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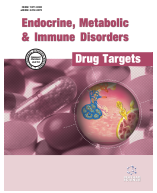
REVIEW ARTICLE



Metabolic Association between Leptin and the Corticotropin Releasing Hormone

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Abstract: ***Objective:*** In healthy individuals, leptin is produced from adipose tissue and is secreted into the circulation to communicate energy balance status to the brain and control fat metabolism. Corticotropin-Releasing Hormone (CRH) is synthesized in the hypothalamus and regulates stress responses. Among the many adipokines and hormones that control fat metabolism, leptin and CRH both curb appetite and inhibit food intake. Despite numerous reports on leptin and CRH properties and function, little has been actually shown about their association in the adipose tissue environment.

Methods: In this article, we summarized the salient information on leptin and CRH in relation to metabolism. We also investigated the direct effect of recombinant CRH on leptin secretion by primary cultures of human adipocytes isolated from subcutaneous abdominal adipose tissue of 7 healthy children and adolescents, and measured CRH and leptin levels in plasma collected from peripheral blood of 24 healthy children and adolescents to assess whether a correlation exists between CRH and leptin levels in the periphery.

Results and Conclusion: The available data indicate that CRH exerts a role in the regulation of leptin in human adipocytes. We show that CRH downregulates leptin production by mature adipocytes and that a strong negative correlation exists between CRH and leptin levels in the periphery, and suggest the possible mechanisms of CRH control of leptin. Delineation of CRH control of leptin production by adipocytes may explain unknown pathogenic mechanisms linking stress and metabolism.

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1. INTRODUCTION

1.1. LEPTIN

Leptin is one of the main adipokines produced by White Adipose Tissue (WAT), and is the main regulator of food intake and energy expenditure, acting at various sites within the Central Nervous System (CNS) [1-3]. Its existence was hypothesized already in the 1950s, however, its gene was cloned almost 40 years later [3, 4]. In humans, leptin is the product of the gene lep that is located on chromosome 7q31.3, encoding a protein consisting of 167 amino acids with a molecular weight of 16kDa [5, 6]. The amino acid sequence of leptin is highly conserved between mammals; orthologs of leptin have been identified in amphibians, reptiles and fish, with major differences in their primary amino acid sequences. However, the function of leptin is conserved between species because of preserved secondary and tertiary structures that allow the formation of disulfide bonds [7]. Leptin belongs to the family of type I helical cytokines, and is related to growth hormone, prolactin and interleukins [8, 9].

1.2. Leptin Production

Leptin is produced mainly from WAT and is secreted into the circulation. Circulation levels of leptin are proportional to the size of adipose tissue and communicate energy balance status to the brain [10-12]. Leptin expression and circulating levels show diurnal fluctuation and are modified by nutritional status [13]. In healthy humans or animals, fasting decreases leptin levels [14-16], whereas food intake or obesity increases its circulation levels [14-18].

Brown Adipose Tissue (BAT) is another site of leptin synthesis, though the role of leptin in BAT is not fully understood [19-21]. It is hypothesized that it acts in an autocrine/paracrine fashion and that it is involved in ther-

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mogenesis and energy expenditure [22]. The placenta and ovaries are also sites of lep gene expression and leptin production [23-26]. Specifically, leptin production seems to exclusively characterize the syncytiotrophoblast, and its expression is increased following the progression of pregnancy [24, 27-29]. It is hypothesized that leptin may act as a novel growth factor or that it signals energy status between the mother and fetus. Moreover, the placenta also expresses the leptin receptor [24], suggesting that leptin might act in an autocrine fashion. Leptin is also expressed in multiple sites in the murine fetus, including the heart, bones, cartilage, choroid plexus, lungs, kidneys and liver [30]. It has also been identified in the stomach [31] and skeletal muscle of rats [32], in the pituitary of mice and rats [33], in the epithelial cells of human breast tissue [34], in human bone marrow [35], in murine liver [36] and in human leukemic cells [37].

1.3. Regulation of Leptin

The production of leptin is regulated on two levels; a long-term level, where transcriptional control predominates, and a short-term level that is dominated by translational regulation [38].

