Shedding light on the intricate puzzle of ghrelin’s effects on appetite regulation

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**Abstract**

Ghrelin, a hormone primarily produced by the stomach, has a wide range of metabolic and non-metabolic effects. It also stimulates food intake through activation of various hypothalamic and brain stem neurons. A series of recent studies have explored the intracellular mechanisms of the appetite-inducing effect of ghrelin in the hypothalamus, shedding light on the intricate mechanisms of appetite regulation. AMP-activated protein kinase (AMPK) is a key metabolic enzyme involved in appetite regulation. Calmodulin kinase kinase 2 (CaMKK2) has been identified as an upstream kinase of AMPK and a key mediator in the effect of ghrelin on AMPK activity. The fatty acid pathway, hypothalamic mitochondrial respiration, and uncoupling protein 2 have been outlined as downstream targets of AMPK and mediators of ghrelin’s appetite stimulating effect. This short overview summarises the present data in this field.

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AMPK and ghrelin

A new era in the field of appetite research was opened with the identification of AMPK as a major player in the regulation of food intake (Minokoshi et al. 2004). AMPK is a highly conserved enzyme which senses changes in the cellular AMP/ATP ratio, and once activated switches on catabolic, ATP-consuming processes, and switches off ATP-consuming anabolic processes (Kola et al. 2006). AMPK is activated by phosphorylation on the Thr172 residue by upstream kinases LKB1 and calcineurin kinase 2 (CaMKK2; Hawley et al. 2005, Woods et al. 2005), but recent data also suggest a role in certain circumstances for transforming growth factor-β-activated kinase (Suzuki et al. 2004, Hawley et al. 2005, Woods et al. 2005, Momcilovic et al. 2006) and ataxiatelangiectasia mutated gene (Suzuki et al. 2004). Recent data suggest that increased AMP does not directly promote phosphorylation of Thr172 by upstream kinases but rather inhibits the dephosphorylation of AMPK by protein phosphatase-2C-α (PP2C-α; Sanders et al. 2007, Lage et al. 2008). A new model of AMPK regulation, independent of AMP, has emerged with the finding that the cell death inducing DFFA-like effector A (Cidea) by forming a complex with the β-subunit of AMPK, elicits an ubiquination-mediated degradation of AMPK (Lage et al. 2008, Qi et al. 2008).

AMPK seems to mediate the orexigenic and anorexigenic effect of a multitude of hormones and compounds including leptin, insulin, glucose, cannabinoids, adiponectin, glucocorticoids, thyroid hormones, metformin, α-lipoic acid, α-MSH, MT-II, and ciliary neutrophic factor (Kola et al. 2006, Kubota et al. 2007, Kola 2008). Ghrelin is not an exception to this rule. We and others have shown that both central (i.c.v.) and peripheral (i.p.) ghrelin treatment stimulate AMPK activity in the hypothalamus, leading to ghrelin’s well known orexigenic effect (Anderson et al. 2004, Kola et al. 2005). Recently, the signalling pathway of ghrelin’s orexigenic effect in the hypothalamus has been further elucidated with the identification of CaMKK2 as the relevant upstream kinase of AMPK (Anderson et al. 2008), and with the identification of the endocannabinoid–GHSR–Ca2+–endocannabinoids–cannabinoid receptor type 1 (CB1)–Ca2+–CaMKK–AMPK–malonyl-CoA–carbamoylphosphate transaminase 1 (CPT1)–β-oxidation–reactive oxygen species (ROS)–UCP2–NPY/AgRP–food intake (Fig. 1).

