CHAPTER FIVE

Granins and Catecholamines: Functional Interaction in Chromaffin Cells and Adipose Tissue

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Abstract

Catecholamines (CAs) and granin peptides are costored in dense-core vesicles within the chromaffin cells of the adrenal medulla and in other endocrine organs and neurons. Granins play a major functional and structural role in chromaffin cells but are

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ubiquitous proteins, which are present also in secretory cells of the nervous, endocrine, and immune systems, where they regulate a number of cellular functions. Furthermore, recent studies also demonstrate that granin-derived peptides can functionally interact with CA to modulate key physiological functions such as lipolysis and blood pressure. In this chapter, we will provide a brief update on the interaction between CA and granins at the cellular and organ levels. We will first discuss recent data on the regulation of exocytosis of CA and peptides from the chromaffin cells by the sympathetic nervous system with a specific reference to the prominent role played by splanchnic nerve-derived pituitary adenylate cyclase-activating peptide (PACAP). Secondly, we will discuss the role of granins in the storage and regulation of exocytosis in large dense-core vesicles. Finally, we will provide an up-to-date review of the roles played by two granin-derived peptides, the chromogranin A-derived peptide catestatin and the VGF-derived peptide TLQP-21, on lipolysis and obesity. In conclusion, the knowledge gathered from recent findings on the role played by proteins/peptides in the sympathetic/target cell synapses, discussed in this chapter, would contribute to and provide novel mechanistic support for an increased appreciation of the physiological role of CA in human pathophysiology.

1. INTRODUCTION

Chromaffin cells of the adrenal medulla are a primary output of the sympathetic nervous system (SNS) (Fulop, Radabaugh, & Smith, 2005). Firing of action potentials through cholinergic synaptic input from the sympathetic splanchnic nerve excites individual chromaffin cells to undergo exocytosis. Heightened splanchnic firing elevates synaptic acetylcholine (ACh) levels to evoke catecholamine (CA) secretion from the adrenal medulla. Moreover, elevated cell stimulation also leads to the corelease of a second vaso- and neuroactive peptide-transmitter class of stress hormones including enkephalin, neuropeptide Y, atrial natriuretic factor, tissue plasminogen activator, and granin peptides. In particular, granin peptides are emerging as key diagnostic and prognostic biomarkers of several neurological, psychiatric, and neoplastic diseases in humans, while their biological role is being elucidated in mice and cellular models (Bartolomucci et al., 2011; Helle, 2004; Taupenot, Harper, & O'Connor, 2003). The granin family includes chromogranins, secretogranins, and additional related acidic proteins, sharing phylogenetically well-conserved domains within the primary structure. The granin family currently includes eight members, namely, chromogranin A (CgA), CgB, secretogranin II (SgII), SgIII, HISL-19 antigen, 7B2, NESP55, VGF, and ProSAAS. Although granins play a major functional and structural role in chromaffin cells, they are ubiquitous proteins in secretory cells of the nervous, endocrine, and immune

systems, where they regulate a number of cellular functions including protein sorting, granulogenesis, and prohormone convertase (PC) activity, as well as preventing uncontrolled osmotic swelling of secretory vesicles. Furthermore, recent studies also demonstrate that granin-derived peptides can functionally interact with CA to modulate key physiological functions such as lipolysis (Bandyopadhyay et al., 2012; Possenti et al., 2012) and blood pressure (Biswas et al., 2012; Mahapatra et al., 2005).

In this chapter, we will provide an update on the interaction at the cellular and organ level between CA (released from adrenal medulla and/or the sympathetic nerve terminals within the target tissues) and granin-propeptides or granin-derived peptides. We will first discuss recent data on the regulation of exocytosis of CA and peptides from the chromaffin cells by the SNS with a specific reference to the prominent role played by splanchnic nerve-derived pituitary adenylate cyclase-activating peptide (PACAP) (Section 2). Secondly, we will discuss the role of granins in storage and regulation of exocytosis in large dense-core vesicle (LDCV) (Section 3). Then, we will discuss the role played by two granin-derived peptides on lipolysis and obesity, namely, the CgA-derived peptide catestatin (CST) (Section 4) and, finally, the VGF-derived peptide TLQP-21 (Section 5). Remarkably, despite being structurally and functionally unrelated, CST and TLQP-21 activate a very similar prolipolytic pathway in adipocytes by modulating norepinephrine-induced lipolysis (Bandyopadhyay et al., 2012; Possenti et al., 2012). This chapter has been inspired by the symposium 'Granins, Catecholamines, and the Chromaffin Cell' held at the *X International Catecholamine Symposium (XICS)* in Pacific Grove, CA, September 9–13, 2012. It is beyond the scope of this chapter to provide a comprehensive overview of the current knowledge on granin peptides, CA, and chromaffin cells. However, we sincerely hope that the succeeding chapters will provide readers a fresh view of a field, which is undergoing a remarkable innovation in the recent years.

