Human Pituitary Growth Hormone (hGH)

Mass Spectrometry, Quantification, Isoforms, PTM’s, Future Trends

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Anterior Pituitary: Rich neurochemistry. Master regulatory gland in the body. Five different cell types: Each cell type is under close hypothalamic control, and secretes a specific hormone. Somatotrophs: 35-45%.

Long-Term Goals of this Research Program

Elucidate molecular mechanisms in the anterior human pituitary:

- Controls.

- Adenomas (microadenomas and macroadenomas):
  - Need accurate molecular classification.
  - Need effective pharmacology.
  - Model system for more-aggressive tumors*.


Functional categories of 56 differentially expressed proteins (DEP's) in human pituitary adenomas.

Controls (n = 8); NF- (3); LH+ (3); FSH+ (3); FSH+ + LH+ (3); unknown (3); prolactinomas (4).


**A. Down-regulated proteins (34):** I, neuro-endocrine and hormones; II, cytokine and cellular signal-related proteins; III, cellular defense and stress resistance; IV, mRNA splicing, transport or translation-related enzyme; V, DNA-binding proteins; VI, Metabolic enzymes; VII, Immunologic regulation proteins and tumor-related antigen; VIII, Transport proteins; IX, cell proliferation, differentiation, apoptosis-related proteins; X, others.

**B. Up-regulated proteins (22):** I, metabolic enzyme-related proteins; II, energy metabolism; III, cellular signal proteins; IV, cell cycle, cell growth and proliferation proteins; V, cellular defense response; VI, protein folding-related protein; VII, others.

Function: Cancer, endocrine system development, and organ morphology.
Growth Hormone (hGH)

- **Pituitary** (acidophilic somatotrophs):
    - 22 kDa. 191 amino acids.
      - 20 kDa. 176 amino acids.
      - 18 kDa. 153 amino acids.
      - 17 kDa. 145 amino acids.
  - Oligomers.
  - Protein-bound (GHBP).
  - PTM's: phosphorylation, glycosylation, deamidation, acetylation, oxidation, other.

- **Placenta** (villous syncytiotrophoblasts):
  - hGH-V genes: Chromosome 17q22-24.
    - chorionic somatomammotropin-like gene;
    - chorionic somatomammotropin gene A;
    - somatomammotropin gene B;
    - GH-variant gene.
GH Axis
Cone, et al. in Endocrinology, Larsen, et al. eds. 2003
Growth Hormone Signaling

- Growth Hormone (GH) binds to the GH receptor (GHR)
- JAK2 phosphorylates STAT1, STAT3, and STAT5
- PI3K and PDK1 activate Akt/PKB pathway
- SHAP1 binds to STAT5
- GLUT4 translocation
- Protein synthesis
- Cell survival
- Gene expression
- Glucose uptake

Key molecules:
- PI3K
- MAPK
- p90RSK
- CEBP
- STAT1, STAT3, STAT5
- SHP1
- IGF1, IGF2
- PIP2, PIP3
Pituitary hGH Isoforms
1 (88%); 2 (8%); 3 (3%); 4 (1%)

**Isoform 1**
191 a.a., \( M_r = 22129.1 \) Da
*FPTI PLSR: \( m/z = 930.5 \)
*FPTI PLSRLFDNAML: \( m/z = 1891.0 \) (MSO: 40; 1907.0)

**Isoform 2**
176 a.a., \( M_r = 20274.0 \) Da
*LHQLAFDTYQEF*NQQTSLCSFSEIPTPSNR: \( m/z = 3470.6 \) (Cys_CAM: 64; 3527.7)
*AHRHLHQALAFDTYQEF*NQQTSLCSFSEIPTPSNR: \( m/z = 3834.8 \) (Cys_CAM: 64; 3891.9)
*LHQLAFDTYQEF*NQQTSLCSFSEIPTPSRNREETQK: \( m/z = 4213.9 \) (Cys_CAM: 64; 4271.0)
*FPTI PLSR: \( m/z = 930.5 \)
*FPTI PLSRLFDNAML: \( m/z = 1891.0 \) (MSO: 40; 1907.0)

**Precursor**
Human growth hormone (hGH) (Swiss-Prot No: P01241); 217 a.a., \( M_r = 24847.3 \) Da

1 11 21 31 41 51
| MATGSRLLL LAFGLCLLPW LQEGSAFPTI PLSRLFDNAML | LRAHRLHQLA PFDTYQEF EAA | 60
| YIPKEQKYSF LQNPQTSLVF SEIPTPSRN EEETQKSNLE LLRISLLLIQ SWLEPVQFRL | 120
| SVFANSLVYG ASDSNYDLYL KDLEGQITL MGRLDENSPT TQIFKQTYS KFDTNHND | 180
| ALLKNYGLLY CFSKDMKVE TFLRIVQCRS VEGSCGF |

