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Regulation of adipose tissue and skeletal muscle substrate metabolism by the stomach-derived hormone, ghrelin

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Ghrelin is a stomach-derived hormone and a potent appetite stimulant. Ghrelin has recently harbored interest as a potential regulator of carbohydrate and lipid metabolism in skeletal muscle and adipose tissue; however, *in vivo* ghrelin administration is confounded by secondary effects. The assessment of the direct metabolic effects of acylated (AG) and unacylated (UnAG) ghrelin is a relatively new area of research. In isolated adipocytes and muscle, ghrelin has demonstrated antilipolytic effects. In muscle, ghrelin has been shown to acutely stimulate fat oxidation, which may protect the muscle from the insulin-desensitizing effects of high fatty acid concentrations. The effects of ghrelin directly on muscle glucose uptake are controversial. Whether ghrelin can be utilized therapeutically for conditions such as type 2 diabetes will depend on our better understanding of ghrelin's independent effects on muscle and adipose tissue metabolism, and whether this can predict ghrelin's effects when administered *in vivo.*

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Background

Introduction

Ghrelin, the 'hunger hormone', was isolated from the stomach of rats in 1999 [1]. Ghrelin was first documented to stimulate growth hormone (GH) release before establishing its effects on food intake. It is this potent stimulation of GH release that confounds the interpretation of ghrelin's physiological effects *in vivo*. GH has numerous metabolic effects which include an increase in hepatic glucose production and increased lipolytic sensitivity of adipose tissue [2,3]. Ghrelin is the only known orexigenic, endocrine hormone, stimulating food intake in both animals [4,5] and humans [6] Ghrelin's action in the central nervous system opposes that of leptin, an adipose-derived hormone that signals for satiety [7]. In recent years, research has begun to unravel potential effects of ghrelin on peripheral tissue metabolism, including skeletal muscle and adipose tissue (Figure 1).

Ghrelin originates mainly from endocrine P/D1 cells in humans and A-like cells (often termed X/A-like cells) in rats [8], and is secreted directly into blood vessels supplying the systemic circulation [9]. Ghrelin can be further modified at Ser^3 by an octanoyl group to produce the acylated form [10]. The enzyme ghrelin-*O*-acyl-transferase (GOAT) catalyzes this post-translational modification with the provision of octanoyl-CoA [11•,12,13]. Ghrelin circulates in two forms: i) acylated (AG) and ii) des-acyl, or unacylated ghrelin (UnAG). UnAG was first believed to have little to no biological activity, but a significant body of work [14–17] including our own [18•,19,20•] has demonstrated otherwise. Ghrelin isoform concentrations appear to be controlled by a balance between ghrelin synthesis and release from gastric cells, acylation by GOAT and deacylation by circulating enzymes

Ghrelin kinetics can generally be described as a large preprandial rise and immediate postprandial return to baseline. There are a multitude of factors potentially impacting these patterns including the autonomic nervous system, gastric emptying rate, and intestinal osmolarity [21–23]. In healthy humans postprandial ghrelin concentrations decrease in a manner that is proportional to the total ingested caloric load [21]. When comparing isoenergetic meals, ghrelin kinetics may also depend on the predominant macronutrient being consumed [21,24•,25]. In healthy individuals, carbohydrate appears to be most effective in returning ghrelin levels to baseline [25], while protein appears to suppress ghrelin levels the longest [21,26].

Ghrelin promoter activity is stimulated in gastric cells by the fasting hormones glucagon (discovered in humans) and norepinephrine (discovered in rats) [23,27], suggesting that ghrelin production is largely driven by energy deficit. In conditions of prolonged energy deficit (anorexia, cachexia), total circulating ghrelin concentrations are increased, and conversely are decreased during states of energy surplus, that is, obesity [28–30]. However,

