Differential distribution of VGF-derived peptides in the adrenal medulla and evidence for their selective modulation

Filomena D'Amato¹, Barbara Noli¹, Carla Brancia¹, Cristina Cocco¹, Giovanna Flore², Maria Collu², Paola Nicolussi³ and Gian-Luca Ferri¹

¹NEF-Laboratory, Department of Cytomorphology, ²Department of Neuroscience, University of Cagliari, I-09042 Monserrato (Cagliari), Italy

³Istituto Zooprofilattico Sperimentale, I-07100 Sassari (SS), Italy

(Correspondence should be addressed to G-L Ferri; Email: ferri@unica.it)

Abstract

While *vgf* gene knockout mice are hyperactive and hypermetabolic, surprisingly the TLQP-21 brain VGF peptide increased energy consumption, suggesting that opposing regulatory effects could be exerted by peptides alternatively cleaved from the VGF precursor. Using antisera to the VGF precursor C-terminus and three cleavage products, we revealed a distinct differential distribution in adrenal, certain peptides (VGF_{422–430}: PGH peptides) being found throughout bovine and swine medulla, while C-terminus and TLQP peptides were confined to adrenaline cells in the above species and in rat and C-terminally shortened forms (VGF_{604–612}: HVLL peptides) to nor-adrenaline cells. Random abattoir samples of bovine and swine adrenal contained 520 ± 40 and 450 ± 60 pmol/g (mean $\pm$ S.E.M. respectively) of C-terminus peptides and similar or lower amounts of others. Upon gel chromatography, *bona fide* VGF precursor, ~ 7.5 and

~ 3.5 kDa forms were revealed by C-terminus assays, HVLL peptides being limited to small fragments. TLQP peptides included ~ 7.5 kDa form and peaks accounting for TLQP-21 and predicted TLQP-30 and TLQP-42. Low molecular weight (MW) PGH peptides were revealed, together with a high MW form possibly encompassing the VGF precursor N-terminus. In acutely stressed swine, a striking increase was seen for C-terminus and TLQP peptides, with no significant differences for PGH peptides. A similar response was found in rat TLQP peptides showing a major increase upon an acute swimming stress and 30 min thereafter. A differential processing of the VGF precursor encompassing many areas of its primary sequence and selective modulations of its derived peptides occur in adrenal medullary cells, possibly relevant to adaptive homeostatic responses.

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Introduction

The *vgf* (non-acronymic) gene was discovered because of its distinct, selective up-regulation by nerve growth factor in the PC12 rat pheochromocytoma cell line (Levi *et al.* 1985) and proved to be selectively expressed in a range of neuroendocrine cell types (Ferri *et al.* 1992, Salton *et al.* 2000, Levi *et al.* 2004). The primary gene product, VGF protein or VGF precursor, is composed of 617 or 615 amino acids (in rat/mouse and man respectively, $>85\%$ identity, ~ 65 kDa calculated molecular weight (MW)) and migrates as ~ 90 kDa MW band in western blot (Salton *et al.* 2000). The primary sequence of VGF shows at least ten short stretches of basic amino acid residues, highly conserved across species, which could be target sites for endoprotease activity, with cleavage to smaller VGF peptides (Salton *et al.* 2000). The 30 amino acid 'peptide V' was isolated from bovine posterior pituitary (Liu *et al.* 1994), and, upon sequencing of the relevant region of human (Canu *et al.* 1997, Salton *et al.* 2000) and bovine VGF (accession No. XP_875466.2), proved to correspond to the 30 amino acid portion of VGF

comprised between the Arg₅₈₄–Arg₅₈₅ (numbering according to human VGF) putative cleavage site and the C-terminus. In rat brain, lower MW peptides (10, 18 and 20 kDa) were detected by an antiserum against the C-terminal VGF nonapeptide (Trani *et al.* 1995, 2002). Processing at the rat VGF Arg₅₅₃–Pro₅₅₄–Arg₅₅₅ cleavage site resulted in further VGF peptides isolated from rat brain and called TLQP peptides from their N-terminal sequence (Trani *et al.* 2002). When VGF peptide release in response to secretory stimuli was studied *in vitro*, low MW C-terminal peptides were released preferentially, as opposed to high MW forms (Possenti *et al.* 1989, 1999, Trani *et al.* 1995).

VGF-deficient (knockout) mice are hyperactive and hypermetabolic, with altered energy homeostasis, decreased body weight and especially body fat, and a deranged hypothalamic response to feeding (Hahm *et al.* 1999, 2002). Nonetheless, intracerebroventricular injection of the VGF precursor-derived TLQP-21 brain peptide increased resting energy expenditure, body temperature and circulating adrenaline levels (Bartolomucci *et al.* 2006), indicative of a stimulatory action on the autonomic nervous system and

adrenal medulla. In addition, when TLQP-21-treated mice were fed a high-fat diet, their expected increase in body weight and white adipose tissue was prevented (Bartolomucci *et al.* 2006). On the whole, *vfg* gene inactivation and the administration of its derived TLQP-21 peptide product showed surprising similarities, suggesting that different VGF peptides, out of the many which are or can be produced by differential cleavage of VGF, may well have opposing activities.

In the rat adrenal, immunoreactive medullary cells were seen using antisera to VGF-fusion proteins encompassing extended portions of the VGF precursor (Ferri *et al.* 1992), while VGF mRNA was low compared with pituitary and hypothalamus (Salton *et al.* 1991, Ferri *et al.* 1992). Upon reserpine treatment, VGF mRNA showed a striking increase lasting for days 1–5 (earliest and latest time points studied), probably mediated by sympathetic stimulation via the splanchnic nerve (Laslop *et al.* 1994). In western blot of rat adrenal extracts, a rat VGF C-terminus (VGF_{609–617}) antiserum revealed intact VGF, but little low MW forms (Trani *et al.* 1995). In humans, an immunocytochemical study using human VGF C-terminus and rat VGF_{422–430} antisera showed labelled medullary cells from the tenth week of gestation, as well as in adult adrenal and its medullary tumours (Rindi *et al.* 2007).

