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PEGylation of Neuromedin U yields a promising candidate for the treatment of obesity and diabetes

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ABSTRACT

Neuromedin U (NMU) is an endogenous peptide, whose role in the regulation of feeding and energy homeostasis is well documented. Two NMU receptors have been identified: NMUR1, expressed primarily in the periphery, and NMUR2, expressed predominantly in the brain. We recently demonstrated that acute peripheral administration of NMU exerts potent but acute anorectic activity and can improve glucose homeostasis, with both actions mediated by NMUR1. Here, we describe the development of a metabolically stable analog of NMU, based on derivatization of the native peptide with high molecular weight poly(ethylene) glycol (PEG) ('PEGylation'). PEG size, site of attachment, and conjugation chemistry were optimized, to yield an analog which displays robust and long-lasting anorectic activity and significant glucose-lowering activity in vivo. Studies in NMU receptor-deficient mice showed that PEG-NMU displays an expanded pharmacological profile, with the ability to engage NMUR2 in addition to NMUR1. In light of these data, PEGylated derivatives of NMU represent promising candidates for the treatment of obesity and diabetes.

1. Introduction

With the increasing global prevalence of obesity, the interest is growing in pharmacological therapies to reduce appetite and increase energy expenditure, and gut peptides which regulate energy homeostasis represent attractive leads.[1–3] Neuromedin U (NMU) is a highly conserved peptide that is implicated in a number of physiological processes including nociception, stress, inflammation, blood pressure, feeding, energy homeostasis, and glycemic control.[4,5] Originally isolated from porcine spinal cord based upon its ability to contract rat uterine smooth muscle[6], NMU has since been found in several species.[7–11] It is distributed in the body both peripherally and centrally,[9,12–14] where it binds to two high affinity G-protein coupled receptors (GPCRs), NMUR1 and NMUR2, with complementary tissue distribution:[4,15–21] NMUR1 is predominantly expressed in the peripheral tissues, particularly the gastrointestinal tract, pancreas, uterus and testes,[18,19] whereas NMUR2 is mainly expressed in the central nervous system (CNS) with the highest levels in the hypothalamus, hippocampus, spinal cord and paraventricular nucleus.[14,15,17]

Although the pharmacology of NMU is not yet fully elucidated, a growing body of evidence highlights its role in the regulation of energy homeostasis. NMU-deficient mice develop obesity characterized by hyperphagia, reduced energy expenditure and hyperglycemia,[22] while NMU-overexpression in mice results in a lean phenotype with improved glucose homeostasis.[23] Moreover, an association has been recently found between NMU gene variants and excess body weight in humans.[24] Central administration of the peptide reduces food intake and body weight in rodents and birds,[17,25–28] and increases locomotor activity and core body temperature in rodents.[25]

The effects of centrally administered NMU are primarily mediated by NMUR2.[29] However, chronic intracerebroventricular infusion of NMU in NMUR2-deficient mice still showed a significant (albeit small) reduction in body weight, an effect which we hypothesized to depend on agonism at peripheral NMUR1 receptors.

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Accordingly, we showed that both acute and chronic peripheral administration of NMU resulted in a significant reduction in cumulative food intake and body weight, and that this effect is NMUR1-mediated.[30] Importantly, the anorectic effect of NMU did not exhibit tachyphylaxis, as observed for other peptides such as CCK or Amylin;[31,32] rather, the sustained activity observed upon chronic infusion was similar to other peptides which are being considered for therapeutic development, like GLP-1 and oxyntomodulin.[33] Finally, independent of its weight loss effects, peripherally administered NMU markedly improved glucose tolerance in diet-induced obese (DIO) mice.[30] The underlying mechanism of how NMU contributes to the regulation of body weight, feeding and glucose homeostasis is presently unclear.

Collectively, these findings suggest the possibility of developing NMU for the treatment of obesity and diabetes. To this end, engineering of the native peptide is required to overcome the poor pharmacokinetic properties typical of peptide hormones: the half-life of NMU after subcutaneous (sc) injection is less than 5 min.[30]

Here, we describe the development of a metabolically stable analog of human NMU with potent and long-lasting efficacy upon peripheral administration, obtained through conjugation of poly(ethylene) glycol (PEG) to the native peptide.

2. Materials and methods

A list of all the NMU Analogs discussed in this manuscript is given in Table 1, and their analytical data are reported in Table 2.

2.1. Peptide synthesis and purification

Solid-phase peptide synthesis via Fmoc chemistry was performed on ABI433 (Applied Biosystems) or Symphony (Protein Technologies Inc.) synthesizers. The resin was aminomethylated polystirene resin (100–200 mesh, 0.41 mmol/g) (Novabiochem) derivatized with the modified Rink linker *p*-[(R,S)-α-[9H-Fluoren-9-yl-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid.[34,35] Amino acids were used as 0.5 M solutions in DMF, containing equimolar HOBt (Hydroxybenzotriazole). Activation was with equimolar HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and a 2-fold molar excess of DIEA (*N*,*N*-diisopropylethylamine) in DMF or NMP. The acylation

Table 1
In vitro activity at the human NMUR1/R2 receptors of NMU and its PEGylated analogues

Peptide		Sequence[a]	EC_{50}[b] (nM)	
			hNMUR1	hNMUR2
1	hNMU	FRVDEEFQSPFASQSRGYFLFRPRN	2.3 ± 1.1	1.4 ± 0.7
2	Ac-NMU(19-25)	Ac-FLFRPRN	1.5 ± 0.8	2.6 ± 1.2
3	PEG40-NMU	**PEG40-**FRVDEEFQSPFASQSRGYFLFRPRN	125 ± 16	147 ± 21
4	C(PEG40)-NMU	Ac-**C(PEG40)**-FRVDEEFQSPFASQSRGYFLFRPRN	36 ± 16	109 ± 35
5	Ac-NMU	Ac-FRVDEEFQSPFASQSRGYFLFRPRN	4.8 ± 1.4	2 ± 1.2
6	C(NEM)-NMU	Ac-**C(NEM)**-FRVDEEFQSPFASQSRGYFLFRPRN	8.9 ± 2	5.2 ± 0.8
7	NMU-Cys4(PEG40)	Ac-FRV**C1**EEFQSPFASQSRGYFLFRPRN	97 ± 45	228 ± 75
8	NMU-Cys8(PEG40)	Ac-FRVDEEF**C1**SPFASQSRGYFLFRPRN	212 ± 137	722 ± 322
9	NMU-Cys12(PEG40)	Ac-FRVDEEFQSPF**C1**SQSRGYFLFRPRN	308 ± 162	659 ± 203
10	NMU-Cys16(PEG40)	Ac-FRVDEEFQSPFASQS**C1**GYFLFRPRN	980 ± 163	>1000
11	C(PEG40)-NMU(7-25)	Ac-**C1**FQSPFASQSRGYFLFRPRN	227 ± 48	224 ± 62
12	C(PEG40)-NMU(12-25)	Ac-**C1**ASQSRGYFLFRPRN	434 ± 126	>1000
13	C(PEG40)-NMU(19-25)	Ac-**C1-Ttds-**FLFRPRN	>1000	>1000
14	C(PEG20)-NMU	Ac-**C(PEG20)**-FRVDEEFQSPFASQSRGYFLFRPRN	20 ± 8	37 ± 18
15	C(PEG5)-NMU	Ac-**C(PEG5)**-FRVDEEFQSPFASQSRGYFLFRPRN	5.8 ± 1.1	12 ± 2.3