Glossary
ACC: Acetyl-CoA carboxylase
AG: Acylated ghrelin
AMPK: AMP-activated protein kinase
AS160: Akt-substrate of 160 kilodaltons
AT: Adipose tissue
CNS: Central nervous system
CPT-1: Carnitine palmitoyl-transferase-1
CRF-2R: Corticotropin-releasing factor-2 receptor
DAG: Diacylglycerol
EDL: Extensor digitorum longus
ERK: Extracellular signal-related kinase
FA: Fatty acid
GH: Growth hormone
GHS-R: Growth hormone-secretagogue receptor
GOAT: Ghrelin-*O*-acyltransferase
HSL: Hormone-sensitive lipase
ICV: Intracerebroventricular
IRS: Insulin receptor substrate
T2D: Type 2 diabetes
TAG: Triacylglycerol
UnAG: Unacylated (des-acyl) ghrelin
WAT: White adipose tissue

challenging the notion that ghrelin is truly a fasting hormone is about some data in humans which have shown that concentrations of AG decline, while UnAG remains relatively stable when participants are fasted for ~42.5 hour [31]. This is in agreement with other findings in 2–3d fasted humans where no significant change in total ghrelin was observed [32]. Taken together, these findings suggest that ghrelin may not be a fasting hormone *per se*.

Ghrelin receptor

Two ghrelin receptor (GHS-R1) subtypes exist: full length GHS-R1a and a truncated GHS-R1b [33]. GHS-R1a is the functionally active receptor whereas GHS-R1b lacks the capacity for high-affinity ligand binding and signal transduction [33,34]. However, recent work has shown that GHS-R1b may be essential in the regulation of ghrelin-induced GHS-R1a trafficking and signalling [33,34]. GHS-R1a is predominantly expressed in the hypothalamus and pituitary gland; however, GHS-R1a mRNA is also detectable in several peripheral tissues [35,36]. Interestingly, GHS-R1a content changes with aging, suggesting that ghrelin action may be altered in different physiological states [37].

The role of ghrelin in the regulation of peripheral glucose and lipid metabolism

Regulation of insulin secretion, insulin action and signalling by ghrelin

In both rats and humans, AG may modulate insulin release from the pancreas, although findings have been equivocal [38–40]. AG has been shown to increase cytosolic Ca^{2+} and stimulate insulin secretion in isolated pancreatic islets from rodents, but only at 'permissive' glucose concentration of ~8 mM [40,41]. However, when rat pancreas is perfused *in situ* ghrelin has been observed to blunt glucose-stimulated insulin secretion [3842•] Similarly, acute AG infusion in humans reduces insulin secretion, but not when co-infused with UnAG [39,43]. There is some evidence that AG may stimulate the release of the incretin, glucagon-like peptide-1 [44], although this has recently been challenged [45•].

