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Regulation of peripheral tissue substrate metabolism by the gut-derived hormone ghrelin

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ABSTRACT

Ghrelin increases in the circulation prior to entrained mealtimes, with the acylated (AG) form functioning to stimulate food intake and growth hormone release. Acutely, AG induces whole-body insulin resistance, potentially to maintain glycemia between meals. Alternatively, chronic administration of both AG and the unacylated isoform of ghrelin (unAG) is associated with improved skeletal muscle insulin sensitivity as well as reduced intramuscular lipids and inflammation. This may be due to effects on lipid metabolism, with ghrelin promoting storage of fat in adipose and liver while stimulating oxidation in skeletal muscle, preventing ectopic lipid accumulation. This is of specific relevance in the handling of meal-derived lipids, as ghrelin rises preprandially with effects persisting for 2–3 h following exposure in skeletal muscle, coinciding with elevated plasma FFAs. We hypothesize that ghrelin acts as a preparatory signal for incoming lipids, as well as a regulatory hormone for their use and storage. The effects of ghrelin on skeletal muscle are lost with high fat diet feeding and physical inactivity, potentially being implicated in the pathogenesis of metabolic disease. This review summarizes the metabolic effects of both ghrelin isoforms on peripheral tissues including the pancreas, adipose, liver, and skeletal muscle. Additionally, we speculate on the physiological relevance of these effects in vivo and suggest that ghrelin may be a key regulatory hormone for nutrient handling in the postprandial state.

1. Introduction

Ghrelin is classically known as the "hunger hormone", rising in circulation prior to entrained mealtimes to stimulate food intake through interactions with hypothalamic neurons [1–4]. The central orexigenic function of ghrelin is attributed to its acylated (AG) form [5–7]. Traditionally, AG has been considered the only biologically active ghrelin isoform. However, both AG and unacylated ghrelin (unAG) have recently been the focus of a growing body of research for their effects on peripheral tissue metabolism (Fig. 1) [8–15]. In this review, we summarize the metabolic effects of both AG and unAG on peripheral tissues. In addition, we suggest that ghrelin serves a physiological role as a prominent regulatory signal for the handling of mealtime nutrients, and that the disruption of this process may contribute to the development of metabolic disease.

2. Overview of ghrelin and its kinetics

Ghrelin, a 28-amino acid peptide, was originally isolated from the rat stomach following its identification as the endogenous ligand for the growth-hormone secretagogue receptor (GHSR) [16]. Rat and human ghrelin are highly homologous, with both undergoing octanoylation modification at the serine 3 residue by ghrelin O-acyltransferase (GOAT) [16,17]. This distinguishes between the unacylated and acylated isoforms of ghrelin, the latter which is responsible for the well-characterized central functions to stimulate food intake [4] and growth hormone (GH) release [16].

In humans, ghrelin is produced largely by endocrine P/D1 cells of the gastric fundus [18]. In circulation, unAG is the more abundant isoform, representing 65–80% of total circulating ghrelin in healthy humans [19–21]. Ghrelin exhibits a distinct rhythm in the circulation, rising prior to entrained meals followed by a reduction upon feeding [22,23]. These fluctuations are associated with scheduled mealtimes [24,25], and differ with individual eating patterns [26].

Ghrelin release in response to mealtimes is facilitated by the parasympathetic nervous system, likely through vagus nerve transmission [27]. Treatment with norepinephrine [28] and propranolol (β-adrenergic receptor (β-AR) agonist) [27] also increase ghrelin secretion, suggesting that the sympathetic nervous system may stimulate its release under certain conditions. Ghrelin also has a reciprocal relationship with insulin postprandially. Hyperinsulinemic conditions have

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Abbreviation list

AC	Adenylyl cyclase
ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
AG	Acylated ghrelin
AKT	AKT/Protein Kinase B
AMPK	Adenosine monophosphate-activated protein kinase
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
AUC	Area under the curve
β-AR	β-adrenergic receptor
CAMKII	Calcium-calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CPT-1	Carnitine palmitoyltransferase-1
CRF-2R	Corticotropin releasing factor receptor 2
DAG	Diacylglycerol
Db-cAMP	Dibutyryl-cyclic adenosine monophosphate
EDL	Extensor digitorum longus
EGP	Endogenous glucose production
FABPc	Fatty acid binding protein - cytosol
FABPpm	Fatty acid binding protein - plasma membrane
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase/cluster of differentiation 36
FFA	Free fatty acid
FAO	Fatty acid oxidation
GH	Growth hormone
GHS	Growth hormone secretagogue
GHSR	Growth hormone secretagogue receptor
GLUT4	Glucose transporter protein 4
GOAT	Ghrelin O-acyltransferase
GSK	Glycogen synthase kinase
G6Pase	Glucose-6-phosphatase
HFD	High fat diet
HOMA-IR	Homeostatic model of Assessment for Insulin Resistance
HSL	Hormone sensitive lipase
ICV	Intracerebroventricular
IR	Insulin receptor
IRS	Insulin receptor substrate
LFD	Low fat diet
LPL	Lipoprotein lipase
MAG	Monoacylglycerol
MGL	Monoacylglycerol lipase
mTOR	Mammalian target of rapamycin
NE	Norepinephrine
PDE	Phosphodiesterase
PDH	Pyruvate dehydrogenase
PEPCK	Phosphoenolpyruvate carboxykinase
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
Pparγ2	Peroxisome proliferator-activated receptor γ2
SCD-1	Stearoyl-Coenzyme A desaturase-1
SREBP1	Sterol regulatory-element binding protein
TAG	Triacylglycerol
TCA	Tricarboxylic acid cycle
UCP	Uncoupling protein
UnAG	Unacylated ghrelin
WAT	White adipose tissue

been shown to reduce circulating ghrelin levels in humans [29,30], suggesting a role of insulin in its postprandial suppression. In line with this, lipid ingestion results in a smaller decrease in circulating ghrelin levels when compared to carbohydrates and amino acids [31–33]. Deacylation enzymes are thought to exist in the circulation, potentially regulating the ratio of ghrelin isoforms [21,34], however this area of research is ongoing and outside the scope of this review. Only AG is able to stimulate GH release; therefore, investigating the regulation of ghrelin acylation by GOAT, as well as the processes that control its deacylation, may provide insight into the importance of endogenous GH