Ghrelin stimulates CaMKK2

The ghrelin receptor GHSR, a Gq-coupled receptor, increases intracellular Ca2+ via PLC and PKC activation and the rise in intracellular Ca2+ could in principle lead to CaMKK2 activation. It was therefore postulated that the stimulatory effect of ghrelin on hypothalamic AMPK might be mediated through CaMKK2 (Anderson et al. 2008). The mRNA for CaMKK2 was found to be highly expressed in the ARC, ~20–40-fold more than in other hypothalamic areas. CaMKK2 knockout mice (CaMKK2-KO) showed reduced expression of NPY and AgRP, but no differences in pro-opiomelanocortin (POMC) expression, suggesting a role for CaMKK2 only in the orexigenic NPY neurons. Modulation of AMPK activity has been shown to regulate NPY expression (Minokoshi et al. 2004). In agreement with the above results, hypothalamic AMPK activity was also reduced in the CaMKK2-KO mice (Anderson et al. 2008). Systemic ghrelin injection, which increased food intake in wild-type (WT) animals, failed to do so in the CaMKK2-KO mice. In addition, STO-609, a selective CaMKK2 inhibitor, inhibited NPY expression and reduced food intake and body weight in WT animals. A key finding of this study was the demonstration of a unique complex comprising CaMKK2, AMPK-α/β, Ca2+, calmodulin, and acetyl-CoA carboxylase (ACC). CaMKK2 forms a stable complex containing the α and β subunits of AMPK (but not the γ subunits), although this stable complex between CaMKK2 and AMPK is not necessarily required for the kinase–substrate (i.e., CaMKK2–AMPK) interaction (Anderson et al. 2008).

The finding that CaMKK2 is the upstream AMPK kinase in the hypothalamus, mediating the stimulatory effect of ghrelin on the enzyme, suggests the possibility that CaMKK2 might be involved in the effects of ghrelin in other tissues. This also brings up the interesting question as to why ghrelin, which acts through a Gq-coupled receptor and consequent increases in intracellular Ca2+ in all tissues, has a tissue-specific effect on AMPK, stimulating it in the hypothalamus and myocardium, and inhibiting it in liver and adipose tissue (Kola et al. 2005)? We also need to bear in mind the fact that several other AMPK regulators also have a tissue-specific effect, most notably leptin. Ghrelin might affect PP2C-α, a known dephosphatase of AMPK, and the final effect on AMPK activity, could be due to an altered balance between CaMKK2-induced activation and PP2C-α-induced inactivation of AMPK. While the study from Anderson et al. (2008) clearly shows that ghrelin-induced AMPK activation is dependent on an increase in intracellular Ca2+ and consequent activation of CaMKK2, another recent report suggested that ghrelin increases intracellular Ca2+ levels in the ARC NPY neurons via AMPK-mediated signalling, as the AMPK inhibitor, compound C, was able to suppress ghrelin’s effect on intracellular Ca2+ levels (Kohno et al. 2008). Thus, changes in intracellular Ca2+ can activate CaMKK2 which then seems to modulate AMPK activity, while on the other hand AMPK status seems to influence intracellular Ca2+ levels, suggesting a positive feedback loop between AMPK and Ca2+. However, we believe that the majority of the data support CaMKK2 activation. It was therefore postulated that the stimulatory effect of ghrelin on hypothalamic AMPK might be mediated through CaMKK2 (Anderson et al. 2008). The mRNA for CaMKK2 was found to be highly expressed in the ARC, ~20–40-fold more than in other hypothalamic areas. CaMKK2 knockout mice (CaMKK2-KO) showed reduced expression of NPY and AgRP, but no differences in pro-opiomelanocortin (POMC) expression, suggesting a role for CaMKK2 only in the orexigenic NPY neurons. Modulation of AMPK activity has been shown to regulate NPY expression (Minokoshi et al. 2004). In agreement with the above results, hypothalamic AMPK activity was also reduced in the CaMKK2-KO mice (Anderson et al. 2008). Systemic ghrelin injection, which increased food intake in wild-type (WT) animals, failed to do so in the CaMKK2-KO mice. In addition, STO-609, a selective CaMKK2 inhibitor, inhibited NPY expression and reduced food intake and body weight in WT animals. A key finding of this study was the demonstration of a unique complex comprising CaMKK2, AMPK-α/β, Ca2+, calmodulin, and acetyl-CoA carboxylase (ACC). CaMKK2 forms a stable complex containing the α and β subunits of AMPK (but not the γ subunits), although this stable complex between CaMKK2 and AMPK is not necessarily required for the kinase–substrate (i.e., CaMKK2–AMPK) interaction (Anderson et al. 2008).

