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Site-Specific ^{18}F-Labeling of the Protein Hormone Leptin Using a General Two-Step Ligation Procedure

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Abstract: The protein hormone leptin acts to regulate body fat and energy expenditure. Resistance to this hormone is implicated in human obesity and its pathophysiological consequences. In order to gain insight into the mechanism of leptin resistance, an ^{18}F-labeled derivative was developed to study the biodistribution of the hormone using positron emission tomography (PET). A two-step, site specific ligation approach was developed for this purpose, in which an aminooxy-reactive group was incorporated at the C-terminus of leptin using expressed protein ligation (EPL), which was subsequently derivatized with [^{18}F]fluorobenzaldehyde using an aniline-accelerated radiochemical oximation reaction. The modified hormone was shown to be biologically active *in vitro* and *in vivo*, and it was applied to PET imaging in *ob*/*ob* mice. These protocols will allow for the routine production of site-specifically ^{18}F radiolabeled leptin, as well as other proteins, for use in PET imaging in systems from mouse to man.

Leptin is a polypeptide hormone, secreted principally by white adipose tissue, which acts in a homeostatic feedback loop to regulate body fat and energy stores.[1,2] Leptin is produced in proportion to adipose tissue mass and functions as the afferent signal in a negative feedback loop that maintains constancy of body weight. Humans and *ob*/*ob* mice lacking a functional leptin gene show profound hyperphagia and obesity, which is corrected by the administration of the hormone. However, in most cases of human obesity and also in diet induced obese animals, serum leptin levels are high and there is a lack of responsiveness to exogenous hormone.[3] Based on this, most human obesity has been suggested to be a result of leptin resistance, analogous to insulin resistance in type 2 diabetes.[1,2,4] While the molecular basis of leptin resistance is largely unknown, several mechanisms have been proposed which predict altered biodistribution of the hormone *in vivo*, by alteration of peripheral or central binding sites, limited transport to binding sites in the central nervous system, or binding to circulating factors which inhibit leptin activity.[5–7] One approach to evaluate these hypotheses would be to study the localization of the protein in live animals. For example, by systematically studying leptin biodistribution using an *in vivo* imaging modality such as positron emission tomography (PET) in animal model systems and in humans, it may be possible to dissect the mechanisms underlying leptin resistance *in vivo*.

PET has emerged as a powerful tool for *in vivo* imaging in clinical medicine as well as in basic research. PET imaging is complementary to other *in vivo* imaging techniques such as single photon emission computed tomography (SPECT), fluorescence imaging, bioluminescence imaging, and magnetic resonance imaging (MRI), and in particular it offers good resolution, high sensitivity, ready translation to clinical studies, and ease of quantification.[8] PET requires that a probe of interest be labeled with a positron emitting nuclide, such as ^{18}F, ^{11}C, or ^{68}Ga.[9] ^{18}F is a particularly desirable radionuclide for PET imaging owing to its intermediate half-life of 109.7 min, its routine availability by biomedical cyclotron production, and the high resolution of images obtained. Proteins and peptides are widely used tracers in PET, and a variety of techniques, principally based on the incorporation of radiometals, have been

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developed to label them.[10] Indeed, a ^{68}Ga-containing leptin conjugate has been generated for localized PET analysis of leptin pharmacology in the spinal cord.[11] The interest in peptide imaging and the superior imaging properties of ^{18}F has led to the development of several approaches for labeling synthetic peptides with this isotope.[12–23] Protein labeling using ^{18}F synthons targeting lysine[24–26] or cysteine[27] residues has also been developed and applied to a variety of substrates. However, in contrast to the aforementioned peptide labeling approaches, these protein labeling techniques give rise to a variety of labeled products, and it is difficult to characterize the structure or function of the tracer. Site specific labeling of proteins is particularly desirable as it allows unambiguous biophysical and activity-based characterization of a homogeneous product, leading to reproducibility in labeling and imaging.[28] Thus, the generation of site-specifically labeled proteins for molecular imaging has become an area of active research.[28]

Herein we describe a two-step ligation approach in which an aminooxy group is introduced site specifically at the C-terminus of leptin using expressed protein ligation (EPL). Importantly, this procedure provides semisynthetic access to this protein hormone, potentially allowing for a variety of modifications to be installed at the C-terminus. The aminooxy-leptin derivative was then further modified with [^{18}F]4-fluorobenzaldehyde (FBA), a previously described synthon. In order to allow the radioconjugation to proceed in the mild conditions required to preserve the function of leptin, the oximation reaction was accelerated by the addition of aniline as a catalyst. Additionally, a ^{19}F labeled derivative was prepared, which was biologically active *in vitro* and *in vivo*. Finally, the ^{18}F derivative was applied to PET imaging in *ob*/*ob* mice, demonstrating rapid uptake of the hormone in the cortex of the kidney. These results are of general importance as they establish a broadly applicable chemical strategy for site specific F-18 labeling of proteins, on a scale suitable for PET imaging for both animal model systems as well, potentially, as in human studies.