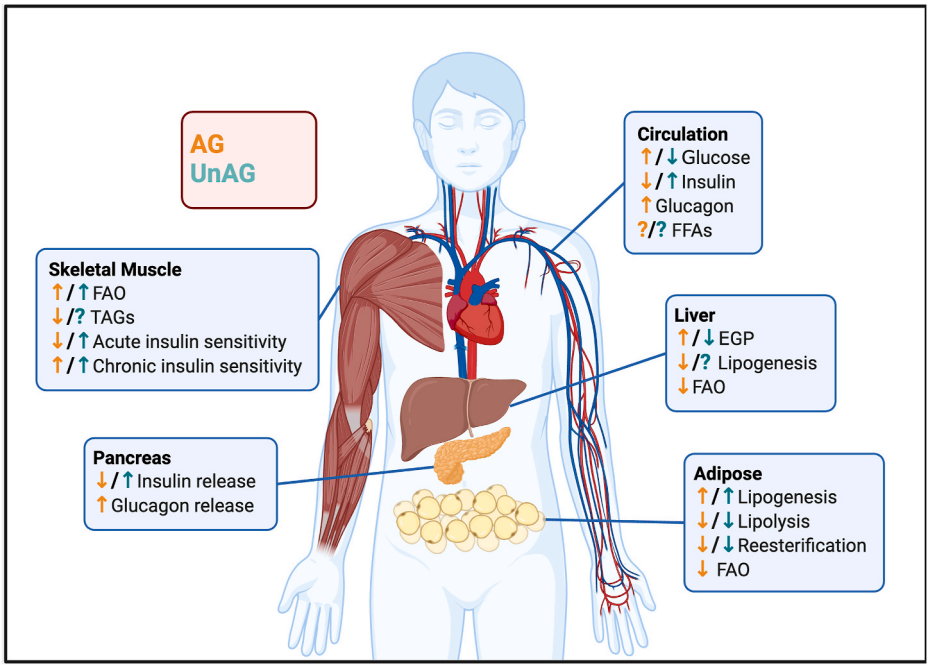

Fig. 1. An overview of the effects of ghrelin on peripheral tissues. ↑, increase; ↓, decrease; ?, unknown/unclear effect; FAO, fatty acid oxidation; TAGs, triacylglycerols; EGP, endogenous glucose production. Created with BioRender.com.

release in response to ghrelin.

3. Ghrelin receptors

GHSR is the primary receptor through which ghrelin signals. GHSR was first identified as the receptor responsible for mediating the GH-releasing effects of synthetic growth hormone secretagogue (GHS) molecules [35], representing a previously unknown hormonal pathway regulating the release of GH. GHSR has two isoforms, GHSR1a and GHSR1b, with only GHSR1a active for GHS binding [36]. While GHSR1b is expressed widely [37], GHSR1a mRNA is detected in limited tissues, including the pituitary, hypothalamus, pancreas, adipose tissue, and liver [35,37–39]. In addition to GH-release, GHSR1a mediates the orexigenic action of AG in the arcuate nucleus [4,40,41]. GHSR binding is unique to the acylated isoform of ghrelin [16].

In addition to GHSR, the corticotropin releasing factor receptor 2 (CRF-2R) has been suggested to mediate some of the peripheral effects of ghrelin, particularly in skeletal muscle. The existence of an alternative ghrelin receptor was first proposed by Baldanzi et al. [42]; both AG and unAG exerted protective effects on cardiomyocytes, despite only AG activating GHSR-1a, and GHSR-1a being undetectable in the cell line used. CRF-2R was implicated in ghrelin signaling in muscle by Gershon and Vale [43]. Treatment with AG was able to increase glucose uptake in C2C12 myocytes through CRF-2R, and both ghrelin isoforms were able to bind and upregulate CRF-2R mRNA [43]. CRF-2R is likely to be, at least in part, responsible for mediating the effects of both ghrelin isoforms in muscle.

4. Ghrelin and glucose metabolism

4.1. Insulin and glucagon release from the pancreas

Glucose stimulates the release of insulin from pancreatic β-cells via an increase in the ratio of ATP/ADP. This results in the closure of ATP-sensitive potassium channels and subsequent depolarization of β-cells, increasing intracellular Ca^{2+} and the exocytosis of insulin granules [44]. It is generally accepted that AG has an inhibitory effect on glucose-stimulated insulin secretion from the pancreas, although a few studies do not show this [45–48]. The inhibitory effect of AG on insulin release has been observed in both rodents [49–52] and humans [8,53–57], with AG causing a reduction in circulating insulin despite an increase in glucose. Additionally, administration of AG dose-dependently reduces acute insulin response to a glucose bolus [58]. This suppression of insulin release with AG has been measured along with a reduction in C-peptide, indicating this change is independent of insulin clearance [58].