2. PACAP AS A PRIMARY EFFECTOR OF THE ACUTE SYMPATHOADRENAL STRESS RESPONSE

Firing of action potentials through cholinergic synaptic input from the sympathetic splanchnic nerve excites individual chromaffin cells. This in turn causes Ca^{2+} influx through voltage-gated channels and Ca^{2+}-dependent fusion of CA and peptide transmitter-containing LDCV with the cell surface and their release into the circulation (Aunis, 1998).

At sympathetic tone, low-frequency neuronal excitation of chromaffin cells evokes the release of CA at a modest rate. In the circulation, the secreted CA contributes to physiological regulation of the 'rest and digest' metabolic status. Example homeostatic functions include regulation of vascular tone, enteric activity, and insulin release to achieve an overall state of energy storage. Thus, a second potent noncholinergic stimulatory mechanism must exist under the sympathetic stress response (Wakade, 1988). A candidate excitatory secretagogue is the peptide-transmitter PACAP. PACAP was first isolated from the pituitary gland but is found throughout a wide range of tissues, including the splanchnic nerve that innervates the adrenal medulla. PACAP acts through a G protein-coupled receptor and has been shown to modulate a broad array of neuronal activity (Smith & Eiden, 2012). Chromaffin cells express the high-affinity PACR1 receptor and respond to exogenous PACAP with an immediate and persistent release of CA (Hamelink et al., 2002). Indeed, PACAP$^{-/-}$ mice fail to mount a normal stress response and die under insulin shock or physical restraint (Smith & Eiden, 2012). Thus, PACAP-evoked stimulation of the adrenal medulla must be linked to a Ca^{2+} influx to support secretory granule fusion, increase the release of CA, and initiate the release of peptide transmitters to formulate the sympathoadrenal stress response. In this proceeding report, recent experimental support for PACAP-evoked Ca^{2+} entry into adrenal chromaffin cells and general electrical remodeling of the adrenal medulla, each to support adrenal hormone secretion under diverse physiological conditions, are considered.

2.1. PACAP-evoked Ca^{2+} entry

Upon stimulation with exogenous PACAP or by endogenous PACAP release through splanchnic stimulation, adrenal chromaffin cells exhibit a change in basic electrophysiological behaviors. The resting membrane potential depolarizes by an average of 15 mV from a normal resting potential of approximately −65 to −50 mV (Hill, Chan, Kuri, & Smith, 2011). This membrane depolarization has been shown to depend on the phospho-regulated influx of external Na^+ but is insensitive to blockers of voltage-gated Na^+ channels (Mustafa, Grimaldi, & Eiden, 2007; Tanaka, Shibuya, Nagamoto, Yamashita, & Kanno, 1996). Candidate routes for this regulated Na^+ influx include an activation of the Na^+/Ca^{2+} exchanger (NCX) or an increased conductance through TRP channels, with further investigation necessary to isolate either candidate. A second phospho-regulated

PACAP-evoked change in chromaffin cell's electrical properties describes the recruitment of a low voltage-activated (LVA) Ca^{2+} conductance (Smith & Eiden, 2012). Pharmacological and kinetic analysis of the Ca^{2+} conductance is most consistent with an initial recruitment of a T-type Ca^{2+} channel (Hill et al., 2011). Both the depolarization and the recruitment of the LVA Ca^{2+} conductance are an absolute requirement for rapid PACAP-evoked adrenal excitation; block of either results in the block of acute PACAP-mediated secretion. Thus, PACAP stimulation bypasses the canonical ACh-mediated excitation observed under sympathetic tone; the chromaffin cell does not fire an action potential and the high voltage-activated channels shown to be responsible for ACh-evoked secretion (P/Q- and R-type Ca^{2+} channels) are not necessarily involved in the PACAP-triggered excitation path.

2.2. PACAP-evoked intercellular coupling

Adrenal chromaffin cells receive heterogeneous innervation from the splanchnic nerve, with anywhere between a single and 4 or 5 synaptic contacts per cell. Thus, splanchnic stimulation does not excite all cells of the medulla equally. However, intercellular electrical coupling has been demonstrated to be modulated under the sympathetic stress response. Specific stressors have been shown to increase intercellular gap-junction conductance (Colomer, Lafont, & Guerineau, 2008). Such an increase facilitates the spread of depolarization from highly innervated chromaffin cells to lesser innervated cells. PACAP again plays a role in this tissue-level electrical remodeling. Second messenger-signaling processes activated by PACAP excitation result in increased gap-junction coupling and thus facilitate electrical excitation within the adrenal medulla and thereby increase overall adrenal secretory output.

In this manner, stress-evoked release of PACAP from the splanchnic synapse acts at both the cell and tissue level to increase excitation of the adrenal medulla. Stimulation of the PACR1 receptor results in a membrane subthreshold for action potential stimulation but sufficient to gate LVA Ca^{2+} channels. In parallel, there is a phospho-dependent recruitment of a normally silent cohort of T-type LVA Ca^{2+} channels that facilitate entry of Ca^{2+} to support granule fusion and exocytosis. Furthermore, PACAP excitation results in the phospho-regulated increase in intercellular electrical coupling within the medulla to facilitate overall excitation and support elevated secretion under the sympathoadrenal stress response.

