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The Role of the Neuropeptide Y-2 Receptor in Liporemodeling: NPY Mediated Adipogenesis and Adipose Graft Maintenance

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Abstract

Background—Neuropeptide Y (NPY) is a signaling molecule that was recently found to stimulate adipose tissue growth in vitro via a peripherally acting mechanism involving NPY type 2 receptor (Y2R) found on adipocytes and endothelial cells. This study aims to evaluate the translational applications of a Y2R agonist for autologous fat grafting in plastic surgery.

Methods—Murine and primate animal models were used to investigate the proliferative effects of NPY on adipose tissue. The effect of applying NPY to subcutaneous tissues in mice and monkeys was assessed by MRI, histology, and immunohistochemistry. The effect of NPY on human fat xenograft survival and vascularity in athymic mice was measured by ultrasonography and immunohistochemistry. Six animals per group were used in murine experiments, and two animals were used in the pilot primate study.

Results—NPY stimulated growth of adipose tissues when applied subcutaneously in mice and monkeys, and increased human fat xenograft survival and vascularity in athymic mice at three months.

Conclusion—These data provide in vivo evidence for a critical role for NPY-Y2R interactions in adipogenesis, and suggest Y2R as a potential target for agonist compounds that can be used to enhance fat graft survival or stimulate de novo adipogenesis

Introduction

The ability to reliably and predictably graft human adipose tissue would have enormous benefit for plastic surgery patients. Unfortunately, autogenous fat grafting remains unpredictable in the hands of many plastic surgeons. Our previous work has shown that NPY may provide a mechanism to improve the long term volume maintenance of grafted fat.¹

NPY is a naturally occurring signaling molecule that co-localizes with the sympathetic nervous system and is highly conserved across species.² In humans, the cell membrane receptor for NPY has five subtypes, Y1-Y5. The pathway involving interactions between NPY and its type 2 receptor (Y2R) plays an important role in an animal's ability to store fat in times of stress.¹

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The Y2R is expressed on human endothelial cells and adipocytes in peripheral and visceral fat depots, where it has been found to induce proliferation of these cells as well as differentiation of preadipocytes into adipocytes.^{1,3} Based on these findings, it is hypothesized that stimulation of Y2R by NPY would result in localized proliferation of adipose tissue in animal models of fat grafting. The purpose of this manuscript is to focus on the translational applications of NPY as adjunct growth factors for autogenous fat grafting in cosmetic and reconstructive surgery.

Methods

Experimental Design

In one arm of the study, we investigated the effect of local NPY administration via subcutaneous pellet delivery system on de novo fat synthesis in wild-type and genetically obese mice, as well as in primates. De novo adipogenesis was measured at 2 weeks and 3 months using MRI quantification of the total body fat and separate specific fat depots.

In a second arm of the study, we investigated the effect of the NPY on human fat graft survival in athymic mice. Freshly harvested human fat was grafted into nude mice along with simultaneous implantation of either NPY-containing or a blank pellet. Fat graft survival and vascularization were measured at 2 weeks and 3 months using ultrasonography and immunohistochemistry. Fluorescence in-situ hybridization was used to determine fat graft composition (human vs murine cells) at 3 months.

The use of animals and human tissue samples in this study was approved by the Institutional Animal Care and Use Committee and the Institutional Review Board (GUACUC# 06-075, IRB# 2004-158), respectively, at the Georgetown University Medical Center.

Animals

Male wild-type C57BL/6 (WT) mice 6-8 weeks old were purchased and used from JAX laboratories. Male athymic mice were purchased from Taconic and used when 6-8 weeks old. Two female rhesus macaque primates (generous gift from Dr. James Gnadt) were used. Light/dark cycles were 12 hrs (6am-6pm light). Husbandry was provided by Georgetown University Animal Facility.