1.3.1. Regulation of Leptin at the Transcriptional Level

The proximal promoter of the lep gene had been characterized in humans and mice [39, 40]. A classic TATA box has been identified along with binding sites for transcription factors that include C/EBP, Sp-1, GR and CREB, and an E box element, which is the binding site of transcription factor SREBP1c [41-44]. The transcription factor AP-2β binds to the promoter of the lep gene and inhibits leptin expression [45]. In addition, a cis-regulatory element has been identified that is specific for adipocyte lep gene expression; the transcription factor FOSL2 that binds specifically to this element constitutes an important regulator of leptin expression in adipocytes [46, 47].

Changes in hormonal or neural signals induced by long-term alterations in nutritional status play an important role in long-term regulation of leptin production. For example, in obesity, leptin mRNA levels are increased [48, 49]. Moreover, it has been observed that signals associated with a positive energy balance, such as elevated insulin, increase leptin mRNA levels, whereas those associated with energy deficiency, such as sympathetic nervous system activation by starvation, decrease leptin mRNA [50, 51]. However, even though there are numerous reports documenting a long-term regulation of leptin production by hormonal, nutritional and neuronal signals, the mechanisms underlying leptin regulation at the transcriptional level remain mostly undefined [38].

1.3.2. Regulation of Leptin at the Post-Transcriptional/ Translational Level

The short-term regulation of leptin production seems to occur at the post-transcriptional level, and particularly at the translational level, because whereas leptin levels change within hours, this variation is not associated with a corresponding change in leptin mRNA levels [38]. A number of factors participate in the regulation of leptin production and secretion, including inflammatory cytokines [52, 53], glucocorticoids [54, 55], insulin [56], glucose [57] as well as leptin itself [37, 58].

1.4. Signaling Pathways Involved in Leptin Regulation

Insulin increases leptin levels, and this effect is mediated through the PI3K/Akt/mTOR pathway (PI3Ks and their downstream mediators Akt and mTOR constitute the core components of the PI3K/Akt/mTOR signalling cascade that regulates cell proliferation, survival and metabolism). The use of inhibitors against PI3K, MEK1/MEK2, Akt or mTOR inhibits insulin-stimulated leptin release by adipocytes without altering leptin expression levels [54] or leptin biosynthesis [59]. Expression of a constitutively active Akt in 3T3-L1 adipocytes led to a 20-fold increase in secreted leptin protein levels, without affecting mRNA levels [60]. Additionally, activation of mTOR complex 1 by overexpression of Rheb or inhibition of AMPK increases leptin translation [61]. Amino acids, like leucine, also increase leptin [62], which also occurs through activation of mTOR [62]. The interplay between AMPK and mTOR lies at the center of energy balance regulation [63] which provides a mechanism to link leptin synthesis to energy availability [38]. AMPK is activated when cells are low on energy, as during fasting, which inhibits mTOR and possibly leptin production. Thus, the ability of nutrients to increase leptin levels might be, at least in part, due to the inhibitory effect of AMPK [38]. Nevertheless, more studies are needed to elucidate the role of the PI3K and AMPK signaling pathways in the regulation of leptin.

Activation of the cAMP-PKA signaling pathway (that differentially regulates cell metabolism, growth and differentiation depending on the cell type) seems to reduce the expression of leptin mRNA in adipocytes and inhibit leptin secretion [64, 65]. It is possible that the elevation of cAMP levels mediates the inhibitory effect of β-adrenergic receptors over leptin [66, 67], which counteracts the positive effects of insulin. On the other hand, insulin may decrease cAMP levels through activation of phosphodiesterase 3B (PDE3B), which hydrolyses cAMP, and this is achieved through PI3K signaling [68, 69]. This pathway is also active in adipocytes mediating insulin-stimulated lipolysis [70] and leptin and adiponectin secretion [66]. Elevation of intracellular cAMP inhibits mTOR activity, as well as phosphorylation of 4E-BP1 and S6K, which are downstream targets of mTOR [71-73]. Additionally, the interplay between cAMP and AMPK seems to mediate the inhibitory effect of β-adrenergic receptors [74-76]. Similarly, in human Mesenchymal Stem Cells (MSCs), the activation of cAMP-PKA pathway negatively regulates leptin [77, 78], which is mediated by activation of CREB, and is related to differentiation of MSCs towards adipocytes, osteoblasts or other cells. However, the mechanisms that regulate leptin production through the interplay of PKA, AMPK and mTOR signaling pathways remain mostly unknown.