AG has no effect on insulin release under basal non-stimulatory glucose concentrations [49–52,58]. When directly measured in isolated mouse islets, no effect of AG was observed at 3.3 or 5.5 mM glucose, with a suppressive effect evident at 8.3, 11.1, and 22.2 mM [51]. AG inhibits glucose-induced Ca2+ oscillations through a GHSR-dependent mechanism [50,59], however this exact pathway remains to be elucidated. No direct effect of unAG on insulin secretion in humans has been observed [8,57], however unAG has been reported to oppose the insulin-suppressing effects of AG [8].

The effects of AG on glucagon have also been controversial with several studies showing no change in glucagon release [47,49,50,56,60]. However, a comprehensive study by Chuang et al. found that AG administration in mice dose-dependently increased plasma glucagon independent of changes in insulin [61]. Additionally, AG was shown to stimulate glucagon from isolated islets and α-cells [61]. A stimulatory effect is also supported in humans, with AG administration countering the suppression of glucagon in hyperinsulinemic conditions [62]. Plasma glucagon levels remain unchanged following unAG administration [60].

4.2. Whole body insulin sensitivity and glucose tolerance

Infusion of AG into healthy human subjects consistently results in an increase in plasma glucose [8,11,53,54,56,57,63,64]. Furthermore, a positive relationship between HOMA-IR and AG has been identified [65], as well as a negative association between insulin resistance and unAG, and the ratio of unAG/AG [65,66]. AG was first directly implicated in the regulation of whole body insulin sensitivity by Gauna et al. [64], with AG administration resulting in a decrease in insulin sensitivity estimated from glucose, insulin, and free fatty acid (FFA) AUCs for 4 h following a meal. Accordingly, Vestergaard et al. [11] reported AG administration to increase both plasma glucose and FFAs with no changes in circulating insulin, suggesting a reduction in insulin sensitivity. Euglycemic-hyperinsulinemic clamp testing has been somewhat inconclusive, with one study showing AG to increase the rate of glucose infusion required to maintain euglycemia [67] indicating an insulin sensitizing effect. However, the majority of studies have shown AG to reduce glucose disposal rates [62,68–70], implying a worsening of insulin sensitivity. This reduction in insulin sensitivity has been measured independently of changes in endogenous insulin, and when GH release is largely suppressed by somatostatin administration [68]. Additionally, this diabetogenic effect is not due to acute changes in circulating FFAs, as it persists when lipolysis is pharmacologically suppressed [70].

In contrast, unAG administration alone is not typically associated with changes in plasma glucose and insulin concentrations [8]. However, coadministration of unAG with AG has been shown to abolish the hyperglycemia associated with AG administration [8,64]. Additionally, transgenic mice overexpressing unAG are more insulin sensitive [71]. These findings overall indicate that AG may impair glucose handling, with unAG having the ability to counter these insulin-desensitizing effects.

4.3. Skeletal muscle insulin signaling and glucose uptake

Skeletal muscle is the primary site for insulin-mediated glucose disposal, with up to 80% of a glucose load taken up by this tissue in the hyperinsulinemic condition [72]. Both ghrelin isoforms have been identified as potential modulators of glucose uptake and insulin signaling in skeletal muscle. Repeated administration of UnAG for 4 days has been shown to increase AKT phosphorylation and insulin-stimulated glucose uptake in the gastrocnemius muscle of mice [13]. Furthermore, unAG reduces fasting blood glucose and improves insulin signaling in *db/db* mice when administered over a 10-day period [73]. These findings are in agreement with unAG administration improving whole-body insulin sensitivity [8,64,71]. Alternatively, the direct effects of AG on skeletal muscle glucose metabolism do not consistently reflect the insulin-desensitizing effects reported at the whole body level. Following 4 days of injections, Barazzoni et al. [12] reported AG to increase AKT phosphorylation and GLUT4 mRNA in rat soleus muscle. It is possible that repeated treatments with AG result in some AG being endogenously deacylated to unAG, and the insulin-sensitizing effects of this isoform being observed. However, the relative amounts of each ghrelin isoform was not measured [12].

In order to minimize the confounding effects of AG administration in vivo (e.g., GH release, AG deacylation), several studies have utilized isolated cell cultures and muscle incubations to assess its direct influence on skeletal muscle glucose metabolism. In agreement with the findings of Barazzoni et al. [12], a 72-h AG treatment was found to increase insulin-stimulated GLUT4 translocation and subsequent glucose uptake in C2C12 cells [43]. In contrast, work from our own lab using isolated oxidative soleus and glycolytic EDL muscles found no acute (i.e., 1 h) impact of either AG or unAG on glucose uptake or AKT phosphorylation under basal or insulin-stimulated conditions [74]. The inconsistency in these findings may indicate a potential dissociation between the acute and more chronic effects of AG on skeletal muscle glucose metabolism. Improvement of insulin sensitivity observed with chronic treatment may

be due in part to the effects of ghrelin on lipid metabolism and inflammation [9,15,75–78]. This will be discussed later in the review.

4.4. Hepatic insulin sensitivity and glucose production

A potential effect of AG on hepatic glucose metabolism was first identified in hepatoma cells, with AG treatment reducing insulin action, including insulin's suppression of PEPCK [79]. Further studies showed that AG directly increases glucose output in primary hepatocytes [80], as well as endogenous glucose production (EGP) and the expression of glucogenic genes (i.e., PEPCK, G6Pase) when administered in rodents [67,81]. These effects also occur chronically, with 4 days of twice-daily AG administration reducing hepatic AKT and GSK phosphorylation, and increasing G6Pase expression [9,12]. Ghrelin's regulation of EGP in vivo has been suggested to be the result of a gut-brain-liver axis [81], however studies using isolated hepatocytes suggest a direct effect [79,80]. These findings have yet to be confirmed in humans, with no effect of AG on EGP being observed [68]. UnAG is able to antagonize the effects of AG on EGP as well as suppress it when administered independently [80].