2.3. Regulation of adrenal peptide-transmitter secretion: a role for PACAP?

Previous studies have shown a differential activity-mediated transmitter release of CA versus peptide transmitters from the adrenal medulla with CA secreted in a graded fashion across all sympathetic activity levels but peptide transmitters only under elevated firing conditions. The mechanism for this behavior was not clear in that CA and peptide transmitters are copackaged in the same secretory granules. Work from our group (Fulop et al., 2005), as well as others (Perrais, Kleppe, Taraska, & Almers, 2004), has provided a simple size-exclusion hypothesis for this differential release of transmitter classes. Under basal stimulation, selective secretion of small, soluble CA is achieved by release through the restricted fusion pore of a transient Ω-form kiss and run exocytic event. The peptide transmitter-containing dense granule core does not pass through the narrow fusion pore and is left in the lumen of the granule, to be reinternalized by endocytosis. Heightened stimulation leads to the expansion of the fusion pore, maximizing CA release (Elhamdani, Azizi, & Artalejo, 2006) and facilitating release of the peptide transmitters contained within the gelatinous granule core. Moreover, the activity-dependence of splanchnic PACAP-mediated adrenal excitation correlates to adrenal peptide-transmitter secretion. Beyond this simple behavioral correlation, no directed experiments have been conducted to specifically test the dependence of adrenal peptide transmitter release with splanchnic-adrenal PACAP excitation. However, such a scenario is an attractive potential mechanism for the physiologically diverse dual roles played by the adrenal medulla. Separation of homeostatic control of cardiac and vascular function and circulating glucose levels under sympathetic tone from stress-evoked acute hormonal responses to facilitate escape or defense under stress would make regulation within each process more achievable. For example, the Ca^{2+} influx experienced under nicotinic stimulation is expected to be quite different than under PACAP-evoked stimulation. Pulsatile influx in response to single action potential stimuli would result in a temporally and volume-limited discrete superthreshold Ca^{2+} domains to support individual secretion events (Neher, 1998). However, such microdomains are not expected to extend in space or time to evoke the activation of cytosolic messenger factors that evoke fusion pore dilation to achieve peptide-transmitter secretion. PACAP-evoked stimulation, on the other hand, is shown to evoke a sustained Ca^{2+} influx that is expected to elevate Ca^{2+} generally throughout the cytosol (Kuri,

Chan, & Smith, 2009; Tanaka et al., 1996). Indeed, evidence for such a segregation of Ca^{2+} pools to support secretion on the granule scale from more global cytosolic processes that modulate granule mobility and availability is described in the literature for many secretory cell types (i.e., for chromaffin cells (Smith, 1999)). Thus, an intriguing possibility that chromaffin cells of the adrenal medulla possess two separate – and therefore, separately regulable – processes to meet the very different physiological roles under the homeostatic 'rest and digest' versus sympathoadrenal 'fight or flight' stress status is hypothesized. The possibility that ACh versus PACAP-evoked excitation of the adrenal medulla represents a tipping point that translated sympathetic firing patterns into diverse regulable differential hormonal profiles, composed of combinations of CA and adrenal-derived peptide transmitters is defined. This potential duality will act as motivation for further guided examination of mechanisms responsible for physiology of sympathoadrenal signaling.

3. CHROMOGRANINS THE KEY PROTEINS IN THE STORAGE AND REGULATION OF EXOCYTOSIS IN LDCV

Along the last years, there has been a considerable amount of work on the contribution of CgA and CgB in amine cargo and exocytosis. The ability of secretory vesicles to actively accumulate enormous concentrations of solutes has intrigued scientists for decades. This process is crucial in cells whose primary function is to efficiently secrete substances, such as neurotransmitters and hormones, as few exocytotic events can provoke sufficiently large secretory responses (Helle, Reed, Pihl, & Serck-Hanssen, 1985, Machado et al., 2010, Nanavati & Fernandez, 1993). Granins also exhibit pH-buffering capacities derived from their acidic characteristic, thus helping to concentrate soluble products for secretion. For these reasons, they are currently considered to be high-capacity and low-affinity buffers. For example, CgA can bind 32 mol adrenaline per mol with a K_d of 2.1 mM (Videen, Mezger, Chang, & O'Connor, 1992). Similar interactions also occur with other soluble species such as Ca^{2+} (Yoo, 2010) and ATP. This matrix probably corresponds to the electron-dense core observed in electron microscopy images (Ehrhart, Grube, Bader, Aunis, & Gratzl, 1986). The strong accumulation of vesicular solutes is probably the main mechanism used to reduce the pressure produced by the concentration delimited in the vesicular membrane. In the limited space of a vesicle, amines ($\approx$800 mM), Ca^{2+} ($\approx$40 mM), ATP ($\approx$200 mM), and other solutes like ascorbate are