[a] Ac, acetyl; NEM, *N*-ethyl-maleimide; PEG40, branched PEG, size 40 kDa; **C1** = Cys(PEG40); **Ttds** = 1-amino-4,7,10-trioxa-13-tridecamine succinimic acid; **C(PEG20)** = Cys(PEG20), **C(PEG5)** = Cys(PEG5), where PEG20 and PEG5 = linear PEG, size 20 and 5 kDa, respectively; all peptides are amidated at the C-terminus.
[b] FLIPR assay.

Table 2
Analytical data for the NMU analogs of Table 1

Peptide	Gradient[a]	Column[b]	RT[c] (min)	Purity[d]	MW found (Da)	MW calc (D)
1	20–40(20′)-80(3′), 1 mL/min, RT	A	15.32	>95%	3081.00	3080.44
3	20–20(1′)-60(5′)-80(1′), 0.4 mL/min, 50 °C	B	4.68	>97%	46282.73[e]	45893.61[e]
4	25–50(20′)-80(3′), 1 mL/min, RT	C	15.83	>97%	46095.57[e]	46226.63[e]
5	20–40(20′)-80(3′), 1 mL/min, RT	C	15.85	>95%	3121.00	3120.50
6	15–35(20′)-80(3′), 1 mL/min, RT	C	14.29	>95%	3350.00	3350.75
7	30–60(6′)-80(1′), 0.6 mL/min, 50 °C	D	4.12	>97%	45769.32[e]	45710.62[e]
8	30–60(6′)-80(1′), 0.6 mL/min, 50 °C	D	3.94	>97%	46318.52[e]	45307.60[e]
9	20–40(5′)-80(1′), 0.6 mL/min, 50 °C	D	3.76	>97%	45726.86[e]	45798.56[e]
10	30–60(6′)-80(1′), 0.6 mL/min, 50 °C	D	3.81	>95%	45776.31[e]	45796.34[e]
11	20–60 (5′)-80(1′), 0.6 mL/min, 50 °C	B	4.68	>97%	45747.21[e]	45674.38[e]
12	20–60(5′)-80(1′), 0.6 mL/min, 50 °C	B	4.67	>97%	45167.71[e]	45069.28[e]
13	20–60(5′)-80(1′), 0.6 mL/min, 50 °C	B	4.71	>95%	44910.43[e]	44619.12[e]
14	25–50(20′)-80(3′), 1 mL/min, RT	C	15.97	>97%	24606.95[e]	24525.60[e]
15	30–60(20′)-80(3′), 1 mL/min, RT	C	10.02	>95%	8951.30[e]	8925.98[e]

[a] % of eluent B (time) flow rate, temperature.
[b] A = Phenomenex Jupiter C18 150 × 4.6 mm 5 μm 300 Å, B = UPLC waters acquity BEH C18 100 × 2.1 mm 1.7 μm 300 Å, C = Reprosil-Pur C4 150 × 4.6 mm 5 μm 300 Å, D = UPLC waters acquity BEH C4 100 × 2.1 mm 1.7 μm 300 Å.
[c] RT, retention time.
[d] By HPLC.
[e] Average MW.

reactions were performed for 60 min with 4-fold excess of activated amino acid over the resin free amino groups. N-terminal acetylation was performed with 10-fold excess of acetic anhydride in DMF. The following side chain protecting groups were used: *tert*-butyl (O^tBu) for Asp, Glu, Ser and Tyr; trityl (Trt) for Asn, Cys and Gln; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg. Fmoc-Ttds (13-amino-4,7,10-trioxa-tridecayl-succinamic acid) was purchased from Polypeptide Laboratories (Strasbourg, FR). Cleavage was with 88% trifluoroacetic acid (TFA), 5% phenol, 2% triisopropylsilane and 5% water[36] for 1.5 h at room temperature. After filtration, the peptide was precipitated with cold methyl-*t*-butyl ether, centrifuged, washed twice with fresh cold methyl-t-butyl ether, dried, resuspended in 20% acetonitrile/water, and lyophilized.

The crude peptides were purified by reversed-phase HPLC on Waters RCM Delta-Pak™ C_4 cartridges (40 × 200 mm, 15 μm) using as eluents (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. The following gradient of eluent B was used: 20%–20% (5 min) and 20%–35% (20 min), flow rate 80 mL/min. Analytical HPLC was performed on an Alliance Waters Chromatograph, using Phenomenex Jupiter C_4 (150 × 4.6 mm, 5 μm, rt) or ACE C_4 300 Å (150 × 4.6 mm, 3 μm, t = 45 °C) columns with the following gradient: 20%–40% B (20 min)-80% (3 min), flow rate 1 mL/min. Alternatively, analytical characterization was performed on a Waters Acquity UPLC, using a BEH 130 Å C_{18} column (2.1 × 100 mm, 1.7 μm), with the following gradient: 20%–40% B (4 min) −80% (1 min), flow rate 0.4 mL/min, 45 °C. The structure of the purified (⩾97%) peptides was confirmed by electrospray mass spectrometry on a Micromass LCZ platform.

2.2. Alkylation with *N*-ethylmaleimide (NEM)

The control peptide **6** (Table 1) with a blocked thiol group was prepared by alkylation with 1.5 molar excess of NEM in 0.2 M sodium phosphate, 8 M urea, 4 mM EDTA, pH 6.5, for 1 h. The peptide was purified and characterized as described above.

