, malonyl-CoA synthesis has been shown to be increased in the situations of diabetes and insulin resistance(122). Acetyl-CoA carboxylase knockout mice show enhanced insulin sensitivity and were prevented from fat-rich diet-induced obesity(123).

Chronic muscle contraction (i.e. exercise) remains as the pivotal preventive therapy against peripheral insulin resistance development. Voluntary exercise stimulates blood glucose uptake, contributing to glucose homeostasis as well as a non-insulin-dependent mechanism(110,124). In addition, exercise raises the expression of several glycolytic and oxidative enzymes involved in glucose and FA oxidation(125–127) contributing to high exercise and resting FA oxidation by the enhancement of TAC flux(117,126,129). In addition to the potential effect upon oxidative enzyme activities, exercise may contribute to a high mitochondrial FA oxidation capacity by regulating TAC flux and intermediate concentrations. Exercise also improves skeletal muscle antioxidant capacity, reducing the installation of oxidative stress-induced mitochondrial impairment(130). During moderate- to high-intensity exercise, TAC anaplerosis is increased and has a pivotal role in maintaining muscle contraction efficiency(21,22). TAC anaplerosis exerts an important role in regulating IMTG oxidation. The failure of this regulation might be implicated in peripheral insulin resistance development. In glycogen-depleted muscle, TAC concentration is maintained at the same level as during high glycogen store conditions during prolonged exercise(131). Under muscle glycogen-depleted exercise, FA metabolism is substantially favoured, suggesting a close relationship between anaplerosis and FA oxidation. However, acute down-regulation of TACI does not reduce oxidative ATP synthesis during exercise(132). Therefore, the anaplerosis process may not be directly associated with oxidative ATP production in muscle skeletal cells. Further studies are required to investigate the meaning of a possible link between anaplerosis, FA oxidation control and insulin resistance in skeletal muscle.

Protein malnutrition and peripheral insulin resistance

Protein-deficient diets have been shown to markedly affect skeletal muscle function. Several reports indicate that undernourished rats present low muscle weight and impaired
morphological, metabolic and functional developments\(^{133-135}\). Moreover, offspring from protein-malnourished dams also shows altered muscle structure and function that can affect animal posture and locomotion\(^{35}\). Lehnert et al.\(^{136}\), using complementary DNA microarrays, showed a 2- to 6-fold reduction in the expression of several muscle structural proteins and metabolic enzymes in cattle submitted to severe undernutrition. Suppressed muscle growth in undernourished rats occurs probably due to a reduction in muscle and blood insulin-like growth factor-I content\(^{137}\). Based on the aforementioned, one would expect that a protein deficiency-induced decrease of muscle size and function would result in reduced muscle glucose and FA metabolism and predisposing to obesity and type 2 diabetes\(^{138,139}\).

Protein malnutrition shows diverse effects during the life-span concerning peripheral insulin resistance development. Short-term protein undernutrition effects have been previously demonstrated to enhance muscle insulin sensitivity\(^{140}\). This effect has been shown to occur by improvement in several steps of the insulin cascade, such as increased p38 mitogen-activated protein kinase-induced GLUT-4 translocation, high levels of insulin receptor and IRS-1 tyrosine phosphorylation as well as increased IRS-1–PI3 kinase–Akt insulin signalling steps have been demonstrated to be altered in low-birth-weight men and rats\(^{147,151,152}\). Conversely, long-term protein undernutrition has the opposite effect, mainly when higher amounts of the nutrient become available\(^{144}\). Adult rats submitted to protein restriction in early life, or offspring from undernourished mothers, develop hyperinsulinaemia, peripheral insulin resistance and type 2 diabetes\(^{27,145-147}\). These alterations have been demonstrated to affect the second generation\(^{148}\). Indeed, evidence points to a positive correlation between reduced fetal growth, a well-known protein-restriction feature, and peripheral insulin resistance development\(^{34,149,150}\). In contrast to short-term effects, insulin signalling seems to be compromised in the adult stage of early protein undernourishment. Reduced protein kinase C-\(\zeta\) expression has been reported in rats and young men. In addition, skeletal muscle PI3 kinase–Akt insulin signalling steps have been demonstrated to be altered in low-birth-weight men and rats\(^{147,151,152}\).