In view of the crucial involvement of the adrenal in the adaptive responses and in the regulation of energy homeostasis, we aimed to study the VGF peptides it contained. In order to specifically address VGF processing and its derived peptides, we used antisera to the C-terminus of rat and human VGF, as well as to three amino acid stretches adjacent to demonstrated or putative VGF precursor cleavage sites.

Materials and Methods

Tissue samples and processing

Adrenal glands were collected at random from a local abattoir from bovine (male and female, 7–11 months, $n=14$) and swine (female and castrated male, 7–9 months, $n=22$). Samples of liver and spleen were taken as control tissues. All animals were from local farms (travel time to abattoir 0.5 to ~3 h) and routinely received a limited amount of food on the day preceding slaughter to facilitate butchery procedures. In view of the stress involved in transportation and animal regrouping, we also identified a group of swine ($n=102$, 65–7 months, raised at one and the same farm by crossing Pietrain halothane free sires with Landrace sows), which was to be stabulated at a local abattoir site for over 3 days after transportation. Animals were led to a clean butchery line early on the following Monday, and adrenals were taken from animals butchered first ($n=6$) and last ($n=6$), so that animals stayed in line for 1–5 and 25–30 min respectively, before being stunned with electricity and ejugulated. In connection with butchery procedures, tissues were made available between 40 and 55 min after ejugulation in all cases.

Individual rats from a first random sacrifice group were taken from their cages (two to four rats/cage) in sequence and were killed by decapitation ($n=10$, Sprague–Dawley rats of either sex, 250–300 g body weight). The effect of stress was further studied in rats (250–350 g body weight of either sex: female rats underwent vaginal smears and were used when in dioestrus). These were either rapidly moved to a killing box containing diethyl ether vapour ($n=10$, minimum stress group: rats from each two to three animals per cage were moved to the killing box at once within 5–10 s), similarly killed after an acute swimming stress (5 min duration, 25 °C water, $n=12$, acute stress group) or after the same stress followed by a 30 min recovery period ($n=6$, 'stress + recovery' group). Experimental protocols were approved by the Ethical Committee at the University of Cagliari and were performed in accordance with the care and use of animals approved by the American Physiological Society and EEC Council Directive of 24 November 1986 (86/609).

For immunocytochemistry, thin slices of bovine/swine adrenal (2–3 mm) or rat adrenals (cut into two) were immersion-fixed in paraformaldehyde (40 g/l in PO₄ buffer, 0.2 mol/l (pH=7.2); 3 h at 0–4 °C), washed with PBS containing 70 g/l sucrose and 0.2 g/l NaN₃, oriented in aluminium foil moulds with cryoembedding media and snap frozen (Cocco *et al.* 2003), hence stored in a liquid nitrogen tank (vapour phase). Cryosections (5–7 µm) were obtained using a Microm HM-560 cold-blade cryomicrotome, collected on slides pre-treated with poly-L-lysine and stored as above.

For peptide extraction, adrenal samples were weighed, coarsely minced with a scalpel, dropped into pre-heated polypropylene tubes containing distilled water (~10 ml/g tissue), kept in a vigorously boiling water bath for an additional 10–15 min, hence homogenized using an Ultra Turrax (3–5 min; Ika-Werke, Staufen, Germany) and centrifuged (10–15 min, 3000 g). Rat adrenals, and some parallel samples from bovine and swine, were homogenized fresh in the presence of protease inhibitor cocktail (Sigma), briefly spun, hence supernatants were boiled as above. Extracts were kept frozen until used (–20 °C or lower).

VGF peptide antibodies

VGF antisera were raised against peptide sequences at the VGF C-terminus or adjacent to three dibasic cleavage sites (Fig. 1, while all antisera are listed in Table 1). At the C-terminal end of rat and mouse VGF, a His₆₁₅-Arg₆₁₆-Pro₆₁₇ sequence is found, as opposed to Arg₆₁₃-Arg₆₁₄-Pro₆₁₅ in man (Salton *et al.* 2000), hence antisera were raised against rat VGF_{609–617} (Ferri *et al.* 1995) and human VGF_{607–615} (Brancia *et al.* 2005) conjugated via their N-terminal tyrosine. Since the above Arg₆₁₃-Arg₆₁₄ sequence might also function as a cleavage site, the preceding decapeptide (human VGF_{603–612}) was synthesized and conjugated at its N-terminus (Biomol, Exeter, UK). TLQP peptides were isolated from rat brain and proved to be cleaved from VGF at

Figure 1 VGF and antisera. Peptides and fusion protein used to raise antisera and their short names (see Table 1) are plotted onto a schematic outline of the VGF precursor. Arrowheads are directed according to expected antiserum reactivity; 'R' demonstrated or putative processing sites (two or more Arg/Lys basic residues); 'pR', Arg-Pro-Arg processing site (Trani *et al.* 2002); 'agR', putative processing-amidation site, Ala-Gly-Arg-Arg; last 3 C-terminal amino acids are indicated in single-letter notation ('Arg' residue, differing in human versus rat/mouse, is underlined); 'hu', human; 'hu C-terminus -3aa', human VGF₆₀₃₋₆₁₂, identical to rat VGF₆₀₅₋₆₁₄.