2.3. PEGylation

For PEGylation at the N-terminal amino group of peptide 3, the peptide precursor 1 (10 mg, 3.2 μmoles) was dissolved at 6.7 mg/mL concentration in 0.2 M HEPES, pH 7.3., and reacted with 3-fold molar excess (413 mg) of 40 kDa methoxy-PEG-*N*-hydroxysuccinimide (SUNBRIGHT GL2-400GS2, NOF Corp.), dissolved in water at 100 mg/mL. The conjugation reaction was monitored by HPLC, and was complete after 14 h. The solution was acidified with 1% formic acid, and the PEGylated peptide was purified by sequential cation-exchange chromatography (IXC) and size-exclusion chromatography (SEC).

For PEGylation through formation of a thioether bond, the Cys-containing peptide precursors were reacted with branched 40 kDa methoxy-PEG-maleimide (SUNBRIGHT GL2-400MA, NOF Corp.) (peptides 4 and 7–13), linear 20 kDa methoxy-PEG-maleimide (SUNBRIGHT ME-200MA, NOF Corp.) (peptide 14), or 5 kDa methoxy-PEG-maleimide (SUNBRIGHT ME-050MA, NOF Corp.) (peptide 15). In a typical preparation, the peptide precursor was dissolved at 10 mg/mL concentration in 0.15 M sodium phosphate, 8 M urea, 4 mM EDTA, pH 6.5, and reacted with a 1.1-fold molar excess of PEG reagent dissolved in water at 100 mg/mL. The reaction was monitored by HPLC or UPLC, and was typically complete after 30 min. The solution was acidified with 1% formic acid, and the PEGylated peptide was purified by sequential IXC and RP-HPLC.

IXC was carried out on MacroCap SP (GE Healthcare) column (26 × 120 mm) with a linear 0–0.6 M gradient of NaCl in 3.5 column volumes in 0.05% formic acid; the flow rate was 1 mL/min for column loading and 6 mL/min for gradient elution. SEC was carried out on TSK-HW50 (Tosoh) column (21 × 700 mm) in 0.1% (w/v) acetic acid, 30% acetonitrile, flow rate 1 mL/min. RP-HPLC was performed on a semi-preparative Waters RCM Delta-Pak™ C_4 cartridge (40 × 100 mm, 15 μm), using as eluents (A) 0.2% acetic acid in water and (B) 0.2% acetic acid in acetonitrile and the following gradient: 5%–5% B (5 min)–50% B (10 min) −80% B (2 min), flow rate 80 mL/min.

The purified PEGylated analogs were characterized by amino acid analysis (AAA), RP-HPLC and MALDI-Tof Mass Spectrometry. Purity by HPLC was ⩾98%. The concentration of the PEGylated peptide solutions used for in vitro and in vivo studies was determined by quantitative AAA.

2.4. In vitro functional assay (FLIPR)

The NMU receptors signal primarily through Gαq/11 proteins; therefore FLIPR, a calcium mobilization assay, was used to measure functional activity using cell lines stably expressing the human or mouse NMU receptors. Stable cell lines expressing NMUR1 or NMUR2 were plated at a density of 12,000 cells/well overnight on poly-lysine coated 384-well black-walled plates. The following day, the media was removed from the plates and the cells loaded with Fluo-3 (Molecular Probes), a calcium sensitive dye, diluted in FLIPR buffer (1X Hank's buffered saline containing 20 mM HEPES, 0.1% BSA, 2.5 mM probenecid (Sigma) and 1.6 mM TR40). All reagents were from Invitrogen unless otherwise noted. Peptide solutions were resuspended in saline at a stock concentration of 2 mM, and diluted on the day of the experiment in FLIPR buffer to a 4 μM working stock solution. After 90 min incubation at rt, cell plates were loaded onto a FLIPR (Molecular Devices) to monitor cellular fluorescence (excitation = 488 nM; emission = 540 nM) before and after peptide addition. Eight to twelve point dose responses were tested on NMUR-expressing cell lines using FLIPR with 1 μM peptide as the highest dose. The response after peptide addition was taken as the maximum fluorescence units minus the fluorescence immediately prior to stimulation for each well. EC_{50} values were calculated using GraphPad Prism (San Diego, CA) software. All the experiments were run at least in duplicate. Note that the reported endogenous start codon for mouse NMUR1 is a CTG codon[17] but due to low expression/activity in engineered cell lines we used a mouse NMUR1 cDNA clone that utilized the traditional ATG start codon to ensure for better receptor expression and activity in our functional assays.

2.5. Pharmacokinetics

PEGylated NMU was administered intravenously or subcutaneously to C57BL/6 mice and plasma samples were obtained at different times. Plasma concentrations of biologically active peptide were determined by bioassay, measuring NMU-induced formation of inositol-1-phosphate (IP1) in HEK293 cells expressing the human NMUR1 receptor. IP1 was quantified using a homogeneous time-resolved fluorescence (HTRF) assay (IPOne, CISbio International) according to the manufacturer's instructions. Briefly, cells were plated 24 h before the experiment into 96-well poly-L-Lysine coated-plates (Becton Dickinson) at a density of 90,000 cells/well and kept in DMEM medium containing 10% fetal bovine serum, 50 I.U penicillin, 50 μg/ml streptomycin and 2 mM glutamine. On the day of the assay, the medium was removed and 70 μl of stimulation buffer containing diluted plasma samples or peptide standards was added. Following incubation at 37 °C for 1 h, the incubation medium was removed and detection molecules (IP1-d2 conjugate and anti-IP1 cryptate) were added. An aliquot of this mixture was transferred to 96-well half-area white plates (Greiner Bio-one) which were then incubated for 1 h at room temperature. The HTRF signal was read using a Safire2™ reader (Tecan). Results

were calculated from the HTRF ratio given as [emission (665 nm)/emission (620 nm)]. A three-point titration was done with the test plasma samples and a 12-point titration of the peptide was run in naïve plasma to serve as standard. Peptide concentrations in plasma were extrapolated from the standard curve using non-linear regression analysis using XLfit 4.2 (IDBS). Pharmacokinetic parameters were calculated by non-compartmental analysis using WinNonlin (Pharsight).

2.6. Food intake studies

Animal studies were performed as previously described.[30] Briefly, male mice were individually housed under a 12-h light/12-h dark cycle. For knockout and wild type studies, NMU receptor-deficient animals were weight-matched to littermate controls. All protocols for use of these animals were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee (Rahway, NJ). Animals were *ad libitum* fed D12492, a high fat diet composed of 60% Kcal from fat (Research Diets). Male diet-induced obese (DIO) mice were weighed and dosed subcutaneously (sc) 30 min prior to the onset of the dark phase of the light cycle, and provided with a pre-weighed aliquot of food. Mice and food were weighed 18 h (day 1), 42 h (day 2), 66 h (day 3), and 90 h (day 4) after the onset of the initial dark phase. In the chronic studies, food intake and body weight were measured daily. Whole body composition analysis of conscious live mice was conducted using a Quantitative Magnetic Resonance (QMR) method (EchoMR™, Echo Medical Systems). The Minispec (Bruker-Optics) was used as NMR hardware having an applied static magnetic field corresponding to radio frequency of 7.5 MHz.