Early protein restriction has been reported to programme the appetite in late adult life\(^{153}\), reducing serotonin inhibitory action on food intake, and stimulating the preference for fat- rich foods\(^{154,155}\). Furthermore, early protein undernutrition has also been shown to determine fat distribution and reduce physical activity levels, contributing to body and skeletal muscle fat storage in adult life\(^{156}\). The observed differences upon skeletal muscle glucose metabolism after early protein

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**Fig. 4.** Insulin resistance reduces tricarboxylic acid cycle (TAC) flux in peripheral tissues. The shadowed areas indicate the major pathways associated with low anaplerotic and cataplerotic capacity including glycolysis, the TAC and \(\beta\)-oxidation. Glucose 6-P, glucose 6-phosphate; CPT, carnitine palmitoyltransferase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; mME, mitochondrial malic enzyme; ETC, electron transport chain; \(O_2\), superoxide anion.
restriction might be associated with time- and diet-dependent metabolic changes. However, data on the metabolic regulation of protein malnutrition-induced peripheral insulin resistance are scant. To date, most studies have focused on neuroendocrine aspects and some have attempted to investigate skeletal muscle insulin signalling pathway changes. The effects of IMF stores, incomplete FA oxidation and accumulation of metabolites leading to serine kinase activation and mitochondrial function are still unclear.

Short- and long-term protein malnutrition, anaplerosis and peripheral insulin resistance

Concerning metabolic aspects of short-term protein malnutrition, Fagundes et al.\textsuperscript{(157)} reported that young adult rats from low-protein-fed mothers showed low visceral and total body fat content, probably caused by increased lipolysis or decreased lipogenesis which might be related to the observed high plasma catecholamine and low insulin levels compared with control. Pups from low-protein-fed mothers showed reduced serum NEFA and similar skeletal muscle acetyl-CoA carboxylase, FA synthase and carnitine palmitoyl transferase-I expression as compared with control\textsuperscript{(158)}. These results are in line with those reported by Toyoshima et al.\textsuperscript{(159)}. These latter authors reported decreased levels of IRS-1 serine phosphorylation. The lower level of total body and consequently skeletal muscle fat might reduce the activation of specific serine kinases that impair skeletal muscle insulin downstream events. On the other hand, Goshy et al.\textsuperscript{(159)} provided clues for long-term protein restriction to increase body fat content triggering peripheral insulin resistance events. Zhu et al.\textsuperscript{(160)} examined the long-term effects of undernutrition on skeletal muscle of offspring pregnant ewes and reported severe alterations in skeletal muscle metabolism. Despite an enhanced proportion of type 2 muscle fibres, GLUT-4 concentration was decreased and IMTG content was increased. In addition, a reduction in FA oxidation due to an almost 25% decrease in carnitine palmitoyl transferase-I activity, as well as a reduced expression of ATP synthase and antioxidant enzymes, probably by decreased mitochondria density, was reported. In agreement, Park et al.\textsuperscript{(161)} showed reduced mitochondrial DNA content and cytochrome c oxidase subunits I and III expression due to long-term effect of protein malnutrition during gestation and lactation.

