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BIOCHEMISTRY

pNaKtide inhibits Na/K-ATPase reactive oxygen species amplification and attenuates adipogenesis

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Obesity has become a worldwide epidemic and is a major risk factor for metabolic syndrome. Oxidative stress is known to play a role in the generation and maintenance of an obesity phenotype in both isolated adipocytes and intact animals. Because we had identified that the Na/K-ATPase can amplify oxidant signaling, we speculated that a peptide designed to inhibit this pathway, pNaKtide, might ameliorate an obesity phenotype. To test this hypothesis, we first performed studies in isolated murine preadipocytes (3T3L1 cells) and found that pNaKtide attenuated oxidant stress and lipid accumulation in a dose-dependent manner. Complementary experiments in C57Bl6 mice fed a high-fat diet corroborated our *in vitro* observations. Administration of pNaKtide in these mice reduced body weight gain, restored systemic redox and inflammatory milieu, and, crucially, improved insulin sensitivity. Thus, we propose that inhibition of Na/K-ATPase amplification of oxidative stress may ultimately be a novel way to combat obesity, insulin resistance, and metabolic syndrome.

INTRODUCTION

The number of overweight individuals worldwide has markedly grown, leading to an increase in obesity-related health problems and associated morbidity and mortality (1). Oxidative stress in adipose tissue is an important pathogenic mechanism leading to maintenance of the obesity phenotype-associated metabolic syndrome (2–5). It has recently been observed that the development of obesity can be markedly affected by the manipulation of either the systemic redox state (6–11) or the redox state of adipocytes alone (2, 12). Specifically, manipulation of the redox state of adipocytes by the targeted transfection of antioxidant genes to adipocytes alters their phenotype as well as reduces total body fat stores and ameliorates metabolic abnormalities (13, 14).

Dysfunctional adipogenesis is one of the hallmarks of chronic obesity and is characterized by increased lipid accumulation and altered endocrine function of the adipose tissue (15). Peroxisome proliferator-activated receptor γ (PPAR γ) is a master regulator of adipogenesis and activates the expression of genes, such as fatty acid synthase (FAS), to trigger the synthesis of fatty acids and triglycerides (16). Mesoderm-specific transcript (MEST), when up-regulated, results in adipocyte enlargement during adipose tissue expansion (17), which is associated with increased release of inflammatory adipocytokines and enhanced insulin resistance (3). Adipose tissue regulates energy metabolism via secretion of soluble factors such as adiponectin and tissue necrosis factor- α (3). Dysregulated production of these adipocytokines participates in the pathogenesis of obesity-associated metabolic syndrome (3, 18–20).

The Na⁺- and K⁺-dependent adenosine triphosphatase (Na/K-ATPase) is a member of the P-type ATPase family. It resides in the plasma membrane and transports Na⁺ and K⁺ ions across the cell membrane by hydrolyzing ATP. The functional Na/K-ATPase consists of α and β subunits, and several isoforms have been identified for each subunit

(21, 22). Our group has observed that, in addition to the ion pumping function, the $\alpha 1$ subunit also serves a scaffolding function with sarcoma-related kinase (Src), allowing conformational changes in the Na/K-ATPase to effect a signaling cascade. We have specifically identified a NaKtide sequence from the nucleotide-binding (N) domain of this α subunit, which binds the kinase domain of Src, tonically inhibiting its function. However, when the conformation of the $\alpha 1$ subunit is changed by the binding of cardiotonic steroids, this inhibition may be released, resulting in the activation of Src (23–25). The Na/K-ATPase therefore controls signaling by regulating Src through downstream modulation of the epithelial growth factor receptor (EGFR) and ultimately reactive oxygen species (ROS) (26, 27). We have recently observed that the $\alpha 1$ subunit of the Na/K-ATPase can also act as a receptor for some ROS and potentially serve as a feedforward amplifier (28, 29). Moreover, we have designed a cell-permeant NaKtide (pNaKtide) and found it to specifically inhibit cardiotonic steroid-induced Src activation (30–33). Given the importance of the adipocyte redox state in the development and maintenance of obesity, we reasoned that pNaKtide might significantly attenuate Na/K-ATPase-modulated ROS amplification within the adipocyte and attenuate the development of adiposity.

RESULTS

Effect of pNaKtide on adipogenesis in murine preadipocytes

3T3L1 cells were exposed to adipogenic media (16, 34) and treated with increasing doses of pNaKtide. Our results showed that pNaKtide reduced lipid accumulation (Fig. 1) in 3T3L1 cells in a dose-dependent manner. As a control, we exposed these cells to rhodamine-labeled pNaKtide and found that pNaKtide readily passed the cell membrane and resided in the intracellular membrane compartments (fig. S1A). In addition to decreasing the net accumulation of lipid, pNaKtide also increased the amount of lipid in small droplets while decreasing the proportion in large droplets, also in a dose-dependent manner (fig. S2, A and B, respectively). Oxidative stress is one of the consequences of