2.7. Glucose tolerance test studies

PEGylated NMU at 0.01, 0.1 or 1 mg/kg was dosed sc in 5% mannitol buffer 18 h prior to an oral glucose tolerance test (OGTT, 20% dextrose challenge, 10 mL/Kg, 2 g/kg) performed in fasted mice at 10:00 A.M. Mice ($n = 5$/group) were distributed by body weight into treatment groups. Basal blood glucose concentrations at $T = -0.5$ h and $T = 0$ h were determined by glucometer from tail nick blood. Mice were then immediately challenged with dextrose. Blood glucose levels were determined from tail bleeds taken at 20, 40, 60 and 120 min after dextrose challenge. The blood glucose excursion profile from $T = 0$ to $T = 120$ min was used to integrate an area under the curve (AUC) for wild-type mice treated with PEG-NMU.

2.8. NMU receptor-deficient mice

NMUR1-deficient (*Nmur1*$^{-/-}$) and NMUR2-deficient (*Nmur2*$^{-/-}$) mice were obtained as previously described.[29,30] NMUR1- & NMUR2-deficient mice (*Nmur1&2*$^{-/-}$) mice were generated by crossing N6 *Nmur1*$^{-/-}$ mice to N7 *Nmur2*$^{-/-}$ mice. *Nmur1&2*$^{-/-}$ mice were fertile, and exhibited no gross histological or pathological abnormalities.

2.9. Statistical analysis

All values are mean ± SEM. The data were analyzed using a two-tailed unpaired Student's *t* test; *p* values $\leqslant 0.05$ were regarded as significant and are denoted with an asterisk.

3. Results and discussion

To produce a metabolically stable, long-lasting analog of NMU we explored conjugation to poly(ethylene) glycol (PEGylation). PEGylation is a well-established strategy to increase the clinical efficacy of drugs and numerous examples exist of its application to peptides.[37–42] The highly hydrated shell of PEG increases the hydrodynamic radius of the conjugate thus decreasing the rate of renal clearance, and simultaneously restricts accessibility to proteolytic enzymes: this combined effect prolongs the circulation time of the conjugate.[43] By the same token however, PEG conjugation is typically associated with a reduction of biological potency at the receptor, caused by steric hindrance of the polymer.[44]

The key to successful application of PEGylation therefore resides in striking the right balance between the improved pharmacokinetics and the reduced receptor potency of the conjugated peptide.[44] We set out to do so for NMU through exploration of a number of variables like PEG size, PEG structure, PEG site of attachment, and PEGylation chemistry.

3.1. PEGylation of NMU. Optimal site of attachment

Human NMU consists of 25 amino acids (Table 1). While the N-terminal region of the peptide differs in length and/or amino acid composition between species, the C-terminal region displays remarkable sequence conservation: the C-terminal pentapeptide is identical across all species, and the C-terminal heptapeptide NMU$^{19–25}$ is conserved in mammals.[4] This conserved segment has been identified as the minimal region able to recapitulate the in vitro potency of the full length peptide on both NMUR1 and NMUR2[4–6,17,45,46] (Table 1). Based on this and in the absence of any structural information about the peptide active conformation, we first explored the N-terminus as the site for PEG attachment. We selected a branched PEG of 40 kDa (PEG40), since this size is the most efficacious to reduce kidney filtration of PEGylated peptides.[38,47,48] Two conjugation chemistries were explored: (i) reaction of the peptide with branched PEG40 N-hydroxysuccinimide, taking advantage of the absence of any amine functionality in the peptide other than the N-terminal amine (PEG40-NMU, 3), and (ii) introduction of an extra-Cys residue at the N-terminus and chemoselective reaction with maleimido-activated PEG (C(PEG40)-NMU, 4). Both PEGylated derivatives were full agonists of the human NMUR1/NMUR2 receptors, with comparable functional EC_{50} potencies (Table 1). However, depending on the receptor subtype, these values were 15–100 fold higher than those of native NMU (1) and of the non-PEGylated peptide controls, namely the N-terminal acetylated NMU (Ac-NMU, 5) and the acetylated peptide with the cysteine residue alkylated with *N*-ethyl maleimide (C(NEM)-NMU, 6) (Table 1). This decrease in receptor potency was expected, and is in line with what is typically observed for other PEGylated peptides and proteins.

We then explored PEGylation at internal positions of the sequence. Four PEGylated peptides were prepared, in which the PEGylation site was scanned every four residues along the sequence up to the C-terminal region$^{17–25}$, which was unaltered. In these analogues, PEGylation was achieved by mutating the amino acid at positions 4, 8, 12 and 16 into cysteine, to enable reaction with maleimide-activated PEG. The activity of internally PEGylated analogues 7–10 at the human NMUR1/R2 receptors is shown in Table 1. The peptides show progressively diminished potency as the site of PEGylation shifts from the N-terminus towards the C-terminus, with EC_{50} values at the human receptors in the following order: PEG40-NMU (**3**) < NMU-Cys^4(PEG40) (**7**) < NMU-Cys^8(PEG40) (**8**) < NMU-Cys^{12}(PEG40) (**9**) < NMU-Cys^{16}(PEG40) (**10**).

Furthermore, we prepared three PEGylated analogues with progressive shortening of the NMU sequence starting from the N-terminus, that is NMU(7–25), NMU(12–25), and NMU (19–25) (**11**–**13**, Table 1). PEGylation of these shortened analogues was achieved by introduction of an extra-Cys residue at the N-terminus and reaction with maleimido-activated PEG, as was done with analogue **4**. In the shortest analogue, corresponding to the minimal

bioactive region of NMU, a spacer (Ttds) was introduced between the N-terminal Cys and the peptide to minimize the shielding effect of the PEG.

The shortened PEGylated analogs showed a similar trend to the internally PEGylated series, with the largest loss of potency observed for the shortest analog C(PEG40)-NMU(19–25) (**13**).