4.5. Physiological role of acylated ghrelin for blood glucose control

To summarize, AG acutely reduces insulin sensitivity in skeletal muscle and liver, reducing glucose uptake and increasing EGP. Along with its effects on pancreatic hormones, AG appears to promote hyperglycemia through several mechanisms, a finding that has been consistently reported in the literature [8,11,53,54,56,57,63,64]. It is important to remember, however, that ghrelin is elevated in the circulation in the postabsorptive state and prolonged fasting conditions, when blood glucose must be maintained to prevent hypoglycemia. It is tempting to suggest that AG may have physiological relevance as a blood glucose regulator during times of an energy deficit, such as between meals (Fig. 2). Notably, GOAT-null mice, which do not synthesize AG, are unable to maintain blood glucose in prolonged fasting conditions, which decrease to life-threatening levels [82]. Moreover, GHSR-null mice show reduced plasma glucose levels in prolonged fasting conditions compared to controls [61]. The physiological relevance of the ratio of ghrelin isoforms should also be examined, given the opposing effects of unAG on AG action. Clarifying the role of both ghrelin isoforms in the regulation of glycemia between meals remains an area for further investigation.

5. Ghrelin and lipid metabolism

5.1. Adipose and liver lipogenesis

Due to its orexigenic effects, chronic AG treatments lead to an increase in body weight and the expansion of adipose tissue [4]. However, treatments with AG have also been shown to increase fat mass independently of food intake [10,38,83]. It has been demonstrated in isolated adipocytes that direct treatment of AG increases the expression of lipogenic genes encoding LPL, ACC, and FAS, accompanied by the accumulation of lipids [84]. Similar effects are seen in rodent white adipose tissue (WAT) with AG administration in vivo [10,85]. AG may also have adipogenic effects, increasing the expression of perilipin in differentiating adipocyte cultures, which is only present in mature adipocytes [84]. AG treatment is also associated with a reduction of CPT-1 mRNA in WAT and UCP1/3 expression in brown adipose tissue, potentially reducing fat oxidation and further promoting lipid storage [10]. Similar to its effects in adipose tissue, repeated AG administration in rats increases the hepatic expression and activity of several key enzymes in lipogenesis while reducing those involved in fatty acid oxidation (FAO) [9,86]. This is accompanied by an increase in hepatic triacylglycerol (TAG) content, independent of food intake [9]. Chronic AG infusion in rodents can also cause hepatic steatosis [38].

In both adipose tissue and the liver, AG increases the expression of the transcription factors SREBP1 and PPARγ [38,39,84,87], potentially mediating the effects of ghrelin on lipogenic gene expression. This effect is likely the result of mTOR signaling, which upregulates these transcription factors in both adipose tissue and the liver [39,88]. Moreover, in cultured hepatocytes, the effects of AG on lipogenesis are abolished with the mTOR inhibitor rapamycin [39]. In both adipose and hepatic

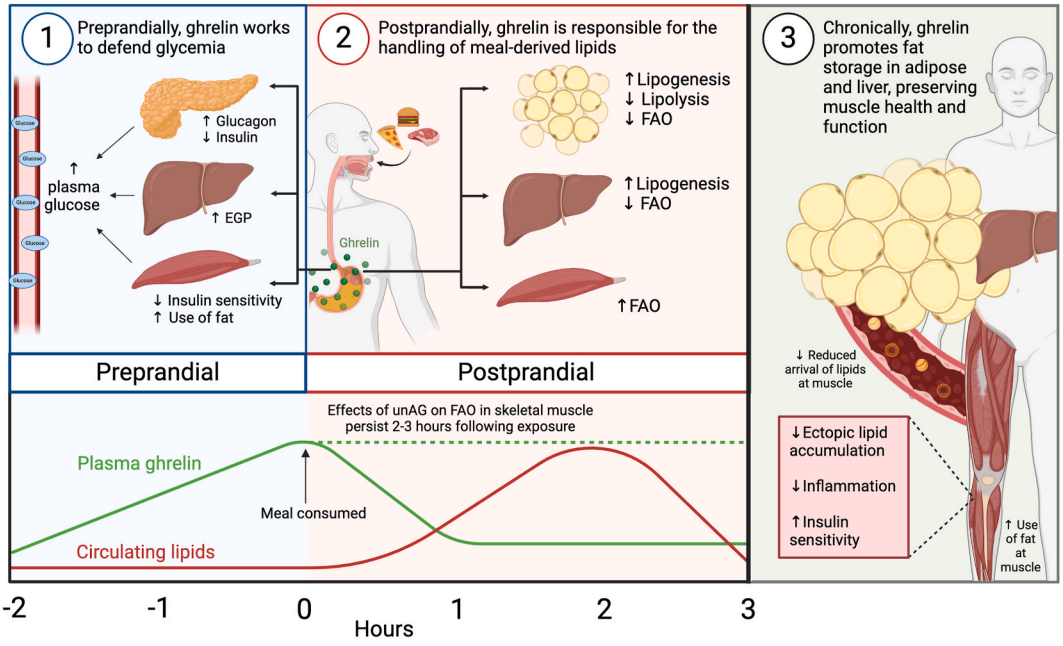

Fig. 2. Proposed physiological roles of ghrelin. A preprandial rise in ghrelin is proposed to maintain normoglycemia in the postabsorptive state by decreasing insulin release and sensitivity, increasing EGP and reliance on fat. Postprandially, ghrelin is proposed to facilitate the storage of meal-derived lipids in the adipose and liver, while increasing oxidation in skeletal muscle. The effects of unAG on skeletal muscle FAO persist for 2–3 h following exposure, aligning with the rise of meal-derived lipids in the circulation. Chronically, the summation of these acute effects is proposed to preserve insulin response by reducing ectopic lipid accumulation. EGP, endogenous glucose production; FAO, fatty acid oxidation; unAG; unacylated ghrelin. Created with BioRender.com.