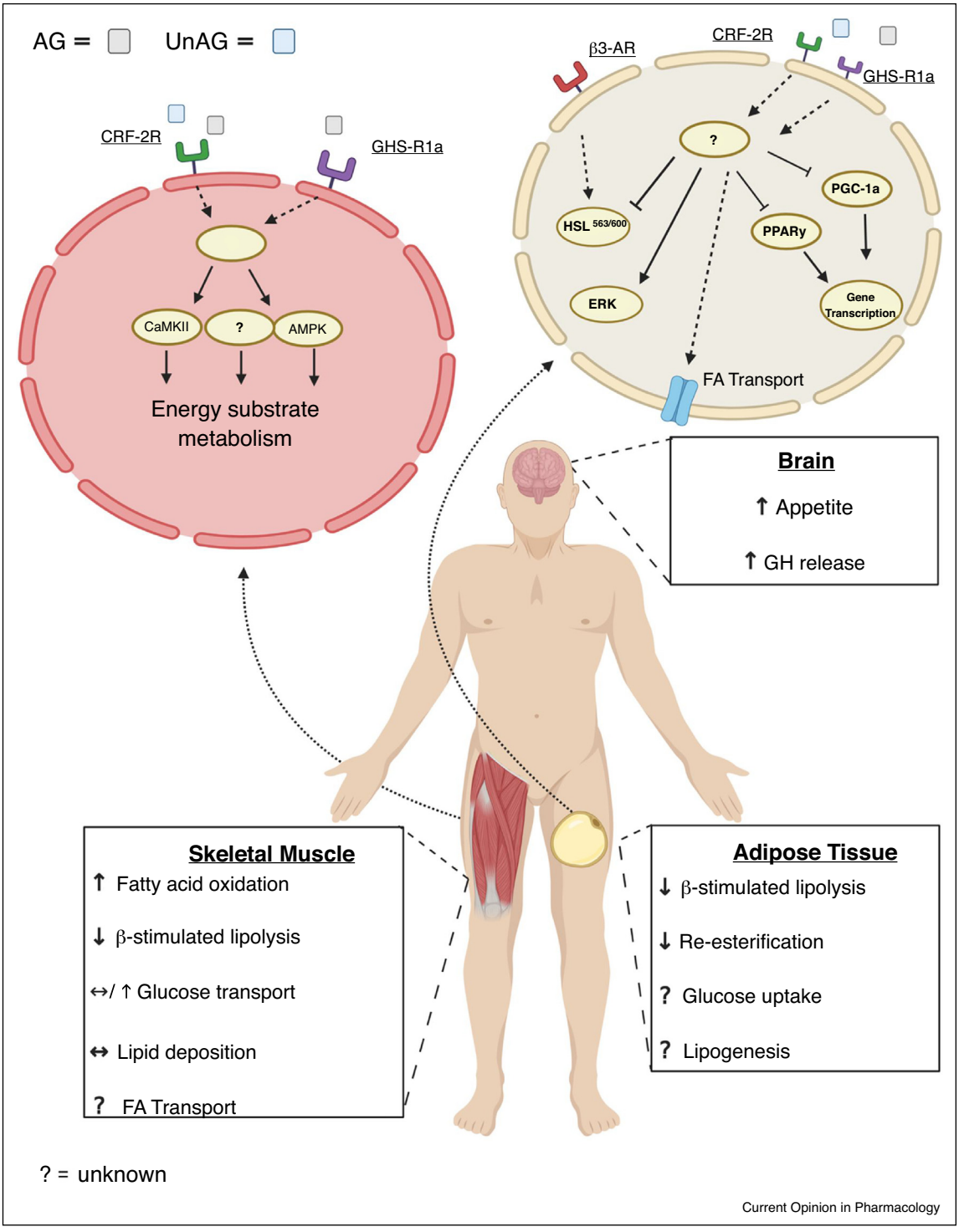
Examination of the direct effects of ghrelin on insulin signalling remains sparse. A few studies have examined key proteins of the insulin signalling cascade (IRS-1, Akt, Akt-substrate 160 (AS160)) in response to ghrelin administration, although these are often measured following chronic ghrelin administration. Four days of subcutaneous AG injection in rodents has been shown to increase levels of activated/phosphorylated Akt in soleus muscle [46], while UnAG injection increased the activation of both IRS-1 and Akt in gastrocnemius muscle [17]. However, when acutely administered to isolated muscle (both glycolytic and oxidative), no change in Akt phosphorylation was observed in response to either isoform [19]. Reductions to whole body insulin action following AG infusion *in vivo* have been attributed to the secondary rise in GH, and subsequent elevation in FAs which can acutely impair insulin action [47,48]. However, the role of GH in mediating AG's effect on whole body insulin action is controversial as AG infusion can cause peripheral insulin resistance in hypopituitary patients [49] who lack the ability to produce GH. Surprisingly, reductions in whole-body insulin action as a result of AG administration are not necessarily paralleled by a decline in muscle Akt phosphorylation [49,50] and in some studies this has not been assessed [51] Finally, in healthy humans, the acute infusion of AG has been shown to have no effect on Akt or AS160 phosphorylation in the presence of insulin [49,50]. Overall, the effects of ghrelin isoforms on whole body insulin action and signalling are unclear.

Regulation of glucose uptake in muscle by ghrelin

In C2C12 myocytes, both AG and UnAG stimulate glucose uptake in a dose-dependent manner [16]. However, in this study muscle cells were cultured with ghrelin for up to 24 hour which questions the physiological relevance [16]. Also, if a meal were to be missed, the direct stimulation of muscle glucose uptake by ghrelin *in vivo* would seem counterintuitive, potentially leading to ghrelin-induced hypoglycemia. In fact, emerging evidence suggests that AG is part of a counterregulatory response to defend against hypoglycemia [52•] and AG continually rises in response to a single missed meal, in obese individuals [53]. Two studies have provided evidence that chronic muscle exposure to AG may increase muscle glucose transporter 4 (GLUT4) gene expression [16,46], although this was not assessed at the protein level.