3.2. PEGylation of NMU. Optimal PEG size

Having established the N-terminus as the optimal placement for PEG, we then explored N-terminal conjugation with different size PEGs. Although 40 kDa PEG is generally needed to endow on a peptide a hydrodynamic radius beyond the threshold for glomerular filtration, examples are reported in which the optimal PEG size for in vivo efficacy is smaller.[37,42] Therefore, we prepared NMU derivatives with 20 kDa and 5 kDa PEG (PEG20 and PEG5), via addition of an N-terminal Cys and reaction with maleimide-PEG (**14** and **15**, Table 1). As expected, the smaller the PEG size, the lower the steric hindrance, and the lower the EC_{50}: C(PEG5)-NMU (**15**) was only 2.5-8-fold less potent than the unconjugated peptide at hNMUR1/R2, while C(PEG20)-NMU (**14**), showed intermediate potency between C(PEG5)-NMU and C(PEG40)-NMU.

3.3. In vivo efficacy of PEGylated NMU analogs

While comparison of the in vitro potency of Peptides **7**–**13** with Peptides **3** and **4** is sufficient to select the latter because they all feature PEG of the same size and are expected to have similar pharmacokinetic properties, the situation is different for Peptides **14**–**15**, which must be compared with Peptides **3**–**4** in vivo to determine which peptide has the best overall properties.

Table 3 shows the results of a comparative acute food intake (FI) study in diet-induced obese (DIO) mice. In the study, we also included native NMU (Peptide 1) and the sequentially shortened analogs **11**–**13**. In addition to the FI data, the table shows the EC_{50} of the peptides at the mouse NMUR1/R2 receptors, which is generally similar to the potency observed at the human receptors.

As previously reported (30), treatment of DIO mice subcutaneously (sc) with a single 10 mg/kg dose of the native peptide was highly efficacious, but the effect on feeding peaked at 2 h and was short-lived; a 45% reduction in FI was measured at day 1, with no effect at day 2 and 3. By contrast, PEG40-NMU (**3**) and C(PEG40)-NMU (**4**) induced a prolonged reduction of FI, which was still apparent on the third day. The 7-amino acid deleted analog C(PEG40)-NMU(7–25) (**11**) showed comparable results, while further deletion of 5 amino acids in C(PEG40)-NMU (12–25), (**12**) reduced the duration of efficacy in vivo to two days. The shortest PEGylated analog, C(PEG40)-NMU (19–25), (**13**) showed only marginal efficacy on day 1, in line with the dramatic decrease of receptor potency (Table 3).

Because of the large difference in molecular weight due to the different PEG sizes, the two analogs C(PEG20)-NMU and C(PEG5)-NMU were tested at doses equimolar with 10 mg/kg of PEG40-NMU, that is at 5.2 and 2 mg/kg, respectively. C(PEG20)-NMU[14], was considerably more efficacious than PEG40-NMU on day 1 (99% versus 51% FI reduction), equipotent on day 2, but the effect was lost on day 3. This likely results from the balance of higher receptor agonism [C(PEG20)-NMU is >10-fold more potent on mNMUR1 and twofold more potent on mNMUR2] and poorer pharmacokinetic profile, both due to the smaller PEG size. C(PEG5)-NMU (15) exhibited good acute efficacy (79% food intake reduction) but no significant effect was detected beyond day 1. C(PEG5)-NMU has EC_{50} values close to native NMU, but conjugation with 5 kDa PEG does not seem to significantly improve the pharmacokinetic profile of the peptide.

Taken together, these data indicated that derivatization of NMU at the N-terminus with a 40 kDa PEG represents the most appropriate solution to achieve metabolic stability and prolonged duration of action. For further characterization, the PEG40-NMU derivative (3) was selected over the similarly active C(PEG40)-NMU (4) and C(PEG40)-NMU(7-25) (11), because of the recently reported potential of cys-maleimide derivatives for slow release of the drug upon exposure to plasma proteins.[49]

3.4. Pharmacokinetics of PEG40-NMU

The pharmacokinetics of PEG40-NMU (3) was evaluated in lean C57BL/6 mice upon subcutaneous and intravenous administration, at 10 and 1 mg/kg, respectively (Fig. 1). Plasma peptide levels were assessed with a functional bioassay that measures the functional response of the active peptide on human NMUR1-expressing cell lines.

Unlike wild type NMU (1) that has a half-life of < 5 minutes in mice (30), PEG40-NMU (3) showed an excellent pharmacokinetic profile, with 47% s.c. bioavailability, a half-life of 25 h, and a clearance of 0.03 ml/min/kg.

3.5. Efficacy of PEG40-NMU upon chronic dosing

The pharmacokinetics (Fig. 1) and the acute efficacy studies (Table 3) indicated that PEG40-NMU (3) is suitable for administration every other day (Q2D). We then evaluated the effect of PEG40-NMU upon repeated administration. *Ad libitum* fed male DIO C57BL/6 mice were dosed sc with 1, 3 and 10 mg/kg of Peptide 3 Q2D for 10 days (Fig. 2). Daily FI in the treatment groups was profoundly reduced in a dose-dependent manner for the first

Table 3
In vitro potency and in vivo activity of NMU analogues in diet-induced obese (DIO) mice

Peptide		Sequence[a]	EC_{50}[b] (nM)		% Food intake reduction[c]		
			mR1	mR2	18h	42h	66h
1	hNMU	FRVDEEFQSPFASQSRGYFLFRPRN	0.8 ± 0.3	0.5 ± 0.1	45	–	–
3	PEG40-NMU	PEG40-FRVDEEFQSPFASQSRGYFLFRPRN	65 ± 13	35 ± 15	51	57	22
4	C(PEG40)-NMU	Ac-C(PEG40)-FRVDEEFQSPFASQSRGYFLFRPRN	110 ± 11	16 ± 9	57	62	23
11	C(PEG40)-NMU(7-25)	Ac-**C1**FQSPFASQSRGYFLFRPRN	134 ± 13	185 ± 23	67	65	23
12	C(PEG40)-NMU(12-25)	Ac-**C1**ASQSRGYFLFRPRN	99 ± 18	363 ± 134	71	40	–
13	C(PEG40)-NMU(19-25)	Ac-**C1-Ttds-**FLFRPRN	>1000	>1000	11	–	–
14	C(PEG20)-NMU	Ac-**C(PEG20)**-FRVDEEFQSPFASQSRGYFLFRPR	8.6 ± 4	65 ± 22	99	65	–
15	C(PEG5)-NMU	Ac-**C(PEG5)**-FRVDEEFQSPFASQSRGYFLFRPRN	nd	nd	79	–	–

[a] Abbreviations as in Table 1.
[b] FLIPR assay; nd, not done.
[c] Food intake reductions in diet-induced obese (DIO) mice administered a single subcutaneous administration at 10 mg/kg of peptide. Peptides 14 and 15 were tested at 5.2 and 2 mpk, respectively, which is a dose equimolar to 10 mg/kg for the 40 kDa-PEGylated peptides. NMU was dosed at 3250 nmol/kg, whilst the PEGylated peptides were dosed at ~220 nmol/kg.