Experimental Section

Semisynthesis of Aminooxy-Leptin 4. Leptin(1−146) α-thioester **1** (typically 10−20 mg, prepared as described in the Supporting Information) was dissolved to a concentration of 20 mg/mL in 6 M guanidine hydrochloride, 100 mM sodium 2-mercaptoethane sulfonate, 200 mM sodium phosphate, and 150 mM sodium chloride, pH 7.2. Peptide **2** (10−20 mg, 25 equiv, prepared as described in the Supporting Information) was added to 10 mg/mL, and the pH was adjusted to 7.2 using 1 N NaOH. After overnight reaction at room temperature, the crude mixture containing unfolded aminooxy-leptin derivative **3** was diluted to 0.25 mg/mL in 6 M guanidine hydrochloride, 20 mM tris pH 7.4, 5 mM dithiothreitol. The product was then dialyzed at 4 °C sequentially against 6 M urea, 2.5 mM dithiothreitol, 2.5 mM 2-mercaptoethanol, 500 μM aminooxyacetic acid, 20 mM tris pH 7.4; then against 4 and 2 M urea containing 5 mM 2-mercaptoethanol, 500 μM aminooxyacetic acid, 20 mM tris pH 7.4; then against 500 μM aminooxyacetic acid, 20 mM tris pH 7.4; and finally overnight against 20 mM tris pH 7.4, 5 mM oxidized glutathione, 0.5 mM reduced glutathione, 500 μM aminooxyacetic acid, 150 mM sodium chloride. The crude refolded mixture was purified on a C4 semiprep RP-HPLC column using a gradient of 45−60% buffer B (buffer A = 0.1% TFA in water, buffer B = 90% acetonitrile, 0.1% TFA in water.) Fractions containing pure refolded AOA-Leptin **4** were identified by HPLC and ESI-MS and pooled and lyophilized into 250 μg aliquots. Yield from a 1 μmol scale: 5 mg (0.3 μmol), 30%.

Tryptic Digestion of Aminooxy-Leptin 4 and Recombinant Leptin. 20 μg of sequencing grade trypsin (Promega, Madison, WI) were dissolved in 100 μL of trypsin resuspension buffer (provided by the manufacturer) and incubated at 30 °C for 15 min. The trypsin solution was added to 150 μg of either aminooxy-leptin **4** (dissolved in 15 mM HCl) or recombinant leptin (Amgen, Thousand Oaks, CA), and the pH was adjusted to 8.3 using 1 M sodium bicarbonate. The reaction was incubated at 37 °C for 3 h and analyzed by analytical RP-HPLC on a C4 analytical column using a gradient of 0−100% B. Peaks were collected and identified by ESI-MS.

Synthesis of ^{19}FBA-Leptin 5. Aminooxy-leptin **4** (5 mg, 0.3 μmol) was dissolved in 2 mL of 15 mM HCl. 250 μL of 1 M sodium acetate pH 4.5 were added, followed by 250 μL of 10 mM 4-fluorobenzaldehyde in DMF. The reaction was vortexed and incubated at 4 °C overnight. The reaction was then purified by C4 semipreperative RP-HPLC on a gradient of 46−61% B. Fractions containing pure FBA-Leptin **5** were pooled and lyophilized, yielding 1.7 mg of pure protein (Yield of 34%).

Luciferase Reporter Assay of Leptin Receptor Activation. Ob-Rb-STAT3-luciferase cells (see Supporting Information) were seeded into 24-well plates. At a confluence of 80% the cells were washed and replaced with serum-free medium. After 4 h the medium was changed and replaced by serum-free medium containing dilutions of mouse recombinant leptin or the appropriate semisynthetic derivative. Samples were assayed in duplicate for every dilution. After 24 h cells were collected and luciferase activity measured in cell lysate using a luciferase assay system (Promega).

Leptin Administration to *ob*/*ob* Mice. Leptin or its semisynthetic derivatives were administered continuously with mini osmotic pumps (Alzet, Cupertino, CA, model 2002) with an exchange rate of 450 ng/h to *ob*/*ob* mice. Pumps were filled aseptically according to the manufacturer instructions with either sterile PBS solution or leptin. Recombinant leptin or FBA-leptin **5** were diluted in sterile PBS buffer. Before surgery, pumps were incubated overnight in a