2. CORTICOTROPIN-RELEASING HORMONE

Corticotropin-Releasing Hormone (CRH) is the main regulator of stress responses, and the principal hypothalamic factor regulating the hypothalamic-pituitary-adrenal axis [79-82]. Since its characterization in 1981 by Vale and cowork-

ers [83], two G-protein coupled receptors that bind CRH have been also identified, encoded by different genes. These receptors, namely the type 1 CRH receptor (CRH-R1) and type 2 CRH receptor (CRH-R2), also bind three more peptides related to CRH, the urocortin (UCN)1-3 [84]. CRH exhibits high affinity for the CRH-R1 and low affinity for the CRH-R2. On the contrary, UCN2 and UCN3 bind with high affinity to CRH-R2 but weakly to CRH-R1, whereas UCN1 shows no predilection for either of the receptors.

2.1. Expression sites of CRH and CRH-Rs

CRH and CRH-Rs are mainly expressed in the brain [85, 86]. There are 4 splicing isoforms of CRH-R1 (α, β, c, d) but CRH-R1α is the predominantly expressed and functional isoform that is expressed in specific brain areas and the pituitary corticotroph and mediates the central actions of CRH [87-89]. CRH-R2 has 2 isoforms in rodents (α, β) and 3 in humans (α, β, γ); CRH-R2α and β are expressed in the brain and the periphery, whereas CRH-R2γ is expressed predominantly in the brain. CRH-R2 plays a critical role in the regulation of food intake and energy balance [89, 90-92]. CRH and CRH-Rs are also expressed in peripheral organs and tissues, including the testis, ovary [89], skin [89, 93, 94], skeletal muscle [89, 95, 96] and the adrenals [89, 97], where they participate in the regulation of organ physiology and metabolism through autocrine/paracrine mechanisms [95, 98].

2.2. CRH/CRH-R Signaling

Binding of CRH or UCNs to CRH-Rs induces the recruitment of G-proteins to the receptor. CRH-Rs are highly promiscuous and have the ability to bind and activate multiple Gα subunits, including Gas, Gao, Gaq/11, Gai1/2 και Gaz [99-101]. Subsequent binding and activation of adenylyl cyclase leads to cAMP production, which in turn activates the PKA signaling pathway. It has been documented that activation of the cAMP-PKA pathway by CRH mediates most of the physiological actions of the hormone in the CNS and the periphery. However, in certain tissues, including the placenta and testis, CRH-Rs are unable to activate certain G-proteins (CRH-R activation does not activate adenylyl cyclase pathways or Gi/Gs, although CRH-R activation activates other G-proteins such as Gq, Go and/or Gz), but instead activate alternative signaling pathways, like MAPK or intracellular Ca^{2+}/PKC [102, 103]. In addition, experiments with mouse hippocampal cells and corticotrophs showed that different sources of cAMP are triggered by CRH-activated CRH-R1, and that CREB phosphorylation downstream CRH-R1 takes place in neuronal cells [104, 105].

In various cellular systems it has been shown that activation of the CRH-R1-cAMP-PKA pathway may lead to downstream activation of a variety of signaling molecules, including membrane guanylyl cyclase, the NF-κB transcription factor, the GSK-3β/Wnt/β-catenin pathway, the ERK1/2 kinase and tyrosine hydroxylase [106]. In addition, CRH-Rs may signal through PKA-independent pathways that involve activation of EPACs (guanine nucleotide exchange proteins directly activated by cAMP) [107-109]. Post-translational modifications and coupling with different G-proteins seem to differentiate the signaling through CRH-Rs in various tissues [106].