the rat VGF₅₅₃₋₅₅₅ (Arg₅₅₃-Pro₅₅₄-Arg₅₅₅) processing site (Trani *et al.* 2002), at least their N-terminal five amino acids (TLQPP or Thr-Leu-Gln-Pro-Pro) being identical in human and rat VGF. The rat VGF₅₅₆₋₅₆₅ peptide was synthesized and conjugated at its C-terminus to expose the N-terminal region during immunization (Brancia *et al.* 2005). A fusion protein composed of a 21 amino acid peptide (rat VGF₅₅₆₋₅₇₆ or rat TLQP-21) plus glutathione S-transferase (GST, from *Schistosoma japonicum* species) linked via the peptide's N-terminal 'T' (threonine) was obtained using the pGEX-4T-3 plasmid vector (Amersham Pharmacia), and its derived antiserum was used in immunohistochemistry (Brancia *et al.* 2005). The rat VGF₄₂₂₋₄₃₀ peptide, as well as the human VGF₄₁₉₋₄₂₇ peptide

(which shows a single amino acid difference compared with bovine, as from accession no. XP_875466.2, an Arg residue being found at position -7 from C-terminus in bovine, as opposed to a Gln residue in human), was synthesized (Biomol) and conjugated via an N-terminal tyrosine (Ferri *et al.* 1995).

Immunohistochemistry

Slides were treated with Triton X-100 (Merck; 1 g/l in H₂O, 1 h), washed with PBS (10 mmol/l PO₄, 150 mmol/l NaCl (pH 7.2-7.4)) and incubated overnight (at room temperature) with primary antiserum/a diluted in PBS containing 30 ml/l

Table 1 Primary antisera used

	Short name	Use	Species	Reference/producer
Antigen				
Human VGF ₆₀₇₋₆₁₅	VGF C-term. (hum)	I, E	Rabbit	Brancia <i>et al.</i> (2005)
Rat VGF ₆₀₉₋₆₁₇	VGF C-term. (rat)	I, E	Rabbit	Ferri <i>et al.</i> (1995) and Trani <i>et al.</i> (1995, 2002)
Human VGF ₆₀₃₋₆₁₂	HVLL peptides	I,E	Rabbit	This paper
Rat VGF ₅₅₆₋₅₆₅	TLQP peptides	I,E	G-pig	Brancia <i>et al.</i> (2005)
GST-Rat VGF ₅₅₆₋₅₇₆	GST-TLQP-21	I	G-pig	Brancia <i>et al.</i> (2005) and Bartolomucci <i>et al.</i> (2006)
Human VGF ₄₁₉₋₄₂₇	PGH peptides (hum)	E	Rabbit	This paper
Rat VGF ₄₂₂₋₄₃₀	PGH peptides (rat)	I, E	Rabbit	Rindi <i>et al.</i> (2007); This paper
Dopamine β-hydroxylase	DBH	I	Rabbit	Biomol
Dopamine β-hydroxylase	DBH	I	Mouse	Chemicon
Phenylethanolamine-N-methyl transferase	PNMT	I	Rabbit	Biomol
Phenylethanolamine-N-methyl transferase	PNMT	I	Sheep	Chemicon

GST, Glutathione S-Tranferase; G-pig, guinea pig; use: I, immunohistochemistry; E, ELISA; Biomol, Exeter, UK; Chemicon, Temecula, CA.

normal serum of the second antibody donor species (donkey), 30 ml/l normal serum of the species being immunostained (bovine, swine or rat respectively) and 0.2 g/l NaN_3 . Secondary incubations (60 min, room temperature) were carried out with affinity purified donkey antibodies to the relevant species' IgG, conjugated with cyanine 3 (Cy3), cyanine 2 (Cy2) or AMCA (Jackson ImmunoResearch, West Grove, PA, USA). For double and triple immunostaining, antisera to VGF peptide/s and/or catecholamine synthesizing enzyme/s (Table 1), each raised in a different species, were mixed followed by the relevant mixture of secondary labelled antibodies. Slides were coverslipped with PBS–glycerol (~50/50%) and were observed and photographed using BX41 and BX51 fluorescence microscopes (Olympus, Milan, Italy) equipped with Fuji S2 and S3 Pro digital cameras (Fujifilm, Milan, Italy). Immunocytochemical controls included substitution of each antiserum/antibody, in turn, with PBS, as well as the use of single primary antiserum with inappropriate secondary antibodies. For absorption controls, each VGF antiserum was pre-incubated overnight with its own antigen in a range of concentrations (0.03–100 mol/l, at 0–4 °C), hence was used for the primary incubation. The highest (~2–5) concentrations of homologous antigen resulted in virtually complete prevention of labelling with each of the VGF antisera used.

ELISA

Assays were set up with the six antisera raised against synthetic VGF peptides (Tables 1 and 2). Multiwell plates (Nunc, Milan, Italy) were coated with the corresponding unconjugated synthetic peptide (5–50 nmol/l, in carbonate–bicarbonate buffer (pH 9.6); 4 h at 37 °C or 16 h at 0–4 °C), hence were treated with normal serum from the secondary antibody donor species (PBS–donkey, 90 ml/l). Primary incubations were carried out in duplicate, including relevant standards (0.005–500 nmol/l) or serially diluted samples (100 µl/well incubation volume, in PBS–donkey, 4 h at room temperature under constant agitation). After the relevant incubations (1 h each) with secondary antibody (Jackson) and streptavidin–peroxidase conjugate (Biospa, Milan, Italy), wells were incubated with tetramethylbenzidine substrate (100 µl/well: Sigma or Kem-En-Tec Diagnostics, Taastrup, Denmark), hence the reaction was stopped with HCl (1 mol/l, 100 µl/well) and optical density was measured at 450 nm using a multilabel plate reader (Chameleon: Hidex, Turku, Finland). PBS–Tween (0.5 ml/l Tween 20) was used for washing throughout. For all assays, when standard peptide was added to tissues before extraction, >75% recovery was shown. When parallel boiling–water and fresh–homogenized extracts or bovine/swine samples extracted after a different post-mortem delay (~40–55 min) were compared, results were comparable (within 80–120% of each other). Swine and bovine liver and spleen, extracted as negative control tissues, showed undetectable VGF peptide levels. Various synthetic peptides were used for assay characterization (Table 2).