Data from our own lab indicate that ghrelin does not acutely affect basal or insulin-stimulated glucose uptake

Figure 1



Schematic showing the major observed direct effects of ghrelin on isolated skeletal muscle and adipose tissue. Upward arrow indicates increase, downward arrow indicates decrease, and lateral arrow indicates no change. A question mark indicates that this parameter has not been directly assessed. Created with BioRender.com.

in mature, isolated EDL and soleus skeletal muscle [19]. In contrast, 4d injection of UnAG has been shown by others to enhance insulin-stimulated glucose uptake in rat gastrocnemius, pointing towards a role for prolonged ghrelin exposure in the regulation of muscle's response to insulin [17]. Interestingly, in rat myoblasts, AG prevents palmitic acid-induced impairment of insulin-stimulated glucose uptake, suggesting that the availability of FAs, which are known to affect insulin response and glucose uptake [47], impact ghrelin's regulatory role on glucose metabolism in muscle [54]. These findings are consistent with data in rat soleus muscle from our lab that demonstrate a protective role for both ghrelin isoforms on FA-induced decrements to insulin-stimulated glucose uptake [55•].

In humans, the infusion of AG during a hyperinsulinemic-euglycemic clamp results in a worsening of insulin action, that is, reduced glucose infusion rate [56]. The infusion of UnAG in humans has been purported in the literature as being beneficial toward glucose homeostasis, however this may solely be the case when co-infused with AG [57]. Collectively, more work is required to fully elucidate ghrelin's role on muscle insulin action and glucose uptake. The effects of chronic ghrelin administration in humans are yet to be explored.

Muscle FA metabolism

Fatty acid oxidation, lipolysis, and lipid content

The direct exposure of rat soleus muscle to both ghrelin isoforms independently *in vitro* has been shown to increase oxidation of exogenous FAs [20•,55•]. Whether this also occurs physiologically to stimulate the oxidation of ingested lipids remains to be determined. Additionally, ghrelin's influence on FA utilization may differ between tissues, as evidenced by 4d of subcutaneous AG injections in rats leading to reductions in triacylglycerol (TAG) content in gastrocnemius, but not soleus or liver [46]. Finally, research examining simultaneous administration of both ghrelin isoforms in isolated skeletal muscle has yet to be conducted.

Clinical evidence negatively correlates ghrelin levels, particularly UnAG, with insulin resistance in conditions of elevated FA availability, like obesity and T2D [19,58]. Interestingly, more recent research has investigated the metabolic effects of ghrelin in muscle during elevated FA exposure. Initial findings by Han *et al.* [54] showed that AG was able to reduce TAG content in myocytes incubated in the presence of palmitate. Additionally, glucose uptake was preserved suggesting that ghrelin may protect myocytes from acute palmitate-induced impairment in intracellular glucose uptake [54]. Recently, our lab has extended these findings showing that UnAG also preserves insulin-stimulated glucose uptake and signalling in isolated mature rat skeletal muscle (soleus) during high FA exposure [55•]. This protective effect was likely due to the stimulation of FA oxidation through increased activation of the AMPK/ACC axis [55•]. Interestingly, this protection was lost in soleus muscle isolated from high-fat fed animals, and incubated under identical high FA exposure conditions [55•]. This implies the development of ghrelin resistance (see Ghrelin Resistance section below). Whether any of the beneficial effects of ghrelin on insulin action, in the presence of high FA availability, are attributable to its regulation of lipid intermediate formation such as diacylglycerol (DAG) and ceramides remains uncertain. To date, only one study has investigated the direct effects of ghrelin on FA deposition into lipid pools, with no changes being observed [20•].