tissue, the effect of AG on lipogenesis seems to be direct, mediated by GHSR [38,39]. However, it has been reported that AG does not stimulate lipogenic gene expression in βAR-null mice, indicating these effects are partially mediated by the SNS [10]. In support of a non-GHSR mechanism, unAG has been reported to increase lipogenic protein content as well as mRNA for SREBP1 and PPARγ in cultured human adipocytes [84]. Overall, these findings suggest that administration of both ghrelin isoforms promotes lipid storage as opposed to oxidation in the liver and adipose tissue.

5.2. Lipolysis and reesterification

Adipose tissue lipolysis is stimulated following the activation of β-AR by catecholamines, triggering adenylyl cyclase and the production of cAMP. This results in the activation of PKA and its phosphorylation of HSL [89]. In isolated rat adipocytes, both ghrelin isoforms exhibit antilipolytic effects under conditions that stimulate lipolysis, such as treatment with isoproterenol (β-AR agonist) [87,90,91] and forskolin (increases cAMP) [91]. Both AG and unAG have been shown to reduce β3AR-stimulated phosphorylation of HSL in adipose tissue organ culture, with a corresponding decrease in glycerol release [14]. A similar effect was observed in skeletal muscle, with epinephrine-stimulated lipolysis blunted by both ghrelin isoforms [15]. This suppression of lipolysis is likely mediated through the degradation of cAMP by phosphodiesterases (PDEs), as AG and unAG reduce isoproterenol-induced cAMP accumulation [91]. PDE3D inhibitors and db-cAMP (analog of cAMP not hydrolyzed by PDEs) prevent the lipolytic-suppressive effects of both ghrelin isoforms [91]. Increased adipose tissue AKT phosphorylation by AG and unAG [91,92], as well as the attenuation of their suppressive effects on lipolysis by wortmannin (PI3K inhibitor) [91], provide a potential mechanism for ghrelin to act on adipose tissue via insulin signaling.

In vivo findings regarding the effects of ghrelin on lipolysis are inconsistent. In rats, AG and unAG show no effect on β3AR-stimulated circulating glycerol or FFA concentrations [14]. Conversely, a study by Vestergaard et al. [68] in humans showed infusion of AG to relieve insulin-induced suppression of glycerol release. However, these results may be confounded by a rise in circulating GH [68]. GH is known to cause increased lipolytic sensitivity to catecholamines in adipose tissue [93]. Indeed, when the effects of AG on lipolysis were directly assessed using microdialysis, no change in interstitial glycerol levels was reported [94], suggesting that GH may be confounding whole body results.

To address the confounding issue of GH release in vivo, the effects of AG infusion on lipolysis have been examined in hypopituitary patients [69]. In contrast to the findings in isolated adipocytes [14,87,90], AG infusion caused an 80% rise in FFAs [69]. However, it is important to note that this study found no increase in glycerol. It has been shown ex vivo that both AG and unAG significantly blunt the reesterification of FFAs in adipose tissue [14,95]. This effect of ghrelin on reesterification questions the accuracy of drawing conclusions regarding lipolysis based on FFA levels. Furthermore, there was increased insulin resistance in response to AG, which may have resulted in a reduced clearance of FFAs by muscle, leading to elevated circulating FFA levels [69]. There has been no observed effect on basal (unstimulated) lipolysis by either ghrelin isoform [14,87,90].

Overall, both ghrelin isoforms demonstrate an inhibitory effect on lipolysis. Additionally, ghrelin promotes fat storage in adipose tissue, increasing lipogenesis and adipogenesis. It is interesting that both ghrelin isoforms may modulate FFA release from adipose tissue through reduced reesterification, with a reported increase in circulating FFAs occurring in some studies [47,60,64,96], but not others [38,64]. The physiological significance of these findings is unclear.

5.3. Skeletal muscle lipid metabolism: uptake and oxidation

In mice, daily injections of AG reduce whole-body lipid utilization, measured as an increase in the respiratory exchange ratio [83]. Despite this initial finding suggesting a reduction in muscle fat use with ghrelin, a number of studies from our lab have demonstrated both AG and unAG to directly stimulate palmitate oxidation in isolated glycolytic [15] and oxidative [15,75–77] muscles. Both ghrelin isoforms have a protective effect on insulin-stimulated glucose uptake under high palmitate conditions that would normally result in impaired insulin signaling [75,97]. This protective effect has been attributed to increased FAO as it is associated with a reduction in muscle TAG content [97] and is abolished when muscle is treated with the CPT-1 inhibitor etomoxir [75]. Similar findings have been observed in vivo, with 4 days of twice-daily AG administration reducing TAG content while increasing mitochondrial cytochrome *c* oxidase and citrate synthase content [9]. Daily AG injection has also been shown to completely abolish high fat diet (HFD)-induced muscle TAG accumulation in rats [78].