NPY

NPY and its derivatives were purchased from Bachem Laboratories. One microgram of NPY was dissolved in a slow-release cholesterol pellet (Innovative Research of America) and released in a sustained fashion over 14 days. The pellets were inserted subcutaneously into the abdominal fat pad of mice, which were separated into treatment groups (6 animals per group). Control groups were matched by background strain, age, and gender. The placebo controls contained the pellet without NPY. NPY pellets containing 1 µg of NPY were also inserted subcutaneously into two macaque monkeys. Control pellets without NPY were inserted in each monkey 10 cm away from the NPY pellet. The insertion site was sealed with cyanoacrylate tissue glue.

Human fat implantation in athymic mice

Human fat was obtained from senior author's surgical team. Abdominal fat from a single abdominoplasty patient was harvested using a 3-mm Tulip cannula and a 60 cc syringe. The fat was processed by gently moving over an absorbent Telfa pad and, once the gross oil was removed, it was atraumatically placed into a 10 cc syringe and capped without air for transport to the animal facility, where it was implanted within 30 minutes of harvest. Athymic nude mice (Taconic) were anesthetized and slow-release pellets (Innovative Research of America, Sarasota, FL) inserted subcutaneously. 100 µl of fat was injected as a bolus in close proximity

to the pellet using a 16-gauge Coleman infiltration cannula (Byron Medical) attached to the syringe. The skin wound was closed with cyanoacrylate tissue glue.

Experimental group (6 mice per group) received a human fat xenograft plus NPY pellet implantation as described above. Control animals (sixty mice) received fat xenograft and placebo pellet without NPY. Animals were followed for various time intervals over 3 months prior to harvesting the fat pads.

MRI quantification of adipose tissue

A Bruker 7-Tesla small-animal magnetic resonance imager coil was used to visualize and noninvasively quantitate various fat depots. A 3-dimensional (3D) T1/T2-weighted imaging protocol optimized for high contrast fat-imaging was implemented. This 3-D T1/2W RARE (Rapid Acquisition with Relaxation Enhancement) imaging sequence TE 5.9, TR 200, Rare Factor 8, Flip angle 60, Matrix 256X128X128, 7X3X3cm - 9X3X5cm (Cranial-Caudal $\times$ AP $\times$ LR) produced a reconstructed image that shows fat as the brightest signal while signals from other tissues were relatively suppressed. Quantification of the total body fat and separate specific fat depots were calculated using thresholding and voxel count plugins (by Wayne Rasband) from NIH imageJ software. VolumeJ plugin (by Michael Abramoff) was used to create 3D fat-images. The animal management system that was used in conjunction with the MRI was used to record core, skin, ambient and water blanket temperature measurements that are monitored during the imaging. The water blanket was used to regulate core temperature of the animal during imaging and anesthesia.

Ultrasonography

Fat pads were measured by ultrasound at 2 weeks, 1 month, and 3 months. Mice were anesthetized by isoflurane 1-3% in oxygen and a sterile water based ultrasonic gel was applied to the area (Aquasonic gel, Visual Sonics). The mice were placed in the Visual Sonics mouse holder containing a thermostatically controlled heating pad to maintain mouse body temperature. The imaging was performed with a small animal ultrasound system, Visual Sonics Vivo 660 (55 MHz).

Tissue sample collection and processing for histology

After treatment, fat pads were harvested from either subcutaneous abdominal, intra-abdominal, sternal, or interscapular locations. The total central fat (abdominal region only) weight (FW) and FW to percent of body weight (F/B) were measured. The adipose tissue samples were embedded in OCT tissue freezing media and snap frozen for frozen sections. Some samples were also fixed in Histochoice (Amresco, Solon, OH) or 10% buffered formalin (Fisher) overnight and then paraffin embedded.