Lipolysis

While the evidence that ghrelin can inhibit isoproterenol-stimulated lipolysis in adipose tissue is well established (see below), work in skeletal muscle is relatively sparse. Initial findings suggested that chronic ghrelin treatment may stimulate lipolysis as evidenced by reductions in TAG content in gastrocnemius and cultured myoblasts [54]. Work from our lab in isolated soleus muscle supports an acute, direct anti-lipolytic role for ghrelin as shown by reductions in epinephrine-induced lipolysis and decreased phosphorylation of stimulatory HSL sites Ser^{660} and Ser^{563} [20•]. Taken together, these findings suggest that ghrelin may act to decrease lipolysis and promote the oxidation of exogenous FAs, resulting in an overall shift towards fat utilization in muscle, which in turn may spare glucose. However, this is contradicted by the finding that peripheral administration of AG can cause an increase in the respiratory exchange ratio, indicating greater carbohydrate utilization [59].

Cellular signalling and receptors

Ghrelin receptor expression is abundant in the pituitary gland and arcuate nucleus of the hypothalamus, which account for the hallmark central effects of ghrelin; however, GHS-R1a expression is relatively low in skeletal muscle [35]. Thus, the cellular action of ghrelin in peripheral tissues like skeletal muscle and AT remains unclear. Studies have suggested that CRF-2 may act as a potential alternative receptor for AG in skeletal muscle [16].

Nevertheless, it has been shown that ghrelin may act in part through AMPK activation [20•,60,61], which ultimately reduces malonyl-CoA content to relieve its inhibition on mitochondrial CPT-1. Importantly, inhibition of CPT-1 with etomoxir attenuates ghrelin's ability to stimulate muscle fatty acid oxidation [55•]. Other findings challenge ghrelin as a direct regulator of the AMPK/ACC axis, necessitating studies using AMPK-knockout animals [19,51]. The mechanism by which ghrelin activates the AMPK/ACC axis is unknown.

Adipose tissue metabolism

Lipolysis

In vivo

Infusing AG *in vivo* increases basal and hyperinsulinemic rates of abdominal and subcutaneous interstitial glycerol release, which indicate an increase to AT lipolysis or a potential counter to insulin's antilipolytic effects [51]. Effort is sometimes made to minimize the secondary rise in GH (somatostatin, hypopituitary patients) following AG infusion. Interestingly, the co-infusion of both ghrelin isoforms results in a lowering of systemic FAs more effectively than UnAG alone, suggesting that UnAG may counteract the prolipolytic effects of AG or possibly GH [57].

Ex vivo

More recently, isolated AT has been utilized to directly assess ghrelin's effects in the absence of secondary hormonal responses. However, findings have been varied and measures of subcellular signalling events are often lacking. It has been shown in primary adipocytes that high concentrations of AG blunt the non-selective adrenergic induction (isoproterenol; β-agonist) of glycerol release [15]. The effects of UnAG were not pursued. Data from our own lab expanded these findings by demonstrating that AG and UnAG both attenuate β_3-stimulated (CL 316,243) lipolysis [18•]. These antilipolytic effects of ghrelin were consistent across both subcutaneous and visceral AT depots [18•]. There has yet to be any assessment of simultaneous AG and UnAG administration in isolated AT, or in combination with insulin, which could provide insight into their role *in vivo*.

Signalling

In agreement with the observed ghrelin-mediated reductions in AT lipolysis, one previous report has shown a trend toward antilipolytic signalling at phosphodiesterase upstream of HSL; other work examining HSL following ghrelin treatment did not assess its state of phosphorylation nor did they have a positive control for stimulated lipolysis [62,63]. In AT organ culture, we have demonstrated that ghrelin isoforms attenuate CL-stimulated activation of HSL at its stimulatory sites ($Ser^{563/660}$), but have no effect on the inhibitory (Ser^{565}) phosphosite [18•]. Extracellular signal-regulated kinases (ERK1/2) were also measured, but were unaffected, although this could be due to the timing of measurements [18•]. *In vivo*, however, the co-administration of AG with CL consistently increases ERK activation, particularly in subcutaneous AT [18•]. This could potentially be due to a secondary rise in GH, which has been shown to signal through the ERK pathway in AT [64]. Interestingly, this was not sufficient to drive a further increase in lipolysis with AG administration *in vivo*.