2.3. CRH and Adipocytes

Human adipocytes also express CRH-Rs, suggesting that adipocytes are a primary target of hypothalamic CRH or its related peptides, including UCN2 and UCN3 [110, 111]. Adipocytes express higher levels of CRH-R2 in relation to CRH-R1, as is also the case in other peripheral tissues, like the adrenal and heart. The CRH-R1:CRH-R2 ratio varies according to the type of fat deposit; in particular, CRH-R1 expression is higher in subcutaneous fat whereas CRH-R2 expression is higher in visceral fat [111]. In addition, CRH added in cultured human adipocytes downregulated in a dose-dependent manner the mRNA levels of both CRH-R1 and CRH-R2 [111].

It is documented that CRH downregulates 11β-hydroxysteroid dehydrogenase type 1 activity (a key enzyme that catalyzes the intracellular conversion of cortisone to physiologically active cortisol) in mature human adipocytes and, in addition, it reduces lipolysis in differentiated human adipocytes [112]. CRH-R2 seems to mediate these effects, instead of CRH-R1. Moreover, adipocytes also express the main ligands of CRH-R2, UCN2 και UCN3 [110]. Collectively these data support a functional role for CRH and UCNs in adipose tissue. Furthermore, in WAT, CRH-R2 mediates hypoxia-induced lipolysis *via* activation of the cAMP-PKA signaling pathway [113].

3. CRH AND LEPTIN ASSOCIATION IN ADIPOCYTES

One study of the effect of chronic subcutaneous leptin infusion in *ad lib*-fed or starving rats, showed no significant effect on the paraventricular hypothalamic CRH mRNA levels [17]. In contrast, two other experimental studies showed that leptin directly stimulates CRH secretion by mouse and rat hypothalamic explants, in a dose-dependent manner [114, 115].

The notion that CRH may be directly involved in the regulation of leptin expression and production in human adipocytes is largely untested. Evidence supporting this notion comes from a report showing that activation of CRH-R1 in 3T3-L1 adipocytes during differentiation downregulates leptin production, whereas in fully differentiated adipocytes CRH-R agonists reduce leptin secretion [110]. Demonstration that CRH downregulates leptin production by primary adipocytes comes from our experiments with human preadipocytes and mature adipocytes cultured *in vitro* from biopsies of human subcutaneous abdominal adipose tissue with recombinant CRH, showing that CRH inhibits leptin secretion by mature adipocytes only (Fig. **1**, this work). We also measured plasma CRH and leptin levels in blood samples drawn from healthy individuals to investigate whether CRH downregulation of leptin production by adipocytes is reflected in the concentration of these hormones in the periphery; our results showed a strong negative correlation between the concentrations of plasma CRH and leptin (Fig. **2**, this work).