concentrated, representing the mobile components whose concentration gradients relative to the cytosol are maintained by special carriers. By contrast, CgA and CgB, as well as other proteins and peptides, constitute the immobile components that form the dense core of LDCV that aggregates the majority of solutes. Using amperometry, patch amperometry, and intracellular electrochemistry in chromaffin cells from CgA/CgB-KO mice, it has been demonstrated that the absence of CgA (Montesinos et al., 2008) and CgB (Diaz-Vera et al., 2010) caused important changes in CAs accumulation and in the kinetics of exocytosis. Borges' lab has recently obtained a double CgA/CgB-KO mouse by crossing those strains, which resulted in viable and fertile mice (Diaz-Vera et al., 2012). The CA content in chromaffin LDCV is halved and the secretory response largely reduced. The incubation of cells with L-DOPA showed that the uptake of newly synthesized CA granules is impaired in CgA/CgB-KO cells, as no increase in the quantum size was detected but the free cytosolic catechols increased. This effect was not due to changes in amine transport nor in the synthesis or degradation of cytosolic amines but to the impaired capacity to accumulate more amines. Electron microscopy revealed the presence of giant and highly altered secretory vesicles with little electrodense inner matrix. But, what are the proteins responsible for this tinny electrodense matrix? Proteomic analysis of the enriched LDCV fraction from the adrenal medulla of the CgA/CgB-KO mouse (Diaz-Vera et al., 2012) shows no significant changes in the amount of SgII or other, surprisingly significant amounts of fibrinogen are detected, for which the three chains (α, β, and γ) are only present in the LDCVs of CgA/CgB-KO mice. In addition to its crucial role in clot formation, fibrinogen has been associated with the sorting of constitutively secreted vesicles (Glombik, Kromer, Salm, Huttner, & Gerdes, 1999). However, no other protein appears to be capable of fulfilling the functional role of CgA/CgB as a matrix condenser for soluble intravesicular components. While the concentration of CA accumulated in CgA/CgB-KO chromaffin vesicles was significantly reduced, it remained above to what is required for reaching isotonicity with the cytosol. As such, we cannot rule out the possibility that other components of the vesicular cocktail, such as ATP (Kopell & Westhead, 1982) and/or H^+ (Camacho, Machado, Alvarez, & Borges, 2008, Camacho, Machado, Montesinos, Criado, & Borges, 2006), contribute to the maintenance of amine accumulation. Since their discovery, CgA/CgB have captivated the attention of scientists and they have been implicated in several processes, including granule biogenesis and sorting, the production of bioactive peptides, tumor marking,

and the pathophysiology of neurodegenerative diseases (Bartolomucci et al., 2011). New data from KO mice provides direct evidence implicating CgA/CgB in vesicular storage and in the exocytotic release of CAs. Secretory events are maintained, even in the complete absence of CgA/CgB. Conversely, although LDCV biogenesis is preserved, the saturation of vesicular storage capacity is largely impaired. Protein analysis of the secretory vesicle fraction revealed the compensatory overexpression of CgA in the absence of CgB, and vice versa. Unexpectedly, other proteins that are apparently unrelated with secretion were only present in the adrenomedullary tissue of CgA/CgB-KO animals.

In conclusion, granins are crucial for the aggregation of vesicular content allowing the high concentration of CA and other soluble species. This association is largely responsible for the kinetic of exocytosis found at single-event level.

4. CST (HUMAN $CGA_{352–372}$) INDUCES LIPOLYSIS AND FATTY ACID OXIDATION THROUGH REGULATION OF ADRENERGIC AND LEPTIN SIGNALING

The secretory proprotein CgA gives rise to several peptides of biological importance, which include the dysglycemic hormone pancreastatin (PST: $CHGA_{250–301}$), the vasodilator vasostatin ($CHGA_{1–76}$), and the antihypertensive, antiadrenergic, cardiosuppressive, and angiogenic peptide catestatin (CST: $CHGA_{352–372}$) (Bartolomucci et al., 2011; Helle, 2004; Taupenot et al., 2003). The increased adiposity in hyperadrenergic, hyperleptinemic, and insulin-sensitive CgA-KO mice is believed to be due to resistance to CAs and leptin. Since CST inhibits CA secretion and CA inhibits leptin secretion, we reasoned that CST would reduce obesity by restoring adrenergic receptor (AR) and leptin receptor (Ob-R) sensitivity through normalization of CA and leptin levels (Fig. 5.1).

4.1. CST suppresses lipid accumulation by inhibiting α-adrenergic activity in a leptin receptor-dependent manner

The key hormones that control fat deposition in adipose tissue are CAs, leptin, and glucocorticoids. While leptin inhibits lipogenesis (Buettner et al., 2008; Ramsay, 2003), it can promote lipolysis via stimulation of CA production (Shibuya et al., 2002; Takekoshi et al., 1999). However, it may lead to desensitization of AR. Similarly, glucocorticoids stimulate epinephrine