Some experiments were carried out with swine and bovine plasma/serum as the unknown samples. Unfortunately, a high degree of interference was revealed by plasma/serum proteins, so that further developments will be required before circulating VGF peptides and their release can effectively be addressed. Statistical analysis was carried out by one-way ANOVA, followed by *post hoc* multiple comparison tests or *t*-test (StatistiXL software: www.statistiXL.com).

Chromatography

Extracts (~2 ml) were loaded onto a Sephadex G–50s column (2 cm² × 1 m; Sigma), equilibrated with 50 mM ammonium bicarbonate and eluted with the same buffer. A MW marker kit (MWGF70: Sigma) was used for column calibration. Collected fractions (3 ml) were reduced in volume using a Vacufuge Concentrator (Eppendorf, Milan, Italy). Recovery after chromatography ranged between 80 and 120% for all peptides tested.

Results

Adrenal medullary endocrine cells were labelled for VGF peptides in various numbers and intensities in the species tested, with no staining of cortical cells. Occasional immunoreactive nerve fibres were seen, but will not be addressed further here in view of their small numbers. Of the various VGF antisera used, those against human and rat C-terminus proved highly species selective and were reserved to bovine/swine and rat tissues respectively, while HVLL and TLQP peptide antisera were used across species. The rat PGH antiserum worked well in immunohistochemistry in the three species tested here and in humans (Rindi *et al.* 2007), hence was used throughout, except for ELISA of bovine and swine tissue extracts for which the human PGH antiserum was employed.

PGH immunoreactivity was abundant in the whole adrenal medulla of bovine and swine (Fig. 2A), with a lower number of scattered cells in rat (not shown). Conversely, VGF C-terminus (Figs 2B and F and 3A) and TLQP peptides (Figs 2C, E and 3B), as well as C-terminally shortened forms (HVLL peptides; Figs 2D and 3E) were found in restricted areas in all three species studied. In both swine and rat, the above overall distribution of VGF peptides was consistently observed across 'minimum stress' and stressed animals.

In all three species studied, TLQP-immunoreactive cells were most abundant, the TLQP peptide (Fig. 2C) and GST-TLQP-21 antisera (Fig. 2E) showing comparable cell populations and regional distributions. VGF C-terminus immunoreactivity was confined to a subfraction of TLQP-immunoreactive cells, staining intensity for either peptide being highly varied on a single-cell basis (Fig. 2F (C-terminus) versus E (TLQP)). Conversely, HVLL peptides (Fig. 2D) showed a virtually complementary distribution compared with either TLQP (Fig. 2C) or VGF C-terminus in

Table 2 Elisa characterization

Assay	Peptide	IC ₅₀ (pmol) ^a	% Cross-reactivity
VGF C-term. (hum)	hum VGF ₆₀₇₋₆₁₅	0.5	100
	rat VGF ₆₀₉₋₆₁₇	59	0.8
	hum VGF ₆₀₃₋₆₁₂ (HVLL)	132	0.4
VGF C-term. (rat)	rat VGF ₆₀₉₋₆₁₇	0.02	100
	hum VGF ₆₀₇₋₆₁₅	>200	<0.01
	hum VGF ₆₀₃₋₆₁₂ (HVLL)	>200	<0.01
HVLL peptides	hum VGF ₆₀₃₋₆₁₂ (HVLL)	0.3	100
	hum VGF ₆₀₇₋₆₁₅	28	1.0
	rat VGF ₆₀₉₋₆₁₇	8.7	3.4
TLQP peptides	rat VGF ₅₅₆₋₅₆₄	1.1	100
	rat VGF ₅₅₆₋₅₇₆ (TLQP-21)	0.6	183
	rat VGF ₅₅₆₋₅₆₇ (TLQP-11)	0.9	122
PGH peptides (hum)	hum VGF ₄₁₉₋₄₂₇ (TPGH)	0.5	100
	hum VGF ₄₁₉₋₄₂₈ (TPGH-R) ^b	50	1
	rat VGF ₄₂₂₋₄₃₀ (APGH)	2.5	20
PGH peptides (rat)	rat VGF ₄₂₂₋₄₃₁ (APGH-R) ^b	>1000	<0.05
	rat VGF ₄₂₂₋₄₃₀ (APGH)	0.3	100
	rat VGF ₄₂₂₋₄₃₁ (APGH-R) ^b	>2000	<0.015
	hum VGF ₄₁₉₋₄₂₇ (TPGH)	8.5	3.5
	hum VGF ₄₁₉₋₄₂₈ (TPGH-R) ^b	>2000	<0.015

^apmol per assay well, 100 µl incubation volume.^bFor PGH peptide assays, cross-reaction with VGF was addressed using peptides with an additional Arg residue at their C-terminus.

either bovine or swine. In rat too, HVLL-immunoreactive cells showed no co-localization with TLQP peptides, in spite of their low numbers (all groups).

When adrenaline cells were identified on the basis of their content of the adrenaline synthesizing enzyme phenylethanolamine-N-methyl transferase (PNMT; Fig. 3C), they also labelled with the TLQP peptide (Fig. 3B) or GST-TLQP-21 and VGF C-terminus antisera (Fig. 3A). Conversely, in all three species studied, HVLL immunoreactivity (Fig. 3E) was virtually confined to medullary cells reactive for dopamine β-hydroxylase (DBH) but not for PNMT (Fig. 3D (DBH) versus F (PNMT)), hence identified as nor-adrenaline cells. The picture outlined above was delineated using random abattoir samples of bovine and swine adrenal and random killed rats. When minimum stress and acutely stressed swine and rats were compared respectively, a consistent VGF peptide distribution was revealed in either species' adrenals, with somewhat higher immunostaining intensity for TLQP and C-terminus VGF peptides in stressed animal groups.