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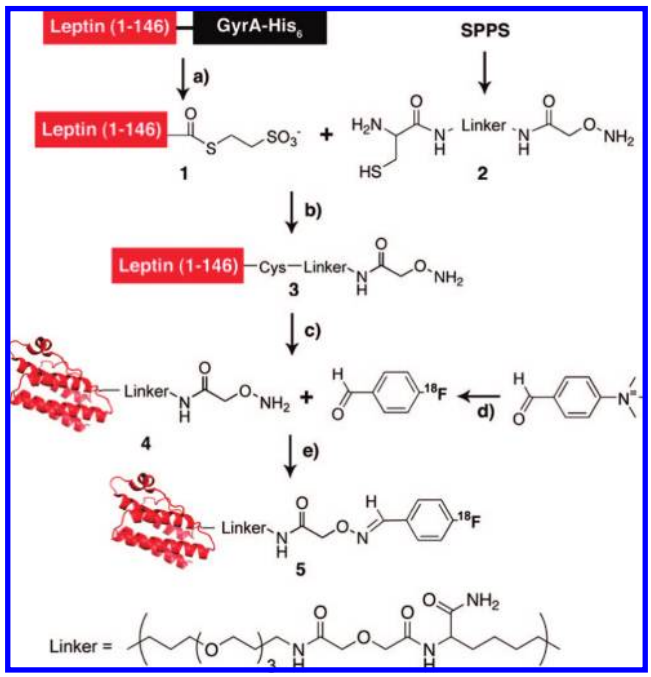
Scheme 1. Synthesis of [^{18}F]FBA-Leptin **5**[a]



[a] Reaction conditions: (a) 100 mM mercaptoethanesulfonic acid, 4 M urea, 150 mM NaCl, 100 mM sodium phosphate pH 7.0, 4 °C, 2 days. (b) 20 mg/mL **1**, 10 mg/mL **2**, 100 mM mercaptoethanesulfonic acid, 6 M Gn-HCl, 150 mM NaCl, pH 7.2, 1 day. (c) Refolding by stepwise dialysis and disulfide exchange. (d) 20 mg of kryptofix, 2 mg of potassium carbonate, 1−2 mg of (4-formylphenyl)trimethylammonium trifluoromethanesulfonate and K^{18}F in DMSO, 120 °C, 8 min. (e) 30 nmol of **4**, 100 mM anilinium acetate pH 4.5, 15 min, 0° C.

sterile 0.9% NaCl solution at 37 °C to achieve an immediate and constant pumping rate at the time of implantation. During surgery mice were anesthetized with isofluorane. Pumps were implanted subcutaneously in the interscapular region of the mice.

Rate of Oxidation. Lyophilized aminooxy-leptin **4** (150 μg, 9.1 nmol) was dissolved in 55 μL of 15 mM HCl, and 15 μL of either 0.5 M sodium acetate pH 4.5 or anilinium acetate pH 4.5 were added, giving a final volume of 70 μL (final concentration of **4** = 125 μM). 2 μL of [^{18}F]FBA in DMF (prepared as described in the Supporting Information) were added to each reaction, which was vortexed and placed on ice. Aliquots of the reaction were spotted on TLC plates at the indicated times and run with 4:1 DCM methanol with 2% acetic acid. [^{18}F]FBA leptin **5** was retained at the origin while [^{18}F]FBA migrated at the solvent front. At t = 30 min, a small portion of the anilinium acetate reaction was injected on a C4 analytical HPLC column and eluted on a 0−100% B gradient over 30 min. The [^{18}F]FBA eluted at 3.5 min while the [^{18}F]FBA-leptin **5** eluted at 21 min on this gradient.

Synthesis of [^{18}F]FBA-Leptin 5. Lyophilized aminooxy-leptin **4** (500 μg, 30 nmol) was dissolved in 100 μL of 15 mM HCl. To this solution was added 25 μL of 0.5 M anilinium acetate, pH 4.5. The aforementioned DMF solution of [^{18}F]FBA was then added, and the mixture was vortexed and incubated on ice for 15 min. The reaction was then purified through two Micro Bio-Spin 6 columns (Biorad, Hercules, CA), which had been pre-equilibrated with PBS according to the manufacturer's instructions. The eluate from the columns was filtered though a 0.22 μM filter. The purity of the oxime product was analyzed both by RP-HPLC using a 0−100% B gradient on a C4 analytical column and by iTLC (ITLC SG silica impregnated glass fiber sheets, Pall, East Hill, NY) using 1% acetic acid in water as an eluant. While the labeling yield as detected by HPLC or iTLC was typically over 50%, the recovered yield was generally 25%, probably due to precipitation of the protein. Starting from 9.25−13 GBq of aqueous K^{18}F, typically 93−200 MBq of [^{18}F]FBA-leptin **5** were recovered with a total