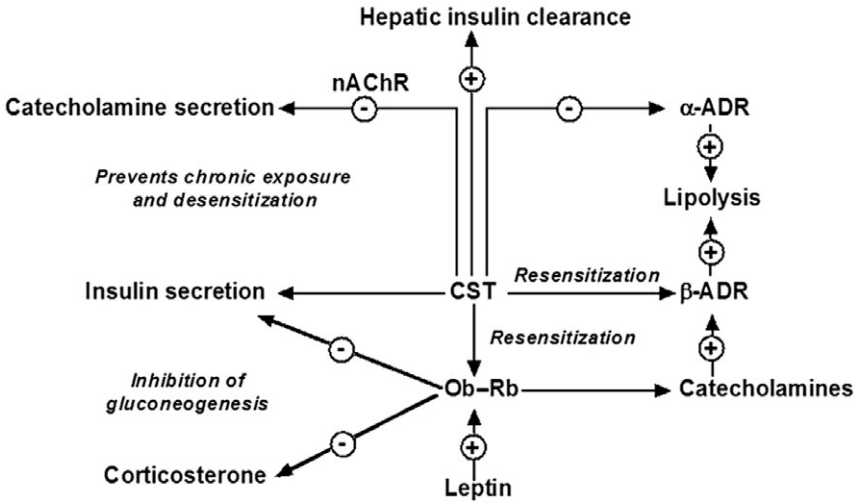

Figure 5.1 Schematic diagram showing the action of the CGA-derived peptide catestatin.

synthesis in the adrenal medulla (Hodel, 2001; Wurtman & Axelrod, 1966); it also leads to desensitization of AR functions, causing fat accumulation. Therefore, ultimately, it boils down to maintenance of AR and Ob-R sensitivity that would dictate the extent of fat mobilization in adipose tissue because leptin via Ob-R would inhibit lipogenesis and CA via AR would promote lipolysis. Desensitization of AR and Ob-R will lead to fat deposition. Our preliminary results led us to believe that CST may help maintain sensitivity of AR and Ob-R. The increased fat mass in hyperadrenergic CgA-KO mice (Gayen et al., 2009) reflects β-ARs desensitization by increased plasma CAs (Mahapatra et al., 2005). Since CAs are known to inhibit leptin secretion (Couillard et al., 2002; Fritsche et al., 1998; Scriba, Aprath-Husmann, Blum, & Hauner, 2000), β-AR desensitization may prevent such inhibition and lead to increased leptin level along with the increased adipose mass as found in CgA-KO mice and other obese models. Chronic hyperleptinemia in turn may desensitize Ob-R and perpetuate the obese phenotype. Therefore, we reasoned that CST could break this cycle and reduce obesity by restoring AR and Ob-R sensitivity through normalization of CA and leptin levels. Indeed, we found that chronic CST administration to obese CgA-KO mice resulted in a dramatic lean phenotype. This decrease in fat content in adipose tissues of CgA-KO mice was accompanied by increased glycerol and nonesterified fatty acid levels in plasma, suggesting increased lipolysis. CST treatment of CgA-KO mice

reduced hyperleptinemia and restored leptin action by reversing the desensitization effect of chronic leptin excess as evidenced by improved leptin signaling via phosphorylation of AMPK and Stat3. Lipolysis is dependent on functions of AR (β1, β2, β3, and α2 subtype) and Ob-R. We observed that CST does not directly stimulate or interfere with the β1-AR and β2-AR-activated cAMP production but suppresses CA production *in vivo* and indirectly reduces β-AR activation. On the other hand, CST can interfere with α2-AR-mediated phospholipase C activation (Bandyopadhyay et al., 2012). Both the nonspecific α-AR antagonist phentolamine and CST potentiated the lipolytic effects of nonspecific β-AR agonist isoproterenol in primary adipocytes. These findings suggest that CST mimics the lipolytic effect of the α1-AR and α2-AR antagonist phentolamine. The fact that CST has phentolamine-like effect and inhibits the action of α1-AR agonist phenylephrine suggests that CST acts by suppressing α-AR signaling. In the liver, CST enhanced expression of genes involved in fatty acid oxidation such as, acyl-CoA oxidase 1 (Acox1), carnitine palmitoyltransferase 1a (Cpt1-α), uncoupling protein 2 (Ucp2), and peroxisome proliferator-activated receptor-α (PPAR-α). In contrast, CST did not alter the expression of lipogenic genes such as sterol regulatory element-binding protein 1 (Srebp-1) and PPAR-γ. While both the liver and adipose tissue in CST-treated mice showed increased palmitate oxidation, there was a decrease in palmitate uptake in adipose tissue, prompting us to believe that CST inhibits the expansion of adipose tissue but promotes fatty acid oxidation in both tissues. Overall, CST appears to promote lipid flux from adipose tissue toward liver for catabolism. CST treatment also reduced body weight and adipose mass in diet induced obesity (DIO) mice without reducing food intake. Interestingly, CST could enhance leptin effects on adipose tissue metabolism and signaling in both DIO and leptin-deficient *Ob/Ob* mice (Bandyopadhyay et al., 2012). Our findings suggest that the reduction in fat mass after chronic CST treatment is due to increased lipolysis and lipid mobilization through CST action on α2-AR and leptin receptor (Fig. 5.1).