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the first possibility as being the primary mechanism, i.e.,
that ghrelin stimulates Ca$^{2+}$ entry and that AMPK is
downstream from this Ca$^{2+}$ signal.

**Ghrelin’s effect via the fatty acid pathway**

The downstream effectors of the ghrelin–GHSR–CaMKK2–AMPK axis in the regulation of feeding have been elucidated by Lopez et al. (2008) who showed that ghrelin regulates feeding through an AMPK-dependent inhibition of the *de novo* fatty acid synthesis pathway in the VMN. Several recent pharmacological and genetic studies have demonstrated that components of the fatty acid metabolism pathways modulate the regulation of hypothalamic feeding (Loftus et al. 2000, Obici et al. 2003, Lopez et al. 2006, Wolfgang et al. 2006, Chakravarthy et al. 2007). AMPK is an important regulator of

the fatty acid biosynthetic pathway: AMPK phosphorylates and inhibits ACC, preventing the production of malonyl-CoA. This stimulates CPT1 and the transport of fatty acids into the mitochondria, ultimately facilitating mitochondrial fatty acid oxidation (Fig. 1).

Ghrelin’s orexigenic effect is AMPK-dependent as both compound C, an inhibitor of AMPK, and adenoviruses harbouring $\alpha$-1/$\alpha$-2 dominant negative AMPK were able to prevent it (Lopez et al. 2008). Ghrelin’s stimulatory effect on food intake was paralleled by a decrease in fatty acid synthase (FAS) mRNA levels in the VMN, as well as a decrease in total hypothalamic FAS activity and protein levels, in fed rats after 6 h of treatment. Co-expression of FAS and GHSR was also found in the ARC and the PVN at lower levels than in the VMN, but no significant changes were found in FAS mRNA levels in these hypothalamic nuclei or in other brain areas.

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**Figure 1** Schematic diagram showing the proposed molecules involved in the appetite-inducing effect of ghrelin. The activation of molecules highlighted in red leads to increased food intake, whereas the activation of molecules highlighted in purple leads to inhibition of food intake. In this figure, we have depicted a simplified, linear relationship among the elements comprising the ghrelin signalling cascade. Clearly, the different components of this cascade can also interact within and outside this pathway, activating other distinct downstream signalling components, here not reported, either sequentially or simultaneously, suggesting a much more complex regulation. This figure has been drawn based on the studies of Andersson et al. (2004), Landree et al. (2004), Kola et al. (2005, 2008), Anderson et al. (2008), Andrews et al. (2008) and Lopez et al. (2008): GHSR, GH secretagogue receptor; CaMKK, calmodulin kinase kinase; CB1, cannabinoid receptor type 1; AMPK, AMP-activated protein kinase; ACC, acetyl-coenzyme A carboxylase; malonyl-CoA, malonyl coenzyme A; FAS, fatty acid synthase; CPT1, carnitine palmitoyl transferase 1; ROS, reactive oxygen species; UCP2, uncoupled protein 2; NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, pro-opiomelanocortin.
Compound C and stereotactic delivery of α-1/α-2 dominant negative AMPK adenoviruses in the VMN also prevented the ghrelin-induced decrease in FAS mRNA levels, demonstrating that the ghrelin effect on FAS mRNA expression is mediated by an AMPK-dependent mechanism (Lopez et al. 2008). The relationship between AMPK and FAS seems to obey the rules of feedback regulation. The study from Lopez et al. places FAS clearly as a downstream target of AMPK, confirmed also by previous studies which have identified FAS as a downstream target of AMPK also in liver (Zhou et al. 2001), whereas previous studies suggested a regulation in the opposite direction as C75, an inhibitor of FAS, reduced AMPK phosphorylation; this probably occurs as a result of increased hypothalamic ATP levels, consequent of increased β-oxidation (Landree et al. 2004; Fig. 1). Nevertheless, it should be kept in mind that C75 is not a very specific inhibitor as it causes non-specific neuronal activation and its anorexigenic effect may not be explained by FAS inhibition alone (Takahashi et al. 2004).