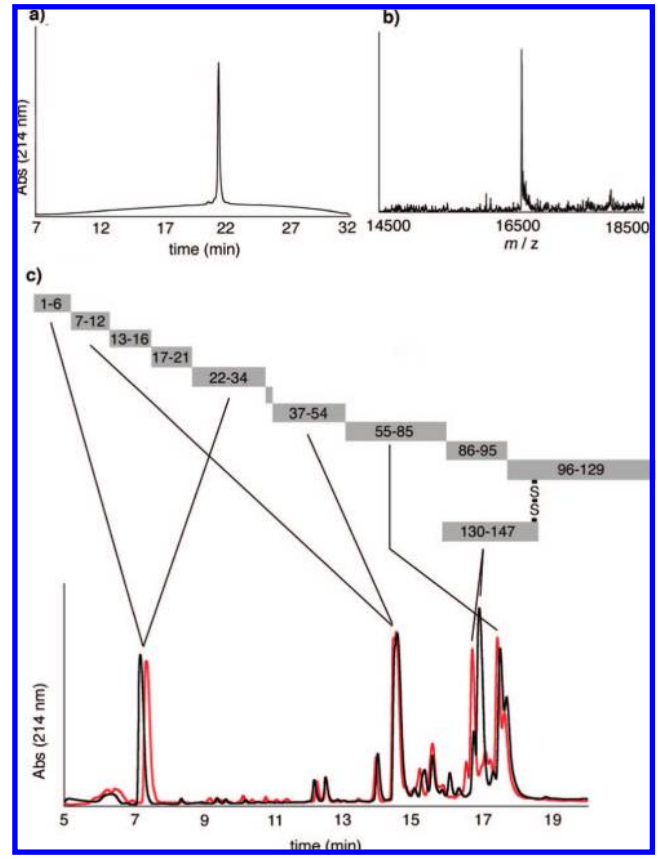


Figure 1. Synthesis and characterization of AOA-Leptin **4**. (a) RP-HPLC chromatogram of **4** on a 0−100% B gradient on a C4 analytical column. (b) ESI-MS reconstruct of AOA-Leptin **4** (calculated mass 16652.2, obtained 16652.2 ± 3.3). (c) Trypsinization HPLC/MS analysis of commercial recombinant leptin (black) and AOA leptin **4** (red). The indicated peaks connected by a line were identified as the corresponding tryptic peptides by ESI-MS. The disulfide bond between C97 and C147 is indicated.

synthesis time of 120 min. The total amount of protein in the final sample was determined using the BCA assay (BCA assay micro plate kit, Pierce, Rockford, MD). The specific activity at the end of synthesis was 9.25−13 GBq/μmol (n = 4).

Cell Binding Experiment. ObRb expressing cells were grown overnight in suspension in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C and 5% CO_2. Cell viability and concentration were then determined using trypan blue staining. Cells were washed with serum free medium and resuspended in HBSS supplemented with 0.1% BSA at a final concentration of 20 million/mL. For each cell binding assay, 750 μL of cell suspension were incubated with a constant amount of radiolabeled leptin corresponding to 80 000 cpm of radioactivity ± 60 μg of cold leptin. The concentration of the labeled leptin was determined using the BCA method. Following incubation of the cells at room temperature for 2 h at constant agitation, the cell suspension was washed twice with 0.5 mL of HBSS with 0.1% BSA and centrifuged at 2000 rpm for 5 min. The radioactivity associated with the cell pellet (cell bound activity) was determined by counting the tubes in a gamma counter with corresponding standards and blanks.

Stability of [^{18}F]-FBA-Leptin 5 *in vivo*. Approximately 20 MBq of [^{18}F]-FBA-Leptin **5** were injected into either *ob*/*ob* or wild type mice via the tail vein. After 20 min, the animals were sacrificed and 500 μL of whole blood were collected. The blood was centrifuged for 15 min at 7000 rpm, and the supernatant was analyzed by C4 analytical RP-HPLC.

Stability of [^{18}F]-FBA-Leptin 5 *in vitro*. Approximately 200 kBq of [^{18}F]-FBA-Leptin **5** in 2 μL were diluted in 100 mM sodium acetate pH 4.5 (10 mM sodium phosphate, 140 mM sodium

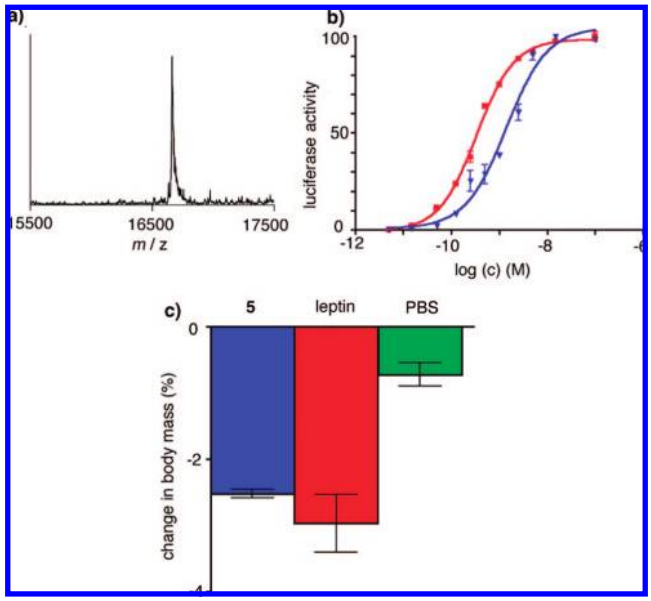


Figure 2. Characterization and biological activity of FBA-Leptin **5**. (a) ESI-MS reconstruct of FBA-Leptin **5** (calculated MS 16758.3, obtained 16760.9 ± 3.5). (b) ObR-STAT3 dependent luciferase assay comparing the activity of FBA-Leptin **5** (blue triangles) and recombinant leptin (red squares), $n = 2 \pm$ sd. (c) Comparison of the weight loss induced by FBA-Leptin **5**, recombinant leptin, and PBS vehicle in *ob*/*ob* mice. Samples were infused at a constant rate of 450 ng/h. The weight of the animals was recorded after 24 h ($n = 4 \pm$ sem).