4.2. Metabolic effects of PST deficiency versus CST deficiency in mice

With the generation of CgA-KO and CST-specific KO mice (Mahata et al., unpublished), it is now possible to distinguish the metabolic effects of PST and CST. While the enhanced insulin sensitivity in obese CgA-KO mice is caused by PST deficiency, obesity due to increased fat mass is due to CST deficiency. Hypertension in hyperadrenergic CgA-KO mice is also caused

by CST deficiency. PST deficiency reduces production of inflammatory cytokines (IL-6 and MCP-1), which supports better insulin sensitivity in *CgA*-KO mice. On the other hand, both PST and CST suppress leptin production. As would be expected, CST deficiency alone in CST-KO mice creates obesity, hypertension, hyperleptinemia, and insulin resistance, all of which get corrected after CST supplementation to CST-KO mice. These results confirm the notion that increased circulatory levels of PST or decreased level of CST would cause insulin resistance and other metabolic disorders.

Age-related developmental changes in CgA-KO and CST-KO mice also reveal an interesting relationship between PST and CST in terms of their metabolic effects. Normally, young mice with low body weight (1–2 months old) are more insulin sensitive than their adult (3–4 months old) counterparts, as judged by glucose tolerance test (GTT) and insulin tolerance test (ITT). Young CST-KO mice (1–2 months old) remain insulin sensitive but become insulin resistant when they are 3–4 months old. However, 3–4-month-old CgA-KO mice, despite of CST deficiency, are more insulin sensitive than wild-type counterparts or CST-KO mice of same age group. PST supplementation to CgA-KO mice causes changes in the phenotype from insulin sensitivity to insulin resistance. It appears that the effects of PST deficiency play predominant role over the effects of CST deficiency in CgA-KO mice. However, by the time they are 6 months old, CgA-KO mice start becoming insulin resistant, suggesting that the deficiency of CST begun to create an impact at an older age overriding the effects of PST deficiency.

4.3. Effects of CST on insulin metabolism

CST-KO mice have higher circulating insulin level compared to wild-type mice. Glucose-stimulated insulin secretion in CST-KO mice is impaired, which goes along with the insulin-resistant phenotype of these mice. Supplementation of CST to CST-KO mice reduces hyperinsulinemia and improves insulin sensitivity. These results suggest that CST may inhibit insulin secretion. Surprisingly, treatment of cultures of insulin-producing cell line, INS-1, with CST did not inhibit insulin secretion. Instead, CST stimulated insulin secretion from those cells independent of glucose challenge. Therefore, it is possible that CST may enhance hepatic insulin clearance and help maintain a balance in insulin level under physiological conditions.

5. VGF-DERIVED PEPTIDE TLQP-21: FUNCTIONAL ROLE OF A NOVEL PROLIPOLYTIC PEPTIDE

After the original identification of VGF gene in PC12 cells, the focus has been for several years the regulation of gene expression and propeptide structure and regulation of neurotrophins (Salton, Ferri, Hahm, Snyder, & Wilson, 2000). More recently, the attention has moved to the biological role of VGF propeptide. Concurrently, VGF peptides/fragments are increasingly investigated as disease biomarkers and druggable targets for human diseases (Bartolomucci et al., 2011). Here, we will briefly summarize the current knowledge on VGF peptides, with a special focus on the structure and function of the internal fragment named TLQP-21, by discussing three major findings: (1) The germline VGF knockout mouse model revealed a major nonredundant role for VGF in obesity and energy homeostasis; (2) TLQP-21 peptide centrally increases catabolic pathways and prevents obesity, while NERP-2 peptide increases food intake; (3) TLQP-21 is localized in sympathetic nerve terminals in the adipose tissue where it exerts a prolipolytic effect.

The first germline $VGF^{-/-}$ mice was developed in 1999 by Salton Lab on a mixed C57Bl6-129/SvJ background (Bartolomucci et al., 2011 for review). A second line was developed more recently by Regeneron Pharmaceuticals Inc. in collaboration with Dr. Salton on a pure C57BL6J background (Watson et al., 2009). The only difference reported between the two knockout lines so far is that the original line in the mixed background showed hyperlocomotion, which is not present in the Regeneron's line. Overall $VGF^{-/-}$ mice are characterized by smaller dimension than wild-type littermates, which resulted in 40–60% lower body weight already at weaning. In addition to being smaller, VGF-deficient mice were lean and hypermetabolic by showing ~50% more O_2 consumption than wild types. As a result, ablation of the VGF gene blocked the metabolic effects of the high-fat diet on body and fat-pads weight. An elegant set of experiments demonstrated that VGF ablation prevents obesity in a variety of neurotoxic or transgenic models including GTG, leptin deficiency, and agouti overexpression. In contrast, targeted deletion of the VGF gene had little influence on the ability of monosodium glutamate (MSG) treatment to increase body weight (Bartolomucci et al., 2011 for review). MSG damages the hypothalamus and SNS, thus suggesting that VGF has a functional role in projections of the SNS that innervate metabolic tissues downstream of