Immunohistochemistry

Tissues were either postfixed with 10% formaldehyde and paraffin-embedded or embedded in tissue-freezing medium and flash-frozen in liquid nitrogen for frozen sections. Immunostaining was performed using the rat anti-mouse cd31 (BD Pharmingen) and mouse monoclonal anti-cd31 (Abcam). To verify the specificity of the antibodies, control experiments were done in parallel staining cultured endothelial cells as well as staining specimens without the primary antibody. Visualization with a streptavidin-biotin complex and horseradish peroxidase technique was used. Images were captured on a Nikon eclipse E 600 microscope (Nikon, Inc., Melville, NY) equipped with epifluorescence, and quantified with imageJ software for vessel staining density.

Identification of cellular origin by species specific fluorescence *in situ* hybridization (FISH)

Mice were euthanatized and the tissue was fixed in neutral buffered formalin overnight and then divided into two portions by cutting down through the skin, graft and musculature along the longest axis of the graft. The cut surfaces of the graft were then imbedded in paraffin blocks with the cut faces outwards and 5 micron sections cut for standard histological staining (H&E) and subsequent analysis.

The species origin of cells within sections of grafted fat was determined using a dual-label FISH technique to be described in detail elsewhere. Briefly, slides were deparaffinized and then digested with Proteinase K (Sigma, St. Louis, MO). The specimens were then allowed to air dry and then hybridized with green fluorescently tagged mouse Cot-1 DNA and red-tagged human Cot-1 DNA (Invitrogen, Carlsbad, CA) for 16-20 hour. The slides then were washed and counterstained with DAPI in mounting media (DAKO, Carpinteria, CA) to visualize cell nuclei. Images of the slides were then captured using a fluorescent dissecting microscope (Nikon, Melville, NY), and red, green and pseudo-dark field images analyzed and combined using MetaMorph software (Molecular Devices Corp., Downingtown, PA).

Statistical analysis

We analyzed the effect of treatment to respective controls in *in-vitro* studies using a one-way ANOVA with Bonferroni's multiple t-test to compare between treatment groups, with $p \leq 0.05$ considered statistically significant. We used unpaired Student *t*-tests to analyze the effects of a single treatment compared to control in immunohistochemistry, with $p < 0.05$ considered statistically significant. All data were normally distributed and presented graphically as mean $\pm$ standard error of the mean. All analyses were performed with Prism 4.03 (GraphPad Software, San Diego, CA).

Results

NPY Stimulates Endogenous Adipose Tissue Growth in Mice

To determine whether local intra-fat injections of NPY increase adipogenesis, adipose tissue deposition was compared between mice injected with NPY-containing pellets versus pellet alone. As measured by MRI, NPY injections into subcutaneous abdominal fat of wild-type mice significantly increased adipose tissue weight and volume compared to controls ($n = 6$ animals per group, $P < 0.05$ tested by one-way ANOVA with Bonferroni's multiple t-test, Figure 1).

NPY Stimulates Endogenous Adipose Tissue Growth in Primates

To assess the effects of NPY in primates, a pilot study with two rhesus monkeys was performed using subcutaneously implanted NPY pellet. Magnetic resonance imaging at three months demonstrated local *de novo* adipogenesis in response to NPY (Figure 2). No adipogenesis was observed with control pellets.

NPY Augments Human Fat Graft Vascularity and Survival in Nude Mice

We have shown previously that human adipose tissue expresses Y2R on adipocytes and endothelial cells¹. For this reason, the effects of NPY injections on human fat xenografts in nude mice were tested next. The NPY pellet, when injected alongside with a human fat xenograft, increased the xenograft's 3-month survival (measured by 3-dimensional ultrasound and histologically) and vascularization (measured by CD31 immunostaining), while xenografts without NPY underwent resorption ($N = 6$ animals per group, $P < 0.05$, Figure 3). Using *in situ* hybridization to differentially stain human and murine adipocytes, we demonstrated long-term survival of human adipocytes within the grafted fat (Figure 4).