Levels of malonyl-CoA are determined by three enzymes: by ACC, which stimulates its synthesis, by malonyl-CoA decarboxylase, which degrades it, and by FAS which converts it to fatty acids. Hypothalamic malonyl-CoA levels decreased 2 h after ghrelin treatment, returning to normal 6 h later. CPT1 was regulated in a similar time-dependent manner, as its activity was increased acutely at 2 h and was restored to normal levels 6 h after treatment. I.c.v. administration of etomoxir, an inhibitor of CPT1, decreased the orexigenic effect of ghrelin at 2 h. This suggests that the stimulatory action of ghrelin on CPT1 activity may be mediated by simultaneous changes in malonyl-CoA levels.

Modulation of AMPK by leptin (Andersson et al. 2004), α-lipoic acid (Kim et al. 2004) or ghrelin (Kola et al. 2005) is associated with decreased (leptin and α-lipoic acid) or increased (ghrelin) phosphorylation of its downstream target ACC, leading to stimulation or inhibition of ACC activity. In support of this mechanism, degradation of malonyl-CoA in the medial basal hypothalamus of rats results in increased food intake and progressive weight gain (He et al. 2006), while inhibition of CPT1 in the hypothalamus suppresses food intake (Obici et al. 2003, Pocai et al. 2006; Fig. 1).

Ghrelin acts via UCP2

The further downstream steps of ghrelin on food intake were explored in a recent study by Andrews et al. (2008). Ghrelin increased the expression of hypothalamic CPT1 mRNA while etomoxir and compound C prevented the ghrelin-induced increase in both NPY and AgRP gene expression. It was suggested that ghrelin stimulates hypothalamic mitochondrial respiration via UCP2. Such an effect seems to mediate the activation of NPY/AgRP neurons, the synaptic plasticity of POMC-expressing neurons, and ghrelin-induced food intake.

UCPs are inner-membrane mitochondrial proteins which modulate energy expenditure via ‘uncoupling’ ATP generation, thereby dissipating the mitochondrial proton gradient necessary for ATP synthesis and generating heat (Wu et al. 1999). Ghrelin has previously been shown to enhance UCP2 mRNA expression in the pancreas (Sun et al. 2006), liver (Barazzoni et al. 2005), and white adipose tissue (Tsubone et al. 2005). In the Andrews’ study, ghrelin was shown to increase hypothalamic UCP2 mRNA expression (Andrews et al. 2008). Mitochondrial respiration (nmol of oxygen consumed per minute per mg protein in isolated hypothalamic synaptosomes) and mitochondrial proliferation (number of mitochondria in NPY neuronal perikarya) were significantly increased in the ghrelin-treated WT mice, whereas no effect was present in UCP2-KO mice. Ghrelin-induced NPY/AgRP mRNA expression and activation of NPY/AgRP neurons – as measured by c-fos expression and increased action potential – were also UCP2-dependent. Ghrelin had no effect on POMC neuronal action potential frequency or on POMC mRNA expression. Ghrelin indirectly hyperpolarizes POMC neurons by activating inhibitory NPY/AgRP inputs (Cowley et al. 2003) and, consistent with this, ghrelin treatment increased inhibitory synapses and significantly reduced the number of excitatory synapses on POMC neurons in WT but not in UCP2-KO. The UCP2-dependent effects on neuronal plasticity and activity translate into ghrelin’s orexigenic effects, as UCP2-KO mice do not increase food intake in response to either i.p. or intra-mediobasal hypothalamic ghrelin. Ghrelin’s effect on AMPK activity was further confirmed by this study, and AMPK seems to lay upstream of UCP2, as the effect of ghrelin on the enzyme was still present in UCP2-KO mice. As proof of the hypothesis that AMPK requires UCP2 to exert an effect on feeding, the effect of the AMPK activator AICAR on food intake was lost in the UCP2-KO mice. The ghrelin-induced increase in UCP2 mRNA levels was diminished by concomitant i.c.v. administration of compound C or etomoxir placing the AMPK-CPT1 pathway upstream of UCP2. In agreement with this, ghrelin increases non-esterified fatty acid in the blood, providing a source of hypothalamic long chain fatty acids CoAs (LCFA-CoAs). There is initially an increase in the hypothalamic LCFA-CoAs after ghrelin treatment, followed by a reduction, suggesting that they undergo mitochondrial β-oxidation. UCP2 has the ability to reduce ROS levels, which are generated during mitochondrial β-oxidation. Concordant with this, ghrelin increased ROS production (as a result of the stimulation of the β-oxidation) in UCP2-KO mice but not in WT mice (where ROS production was stimulated but then buffered by UCP2). The authors propose that ghrelin-triggered ROS production promotes UCP2 mRNA expression and activity, and UCP2 then promotes ROS scavenging. In line with this, in UCP2-KO mice, i.c.v. injection of a ROS-scavenging cocktail (which reduces ROS levels and therefore UCP2 activation) reversed the effects of ghrelin on NPY/AgRP mRNA levels, on mitochondrial uncoupled respiration, on NPY neuronal firing and on food intake. While the effect of leptin on food intake was not impaired in UCP2-KO mice, it would be
interesting to see if other orexigenic or anorexigenic substances utilize the same pathway. We do not know, however, whether the energy released from the β-oxidation is the only way to influence the activity of these neurons. We are also unaware as to how UCP2 activation stimulates NPY/AgRP transcription.