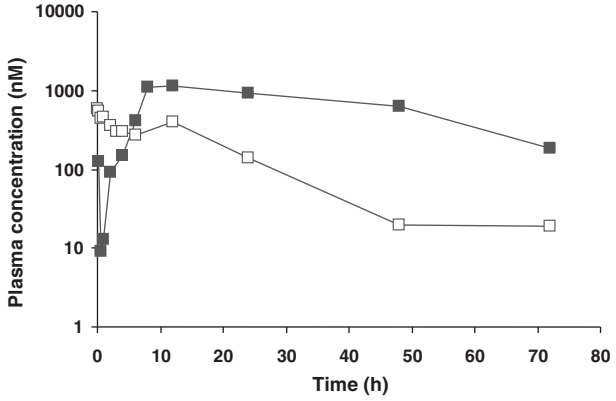

Figure 1. Pharmacokinetics of PEGylated NMU in mouse. Peptide 3 (PEG40-NMU) was administered intravenously (□) or subcutaneously (■) to C57BL/6 mice, respectively at 1 mg/kg and 10 mg/kg in water, and the plasma concentrations monitored with time. The plasma concentration of peptide were determined by bioassay as described in the experimental procedures. The curves represent the mean of three animals.

5 days of the study; by day 6 FI returned to levels comparable to vehicle treated animals (Fig. 2A). We observed the same initial reduction and subsequent restoration of food intake upon continuous dosing of native NMU in DIO mice (30), and similar effects have been reported for other anorexigenic peptides.[37,50,51] However, unlike the effect on FI, BW loss was sustained throughout the course of the study (Fig. 2B). The cumulative reduction in FI after 10 days of treatment was 5.8% and 20.3%, respectively, for the 3 and 10 mg/kg doses (Fig. 2C), while the cumulative reduction in BW was 2.4% and 7.6%, respectively (Fig. 2D). Weight loss was primarily due to a decrease in fat mass (vehicle: 0.6 g; 1 mg/kg: 0.8 g; 3 mg/kg: −1.4 g; 10 mpk: −2.1 g). No significant effect on either parameter was seen for the 1 mg/kg dose. No changes in locomotor activity were observed with the PEGylated peptide (data not shown). The sustained reduction in body weight could be partly due to differences in energy expenditiure, since native NMU has been shown to have acute effects on metabolic rate.[30] Further studies are warranted to better understand these findings. Encouragingly, we did not observe any adverse event during the treatment.

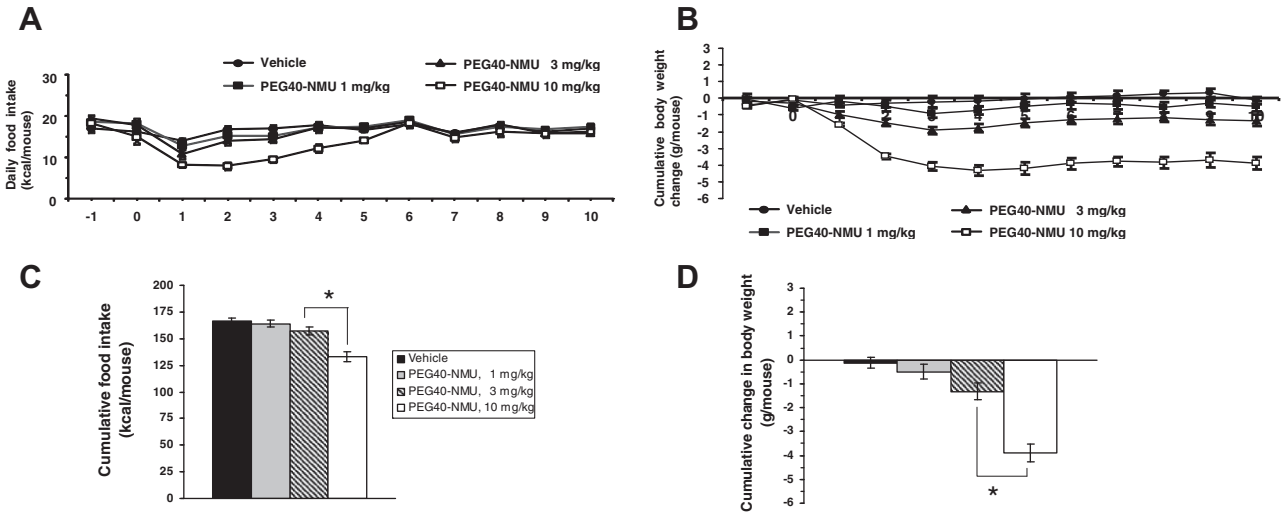

Figure 2. *Ad libitum* fed male DIO C57BL/6 mice were treated with either vehicle or peptide 3 (PEG40-NMU) at the indicated doses every other day for 10 days. (A) Daily food intake; (B) cumulative daily change in body weight; (C) cumulative food intake change at the end of the experiment; (D) cumulative body weight change at the end of the experiment. The asterisk indicates statistical significance (P <0.05) relative to vehicle-treated animals.

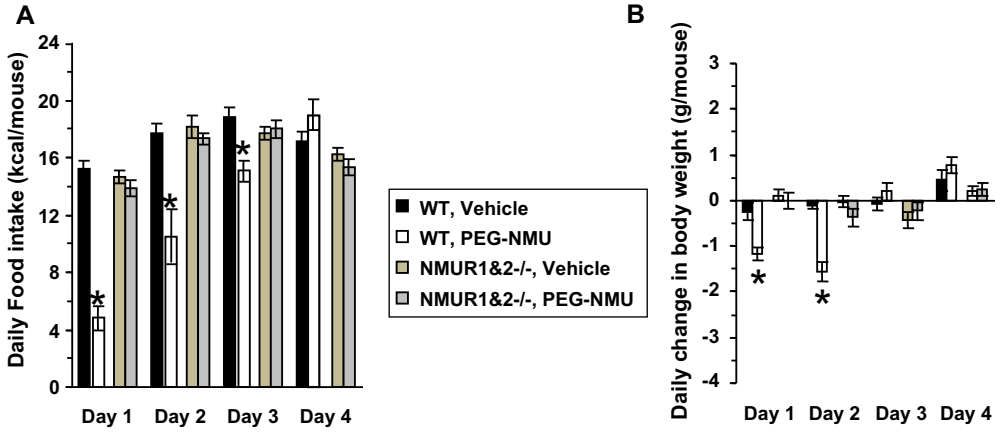

Figure 3. *Nmur1&2*$^{-/-}$, and wild type (WT) mice received a single sc dose of either vehicle or 10 mg/kg PEG40-NMU (3). (A) Daily food intake and (B) daily body weight change. The asterisk indicates statistical significance (P <0.05) relative to vehicle-treated animals.