In ELISA assays, VGF C-terminus and PGH peptides were especially abundant in bovine and swine, while TLQP peptides were well represented in all three species tested (Table 3). Only small amounts of measurable HVLL-like peptides were found, with below detection levels in several samples (Table 3). It ought to be pointed out here that standard samples of bovine and swine adrenals were taken at random from a local abattoir, hence largely under acute stress conditions (see also below). In spite of marked inter-individual variability, especially for TLQP peptides, the latter and C-terminus VGF peptides showed a tendency to increase

in our random sacrifice rats, too, compared with the minimum stress group (Table 3, columns 3 and 4).

Upon gel chromatography, the VGF C-terminus antiserum (Fig. 4A) revealed a major peak close to the void volume, compatible with the expected migration of the VGF precursor, as well as lower MW forms of ~7.5 and 3.5 kDa. These were similar to peptides previously identified in rat brain and named VGF10, for the ~7.5 kDa form (Trani *et al.* 1995, 2002), or from bovine pituitary and named peptide V, for the ~3.5 kDa one (Liu *et al.* 1994). A high MW peak also compatible with VGF was found with the TLQP and HVLL peptide assays (Fig. 4B and D; see also below), at apparent concentrations in keeping with the minor cross-reaction with VGF, for the TLQP peptide antiserum (Brancia *et al.* 2005), and with the human VGF C-terminal nonapeptide, for the HVLL peptide assay (Table 2). Several distinct forms were revealed for TLQP peptides (Fig. 5B), including a major peak of ~7.5 kDa at least partly coincident with the one shown for VGF C-terminus (Fig. 5A), and compatible with a peptide running all the way to VGF C-terminus (~7.4 kDa in rat (Trani *et al.* 2002); ~7.5 kDa, as calculated from human VGF (Salton *et al.* 2000)). The overlapping peaks spanning ~2.5–5 kDa elution positions (Fig. 4B) could account for TLQP-21 (~2.4 kDa in rat (Bartolomucci *et al.* 2006) and ~2.5 kDa as calculated for human VGF (Salton *et al.* 2000)), as well as for the predicted peptides TLQP-30 and/or TLQP-42 that would result from cleavage at VGF₅₈₄₋₅₈₅ or VGF₅₉₅₋₅₉₆ Arg-Arg sites (numbering relevant to human VGF calculated MW ~3.5 and ~48 kDa respectively). The rat PGH antiserum showed minimal reactivity with rat and human C-terminally

Figure 2 VGF peptide immunolocalization. PGH peptides were revealed throughout the (A) adrenal medulla, while (B and F) VGF C-terminus, (D) HVLL peptide and (C and E) TLQP peptide immunoreactivity showed a restricted distribution. In all species, VGF C-terminus peptides were confined to a subfraction of TLQP-immunoreactive cells ((F) VGF C-terminus versus (E) GST-TLQP-21), while TLQP peptide and GST-TLQP-21 antisera showed a similar localization (C and E respectively) with varied labelling intensity. Conversely, C-terminally shortened (D) HVLL peptides and (C) TLQP peptides were virtually complementary in distribution in all species. Swine adrenal, double immunofluorescence (A–B, C–D, E–F); scale bars, 50 μ m.

extended peptides (APGH-R/TPGH-R: <0015% cross-reactivity, Table 2), hence chromatography fractions were assessed with such antiserum in order to minimize detection of VGF. While no reactivity was revealed at the elution

position of *bona fide* VGF precursor, a reproducible peak was found several fractions later, at ~45 kDa elution position (Fig. 4C), compatible with a PGH peptide encompassing the VGF N-terminus or nearby. Small MW peptides were also

Figure 3 Differential distribution in adrenaline versus nor-adrenaline cells. (B) TLQP peptides were shown in almost all cells immunostained for the adrenaline-synthesizing enzyme phenylethanolamine-N-methyl transferase (PNMT; C), many of which also labelled for the (A) VGF C-terminus. Conversely, in all three species studied, (E) HVLL immunoreactivity was confined to medullary cells reactive for dopamine β -hydroxylase (DBH; D) but not for PNMT (F), hence identified as nor-adrenaline cells. Swine adrenal, triple immunofluorescence (A–C, D–F); scale bars, 50 μ m (A–F).

seen in a broader band around 0.8–1.3 kDa (Fig. 4C). The high MW peak shown with the HVLL assay is likely to represent intact VGF, in keeping with the degree of cross-reactivity of VGF C-terminus peptides in such assay (Table 2). Hence, only small heterogeneous fragments would account for adrenal HVLL peptide immunoreactivity (Fig. 4D). In rat adrenal extracts, the TLQP and C-terminus peptides showed a profile comparable with that seen in swine and bovine with similarly migrating peaks, while the HVLL and PGH peptides were not tested in view of their low concentrations.

When adrenal extracts from minimum stress and acute stress swine were compared in parallel assays, clear-cut differences were revealed (Fig. 5). Adrenal extracts from the acute stress group were closely comparable with previous samples of swine adrenals taken at random after butchery from a local abattoir (no significant difference in either VGF C-terminus, TLQP or PGH peptides, while HVLL peptides were not assessed). When the minimum stress animals were set as the reference

control group, a striking fivefold increase was revealed for VGF C-terminus peptides, with an about threefold increase in TLQP peptides and no significant change for PGH peptides (Fig. 5, left panel). A similar pattern was seen in acutely stressed rats (Fig. 5, middle panel), with a clear-cut increase in TLQP peptides, which increased further after a 30-min recovery period (Fig. 5, right panel). Upon gel chromatography, no distinct changes were revealed in the relative proportions of the various molecular forms of TLQP (all species) and C-terminus VGF peptides (bovine and swine), across the minimum stress and stressed animals (not shown).