A crucial point of regulation in skeletal muscle fatty acid metabolism is their uptake at the sarcolemma. We recently assessed the effect of unAG on fat transporters and found that while the stimulation of FAO persists for 2–3 h following an initial acute exposure to unAG, this was not associated with increased translocation of FAT/CD36 or FABPpm to the sarcolemma [77]. It is important to note, however, that in this study, transporter content was measured from prepared giant sarcolemmal vesicles, and therefore excluded t-tubules, which is also an important location for the transport-mediated uptake of glucose and FFAs [98,99]. Furthermore, vesicles were not produced (after harvesting of the muscle tissue) in the presence of FFAs, which are known to directly stimulate fat transport [100] and would more accurately reflect physiological postprandial conditions 2–3 h after a mixed meal. Interestingly, ghrelin has been shown to decrease serum FFA levels following a meal [64]. A direct measurement of FFA transport in giant sarcolemmal vesicles may be valuable to conclusively determine if unAG has an effect on fatty acid uptake or the intrinsic activity of these proteins.

The increase in FAO observed in skeletal muscle with ghrelin has been associated with activation of the AMPK-ACC axis [15,77]. This is similar to ghrelin signaling in the hypothalamus, with intracerebroventricular (ICV) AG administration phosphorylating AMPK and ACC, and with the AMPK inhibitor Compound C abolishing its orexigenic effects [101,102]. This signaling event has been inconsistently detected in muscle in response to ghrelin [9,74,77] potentially due to the transient phosphorylation of these proteins. Further testing is required to confirm the role of AMPK-ACC in mediating ghrelin-stimulated FAO in skeletal muscle.

While the mechanism underlying ghrelin-induced stimulation of FAO is unclear, there remains several possibilities. CPT-1 is part of the rate-limiting entry of FFAs into the mitochondrion. In the hypothalamus of rats, ICV ghrelin administration decreases malonyl-CoA levels with subsequently increased CPT-1 activity [101]. Whether this effect occurs in muscle is an area for further investigation. AG has also been implicated in upregulating UCP2 expression in several tissues including skeletal muscle [9], potentially mediating its effects of FAO by increasing energy expenditure. Lastly, ghrelin has been shown to activate CAMKII in muscle, albeit inconsistently [74], providing opportunities for further research into its role in stimulating FAO.

5.4. Physiological relevance of ghrelin in the handling of meal-derived lipids: more than just a fasting hormone

Ghrelin has typically been considered a fasting hormone, increasing food intake and energy substrate supply [4,6,7,103]. However, this idea is not consistent with the inhibitory effect of ghrelin on FFA release by decreasing lipolysis, or the promotion of fat storage in the liver and adipose tissue. Instead, we hypothesize that ghrelin is functioning as a preprandial signal to prepare peripheral tissues for incoming meal-derived lipids.

It takes 2–3 h for meal-derived lipids to peak in circulation [104]. Insulin's stimulatory effects on FFA transport are no longer evident 1 h

after exposure [100], and there is currently no identified hormone thought to be responsible for the postprandial handling of lipids in skeletal muscle. It has recently been determined by our lab that the acute FAO-stimulating effects of unAG persist at least 2–3 h in isolated rat skeletal muscle following its removal [77]. As ghrelin rises in the circulation prior to entrained meal times [22] with effects that may persist for up to 3 h following its postprandial decrease [77], we hypothesize that ghrelin has a preparatory role in priming tissues for incoming fat, and that these effects continue to persist throughout the period in which lipids are elevated post meal, facilitating their use and storage (Fig. 2). Consistent with this, administration of both ghrelin isoforms 2 h prior to a test meal results in reduced serum FFA levels compared to control conditions [64]. Moreover, a reduction in serum FFAs was consistently observed over a 16 h infusion of unAG, during which two meals were consumed [105]. Further research in this area is warranted.

Skeletal muscle insulin resistance is caused by the ectopic accumulation of lipids, which disrupt the insulin signaling cascade [106]. Increasing the oxidation of FFAs has the potential to be beneficial in maintaining insulin sensitivity. Indeed, chronic treatment with AG has been reported to be insulin sensitizing [12,43]. In ex vivo models, acute treatment of skeletal muscle with both AG and unAG is able to preserve insulin-stimulated glucose uptake in high palmitate conditions that would normally result in acute impairments to the actions of insulin [75, 97]. Additionally, 4 days of twice-daily AG administration in rats is able to prevent intramuscular TAG accumulation and inflammation typically following 30 days of HFD-feeding [78]. These changes were associated with an increase in mitochondrial enzymes, and independent of changes in antioxidant enzymes [78]. Overexpression of unAG also reduces inflammatory cytokines and maintains insulin signaling during HFD-feeding compared to wildtype controls [13]. These benefits may extend to other tissues such as the liver, as HFD-induced hepatic lipid accumulation and inflammation are also attenuated with AG administration [107]. Overall, we hypothesize that ghrelin postprandially shunts the storage of meal-derived lipids to tissues equipped for lipid storage (e.g. adipose tissue and liver) by increasing lipogenesis and decreasing lipolysis, while stimulating FAO in skeletal muscle. Chronically, the summation of these acute effects exerts a protective effect on skeletal muscle function (Fig. 2).