Lipogenesis and re-esterification

WAT is important in the clearance of FAs following a meal and are the primary storage site for TAGs. Some literature supports a lipogenic role for ghrelin, but these findings are often complicated by its orexigenic effects, that is, increases to energy intake [65]. When pair-feeding is employed, increases in total body and fat mass with ghrelin ICV administration over 6d is similar to control, suggesting that increases in food intake are primarily responsible for ghrelin-induced increases in fat mass [65]. Furthermore, some evidence suggests that UnAG may even be anti-lipogenic through the suppression of genes, for example, *pparg*, *ppargc1a* that encode regulators of lipid synthesis [59,66]. Further underlying mechanisms should be pursued to determine whether ghrelin directly regulates lipogenesis in AT. Similarly, ghrelin's effects on FA transport into adipocytes remains to be studied.

Depending on energy status, ~50% of hydrolyzed FAs can be re-esterified back into adipose TAG [67,68]. Ghrelin's effect on FA re-esterification has only been estimated in AT organ culture. This is calculated as the observed FA release subtracted from theoretical maximum for FA release, that is, three FAs for each glycerol [18•]. Interestingly, FA concentrations in media were maintained during adrenergic stimulation, even in the presence of AG and UnAG, which blunted lipolysis [18•]. It is therefore likely that ghrelin inhibited FA re-esterification, although it is uncertain whether ghrelin diverted FAs away from re-esterification by affecting glucose or FA transport, the former being essential for glyceroneogenesis. These findings imply that ghrelin may help to maintain circulating FA levels, even in the face of decreased lipolytic rates. It could be that secondary to its role in reducing AT lipolysis, ghrelin maintains elevated FAs to protect blood glucose when a meal is missed, that is, glucose is not consumed.

Ghrelin resistance

Tissues may become resistant to the effects of several hormones in obesity [69,70]. However, potential changes to ghrelin's effects in the CNS and in peripheral tissue metabolism are largely unknown. The results of studies using diet-induced obesity have observed ghrelin resistance in the CNS following peripheral ghrelin administration [71,72]. Central ghrelin resistance may manifest differently than the peripheral sensitivity to ghrelin, since as little as ~12 hour high-fat diet overfeeding impairs the orexigenic action of ghrelin in mice [73]. With respect to ghrelin signalling in the periphery, initial work by Gershon [16] observed that the stimulatory effects of AG on glucose uptake in C2C12 myocytes could be attenuated using a CRF-2R inhibitor (anti-sauvagine), suggesting that AG may act through this receptor to exert some of its metabolic effects in skeletal muscle. We have shown in our lab that CRF-2R content is reduced in soleus muscle from 6-week high-fat fed rats, which coincides with the

loss of ghrelin stimulation of palmitate oxidation [55•]. These novel findings further implicate this receptor in mediating ghrelin action in muscle and suggest that ghrelin action may be altered in obesity, although more work is warranted.

Conclusion

The neural effects of acylated ghrelin may contribute to the regulation of appetite, energy intake and adiposity. However, more recent findings provide evidence for both acylated and unacylated ghrelin as modulators of peripheral tissue substrate metabolism. The most consistent findings are that ghrelin is antilipolytic in isolated muscle and adipose tissue and stimulates FA oxidation in muscle. The mechanisms that underlie ghrelin's effects in these tissues remain relatively poorly defined. It is intriguing to consider future directions addressing whether UnAG presents a therapeutic strategy to combat obesity or T2D. In addition to a possible resistance of skeletal muscle to ghrelin, circulating UnAG concentrations decline in obesity. Despite this, there are very few registered clinical trials utilizing UnAG as a therapy in the obese. Moving forward, it will be imperative that researchers confirm the presence of ghrelin resistance in skeletal muscle (and perhaps adipose tissue) from obese or diabetic humans. It will be important to characterize whether effective interventions such as exercise or specific nutritional strategies can reverse the impaired ghrelin response [69]. It is also not known whether either of the ghrelin isoforms can influence the secretory function of skeletal muscle and/or adipose tissue. This could also be a potential future area of research.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Daniel T Cervone: Conceptualization, Writing - original draft, Writing - review & editing. **Andrew J Lovell:** Writing - original draft. **David J Dyck:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing, Funding acquisition.

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