Discussion

On the basis of the present study, VGF and derived peptides are well represented in the adrenal gland of several mammalian

Table 3 VGF peptide concentrations (pmol/g wet tissue weight) in adrenal extracts. Values are mean ± s.e.m.

	Bovine random ^a	Swine random ^a	Rat random ^a	Rat minimum stress ^b
VGF C-terminus peptides ^c	520 ± 40	450 ± 60	9 ± 2*	4.9 ± 0.5*
TLQP peptides ^d	160 ± 40	358 ± 40	172 ± 48	97 ± 19
PGH peptides ^e	440 ± 88	210 ± 94	15 ± 7	14.1 ± 0.6
HVLL peptides ^f	<45	<30	<10	<10

n=7 swine, 15 bovine, 10 and 10 rats respectively. *P<0.005, other differences between two rat groups were not significant.

^aRandom samples from abattoir line or sequentially killed rats.

^bAll rats from each cage killed at the same time (see text).

^cHuman and rat C-terminus assays, for swine/bovine, and rat tissues respectively.

^dCalculated as rat TLQP-21 equivalent for all three species.

^eHuman TPGH and rat APGH assays, for swine/bovine and rat tissues respectively.

^fBelow detection in 20–30% of samples studied.

species. VGF has not been sequenced in swine and partly in bovine (accession No. XP_875466.2), bovine C-terminus and PGH peptide regions being almost identical to human VGF, while the TLQP peptide domain remains to be determined. The parallel distribution profiles we found in the three species studied argue for a largely conserved primary structure, relevant to immunohistochemical findings and to intra-species comparative results obtained by immunochemistry. Conversely, numerical values of tissue peptide concentrations measured ought to be considered with caution, pending sequencing of the relevant peptides, hence use of proper standards. In this respect, we used rat TLQP-21 as reference standard for all three species. In rat adrenal, we measured relatively low concentrations of VGF C-terminus peptides compared with those found in mouse brain (100–1500 pmol/g, depending on areas; Chakraborty *et al.* 2006). Pending further characterization of the degree of reactivity of all molecular forms involved, one ought to note that our antibodies were strictly selective for an intact C-terminus, removal of the last three amino acids or substitution of one, resulting in complete loss of reactivity. The AQEE peptide assay used by Chakraborty *et al.* (2006) was selective for an extended, at least 19 amino acid region upstream of the C-terminus, and the molecular forms involved were not determined.

A major finding of the present study is that different VGF peptide profiles were revealed for adrenaline versus nor-adrenaline medullary cells. Altogether, our findings argue for a variety of VGF peptides, derived from extended regions within VGF, being produced and probably released by the former cells. These would probably include a 62 amino acid form running from the TLQP region to the VGF C-terminus (similar to the VGF10, or TLQP-62 peptide found in rat brain; Trani *et al.* 2002), as well as shorter TLQP and C-terminal peptides respectively. The low amounts of small HVLL peptides we found, confined to nor-adrenaline cells, may represent degradation products derived from the C-terminal portion of VGF. Nonetheless, the abundance of PGH peptides throughout swine and bovine adrenaline as well as nor-adrenaline cells (and in fewer corresponding cells in the rat) suggests that such peptides may have a role in the

secretory repertoire of both cell types. PGH peptides share a common sequence and apparent abundance in the adrenal between at least bovine and human, both species also showing intermediate–high MW adrenal PGH peptides encompassing more or less extended domains towards the VGF N-terminus (Rindi *et al.* 2007).

A second major point is the rapid, differential inducibility of VGF peptides in adrenal medullary cells, with a major several fold increase in tissue concentrations of some such peptides, while others showed little change. As mentioned, a clear-cut induction of VGF mRNA was revealed in rat after the administration of reserpine, probably via activation of sympathetic nerves, together with a parallel rise in various secretogranins/chromogranins, as well as neuropeptide Y (Laslop *et al.* 1994). While the latter increase in VGF mRNA lasted throughout the study (days 1–5), the far shorter time frame of the changes we observed points out a very rapid capacity for response, well before novel mRNA synthesis is likely to be involved. Hence, a combination of increased VGF precursor biosynthesis from existing mRNA, as well as VGF precursor processing is likely to have occurred in our experimental set-up. While the above, longer term changes were considered to increase the secretory content of secretory vesicles under prolonged stimulation (Laslop *et al.* 1994), the rapid changes of certain VGF peptides could be involved in a promptly evoked short-term response. Further to the acutely stressed groups, our random sacrifice rats also showed a tendency to increase in TLQP peptides, the high variability observed being possibly related to a highly varied amount of stress, since animals from each cage were randomly killed in sequence. The significance of such higher TLQP and VGF C-terminus peptides and their involvement in local and/or systemic adaptive changes is presently unknown. Investigations focussed onto VGF peptide release into the bloodstream are warranted, aimed at addressing the possible role of such peptides as circulating mediators or hormones, as well as potential biomarkers in various stress conditions. As to the central nervous system counterpart, a significant increase in C-terminus-related VGF peptides was shown in mouse females, but not males, upon exposure to cold or to

Figure 4 Chromatographic profile of VGF peptides. Several antisera (A, B and D) showed a high MW peak compatible with intact VGF. Additional peptides migrated at ~7.5 and ~3.5 kDa, VGF C-terminus peptides at ~7.5 kDa and in the ~5–2.5 kDa region for TLQP peptides, or as low MW forms (1.3–0.8 kDa, PGH and HVLL peptides). The rat PGH peptide antiserum showed no VGF-like forms, in keeping with its very low reactivity with C-terminally extended PGH peptides. Conversely, the same antiserum revealed an intermediate-high MW form (~40–50 kDa; C). 'h C-terminus', human VGF C-terminus peptides; swine adrenal extracts.

a high carbohydrate, high-fat diet (Chakraborty *et al.* 2006). Although pituitary VGF peptides were not studied here in connection with stress, none were revealed in ACTH cells in either sheep or rat, which had been butchered or killed, as our respective random sacrifice swine/bovine (Brancia *et al.* 2005) or rats (Ferri *et al.* 1995). Further investigations of different stress models, as well as addressing a wider range/s of VGF peptides will need to be carried out, focussing on both the hypothalamus–pituitary–adrenal cortex axis, as well as the sympathoadrenal system.