Discussion

Autogenous fat fulfills many of the criteria for the ideal soft tissue filler.^{4,5} The clinical applications for autogenous fat grafting are expanding rapidly with recent articles reporting indications in breast reconstruction, breast augmentation, buttock enhancement, facial rejuvenation, and facial reconstruction.^{6,7,8} However, the popularity of fat grafting is limited by its unpredictability and the need for multiple injections.⁹ If a method to increase the predictability and survival of grafted human fat were identified, it would have tremendous benefit for plastic surgeons and their patients. This study proposes a novel mechanism utilizing the NPY-Y2R pathway to increase the reliability of autogenous fat grafts and generate localized fat de novo without fat grafts through local delivery of NPY.

The in vitro adipogenic and angiogenic effects of NPY on murine and human adipocytes were demonstrated by the authors in a previous study.¹ Based on these findings it was felt that NPY could serve as a powerful tool to enhance the volume of grafted fat in animal models. The preliminary work on the adipogenic effects of NPY in animals was performed in a murine model. Our results demonstrate that NPY is capable of generating de novo adipose tissue through local delivery of the peptide. When a cholesterol pellet that steadily releases NPY for a period of fourteen days is placed into the subcutaneous tissue of the mouse, a halo of new fat is generated around the pellet. This new adipose issue was found to be maintained for at least three months when the mice were sacrificed.

As a test to evaluate the translational potential of NPY in humans, a nonhuman primate model was utilized. Two rhesus monkeys had pellets containing NPY implanted in the subcutaneous region of the abdomen. Adipogenesis was observed on the MRI images after one month despite the fact that the doses of NPY used were identical to those used in the mice. It is felt that if the doses of NPY were increased to account for the increased weight of the primates, an even more profound adipogenic effect would be observed. Although the primate data are preliminary and necessitate additional studies, the adipogenic effects of NPY in both the murine and primate models demonstrate the highly conserved nature of the NPY-Y2R pathway and suggest its translational potential for humans.

An interesting observation in the primate study was that the NPY pellets were placed subcutaneously in the abdomen, and this region did not have an observable subcutaneous fat at the time of implantation. After low doses of NPY had been delivered to this location for fourteen days, there was clinical and MRI evidence of fat around the NPY pellets when the primates were sacrificed at three months. This adipose tissue was not observed around the control pellets. Further studies are needed to investigate whether the NPY acts to stimulate and differentiate mesenchymal stem cells to adipose tissue, increase preadipocyte differentiation, or initiate proliferation of undetectable subcutaneous adipocytes into observable adipose tissue.

To evaluate NPY's effects on human tissue, lipoaspirated human fat was grafted into immune-deficient mice with the addition of NPY. Three months after grafting, the fat pads in the NPY-treated mice were evaluated with 3-D ultrasound, and dissected weights. The three dimensional ultrasound is accurate within 0.1mm, and the authors are confident that they were able to distinguish between the retained grafted human fat and the normal mouse fat because the nude mouse has almost no subcutaneous fat in the region in which we placed the human xenografts. Ninety-nine percent of the fat was retained in all six mice in the NPY groups, whereas 70 percent of the fat was resorbed in the control mice. Because we had previously observed de novo adipogenesis with the addition of NPY, it was possible that the maintained fat could have been a result of NPY's adipogenic effects on the recipient site which could mask resorption of the human fat. To verify that human fat was maintained, we used in situ hybridization to visualize the presence of human DNA within the mouse recipient region. The observed

distribution of human DNA confirmed the presence, and thus maintenance, of human fat in the graft region. In addition to graft volume maintenance, histologic sectioning and ultrasound images demonstrated a homogenous distribution of grafted fat with no observed vacuolization on ultrasound images.

Although the effects of NPY on fat grafting have only been performed in nonhuman animal models, we do have reason to be optimistic about translation to human application. NPY compounds are currently in FDA trials for other applications and to the best of our knowledge, have not been associated with adverse effects.¹⁰⁻¹² In our experiments, no toxicologic effects were observed over the three month study period, nor were any histologic tissue abnormalities noted in the liver, bone, muscle, and kidney. The resting metabolic rate, body temperature, reproductive ability, triglycerides, or serum cholesterol all remained normal. Because NPY is a peptide, it is degraded by local proteases, and it is believed that this local degradation limits its systemic side effects.