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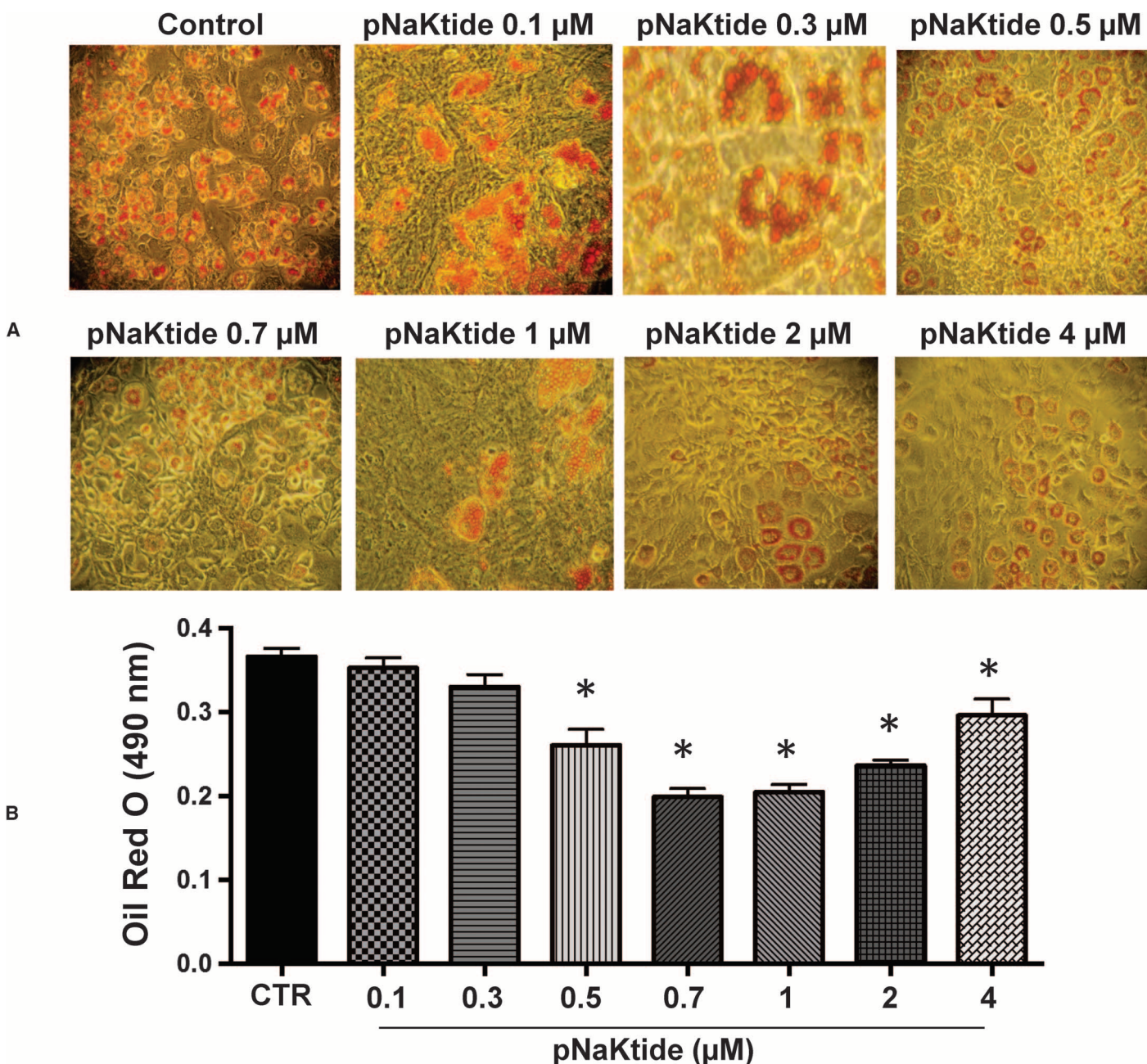


Fig. 1. Effect of increasing pNaKtide concentrations on adipogenesis in mouse preadipocytes. Adipogenesis was measured as the relative absorbance of Oil Red O at day 7 after inducing adipogenesis as described in Materials and Methods. (A) Representative images. (B) Quantitative data expressed as means \pm SE, $n = 7$; * $P < 0.05$ versus control.

Na/K-ATPase activation. Superoxide levels, a marker for oxidative stress, were significantly reduced by pNaKtide in 3T3L1 cells (fig. S2C; $P < 0.05$). No cytotoxic effects of pNaKtide were noted at the studied doses (up to 4 μM) (fig. S2D).

Effect of pNaKtide on adiponectin levels and adipogenic markers in murine preadipocytes

Administration of pNaKtide increased adiponectin levels (Fig. 2A; $P < 0.05$), a known marker of small insulin-sensitive and healthy

adipocytes (35). Furthermore, our results showed that pNaKtide treatment significantly reduced the expression of adipogenic markers, including FAS, MEST, and PPAR γ (Fig. 2, B to D, respectively). Thus, pNaKtide prevented adipocyte dysfunction, an effect that could be attributed to its role in cellular redox through the Na/K-ATPase signal cascade.