The comparatively high amounts of TLQP peptides we found are of special interest, since the increase in energy consumption induced by TLQP-21 (Bartolomucci *et al.* 2006) was at least partly mediated via increased circulating levels of adrenaline. Although peripheral administration of TLQP-21 was apparently devoid of effects, at least in the

Figure 5 Changes in acutely stressed swine and rats versus minimum stress. After being stabulated at a local abattoir for over 3 days, minimum stress swine were butchered first in the day (1–5 min waiting on a clean abattoir line), while acute stress ones waited in line for 25–30 min before being stunned (with electricity) and ejugulated. Rats were subjected to acute stress (5 min swimming) or the same stress followed by a 30-min recovery (stress+rec). A striking increase in tissue TLQP and/or VGF C-terminus peptides was shown in both swine and rats, rat TLQP peptides further increasing after the 30 min recovery period (results expressed as fold change compared with minimum stress animals; means $\pm$ S.E.M.; 'a', $P < 0.01$; 'b', $P < 0.001$; 'c', $P < 0.0001$ compared with relevant minimum stress group; * $P < 0.025$ between two groups indicated; other differences not significant).

Siberian Hamster (Jethwa *et al.* 2007), the comparatively high amounts and molecular heterogeneity of TLQP peptides we found in the adrenal of three mammalian species should not be overlooked. Interestingly, yet to be characterized VGF-immunoreactive peptides were shown in sympathetic neurons (Ferri *et al.* 1998), too, hence it is not unlikely that TLQP-like peptide/s may be present in further endocrine and neuronal systems. A peripheral regulatory role might thus be considered, as recently shown for neuropeptide Y, which is found in both adrenal medulla and sympathetic efferent nerves, and may mediate stress-induced obesity and aspects related to the metabolic syndrome via peripheral mechanisms (Kuo *et al.* 2007).

As to the multiplicity of VGF-derived peptides, it is becoming clear that various heterogeneous and possibly organ- and/or cell type-selective profiles of VGF peptides may exist, as we have shown so far for the sheep pituitary (Brancia *et al.* 2005), as well as the swine and bovine pancreatic islets (Cocco *et al.* 2007). Along a similar line, a striking degranulation of VGF peptides, including PGH peptides, was shown in rat female pituitary gonadotrophs on the morning following oestrus (Ferri *et al.* 1995). In turn, such heterogeneity of VGF peptides implies a complex pattern of cell-specific processing of the VGF precursor, which has been only partly clarified. The VGF10 form found in rat brain could be generated by either of the

neuroendocrine-specific prohormone convertases PC1/3 or PC2 (Trani *et al.* 2002), and probably corresponds to the ~7.5 kDa VGF C-terminus immunoreactive form we found. Conversely, we could not reveal any form corresponding to a larger peptide, named VGF20 and found in rat brain, which was preferentially produced by the PC1/3 convertase (Trani *et al.* 2002). Interestingly, other hormones involved in feeding and metabolic regulatory mechanisms undergo tissue-specific processing, such as pro-glucagon, which can be cleaved by the PC1 enzyme in intestinal L cells to form glicentin, oxyntomodulin, GLP-1 and GLP-2, or is processed by PC2 in pancreatic α cells to yield glucagon and miniglucagon, with the relevant diversity of biological actions (Bataille 2007).

Finally, the possibility of intracellular roles for at least some VGF-derived peptides should be mentioned, as has been shown for various chromogranins and their multiple products, involved in a long list of both intra- and extracellular regulatory mechanisms (Zhang *et al.* 2006).

In conclusion, VGF peptides appear to be widely expressed, differentially processed, as well as rapidly, selectively induced in adrenal medullary cells. Their processing from the single VGF precursor may flexibly provide several peptide hormones and/or intracellular regulators of varied biological activity relevant to adaptive responses.

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References

Bartolomucci A, La Corte G, Possenti R, Locatelli V, Rigamonti AE, Torsello A, Bresciani E, Rizzi R, Pavone F *et al.* 2006 TLQP-21, a novel VGF-derived peptide, increases energy expenditure and prevents the early phase of diet-induced obesity. *PNAS* **103** 14584–14589.

Bataille D 2007 Pro-protein convertases intermediary metabolism: islet hormones, brain/gut hormones and integrated physiology. *Journal of Molecular Medicine* **85** 673–684.

Brancia C, Nicolussi P, Cappai P, La Corte G, Possenti R & Ferri GL 2005 Differential expression and seasonal modulation of VGF peptides in sheep pituitary. *Journal of Endocrinology* **186** 97–107.

Canu N, Possenti R, Rinaldi AM, Trani E & Levi A 1997 Molecular cloning and characterization of the human VGF promoter region. *Journal of Neural Transmission* **68** 1390–1399.

Chakraborty TR, Tkalych O, Nanno D, Garcia AL, Devi LA & Salton SR 2006 Quantification of VGF- and pro-SAAS-derived peptides in endocrine tissues and the brain, and their regulation by diet and cold stress. *Brain Research* **1089** 21–32.

Cocco C, Melis GV & Ferri G-L 2003 Embedding media for cryomicrotomy: an applicative reappraisal. *Applied Immunohistochemistry and Molecular Morphology* **11** 274–280.