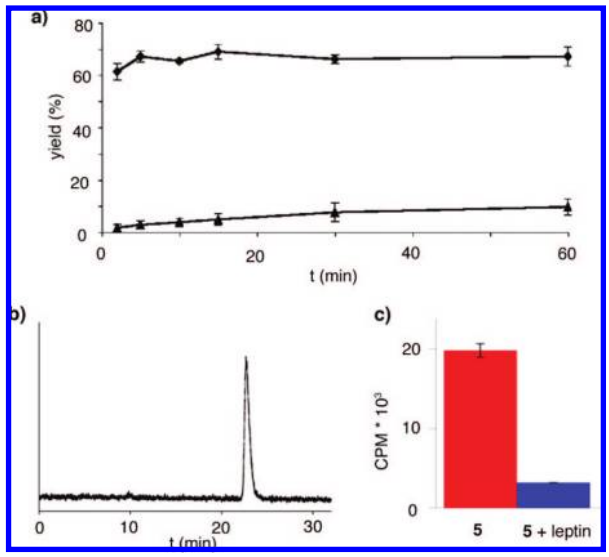


Figure 3. Catalysis of radiochemical oximation of AOA-Leptin **4** with [^{18}F]FBA by aniline and characterization of [^{18}F]FBA-Leptin **5**. (a) Catalysis of the oximation reaction by aniline. 125 μM **4** was incubated with [^{18}F]FBA in the presence of either 100 mM sodium acetate pH 4.5 (▲) or 100 mM anilinium acetate (◆) at pH 4.5 on ice. The radiochemical yield was quantified by TLC and confirmed by HPLC. The results are an average ± standard deviation of four experiments. (b) Radiochemical HPLC of [^{18}F]FBA-Leptin **5**, on a C4 analytical column. (c) [^{18}F]FBA-Leptin **5** was incubated with a cell line overexpressing ObRb in the presence or absence of 5 μM competing leptin (average of two experiments).

chloride, pH 7.4), PBS, or mouse serum. After 60 min a sample of each was injected on a C4 analytical HPLC. Integration of the radiochemical HPLC peaks was used to estimate the purity of the sample, and if no other peaks could be detected above background the sample was assumed to be greater than 95% pure.

PET Imaging Experiments. Imaging was conducted using a MicroPET (Focus TM 220, CTI Concord Microsystems, LLC) camera, with a bore size of 22 cm and an axial field of view of 7.6 cm. This enables imaging of the entire mouse. The resolution at the center of field of view (FOV) is <1.3 mm. Following induction of anesthesia with ketamine and xylazine, each mouse was injected with 3.7−5.4 MBq of [^{18}F]FBA-leptin **5** or ^{68}Ga-DOTA-leptin in the tail vein. The distribution of radioactivity was studied by acquiring dynamic imaging for 30 min. 3D histograms of emission data were generated (span3, rig difference 47) using dead time correction. Images were reconstructed using an OSEM2D algorithm without any attenuation or scatter correction.

Results and Discussion

Design and Synthesis of Aminooxy-Leptin 4. In designing a site-specific leptin ^{18}F labeling protocol, various options were considered. While in principle a cysteine directed labeling approach[29] could produce site specifically ^{18}F labeled leptin, we found that the introduction of additional cysteines in the protein led to difficulties in refolding and protein purification (data not shown). The C-terminus of leptin is solvent exposed in the crystal structure,[30] suggesting that modifications to this region of the protein would not interfere with folding or stability. Moreover, homology modeling and mutagenesis data indicate that the leptin C-terminus is not critical for receptor binding.[31,32] Thus, since the native C-terminal residue of murine leptin is cysteine (Cys147), we designed the two-step semisynthetic strategy shown in scheme 1 in which an aminooxy-derivatized peptide is first attached to the C-terminus of recombinant leptin via expressed protein ligation (EPL)[33,34] and then the lone aminooxy group on the protein derivatized with [^{18}F]-4-fluorobenzaldehyde ([^{18}F]FBA), an ^{18}F synthon which has been previously used to label a variety of substrates through standard bioconjugation methods.[14,17,26,35]

Truncated murine leptin (1−146) α-thioester **1** was generated by thiolysis of the corresponding intein fusion obtained from overexpression in *E. coli* (supporting Figures S1, S2). Note, since leptin is known to be sent to bacterial inclusion bodies upon overexpression,[36] we deliberately fused the hormone to the GyrA intein since this can be efficiently refolded from fusion proteins recovered from the pellet.[37] Following purification, thioester **1** was ligated to cysteine derivative **2** to give leptin analogue **3** (Scheme 1). Compound **2** was synthesized by solid phase methods and contains two reactive moieties, namely a cysteine and an aminooxy group, separated by a short PEG spacer (supporting Scheme S1, Figures S3, S4). In preliminary studies, we found the PEG spacer was required to maintain native-like solubility of the final folded product. We anticipated