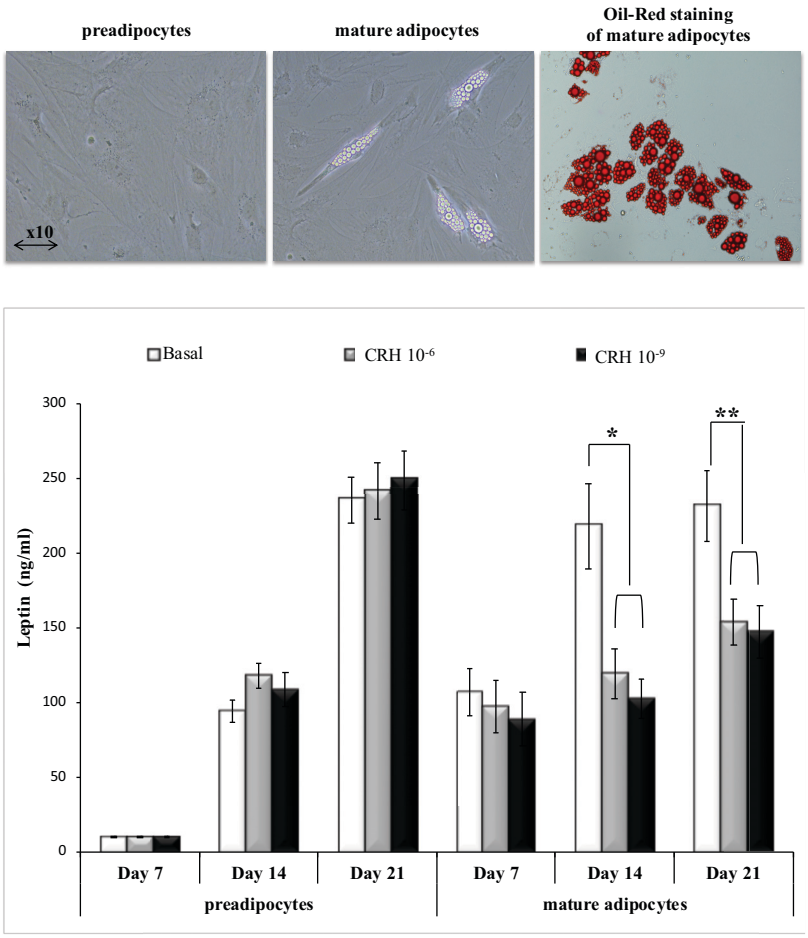

Fig. (1). CRH downregulates leptin production by human adipocytes. **Upper panel**: Primary cultures of preadipocytes and mature adipocytes were developed as described [116] from subcutaneous abdominal adipose tissue of 7 healthy children and adolescents. **Lower panel**: Equal amount of cells (10,000) from each culture at passage 1, were transferred in 24-well plates and cultured in doublicate with recombinant CRH at concentrations 10^{-9}M or 10^{-6}M, during a period of 21 days. Culture supernatants were collected from preadipocyte and mature adipocyte cultures at base line (no CRH added) and at days 7, 14 and 21 of culture. In each sample leptin levels were measured by ELISA. *p<0.05, **p<0.01, Student's t-test [this work].

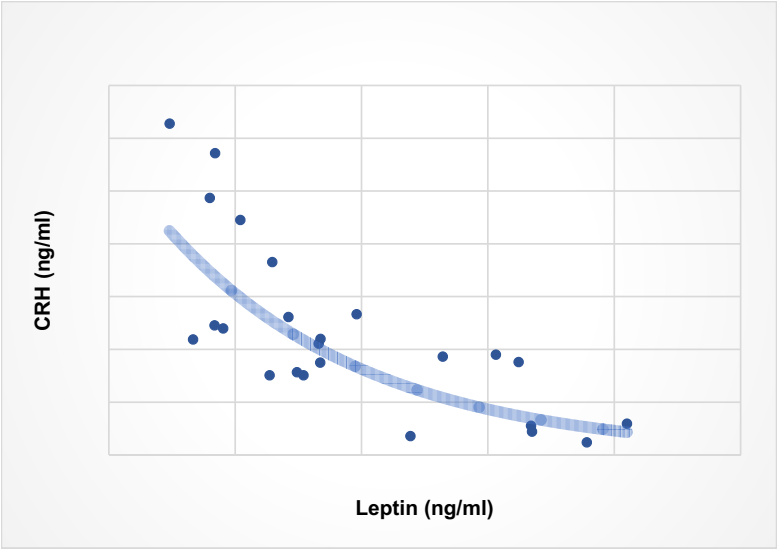

Fig. (2). Correlation between plasma CRH and leptin. Plasma was collected from peripheral blood samples drawn from 24 healthy children and adolescents, 14 male, 10 female, mean age 9 years, age range 4-14 years, mean body mass index 18.98 (SD 3.5), during a routine visit to the Department of Pediatrics of Patras University Hospital for minor problems. Determination of plasma CRH and leptin levels was done by ELISA. Spearman's $R^2 = 0.6231$, $p<0.001$ [this work].