Cannabinoid and ghrelin interaction

Cannabinoids have been identified as yet another important link between ghrelin, AMPK, and appetite. AMPK that has now been confirmed to be involved in the regulatory pathways of many orexigenic and anorexigenic compounds also mediates the orexigenic effect of cannabinoids. Exogenous and endogenous cannabinoids induce food intake through the CB1 (Kirkham & Tucci 2006) and stimulation of hypothalamic AMPK activity (Kola et al. 2005). More intriguing is the role that hypothalamic cannabinoids play in mediating the orexigenic/orexigenic effects of hormones such as leptin and ghrelin (Di Marzo et al. 2001, Kola et al. 2008). Ghrelin and cannabinoid signalling pathways not only converge at the AMPK check-point, but also the interaction seems to take place at several levels. We have shown that ghrelin stimulates hypothalamic 2-AG and anandamide levels, suggesting that the effect of ghrelin on AMPK and the appetite inducing cascade, might be, at least in part, mediated by cannabinoids (Kola et al. 2008). The presence of an intact functional CB1 receptor is required for ghrelin’s effect on AMPK activity and food intake as shown in CB1-KO animals as well as using a CB1 antagonist. Ghrelin inhibits excitatory synaptic input in the PVN and this effect is abolished with a CB1 antagonist as well as via inhibition of pre-synaptic cannabinoid synthesis (Kola et al. 2008). These data suggest that an intact endocannabinoid-CB1 pathway is necessary for ghrelin’s effects.

Summary

Numerous studies aimed to characterize the mechanisms of hypothalamic appetite regulation, and to generate new possible molecular therapeutic targets, have contributed to the exploration of the signalling pathways of ghrelin. While often it is difficult to integrate the results of different studies, it seems that on the ghrelin issue there might be some agreement. The above reported studies lead us to draw a hypothetical intracellular pathway for ghrelin (Fig. 1). Fasting results in increased release of ghrelin from the stomach (the exact mechanism of this remains obscure) leading to increased plasma ghrelin levels, which reach the hypothalamus either via the blood stream directly in areas with no blood–brain barrier, or by crossing the blood–brain barrier via a saturable transport system or via the vagus nerve and the NTS. Ghrelin’s effect on appetite is mediated by an effect both on the hypothalamus and the NTS.

Ghrelin increases Fos expression in the NTS at the level of the area postrema via a catecholaminergic-independent pathway (Faulconbridge 2008). Ghrelin, via GHSR, increases intracellular Ca$^{2+}$ which stimulates the CaMKK–AMPK–ACC–malonyl-CoA–CPT1 pathway, as noted above. However, it also leads to increased levels of intracellular cannabinoids, which additionally stimulate the AMPK–ACC pathway. The final result is increased mitochondrial β-oxidation with increased generation of ROS and stimulation of UCP2, which seems to be a fundamental downstream effector for ghrelin’s modulation of NPY/AgRP neuronal activity. NPY then inhibits both ARC POMC neurons as well as PVN corticotrophin- and thyrotropin-releasing hormone positive anorectic neurons, leading to increased food intake. Intracellular cannabinoids and an intact CB1 receptor are required for the inhibitory effect of ghrelin on PVN neuronal activity.