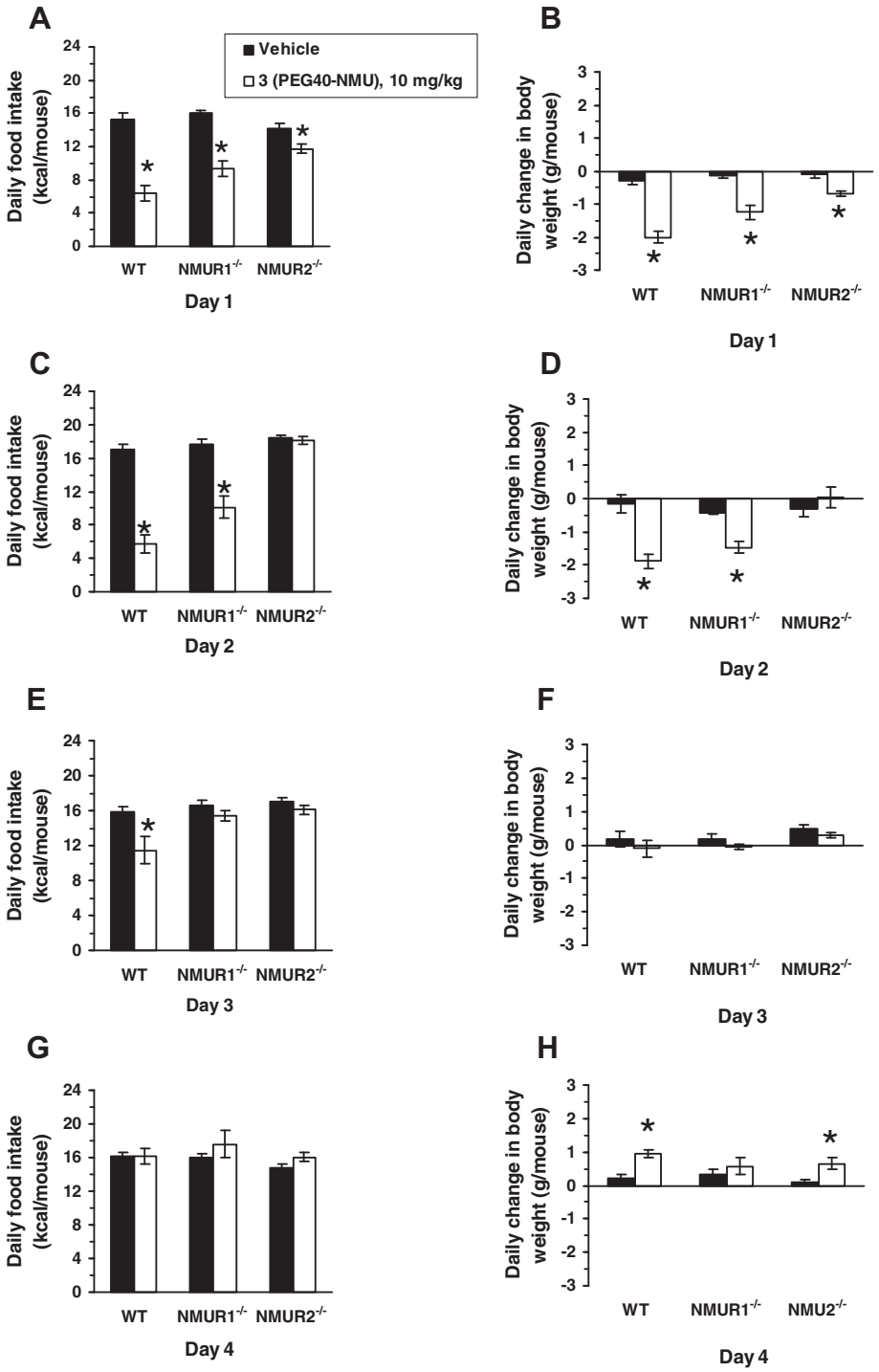

Figure 4. *Nmur1*$^{-/-}$, *Nmur2*$^{-/-}$, and wild type (WT) mice received a single sc dose of either vehicle or 10 mg/kg PEG40-NMU (3). The panels show the daily food intake and body weight change measured after dosing at day 1 (A and B), day 2 (C and D), day 3 (E and F) and day 4 (G and H). The asterisk indicates a significant reduction (P <0.05) relative to vehicle-treated animals.

3.6. Efficacy of PEG40-NMU in NMU receptor-deficient mice

We had previously studied the anorectic action of native NMU using NMUR1-deficient (*Nmur1*$^{-/-}$) and NMUR2-deficient (*Nmur2*$^{-/-}$) mice.[29] We have now produced mice lacking both receptors, NMUR1/2-deficent mice (*Nmur1&2*$^{-/-}$). A comparative study of PEG40-NMU (3) at 10 mg/kg in wild-type (WT) and NMUR1/2-deficent DIO mice showed that, while in WT mice the PEGylated peptide had the usual duration of action (three days post dose for FI and two days post dose for BW), no significant effect was observed in the NMUR1/2-deficent animals (Fig. 3). This demonstrated that, as for the native peptide hormone, the anorectic effect of PEGylated NMU is mediated entirely by interaction with the NMU receptors.