Selak et al.\textsuperscript{(162)}, using the bilateral uterine artery ligation model in pregnant rats to induce intra-uterine growth retardation, reported a decrease of 43% in muscle glycogen content compared with control. This effect was associated with decreased mitochondrial ATP synthesis and reduced pyruvate, α-ketoglutarate, glutamate and succinate oxidation in isolated muscle mitochondria during respiratory state 3. Interestingly, alterations in most of the respiratory chain-linked electron transfer and energy coupling in muscle mitochondria parameters between control and growth-retarded animals were not observed. In this sense, a reduction in TAC flux and FA oxidation would be expected, but unfortunately TACI content was not measured. Evidence for a reduced TAC flux was provided by Lane et al.\textsuperscript{(163)}. These authors showed a decreased NAD\textsuperscript{+}/NADH in growth-retarded rats, despite unaltered activities of mitochondrial isocitrate dehydrogenase and malate dehydrogenase\textsuperscript{(164)}. As discussed earlier, under nutrient deprivation, TAC flux and consequently mitochondrial metabolism are altered. Mehta et al.\textsuperscript{(165)} demonstrated reduced oxidative enzyme activities in growing young monkeys submitted to a low energy intake, being associated with peripheral insulin resistance and type 2 diabetes. However, as proposed for ordinary nutrition conditions, TAC flux might be reduced by impaired specific TAC enzyme activities or anaplerosis capacity, and so further studies are needed to answer the remaining questions.

Elevated oxidative stress has also been reported after protein malnutrition in skeletal muscle. Despite conflicting results, antioxidant enzyme activities vary according to diet protein content, and lipid peroxidation was directly related to their detoxifying capacity\textsuperscript{(161,166)}. Moreover, a twofold reduction in glutathione-S-transferase expression and glutathione content was observed in skeletal muscle from malnourished rats\textsuperscript{(160)}. These effects were observed to be in concert with reduced GPX scavenger capacity\textsuperscript{(167)}.

The long-term metabolic effects of protein undernutrition seem, therefore, to reproduce the effects of obesity in regular-fed subjects and it is reasonable that peripheral insulin resistance and type 2 diabetes in regular feeding and protein undernutrition are induced by the same metabolic alterations. However, earlier, chronic and acute exercise is a powerful tool against peripheral insulin resistance, by its action upon restoration of insulin sensitivity. Nevertheless, early protein undernutrition seems to compromise exercise tolerance in men and rats\textsuperscript{(35,156,168)}.

During adult life, reduced exercise capacity after early protein undernourishment was demonstrated to be associated with reduced intracellular levels of phosphocreatine and inorganic phosphate\textsuperscript{(169)}. This effect was accompanied by a faster depletion of muscle glycogen stores during exercise\textsuperscript{(170)}.

Conclusion

β-Cell elevated TACI concentration plays a key role for oxidative energy production and insulin release. The mechanism is supported by pyruvate metabolism (anaplerosis), mainly involving the enzyme PC. The citrate, malate and isocitrate produced exit the mitochondria to the cytosol (cataplerosis), increasing the cytosolic pyruvate concentration followed by elevated NADPH generation. Protein-deficient diets lead to impaired insulin secretion which is related to the lower mitochondrial oxidative and anaplerotic capacity. It seems reasonable, therefore, that in the protein-deficient state anaplerosis might be compromised, leading to reduced NADH:NAD\textsuperscript{+} and NADPH:NADP\textsuperscript{+} ratios, and thus further investigation is needed. In peripheral tissues, the mismatch between IMTG hydrolysis and mitochondrial β-oxidation increases the intracellular lipid content with detrimental effects on insulin signalling and glucose metabolism in normally fed and protein malnutrition states. In contrast to β-cells, skeletal muscle fibre TAC flux enhancement does not seem to be related to ATP:ADP increase, but with FA oxidation regulation. Despite no clear evidence of impaired anaplerotic and cataplerotic reactions being available in protein undernourishment, it is possible that peripheral insulin resistance could be triggered by reduced anaplerotic replenishment of the TACI, leading to inadequate FA oxidation. In addition, impaired TAC could also be induced by oxidative stress, followed by citrate
cataplerosis and consequent malonyl-CoA accumulation. Therefore, based on the aforementioned data, future studies are needed to better focus on the role played by anaplerosis and cataplerosis reactions upon the control of β-cell insulin release mechanisms and peripheral insulin resistance in protein malnutrition states.

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