hypothalamic/autonomic centers. More recent studies demonstrated a major role of VGF deletion in insulin resistance and glucose tolerance as well as in the morphology and function of the brown and white adipose tissue in mice (Fargali et al., 2012; Watson et al., 2009). The phenotype of the VGF-KO mice illuminated a prominent and nonredundant functional role of this granin protein in the regulation of metabolic functions. However, the VGF gene encodes a precursor protein of 615 (human) and 617 (rat, mice) amino acids (Bartolomucci et al., 2011; Salton et al., 2000). A major feature of VGF is the presence of specific sequence with basic amino acid residues that represent potential cleavage sites for proprotein convertases of the kexin/subtilisin-like serine protease family. The positions within the VGF polypeptide of each of these 10 pairs of basic residues are highly conserved across species. Upon processing, VGF may yield a number of peptides that are stored in dense-core granules and secreted through the regulated pathway (Salton et al., 2000). By convention, the VGF-derived peptides are designated by the four N-terminal amino acids and the total length. Up to now, seven VGF peptides were shown to possess biological activity. Thus, it is difficult to predict or investigate the relative contribution of each VGF peptide based on the phenotype of the VGF-KO mice. Therefore, most of the more recent works used primarily a pharmacological gain of function approach to study their mechanism of action (reviewed in Bartolomucci et al. (2011). Paper published after the publication of the review are noted): TLQP-62 increase the synaptic charge in hippocampal neurons and modulate depression-like behavior and neurogenesis downstream of brain-derived neurotrophic factor; AQEE-30 and LQEQ-19 facilitate penile erection in rats and evoked thermal hyperalgesia; neuroendocrine regulatory peptide (NERP)-1 and NERP-2 dose-dependently suppress vasopressin release, while NERP-1 increases penile erection (Melis, Sanna, Succu, Ferri, & Argiolas, 2012), and NERP-2 modulates insulin release and increases food intake and energy expenditure (Moin et al., 2012); finally, TLQP-21 centrally modulates gastroenteric functions, increases gonadotropin release, and modulates stress-induced CA release (Razzoli et al., 2012), centrally and peripherally regulates energy homeostasis and feeding (Possenti et al., 2012) and inflammatory pain, facilitates glucose-induced insulin release and improves glucose tolerance (Stephens et al., 2012), and, finally, increases the mobilization of intracellular calcium in cerebellum granule cell culture and GH3 cell line (Petrocchi Passeri et al., 2013). We will focus here on the metabolic role of the TLQP-21 peptide.

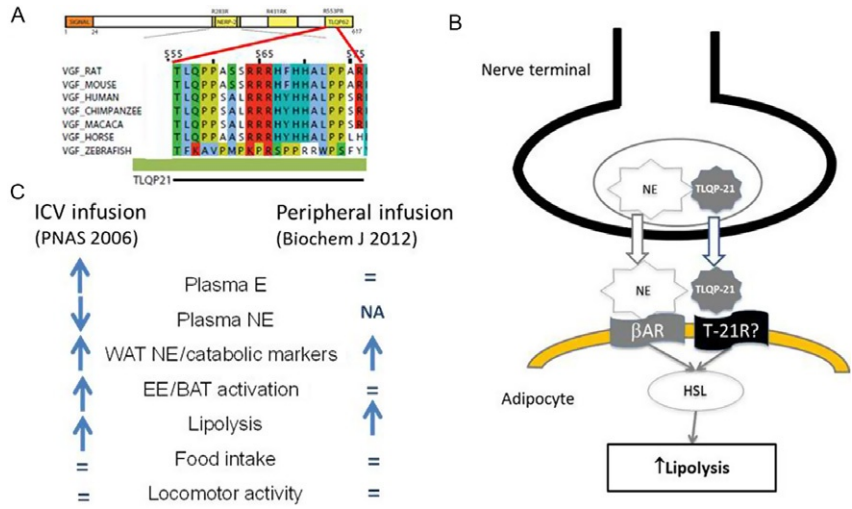

Figure 5.2 Overview of VGF-derived peptide TLQP-21. (A) Primary sequence of VGF showing the amino acid sequence in several mammals and zebrafish. (B) Cartoon of the proposed mechanism of action of TLQP-21 in the mouse adipose tissue. (C) Summary of the central and peripheral metabolic effects exerted by the TLQP-21 peptide.

TLQP-21 has been identified in rat brain homogenates (Bartolomucci et al., 2006; Fig. 5.2). TLQP-21 appears to derive from further processing of TLQP-62, an abundant VGF C-terminal peptide that is produced inside dense-core vesicles by PC1/3. Cleavage occurs at a site that does not conform to the classical PC1/3 dibasic target motif. We also recently demonstrated the presence of both TLQP-21-ir and VGF C-terminal peptides-ir in sympathetic fibers innervating the white adipose tissue (WAT) (Possenti et al., 2012). Anti-TLQP-21-ir and C-terminal peptides-ir colocalized extensively in nerve fibers. However, we also observed fibers that were anti-AQEE-30 positive and anti-TLQP-21 negative (but not vice versa), which support a discrete processing of C-terminal VGF peptides, and that TLQP-21 can be cleaved from its precursor TLQP-62 within neurite secretory vesicles. TLQP-21 is a highly hydrophilic peptide with a molecular mass of 2431 kDa, an isoelectric point of 12.96 and a net positive charge at pH 7. A recent NMR study demonstrated that contrarily to the bioinformatics prediction postulating the existence of an α-helix in the VGF-region of the propeptide that includes TLQP-21 (Garcia et al., 2005), the peptide is present in solution with a random coil while it acquires an α-helix secondary