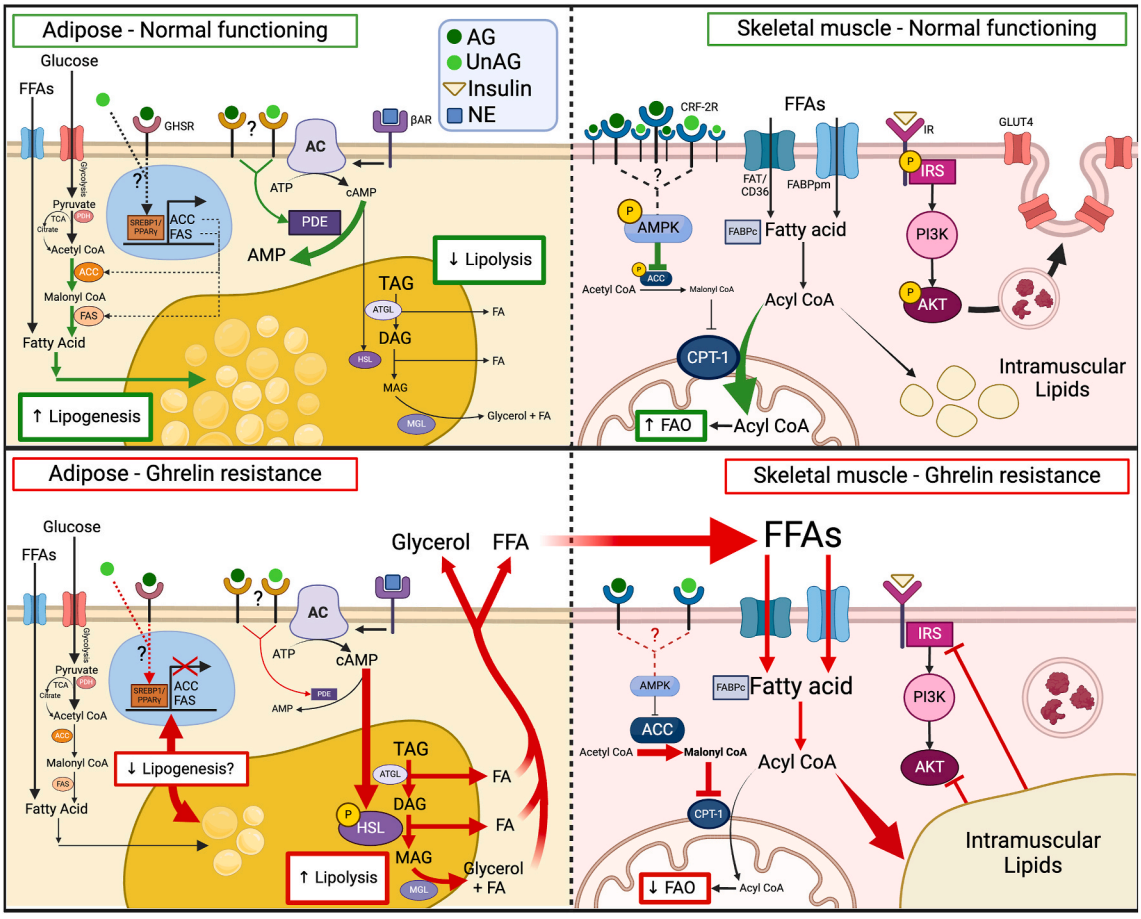

Fig. 3. Disruptions of cellular signaling pathways in adipose tissue and skeletal muscle observed with ghrelin resistance compared with normal functioning. Normally in adipose tissue, AG stimulates lipogenesis, and both isoforms inhibit lipolysis through degradation of cAMP by PDEs. In normal skeletal muscle, AG and unAG stimulate FAO by activation of the AMPK-ACC axis. In ghrelin resistance, AG and unAG are no longer able to suppress lipolysis, resulting in increased FFA release in circulation. In skeletal muscle, both ghrelin isoforms lose their ability to stimulate FAO. With more FFAs arriving at the muscle and less oxidation, intramuscular lipids accumulate and disrupt the insulin signaling cascade. AG, acylated ghrelin; unAG, unacylated ghrelin; NE, norepinephrine; FFA, free fatty acid; GHSR, growth hormone secretagogue receptor; CRF-2R, corticotropin release factor receptor 2; AC, adenylyl cyclase; β-AR, β-adrenergic receptor; PDH, pyruvate dehydrogenase; SREBP1, sterol regulatory element-binding protein; PPARγ, peroxisome proliferator-activator receptor γ; TCA, tricarboxylic acid cycle; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PDE, phosphodiesterase; AMP, adenosine monophosphate; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; MGL, monoacylglycerol lipase; FAT/CD36, fatty acid translocase/cluster of differentiation 36; FABPpm, fatty acid binding protein - plasma membrane; CPT-1, carnitine palmitoyl transferase; FAO, fatty acid oxidation; IR, insulin receptor; GLUT4, glucose transport protein 4; FABPc, fatty acid binding protein cytosol; IRS, insulin receptor substrate; PI3K, phosphoinositide 3-kinase; AKT; protein kinase B. ↑, increase; ↓, decrease. Created with BioRender.com.

6. Peripheral ghrelin resistance

Skeletal muscle becomes less responsive (i.e., resistant) to AG and unAG following chronic HFD-feeding [75,76]. Specifically, 6 weeks of HFD-feeding abolishes the FAO-stimulating ability of both unAG and AG in isolated soleus muscle, as well as the stimulation of the AMPK-ACC axis by ghrelin (Fig. 3) [75]. Implementation of a high-intensity exercise training protocol concurrent with a HFD protects against skeletal muscle ghrelin resistance [76]. Unexpectedly, however, the low fat diet (LFD)-sedentary control animals in this study also became less responsive to unAG following 6 weeks [76]. We speculate that this is due to the prolonged sedentary nature of these animals. However, this study did not have a LFD-exercise group, making it difficult to positively attribute this loss of effect to sedentary behaviour. It is also possible that age- or size-related changes contributed to the loss of ghrelin response. Adipose tissue similarly becomes resistant to both ghrelin isoforms following as little as 5 days of HFD-feeding, as indicated by a loss of ghrelin's ability to blunt β3AR-stimulated lipolysis (Fig. 3) [95]. In contrast to muscle, adipose tissue ghrelin resistance does not seem to be recoverable with exercise [95].