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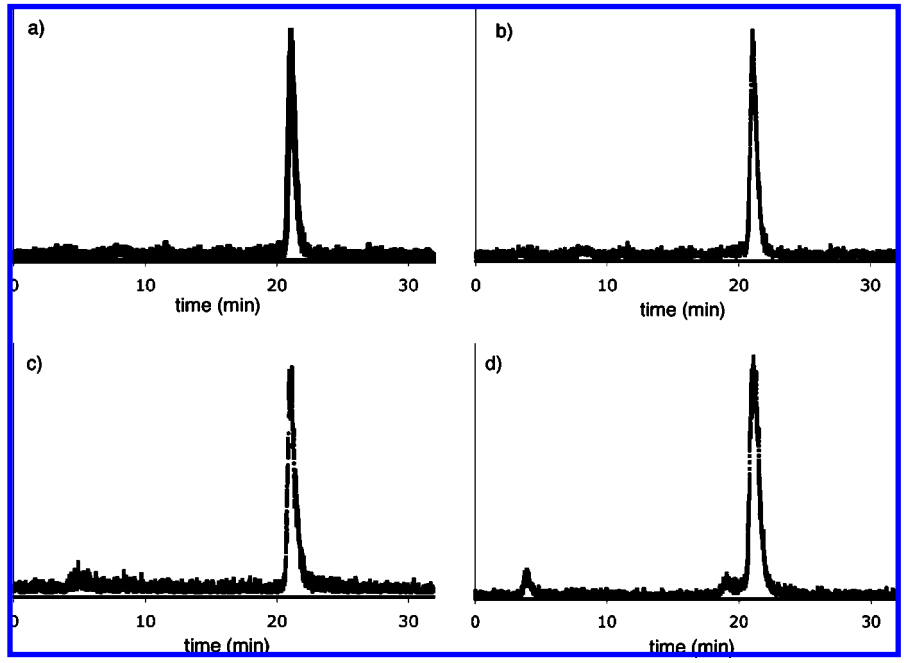

Figure 4. Stability of [^{18}F]-FBA-Leptin **5** *in vivo* and in various buffers. (a) Radiochemical HPLC of serum recovered from wild type mice injected with [^{18}F]-FBA-Leptin **5**. Similar results were obtained from *ob*/*ob* mice. (b−d) Radiochemical HPLC of [^{18}F]-FBA-Leptin after incubation for 60 min in PBS, 100 mM sodium acetate pH 4.5, or mouse serum, respectively. Stability in PBS and sodium acetate was greater than 95%, while in serum it was 93% stable. The initial purity of the labeled product in this experiment was 96%.

that at pH 7 the α-thioester in protein **1** would react preferentially with the cysteinyl group in **2**, via native chemical ligation, due to the relatively slow rates of hydroxymate formation under these conditions.[38,39] The EPL reaction between **1** and **2** was complete after 24 h at which point the crude ligation mixture was refolded using reduced/oxidized glutathione as a redox couple to generate the native disulfide bond between Cys97 and Cys147. The refolded aminooxy-leptin analogue **4** was then purified by RP-HPLC (Figure 1a, b). Aminooxy-leptin analogue **4** gave an identical tryptic digestion pattern to commercially available recombinant leptin as determined by LCMS (Figure 1c). As expected for natively folded leptin, the two C-terminal tryptic peptides in **4** were found to be linked by a disulfide bond, while no reduced tryptic peptides could be found. Interestingly, the mass spectrum of this disulfide-linked C-terminal tryptic peptide contained a peak of mass consistent with loss of aminooxy-acetate. The presence of this fragment, which may have been produced either by trypsinolysis of the aminooxy-acetate or by gas phase fragmentation, provides evidence that protein **4** contains the desired amide bond at the ligation site, since aminooxy-acetate can not be lost from a hydroxymate-containing ligation side product.

Synthesis and Characterization of ^{19}F-Leptin 5. Cold FBA-leptin derivative **5** was generated by overnight treatment of **4** with 1 mM 4-fluorobenzaldehyde at 4 °C in 100 mM sodium acetate buffer, pH 4.5. No side reactions were detected, and the resulting oxime-containing product was purified by RP-HPLC and the identity was confirmed by ESI-MS (Figure 2a). FBA-leptin **5** was then tested for its ability to activate leptin receptor signaling using a cell line stably transfected with leptin receptor ObRb and a plasmid containing luciferase under the control of an STAT3 responsive element. In this assay, **5** induced leptin receptor signaling to a similar extent as commercial recombinant leptin, with a slightly increased EC_{50} (95% confidence intervals 0.26−0.34 nM for recombinant leptin and 1.0−2.0 nM for **5**, Figure 2b). This subtle reduction in potency observed for **5** was not due to the ligation or refolding procedures employed in its preparation as unmodified leptin generated via a similar EPL procedure had an identical EC_{50} compared to commercial recombinant leptin in this assay (Figure S5). Importantly, when administered to *ob*/*ob* mice via subcutaneous osmotic pumps, FBA-leptin **5** had the identical ability to induce weight loss over a 24 h period as commercial recombinant leptin (Figure 2c). We conclude that FBA-leptin **5** is able to bind and activate the leptin receptor and that it is able to induce weight loss in mice, the hallmark of leptin bioactivity.