structure only in presence of target cells expressing its putative receptor (Verardi et al., manuscript in preparation). Following its chemical identification, TLQP-21 was chronically delivered intracerebroventricularly in mice in two conditions, that is, with standard rodent chow and in high-fat diet (Bartolomucci et al., 2006). In mice fed a standard diet, TLQP-21 increased energy expenditure and rectal temperature, an effect that was paralleled by increased serum E or decreased NE level, being instead, independent from food intake, locomotor activity, and fT3 and fT4 serum level. TLQP-21 treatment lowered triglycerides and also increases the expression of catabolic markers in the WAT including increased PPAR-δ, β3-AR, and the brown adipocytes specific UCP1. Interestingly, these changes appear to be independent from changes occurring in major hypothalamic anorexigenic and orexigenic neuropeptides (Bartolomucci et al., 2006). We next hypothesized that TLQP-21 could affect energy balance when energy homeostasis is boosted by a hypercaloric high-fat diet. Indeed, TLQP-21 treatment prevented development of diet-induced obesity and normalized body weight gain and adiposity as well as obesity-induced increase in leptin and decrease in ghrelin. A subsequent study proved that TLQP-21 is particularly effective in preventing diet-induced obesity in a population of fast weight-gaining mice (Bartolomucci et al., 2009). Remarkably, intracerebroventricular administration of another VGF-derived peptide, NERP-2, increased body temperature, oxygen consumption, and locomotor activity in mice with an orexigenic mediated mechanism (Toshinai et al., 2010). At variance with TLQP-21, NERP-2 also increased food intake (Melis et al., 2012; Toshinai et al., 2010).

The VGF gene has a very wide patter on expression, which extends beyond the CNS and includes the sympathetic ganglia and several neuroendocrine cell lines (Salton et al., 2000). In this connection, we recently showed that TLQP-21 peptide is present in sympathetic nerve terminals innervating the adipose tissue, binds with high affinity a yet unknown receptor on adipocytes membranes, and increases lipolysis in the adipocytes downstream of the activation of β-ARs by isoproterenol (Possenti et al., 2012). Pharmacological treatment with TLQP-21 in diet-induced obese mice dose-dependently decreased adipocyte diameter and increased TG lipolysis. Decreased adipocytes diameter and increased lipolysis were paralleled by increased sympathetic tone in the adipose fat pads as demonstrated by increased enzymatic activity/immunoreactivity of tyrosine hydroxylase, the rate-limiting enzyme for the biosynthesis of CA, and the

neurotransmitter norepinephrine in both visceral and subcutaneous fat pads. These results *in vivo* suggest an interaction of NE and TLQP-21 in regulating adipocyte function. In support, TLQP-21 dose-dependently increases isoproterenol-induced phosphorylation of Hormone sensitive lipase (HSL) in 3T3L1 adipocytes and increased lipolysis in mature adipocytes.

Beside its functional role in the WAT, one of the major findings of Possenti et al. (2012) study is the identification of the first receptor-mediated mechanism for a VGF-derived peptide. Research on granin-derived peptides has been so far constrained by the poor understanding of receptor-mediated mechanisms (discussed in Bartolomucci et al., 2011). Here, we identified for the first time a selective and saturable TLQP-21-receptor binding site and demonstrated that the highest affinity and Bmax is present in different adipose fat pad membrane preparations (Possenti et al., 2012). In line with data generated with other cell types (reviewed in Bartolomucci et al., 2011), we proposed that TLQP-21 will bind a seven transmembrane-spanning domain receptor and lead to intracellular calcium mobilization in adipocytes (Possenti et al., 2012). The mechanism of action may be cell specific because a recent study showed that TLQP-21 can increase cAMP in rat pancreatic islets (Stephens et al., 2012).

6. CONCLUSION

In conclusion, recent studies strengthen our appreciation of the CAs/peptide interaction, which occurs at multiple sites and regulates key biological processes having a major pathological significance. The knowledge gathered so far on the role played by granins (e.g., CGA and CGB), their proteolytic products (e.g., CST and TLQP-21), and neuropeptide modulators released by SNS (e.g., PACAP) would contribute to and provide novel mechanistic support for the physiological role of CAs in human pathophysiology. These neuromodulatory peptides have the potentials to be new druggable targets amenable to treat in a safer and more efficacious way disease as diverse as hypertension, adrenal insufficiency, obesity, and type 2 diabetes in which a dysfunctional SNS and CA balance is known to play a major role.

CONFLICT OF INTEREST

The authors declare there are no conflicts of interest.

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