Outstanding questions

In this complex landscape, however, several questions remain to be answered and a few controversies to be clarified.

Anderson et al. (2008) showed the effect of ghrelin on CaMKK2–AMPK to be confined to the ARC, whereas the study from Lopez et al. (2008) showed a ghrelin-activated AMPK–FAS pathway only in the VMN. GHSR is expressed in both nuclei and ghrelin stimulates AMPK activity in both the ARC and the VMN (Anderson et al. 2008, Lopez et al. 2008). Thus, how could the ghrelin-induced AMPK-dependent reduction in FAS activity and expression be restricted only to the VMN? While Lopez et al. could not show a similar situation for the other nuclei, it seems difficult to accept that while ghrelin induces AMPK activity also in other nuclei such as the ARC, no further downstream effects on the fatty acid pathway components are to be seen here. In fact, we have shown increased pACC in whole hypothalamus (Kola et al. 2005) and Andrews et al. (2008) did show an increased CPT1 activity in the ARC following ghrelin treatment.

Does CaMKK truly mediate the stimulatory effect of ghrelin only in the ARC? If this is the case, how could we then explain the increased AMPK activity observed in VMN? Different experimental settings and the difficulty of obtaining specific data for the different hypothalamic nuclei could confound the interpretation of the pathway.

AMPK activation or inactivation seems to have not just tissue-specific but also cell-dependent effects. Losing AMPK in orexigenic AgRP neurons (AgRP α-2-AMPK-KO) leads to reduced body weight, whereas loss of the enzyme in anorexigenic POMC neurons (POMC α-2-AMPK-KO) leads to increased body weight (Claret et al. 2007). This adds further support to the tissue-specific effects mentioned earlier.

Do cannabinoids form a separate parallel signalling pathway converging at the level of the AMPK, or are cannabinoids a
required integrated part of the ghrelin–Ca$^{2+}$–endocannabinoid–CB1–Ca$^{2+}$–CaMKK–AMPK–malonyl-CoA–CPT1–β-oxidation–ROS–UCP2–NPY/AgRP–food intake pathway? It would be interesting to see if cannabinoid antagonists or lack of CB1 receptor would block the effect of ghrelin on UCP2 expression and activity. Do cannabinoids themselves use the same designated pathway to induce food intake?

Increased β-oxidation in the ARC seems to provide the necessary bioenergetics for electrical activity of ARC NPY/AgRP neurons. We do not know, however, how UCP2 activation stimulates NPY/AgRP transcription and activation. Does the same paradigm apply to anorexigenic PVN neurons? Or vice versa, do the intracellular cannabinoids, which modulate electrical activity in the PVN, influence the activity of neurons in the ARC or other nuclei? And finally, is this the only pathway through which ghrelin, possibly cannabinoids and other AMPK activators, lead to increased food intake? Does ghrelin influence neuronal activity only through the AMPK-induced β-oxidation or does AMPK lead to other collateral pathways influencing the final effect of orexigenic and anorexigenic hormones?

While we have restricted ourselves here to a discussion of the hypothalamic effects of ghrelin, another intriguing issue that deserves further attention is the role of central ghrelin, AMPK, and their hypothalamic downstream effectors in the modulation of peripheral metabolism. Central ghrelin treatment influences peripheral adipose tissue metabolism, the modulation of peripheral metabolism. Central ghrelin, the hypothalamic effects of ghrelin, another intriguing issue in that deserves further attention is the role of central ghrelin, AMPK, and their hypothalamic downstream effectors in the hypothalamic fatty acid synthase triggers rapid activation of fatty acid oxidation in skeletal muscle.

As we discover novel pathways and mechanisms, new other aspect of ghrelin’s physiology?

As we discover novel pathways and mechanisms, new questions arise regarding the intricate regulation of appetite. However, the rising impact of obesity on health in both developed and developing societies renders our understanding of these processes of fundamental importance and a major priority for future research.

Declaration of interest

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

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