**Isoform 3**
153 a.a., \( M_r = 17843.3 \) Da
*ISLLLQ"TLMGR: \( m/z = 1357.8 \) (MSO: 113; 1373.8)
*ILLLLQ "TLMGRLEDGSPR: \( m/z = 2112.2 \) (MSO: 113; 2128.2)
*SNLE LRLISLLIIQ "TLMGR: \( m/z = 2183.3 \) (MSO: 113; 2199.3)
*FPTI PLSR: \( m/z = 930.5 \)
*FPTI PLSRLFDNAML: \( m/z = 1891.0 \) (MSO: 40; 1907.0)

**Isoform 4**
145 a.a., \( M_r = 17083.4 \) Da
*ISLILLIQSWLEPV"I I QIFK: \( m/z = 2072.7 \)
*ISLILLIQSWLEPV"IIFI KQTYS K: \( m/z = 2634.5 \)
*SNLE LRLISLLIIQSWLEPV"I QIFK: \( m/z = 2852.7 \)
*FPTI PLSR: \( m/z = 930.5 \)
*FPTI PLSRLFDNAML: \( m/z = 1891.0 \) (MSO: 40; 1907.0)
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<tr>
<th>Spot</th>
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<th>GH splice isoform</th>
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<tr>
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<td>Isoform 3; Isoform 1/2</td>
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<tr>
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<td>24</td>
<td>P01241</td>
<td>Isoform 1</td>
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LC-MS/MS identified two phosphopeptides from hGH in spot 5:

FDTNpSHNDDALLK
SLVYGApSDSNVYDLLK

Neutral-fragment chromatogram:
Only peptides that generated a signal in the MS/MS spectrum at (M+2H-98)²⁺ are displayed.

Endogenous Phosphorylated hGH (phGH)


- phGH:
  - $^{132}\text{pSer}$ and $^{176}\text{pSer}$.
    - $^{132}\text{pSer}$ was also studied in Giorgianni, et al. Proteomics 4, 587-598 (2004).

Endogenous phGH:
  - Unknown biological role.

- in vitro-synthesized pGH.
  - Liberti and Joshi, BBRC 137, 806-812 (1986).

- No other endogenous phGH references found in the literature.
MS/MS Quantification of Peptides

- Synthetic opioid peptide analog: \( \mu \) opioid receptor agonist:
  - Dmt-D-Arg-Phe-Lys-NH\(_2\) = Dmt\(^1\)DALDA. \(2',6'\text{dimethyl-Tyr}\)
    - \(^2\text{H}_5\)-Dmt\(^1\)DALDA internal standard.
    - Ovine plasma. Pharmacokinetics.
    - LC-nanospray-ion trap MS/MS.
    - LC-Q-TOF-MS/MS.

- Endogenous \( \beta \)-endorphin (BE\(_{1-31}\)) in human pituitaries:
  - via NAIIK \(\rightarrow\) NAI transition. NAIIK = tryptic peptide = BE\(_{20-24}\)
    - \(^2\text{H}_4\)-NAIIK internal standard.

- Endogenous methionine enkephalin (ME) in human pituitaries:
  - via YGGFM \(\rightarrow\) YGGF transition.
    - \(^2\text{H}_5\)-ME internal standard.
Heterogeneity Analysis of Human Pituitary Proteomes

- Pituitary control tissue (n=8 tissues).
- 2DGE (triplicate); PDQuest.

<table>
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<td>W</td>
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<tr>
<td>F</td>
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<td>M</td>
<td>55</td>
<td>W</td>
</tr>
<tr>
<td>F</td>
<td>47</td>
<td>W</td>
</tr>
</tbody>
</table>

- 51 differential spots were found:
  - 7 proteins differed with gender; 17 with age; 15 with race; 12 with age and race.
  - Only one protein, hGH, differed with gender, age, and race.

Genotropin. Commercially available.
- Expressed in *E. coli, strain K12*.

Analyzed with 2DGE; MALDI-MS/MS; LC-MS/MS.

Amino acid substitutions and modifications were found:
- dimethyl $^{70}\text{K}$; or $^{70}\text{K} \rightarrow \text{R}$.
- deamidated $^{149}\text{N}$ and $^{152}\text{N}$.
- $^{127}\text{R} \rightarrow \text{K or E}$.
- $^{14}, {125}, {170}\text{M} \rightarrow$ partially oxidized.
- $^{14}\text{M} \rightarrow \text{I}$. $^{14}\text{M}$ influences receptor binding.

Those eight changes (all were partial) occurred at 2% whole protein amount (average $= 0.25\% = 2.5 \text{ ppt}$).

Tryptic peptides for MS/MS quantification of isoform 1:
- 1-6; 91-96; 97-103; 167-171; 172-184; 185-193; 195-198; 199-217.