Although NPY almost eliminates graft resorption in mice, differences in mechanisms across species are commonly encountered in drug development. We have successfully demonstrated the adipogenic effects of the NPY-Y2R pathway in mice, primates, and human adipocytes, which is consistent with the findings that this pathway is highly conserved across species.² The pilot data, showing efficacy of NPY in the primate model and the direct adipogenic effects noted in human fat, suggest that NPY will be effective in a human model.

This study demonstrates two methods through which NPY could provide a means to add soft tissue volume in plastic surgery: as a growth factor to enhance fat graft viability and as an inductive peptide to stimulate adipogenesis without the need for grafted fat. The ability to reliably create or graft fat would have broad applications in both reconstructive and cosmetic surgery.

Reconstructive applications of improved fat grafting include correction of deformities secondary to oncologic breast surgery, congenital anomalies, post-traumatic injuries, or post-ablative head and neck surgery. Cosmetic applications would include the use of fat as a permanent, biocompatible dermal filler that would age with the pliability of the patient's natural tissue. Additionally, fat could be added to the face in volumes that could rejuvenate the face in a cost efficient manner. The volumes necessary for facial rejuvenation are expensive and temporary when using other fillers on the market. Breast augmentation could also be achieved with fat grafting, or even with NPY-induced adipogenesis, if future studies support its safety.

The minimally invasive nature of fat grafting is advantageous in that it could prevent or reduce the need for more invasive operations associated with greater morbidity. We have demonstrated that NPY placed in the subcutaneous tissue or in peripheral fat induces adipogenesis even in the absence of grafted fat, suggesting that local delivery of NPY may achieve the desired contour without the need for fat grafts. Further studies will evaluate optimal dosing and delivery mechanisms in larger animal models to explore this possibility.

Conclusion

The NPY-Y2R pathway has been found to play an important role in the proliferation and maturation of preadipocytes, adipocytes and endothelial cells, and it potentially offers a mechanism to increase the predictability and success of autologous fat grafting. We have shown NPY is capable of creating de novo adipogenesis as well as reducing graft resorption in animal models and human adipose tissue. It remains to be determined if NPY can be safely administered to humans for this indication, but our results offer experimental support for the novel use of NPY-based drugs as candidates for fat grafting in humans. It is hoped that these

results will increase our understanding of fat grafting and lead to techniques that will increase the predictability and minimize the morbidity associated with autologous fat grafting.

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Figure 1. De novo fat synthesis in mice injected with NPY

Intra-fat administration of NPY pellets increased fat pad weight compared to control animals as seen on MRI. 3D-MRI volume reconstruction imaging of the fat in B6.V-Lepob/J mice with cross-sections of subcutaneous abdominal intra-fat where NPY pellets (1 mg/14 d) were administered, compared to controls. N = 6 animals per group, *P < 0.05 tested by one-way ANOVA with Bonferroni's multiple t-test. Error bars represent standard error of the mean. (Bottom panel used with permission from Nature Publishing Group, *Nature Medicine*, Kuo et al., July 2007).

Figure 2. NPY induces fat xenograft survival in a primate model

MRI cross-sections show a thicker fat pad in animals treated with NPY compared to controls.

Figure 3. NPY maintains human fat xenografts in athymic mice

Improved engraftment of human fat with NPY is seen by gross inspection (top panel) and ultrasonography. NPY treated fat injections showed more than twice the retention rate of controls by ultrasound at 2 weeks. Ultrasound imaging shows denser fat xenografts at 2 weeks in mice co-administered NPY (middle panel). Human fat graft retention at 3 months was 99 percent when coinjected with NPY, compared to 30 percent with control (bottom panel). N = 6 animals per group.

Figure 4. NPY maintains original cells contained in the fat xenograft at 3 months
In-situ hybridization of fat pad at three months shows a mixture of human donor (red) and murine host (green) adipocytes.