Radiosynthesis and Characterization of [^{18}F]-FBA-Leptin 5. Synthesis of [^{18}F]FBA was accomplished as previously described,[35] with minor modifications, using the precursor (4-formylphenyl)trimethylammonium trifluoromethanesulfonate. Initial attempts toward oximation of [^{18}F]FBA with leptin analogue **4** failed owing to precipitation of the protein at elevated temperatures required to allow the reaction to proceed in good yield.[14] Jencks and co-workers described catalysis of semicarbazone formation between 4-chlorobenzaldehyde and semicarbazide by aniline and substituted derivatives thereof, and recently Dawson and co-workers found that aniline acted as a catalyst in peptide oxime ligations.[40,41] Thus, we reasoned that aniline should catalyze the radiochemical oximation of [^{18}F]FBA with aminooxy-leptin **4**. Comparison of the rate of oximation of aminooxy-leptin **4** in 100 mM sodium acetate or anilinium acetate at 4 °C revealed that aniline dramatically accelerates the reaction (Figure 3a). Notably, aniline catalysis allows the

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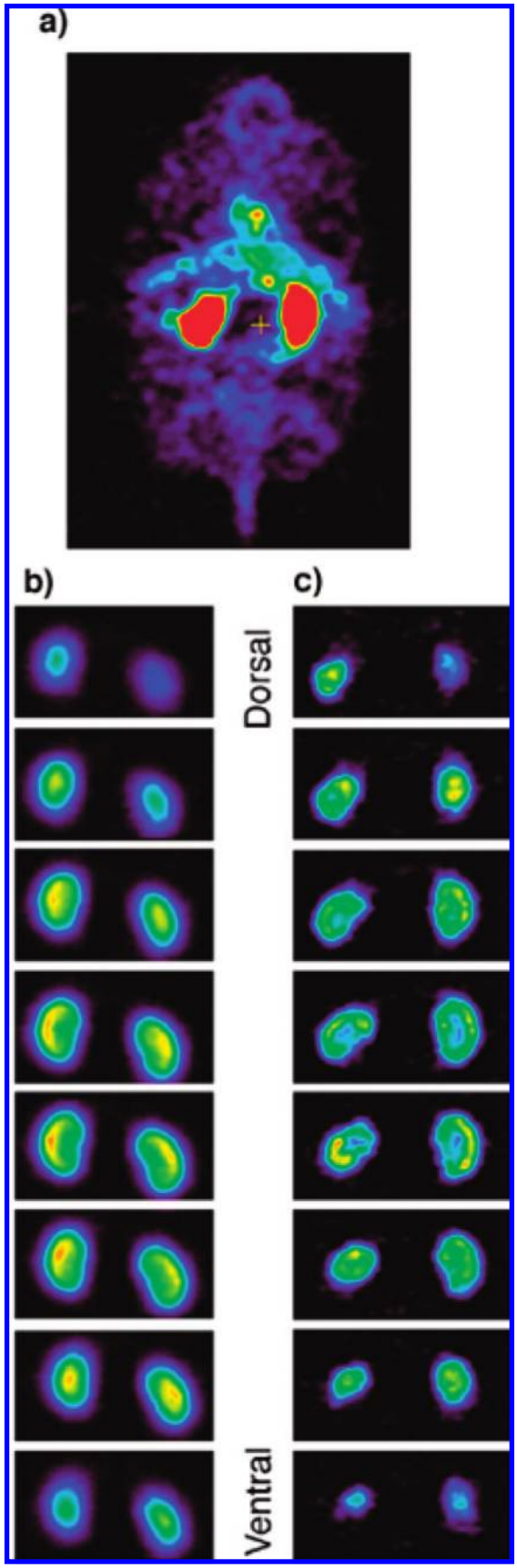

Figure 5. PET images using [^{18}F]FBA-Leptin **5**. (a) Coronal section of *ob*/*ob* mouse injected with **5**. (b) Serial coronal PET images of *ob*/*ob* mouse kidneys, injected with [68Ga]-DOTA-Leptin. (c) Serial coronal PET images of *ob*/*ob* mouse kidneys, injected with **5**.

reaction to proceed in good yield at lower concentration of the aminooxy precursor and at lower temperature than previously described.[14]