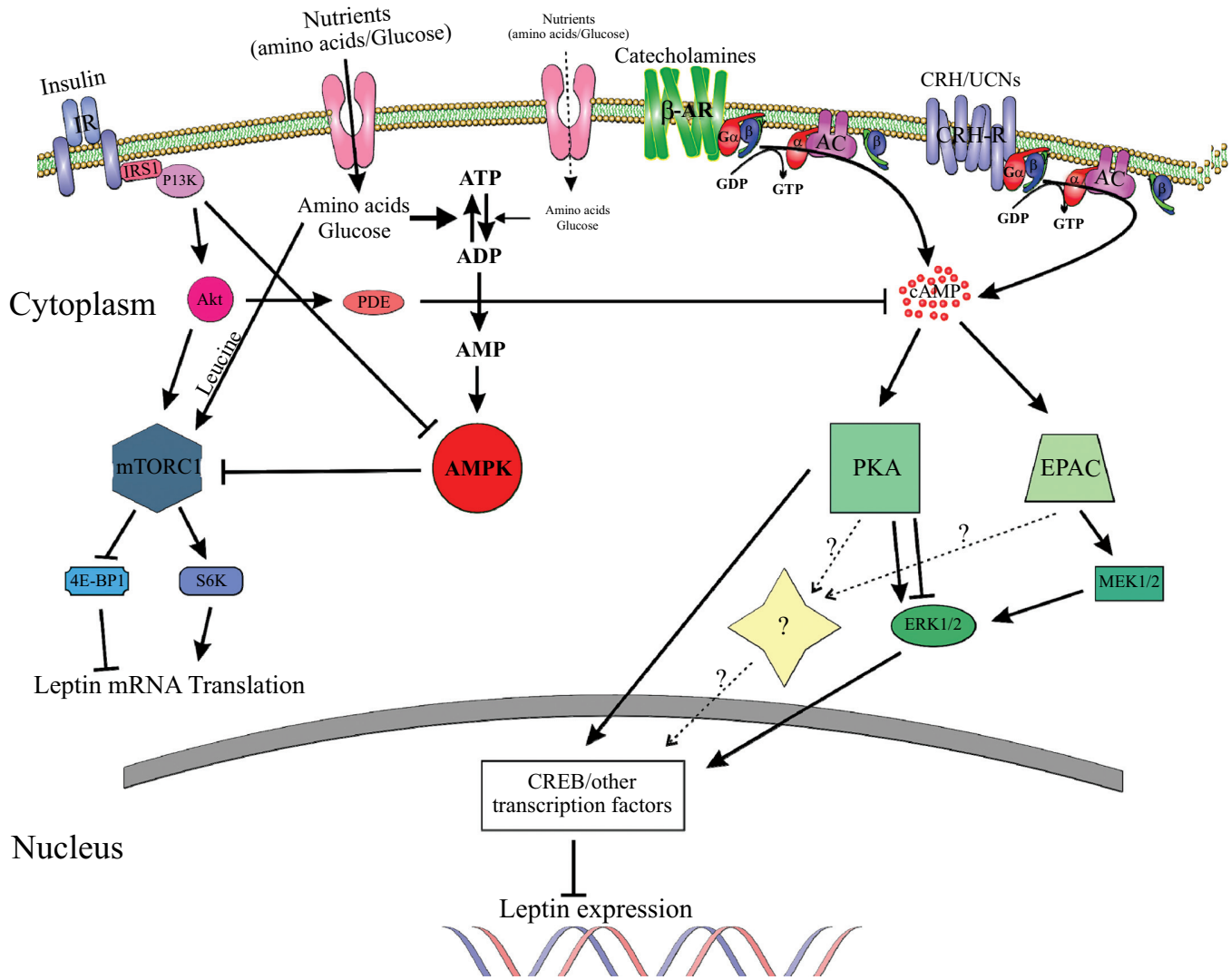

Fig. (3). Molecular pathways involved in leptin regulation in adipocytes. 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; AC, adenylyl cyclase; ADP, adenosine diphosphate; Akt (PKB), protein kinase B; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; cAMP, cyclic AMP; CREB, cAMP response element-binding protein; CRH, corticotropin-releasing hormone; EPAC, guanine nucleotide exchange proteins directly activated by cAMP; ERK, extracellular-signal-regulated kinases; GDP, guanosine diphosphate; GTP, guanosine triphosphate; Gα/β/γ, G-protein alpha, beta, and gamma subunits; IR, insulin receptor; IRS1, insulin receptor substrate 1; MEK1/2, mitogen-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; PDE, phosphodiesterase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; S6K, p70-S6 Kinase 1; UCN, urocortin; β-AR, beta adrenergic receptor. Adapted from [38, 63, 77, 106]. For details see text. The pathway involving CRH is hypothetical and not experimentally proven.