Amino acids deleted from precursor to form isoforms 2, 3, and 4.
Tryptic peptide 7-42 is too large for MS/MS quantification.
Also, use different endopeptidases and/or CNBr.
Wish List and New Horizons for GH Analysis

- Qualitative and quantitative analytical needs:
  - Increase molecular specificity:
    - More % coverage; with amino acid sequence data.
  - Increase detection sensitivity:
    - fmol, amol, zmol.
  - Increase mass range:
    - For intact hGH oligomers with M.W. > 50kDa.

- New horizon:
  - Study ROS/RNS-modified hGH:
    - One in ~10^6 Tyr is nitrated in endogenous proteins.
      - Nitro-hGH (nhGH).
GH-associated nitration of Janus kinase-2 at the $^{1007}Y^{1008}Y$ epitope impeded phosphorylation at this site: Mechanisms for, and impact of, a GH, AKT, and NO synthase axis on GH signal transduction.


- Increase of liver protein tyrosine nitration occurred after GH ingestion within a time-frame that was consistent with GH hyper-responsiveness.
  - Phosphorylation decreased to $\sim2\%$.  (Male calves).
Hypothesis

- hnGH plays a role in GH neuroendocrinology:
  - Search for endogenous hnGH.
    - Nitroproteins are produced via oxidative-stress mechanisms.
    - Athletes have a higher VO$_2$$_{\text{max}}$.
      - And, therefore, also a higher level of oxidative stress.

- It is important to elucidate human pituitary protein pathways to prepare for quantum steps in sports doping and analysis.
Model of nitroproteins and their functions in a cell
Nitroprotein-derived Networks

Adenoma

Function:
Cancer, cell cycle, reproductive-system disease

Control

Function:
Gene expression, cellular development, and connective tissue development and function


testosterone $\rightarrow$ aromatase $\rightarrow$ $\beta$-estradiol
GH Measurement: Analytical challenges

- Discriminate between rGH and hGH.
- Tryptic peptides; stable isotopes; MS/MS.
  - Use to calibrate non-MS analytical methods.
- Carbon isotope-ratio mass spectrometry.
- Glycosylated hGH and rGH (eukaryotic):
  - Any differences in number, structure, antennary structure, etc.?
- Isoforms, hnGH, PTM’s, etc.
- Chemiluminescence immunoassays.
Human growth hormone–transferrin fusion protein for oral delivery in hypophysectomized rats

- ASMS meeting. June 5-9, 2011.
  - Denver. WOG.
Factors to consider in alleged sports doping cases

Factors:

- which hGH 2DGE spot (n = 24)?
  - Isoform; glycosylated hGH; phGH; nhGH (if it exists); p-glycosylated hGH (if it exists).
- intact GH or a tryptic peptide?
- ingestion of GH-stimulating agents (GHRH):
  - Ghrelin, galanin, leptin, serotonin, clonidine, dopamine, ...
    - Metabolically stable synthetic peptide secretagogues.
- heterogeneity factors: age, race, sex.
- neuroendocrinological, enzymatic, and feedback effects:
  - testosterone $\rightarrow$ aromatase $\rightarrow$ $\beta$-estradiol.
  - rGH on [endogenous hGH]:
    - [rGH] versus [hGH] versus [rGH]/[hGH].
Commercial synthesis of a metabolically stable GH secretagogue

- 85% pure:
  - 99% purity costs ~2x more.
- Cost to synthesize 4 mg.
- For a 50 µg dose → 80 doses from 4 mg.
- Synthesis cost: $12 per amino acid.
- Ghrelin (14 a.a.): $168. $2/dose.
- Galanin (28 a.a.): $336. $4/dose.

- A laboratory dedicated to synthesize a selected protein will have much lower costs.
A Proposal to Detect rGH in Athletes

- Analyze completely every preparation of rGH (commercial; black market).

- Expand on heterogeneity study:
  - Study age, race, sex variabilities of hGH.

- Detect every structural difference between endogenous hGH and each sample of rGH:
  - Glycosylation.
  - Amino acid substitutions/modifications that change M.W. or charge:
    - deamidation $\text{CONH}_2 \rightarrow \text{COCO}_2^- 0 \rightarrow -1$
    - $R \rightarrow E +1 \rightarrow -1$

- Perform analysis at the ppt-ppm level.
rGH Analysis

- Currently, no fast, “magic bullet”, or “one-stop” analytical method.
- Requires detailed, fundamental analytical chemistry:
  - Qualitative: amino acid sequence.
    - High specificity.
  - Quantitative.
    - High sensitivity.
- Analytical methods must continuously evolve to match black market rGH production.
Conclusions

The elucidation of basic molecular mechanisms in a human pituitary is needed in hGH research because those mechanisms impact on WADA activities, analytical needs, doping, and sports competition.
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  - RR 14593, 10522, 16679.
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