The mechanism underlying the loss of ghrelin signaling in HFD/sedentary animals is unknown. Six weeks of HFD-feeding has been shown to reduce CRF-2R receptor content in soleus muscle [75], which is the putative receptor through which AG and unAG exert their effects on muscle [43]. Interestingly, there was no reduction in CRF-2R content in LFD-fed sedentary animals, despite the fact that they also demonstrated ghrelin resistance [76]. This suggests that there may be multiple mechanisms underlying the loss of ghrelin's effects on FAO, and further work should aim to dissect the importance of diet and physical activity.

Circulating ghrelin acutely increases in fasted mice immediately following high-intensity treadmill exercise [108]. The relationship between physical activity and ghrelin may shed light on the development and functional significance of ghrelin resistance. Acutely, an increase in ghrelin following exercise may function to prioritize fat utilization and allow glycogen replenishment. It seems logical that this function would be preserved in high exercise situations with frequent glycogen depletion, but may be lost with physical inactivity. Interestingly, glycogen depletion is a known activator of the AMPK-ACC pathway [109], through which ghrelin may also signal to increase FAO. Ghrelin neutralization results in slower replenishment of liver glycogen in fasting conditions following refeeding, suggesting a glycogen sparing effect of ghrelin exists [86]. An examination of ghrelin response following a low-exercise training protocol where glycogen is not depleted, or whether an acute exhaustive bout of exercise can restore ghrelin action, may provide clarification.

A loss of ghrelin's FAO-stimulating effects is associated with both HFD-feeding and sedentary behaviour, and each of these are independently associated with metabolic disease. It is logical to speculate that the loss of ghrelin's effects may be a key event in the development of insulin resistance. As discussed previously, ghrelin may have physiological relevance as a regulatory hormone for the handling of dietary lipids in the postprandial period, promoting storage in adipose tissue. A loss of these effects could result in increased ectopic lipid accumulation in skeletal muscle. Indeed, the inability to stimulate FAO in ghrelin resistance was associated with a loss of ghrelin's protective effects on insulin-stimulated glucose transport in high palmitate conditions [75]. Given this, we speculate that a loss of ghrelin response may exacerbate HFD lifestyle-induced insulin resistance.

7. Challenges and perspectives in ghrelin research

It is important to acknowledge the limitations of using ex vivo research to suggest a physiological role for ghrelin in vivo. To isolate the direct effects of AG on peripheral tissue metabolism from secondary effects due to GH release, researchers have utilized ex vivo models ~~to study the effects of ghrelin~~. These studies have been extremely useful in developing our understanding of the tissue-specific effects of both ghrelin isoforms. However, it is important to be cautious when speculating about the physiological role of ghrelin based on these findings, as they do not reflect the interactions between tissues in vivo. For example, changes in adipose tissue lipolysis by ghrelin may in turn affect FFAs in circulation, and consequently glucose uptake and utilization in skeletal muscle. Additionally, the acylation status of circulating ghrelin is likely regulated by a circulating serum factor in both human and rats, resulting in the rapid deacylation of AG to unAG [34]. Consequently, the infusion of AG in vivo results in a rise in both ghrelin isoforms [21]. This conversion between isoforms remains largely uninvestigated, and the intrinsic regulation of the ratio of AG/unAG, as well as their subsequent effects, may be largely missed in ex vivo work.

It is important to note that while ex vivo models aim to minimize the indirect peripheral metabolic effects due to GH, the release of GH in response to gastrointestinal-derived AG is still physiologically relevant, and the effects of GH are important to consider when hypothesizing the in vivo role of ghrelin. GH elicits numerous direct metabolic effects, such as increasing lipolytic sensitivity to catecholamines in adipose tissue [93], and antagonizing the effects of insulin in several peripheral tissues [110–113]. This may explain some contrasting results in the literature, with AG inhibiting adipose tissue lipolysis ex vivo [15,87,90,91], in contradiction to several studies reporting AG to increase circulating FFAs in vivo [47,60,64,96]. Future work should expand on the isolated direct effects of ghrelin demonstrated in peripheral tissues by determining their significance in vivo.

8. Summary and conclusion

Overall, the literature demonstrates the functions of ghrelin to extend beyond stimulating food intake, with a role in regulating glucose and lipid metabolism in peripheral tissues. AG is acutely associated with a reduction in insulin action, antagonizing insulin release from the pancreas and impairing insulin response in several tissues, potentially to maintain normoglycemia between meals. Chronic treatment with both ghrelin isoforms improves insulin signaling, potentially by optimizing storage of lipids in adipose tissue while increasing skeletal muscle FAO, thereby preventing ectopic lipid accumulation. We have shown that these effects on muscle and adipose tissue can be lost with both chronic HFD-feeding as well as sedentary behaviour. Future research should focus on clarifying the mechanisms underlying the effects of ghrelin in peripheral tissues, and its physiological relevance in the handling of mealtime lipids. Additionally, exploring the loss of ghrelin response in peripheral tissues may further our understanding of the pathogenesis of metabolic disease.

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CRediT authorship contribution statement

Nicole M. Notaro: Writing – review & editing, Writing – original draft, Conceptualization. **David J. Dyck:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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