CRH downregulation of leptin in human adipocytes may be mediated *via* activation of the cAMP-PKA signaling pathway, since: (1) activation of the cAMP-PKA pathway in adipocytes inhibits leptin production, (2) insulin-induced downregulation of cAMP increases leptin synthesis and induces lipolysis, (3) adipocytes express CRH receptors, (4) CRH-R2 activates the cAMP-PKA pathway in adipocytes, (5) CRH inhibits lipolysis in human adipocytes, and (6) CRH-R agonists inhibit leptin in murine 3T3-L1 adipocytes during differentiation and in mature adipocytes. Fig. (**3**) depicts the molecular pathways involved in leptin regulation, including a hypothetical model for leptin regulation by CRH.

Further studies are required to establish whether CRH or other agonists of CRH-Rs are involved in the regulation of leptin in human adipocytes, and under what conditions this happens. In case such a relationship is identified, the next step will be to study *via* which signaling pathway(s) CRH/CRH-Rs regulate leptin. Questions that could be pursued might include the following: Which CRH receptor is involved? Which G-proteins are recruited? Does leptin regulation involve the PKA pathway or other PKA-independent pathway (possible activation of EPACs)? Does mTOR and AMPK mediate leptin regulation *via* CRH-Rs? Which are the downstream factors or kinases activated thereafter? On

which level does leptin regulation *via* CHR-Rs happen (transcriptional, translational, secretion)?

CONCLUSION

Despite numerous reports on leptin and CRH actions, the information on the molecular control of leptin expression and production by adipocytes is still incomplete, and does not include the CRH parameter. The available data suggest that CRH exerts a role in the regulation of leptin in human adipocytes, though the mechanisms involved should be clarified. A plausible regulation loop would entail: increased food intake leading to increased leptin production by adipocytes, leptin stimulation of CRH release by the hypothalamus, inhibition of appetite by both leptin and CRH, and inhibition of leptin secretion from adipocytes by CRH to achieve homeostasis. Derangement of this feedback loop could have a direct bearing on pathogenic mechanisms linking stress and metabolism.

LIST OF ABBREVIATIONS

AC = Adenylyl Cyclase

ADP = Adenosine Diphosphate

Akt (PKB) = Protein Kinase B

AMP = Adenosine Monophosphate

AMPK = AMP-Activated Protein Kinase

ATP = Adenosine Triphosphate

β-AR = Beta Adrenergic Receptor

BAT = Brown Adipose Tissue

CAMP = Cyclic AMP

CNS = Central Nervous System

CREB = CAMP Response Element-Binding Protein

CRH = Corticotropin-Releasing Hormone

CRH-R1 = Type 1 CRH Receptor

CRH-R2 = Type 2 CRH Receptor

4E-BP1 = Eukaryotic Translation Initiation Factor 4E-Binding Protein 1

EPAC = Guanine Nucleotide Exchange Proteins Directly Activated by CAMP

ERK = Extracellular-Signal-Regulated Kinases

GDP = Guanosine Diphosphate

GTP = Guanosine Triphosphate

Gα/β/γ = G-Protein Alpha, Beta and Gamma Subunits

IR = Insulin Receptor

IRS1 = Insulin Receptor Substrate 1

MEK1/2 = Mitogen-Activated Protein Kinase

MSCs = Mesenchymal Stem Cells

MTORC1 = Mammalian Target of Rapamycin Complex 1

PDE3B = Phosphodiesterase 3B

PI3K = Phosphatidylinositide 3 Kinase

PKA = Protein Kinase A

S6K = p70-S6 Kinase 1

UCN = Urocortin

WAT = White Adipose Tissue

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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