Preparative scale ^{18}F labeling of aminooxy-leptin **4** was accomplished by treatment with [^{18}F]FBA in 100 mM anilinium acetate on ice for 15 min and purification of the resulting mixture on a P6 gel filtration column. Using this method, 74−185 MBq (2.5−5.0 mCi) of [^{18}F]FBA-Leptin **5** could be recovered starting from 7.4−11.1 GBq of ^{18}F, with a total synthesis time of ∼120 min (n = 4). The specific activity of the protein varied from 9.25 to 16.65 GBq/μMol, and the radiochemical purity of the protein was greater than 95% as judged by iTLC and HPLC (Figure 3b). The ability of [^{18}F]FBA-leptin **5** to bind to the aforementioned cell line expressing ObRb was tested. We found that the labeled protein bound to the cells when incubated together at room temperature and that binding could be blocked by the addition of an excess of unmodified recombinant leptin (Figure 3c). Additionally, the presence of the toxic catalyst aniline could not be detected in the final preparation of leptin by HPLC.

The stability of the [^{18}F]FBA-leptin **5** was tested both *in vitro* and *in vivo*. Over the course of an hour, the protein was stable in PBS (10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4), 100 mM sodium acetate, pH 4.5, and mouse serum *in vitro* as determined by HPLC analysis (Figure 4b−d). Finally, the stability was tested *in vivo*. In blood recovered from mice, greater than 95% of the radioactivity detected corresponded to intact [^{18}F]FBA-leptin **5** (Figure 4a). Thus, the stability of the protein is sufficient for studies both *in vitro* and *in vivo*. Importantly, this synthesis provides sufficient highly stable radiolabeled protein in good specific activity for studies in small and large animals, as well as in humans, with the biological activity of the protein intact.

PET Imaging of FBA-Leptin 5. We next performed PET imaging experiments in mice. [^{18}F]FBA-leptin **5** (3.7−5.55 MBq) was injected in *ob*/*ob* mice, and they were immediately imaged in a micro-PET scanner. We found that most of the protein rapidly accumulated in the kidneys (Figure 5a and supplemental video 1), in agreement with previous biodistribution studies.[42–44] A key advantage of ^{18}F over other nuclides is the improved resolution of the PET images obtained, up to 5-fold.[45] To illustrate this, we prepared ^{68}Ga-DOTA-leptin using standard NHS-ester bioconjugate chemistry (Supporting Information and Figure S6) and performed an analogous PET study in *ob*/*ob* mice. As expected, the resolution of the micro-PET images obtained with [^{18}F]FBA-leptin was substantially improved over images produced with ^{68}Ga-DOTA-leptin. Thus, while imaging ^{68}Ga-DOTA-leptin demonstrated binding to the kidney in mice (Figure 5b), imaging using [^{18}F]FBA-leptin **5** clearly reveals binding specifically in the cortex of the kidney, with less binding in other regions (Figure 5c). This pattern is consistent with the uptake of the hormone in the cortex of the kidney, in agreement with previous results.[46] Further analysis

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of the biodistribution and PET images of [^{18}F]FBA leptin **5** in various leptin resistant and sensitive animal models is currently underway in our laboratories.

Conclusions

In summary, we have reported the semisynthesis of a site-specifically ^{18}F-labeled leptin derivative, demonstrated its biological activity, and applied this tracer to PET imaging *in vivo*. Expressed protein ligation was used to install an aminooxy group at the C-terminus of leptin, which allowed for derivitization with both [^{19}F]- and [^{18}F]4-fluorobenzaldehyde. To our knowledge, this is the first site-specific ^{18}F-labeling procedure reported for a recombinant protein.[47] The site-specific radiolabeling of leptin allowed for unambiguous characterization of the bioactivity of the labeled product, both *in vitro* and *in vivo*. Furthermore, these protocols provide semisynthetic access to this important hormone, allowing for the introduction of a variety of imaging agents or modifications to improve the pharmacologic properties of the protein. Moreover, it is likely that the semisynthetic protocols developed herein for leptin can be extended to related cytokines of biomedical interest. While oximation with [^{18}F]-FBA failed under previously published conditions, we found that radiochemical oximation of proteins such as aminooxy-leptin **4** is dramatically accelerated by the addition of aniline as a catalyst, allowing high-yielding labeling of the protein at low temperatures. The stability of the tracer was also excellent over the time course used for PET imaging experiments. Additionally, [^{18}F]-FBA-leptin **5** allowed for high resolution PET imaging in *ob*/*ob* mice, demonstrating uptake of the protein in the cortex of the kidney. These methods should be applicable in general to the labeling of challenging protein targets such as leptin, in cases where the biological function of the protein must be preserved during imaging, and in cases where the high specific activity and improved resolution of F-18 is required for robust *in vivo* imaging. Further investigation of the biodistribution of [^{18}F]-FBA-leptin **5** is currently underway in our laboratories in various leptin sensitive and resistant murine and primate models.

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Supporting Information Available: Characterization of leptin thioester **1**, synthetic scheme and characterization of peptide **2**, *in vitro* activity of native leptin produced via an EPL protocol, ESI-MS characterization of DOTA-Leptin, and additional methods and procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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