We then performed studies in single receptor-deficient mice (Fig. 4). At 10 mg/kg, PEG40-NMU (3) was active in both *Nmur1*$^{-/-}$ and *Nmur2*$^{-/-}$ animals, but exhibited different efficacy: In *Nmur1*$^{-/-}$ mice, the effect on FI and BW lasted for 2 days post dose

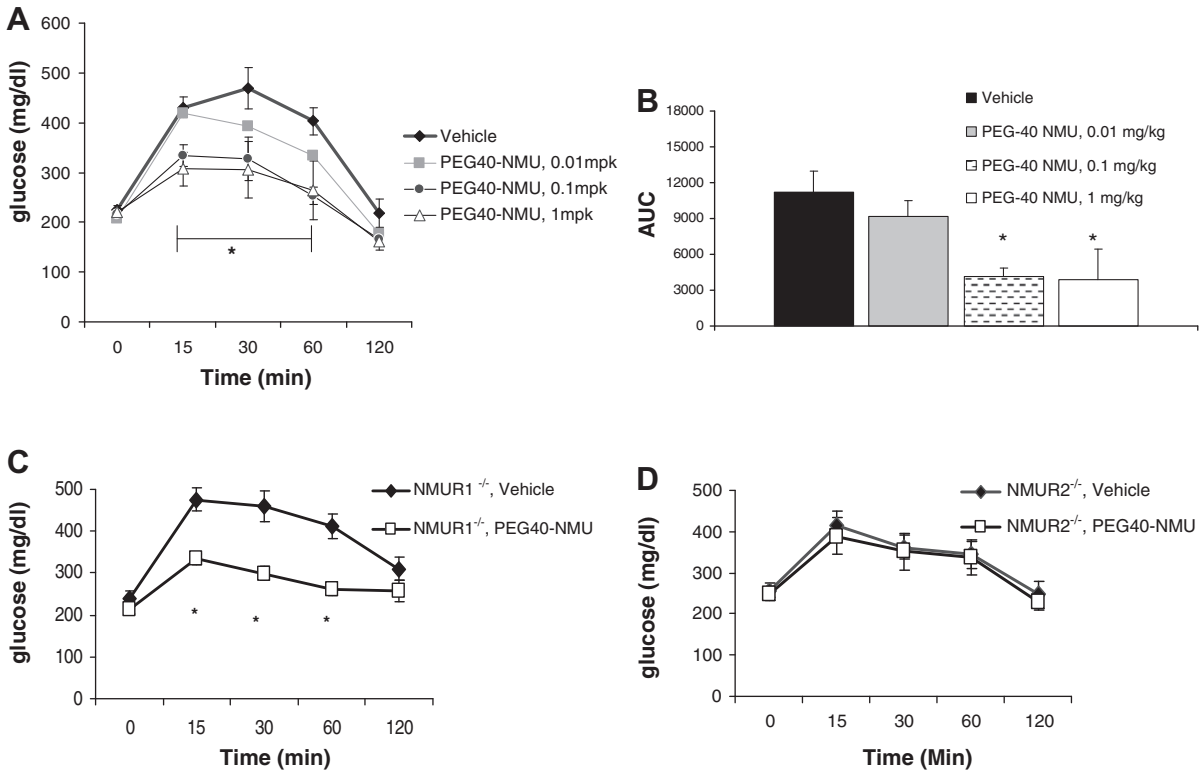

Figure 5. (A). Glucose lowering efficacy of PEG40-NMU (3) in an oral glucose tolerance test (OGTT) in overnight fasted WT DIO mice. (B) Effect of PEG40-NMU on inhibition of glucose AUC in the OGTT. (C) PEG40-NMU (10 mg/kg sc) suppresses glucose excursion in *Nmur1*$^{-/-}$mice. (D) PEG40-NMU (10 mg/kg sc) did not alter glucose excursion in *Nmur2*$^{-/-}$ *mice.* $^{*}P$ <0.05 versus vehicle.

(−41.8% FI and -2.2% BW at day 1, −42.6% FI and −2.1% BW at day 2); in *Nmur2*$^{-/-}$ mice, the peptide was efficacious only on day 1 (−17.3% in FI and −1.2% in BW). In the control WT mice, the PEGylated peptide exerted anorectic effects as usual.

This finding highlights that by peripheral administration, native NMU and PEGylated NMU exert anorectic effects by (at least partially) distinct mechanisms of action. While the anorectic activity of peripherally administered native NMU is exclusively mediated by NMUR1[30], PEG40-NMU exerts its effect by *both* NMUR1 and NMUR2 receptors. Indeed, comparison of the efficacy of PEG40-NMU in *Nmur1*$^{-/-}$, *Nmur2*$^{-/-}$, and WT mice seems to indicate that the three-day efficacy in the latter results from additive agonism on the two receptor subtypes. We hypothesize that the different ability of peripherally administered PEG40-NMU and native NMU to access regions of the brain, where NMUR2 is predominantly expressed, is due to the fast metabolism of the latter peptide. The anorectic activity of PEGylated peptides on receptors expressed in the brain is not unprecedented[37,42], and in the case of NMU PEGylation uncovers the potential for central action of the peripherally administered peptide.

3.7. PEG40-NMU improves glucose tolerance

We had previously demonstrated that peripherally administered NMU improved glucose tolerance during an OGTT[29] and wished to determine if the PEGylated peptide retained this activity. Initial experiments using 10 mg/kg PEG-40 NMU showed a significant improvement in glucose excursion during an oral (OGTT) and intra-peritoneal glucose tolerance test (IPGTT) (data not shown). To determine the minimal efficacious dose, we performed a dose titration of PEG40-NMU in an OGTT. Both 0.1 and 1 mg/kg significantly reduced the blood glucose excursions observed during OGTT (Fig. 5A) and the corresponding area under the curve (Fig. 5B). Significant reductions were observed at 15, 30 and 60 min post glucose challenge. Similar results were observed with an intraperitoneal GTT (IPGTT) (data not shown). To assess which NMU receptors were contributing to the reductions in glucose, we performed OGTTs in *Nmur1*$^{-/-}$ and *Nmur2*$^{-/-}$ mice (Fig. 5C–D). Administration of 10 mg/kg PEG40-NMU significantly reduced glucose excursion in *Nmur1*$^{-/-}$ mice. In contrast, we found that 10 mg/kg PEG40-NMU did not significantly alter glucose excursion in *Nmur2*$^{-/-}$ mice, indicating that the improvement in glucose homeostasis was mediated by NMUR2 and not by NMUR1. These findings are in contrast to what we observed with native NMU (GTT efficacy was mediated by NMUR1) and as we observed for the BW/FI studies, peripheral administration of the PEGylated peptide exhibits an altered pharmacological profile. Additionally, the dose titration study further shows that the MinED for glucose is significantly lower than what is required for body weight.

4. Conclusions

In conclusion, we developed a metabolically stable analog of human Neuromedin U, based on conjugation of the native peptide to poly(ethylene) glycol (PEG). PEG-NMU displays robust and long-lasting body-weight and glucose lowering activity in vivo, and an altered pharmacological profile engaging both central and peripheral receptors. PEG-NMU analogs represent promising candidates for the treatment of obesity and diabetes. Future work towards the development of an NMU-based therapeutic will be directed at better understanding the mechanism of how NMU contributes to energy homeostasis. Moreover, given the other described roles for NMU including the elicitation of the stress reponse (3), it will be important to understand whether any unwanted adverse effects become apparent upon chronic treatment with the peptide.

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