Cocco C, Brancia C, Pirisi I, D'Amato F, Noli B, Possenti R & Ferri GL 2007 VGF metabolic-related gene: distribution of its derived peptides in mammalian pancreatic islets. *Journal of Histochemistry and Cytochemistry* **55** 619–628.

Ferri G-L, Levi A & Possenti R 1992 A novel neuroendocrine gene product: selective *VGF8a* gene expression and immuno-localisation of the vgf protein in endocrine and neuronal populations. *Brain Research. Molecular Brain Research* **13** 139–143.

Ferri GL, Gaudio RM, Cossu M, Rinaldi AM, Polak JM, Berger P & Possenti R 1995 The 'VGF' protein in rat adenohipophysis: sex differences and changes during the estrous cycle and after gonadectomy. *Endocrinology* **136** 2244–2251.

Ferri G-L, Albers KM & Possenti R 1998 Changes in the neurotrophin-inducible protein 'VGF' in mice hyperexpressing NGF. *Social Neuroscience Abstracts* **24** 630.

Hahm S, Mizuno TM, Wu TI, Wisor JP, Priest CA, Kozoc CA, Boozer CN, Peng B, Mcevoy RC, Good P *et al.* 1999 Targeted deletion of the gene indicates that the encoded secretory peptide precursor plays a novel role in the regulation of energy balance. *Neuron* **23** 537–548.

Hahm S, Fekete C, Mizuno TM, Windsor J, Yan H, Boozer CN, Lee C, Elmquist JK, Lechan RM, Mobbs CV *et al.* 2002 VGF is required for obesity induced by diet, gold thioglucose treatment, and Agouti and is differentially regulated in pro-opiomelanocortin- and neuropeptide Y-containing arcuate neurons in response to fasting. *Journal of Neuroscience* **22** 6929–6938.

Jethwa PH, Warner A, Nilaweera KN, Brameld JM, Keyte JW, Carter WG, Bolton N, Bruggaber M, Morgan PJ, Barrett P & Ebling FJ 2007 VGF-derived peptide, TLQP-21, regulates food intake and body weight in Siberian hamsters. *Endocrinology* **148** 4044–4055.

Kuo LE, Kitlinska JB, Tilan JU, Li L, Baker SB, Johnson MD, Lee EW, Burnett MS, Fricke ST, Kvetnansky R *et al.* 2007 Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. *Nature Medicine* **13** 803–811.

Laslop A, Mahata SK, Wolkersdorfer M, Mahata M, Srivastava M, Seidah NG, Fischer-Colbrie R & Winkler H 1994 Large dense-core vesicles in rat adrenal after reserpine: levels of mRNAs of soluble and membrane-bound constituents in chromaffin and ganglion cells indicate a biosynthesis of vesicles with higher secretory quanta. *Journal of Neurochemistry* **62** 2448–2456.

Levi A, Eldridge JD & Paterson BM 1985 Molecular cloning of a gene sequence regulated by nerve growth factor. *Science* **229** 393–395.

Levi A, Ferri GL, Watson E, Possenti R & Salton SRJ 2004 Processing, distribution, and function of VGF, a neuronal and endocrine peptide precursor. *Cellular and Molecular Neurobiology* **24** 517–533.

Liu J-W, Andrews PC, Mershon JL, Yan C, Allen DL & Ben-Jonathan N 1994 Peptide V: a VGF-derived neuropeptide purified from bovine posterior pituitary. *Endocrinology* **135** 2742–2748.

Possenti R, Eldridge JD, Paterson BM, Grasso A & Levi A 1989 A protein induced by NGF in PC12 cells is stored in secretory vesicles and released through the regulated pathway. *EMBO Journal* **8** 2217–2223.

Possenti R, Rinaldi AM, Ferri G-L, Bordoni P, Trani E & Levi A 1999 Expression, processing, and secretion of the neuroendocrine VGF peptides by INS-1 cells. *Endocrinology* **140** 3727–3735.

Rindi G, Licini L, Necchi V, Bottarelli L, Campanini N, Azzone C, Favret M, Giordano G, D'Amato F, Brancia C, Solcia E & Ferri G-L 2007 Peptide products of the neurotrophin-inducible gene vgf are produced in human neuroendocrine cells from early development, and increase in hyperplasia and neoplasia. *Journal of Clinical Endocrinology and Metabolism* **92** 2811–2815.

- Salton SR, Fischberg DJ & Dong KW 1991 Structure of the gene encoding VGF, a nervous system-specific mRNA that is rapidly and selectively induced by nerve growth factor in PC12 cells. *Molecular Cell Biology* **11** 2335–2349.
- Salton SRJ, Ferri GL, Hahm S, Snyder SE, Wilson AJ, Possenti R & Levi A 2000 VGF: a novel role for this neuronal and neuroendocrine polypeptide in the regulation of energy balance. *Frontiers in Neuroendocrinology* **21** 199–219.
- Trani E, Rinaldi AM, Canu N, Ciotti MT, Ferri G-L, Levi A & Possenti R 1995 Tissue-specific processing of neuroendocrine protein VGF. *Journal of Neurochemistry* **65** 2441–2449.
- Trani E, Giorni A, Canu N, Amadoro G, Rinaldi AM, Halban PA, Ferri G-L, Possenti R, Schiniñ ME & Levi A 2002 Isolation and characterization of VGF peptides in rat brain, role of PC1/3 and PC2 in the maturation of VGF precursor. *Journal of Neurochemistry* **81** 565574.
- Zhang K, Rao F, Wen G, Salem RM, Vaingankar S, Mahata M, Mahapatra NR, Lillie EO, Cadman PE, Friese RS *et al.* 2006 Catecholamine storage vesicles and the metabolic syndrome: the role of the chromogranin A fragment pancreastatin. *Diabetes, Obesity and Metabolism* **8** 621–633.

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