Fructose has been shown to induce oxidative stress and increase adipogenesis in adipocytes exposed to adipogenic media (36). As shown in fig. S3, the fructose-induced increase in adipogenesis was prevented

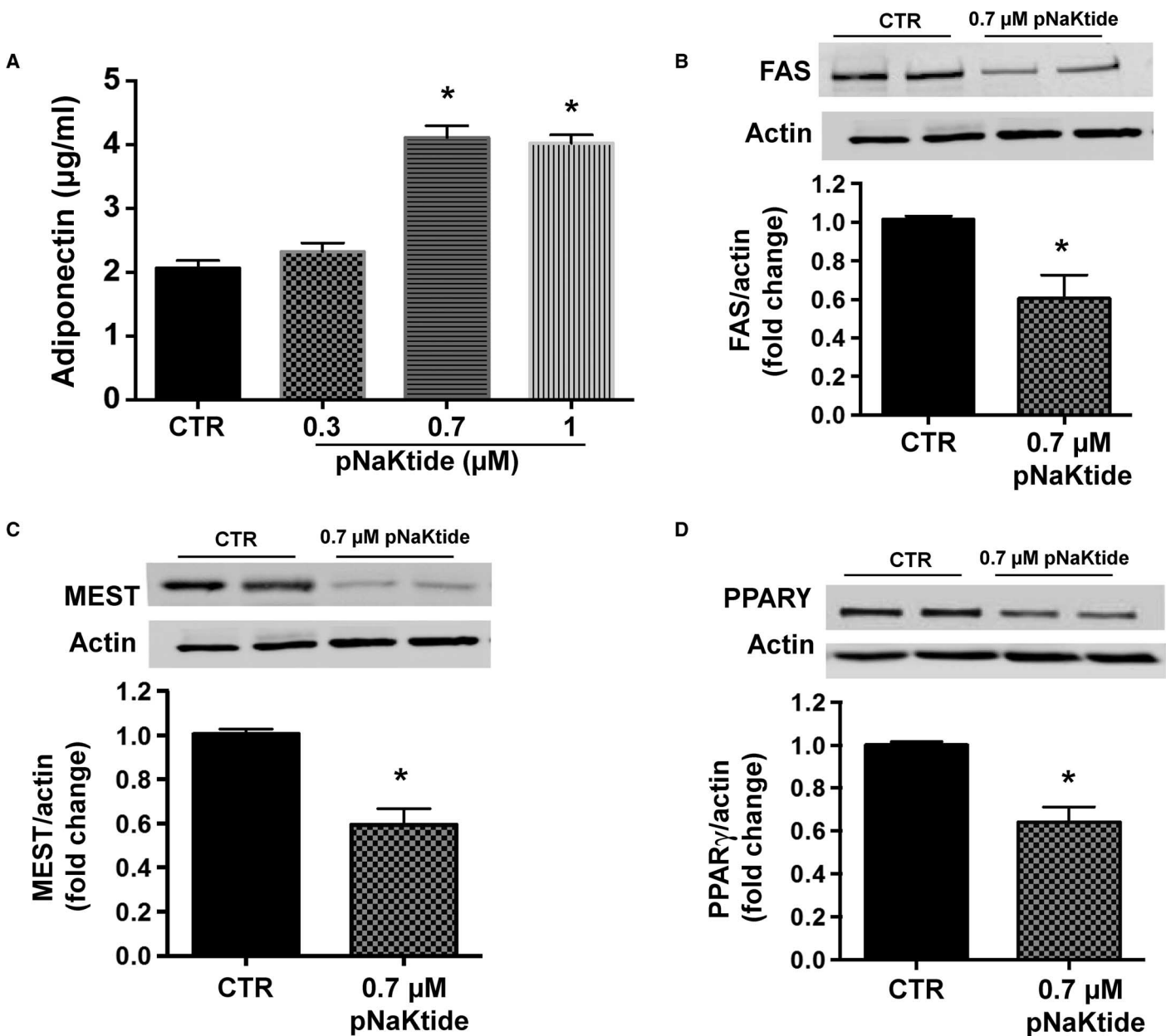


Fig. 2. pNaKtide increased adiponectin levels and decreased adipogenic markers in 3T3L1 adipocytes. (A) Adiponectin levels were determined in conditioned media obtained from 3T3L1 cells after treatment with pNaKtide for 7 days. Cells were treated with varying concentrations of pNaKtide, and 0.7 µM pNaKtide was determined to be the optimal concentration for increasing adiponectin levels. Results are means \pm SE, $n = 4$; * $P < 0.05$ versus control (CTR). (B to D) Expression of (B) FAS, (C) MEST, and (D) PPAR γ was determined by Western blot analysis in 3T3L1 cells after treatment with pNaKtide (0.7 µM) for 7 days. Quantitative densitometric evaluation of protein ratios was done. Data are expressed as means \pm SE, $n = 6$; * $P < 0.05$ versus control.

by treatment with pNaKtide in 3T3L1 cells. Similarly, addition of glucose oxidase to the adipogenic media also increased lipid accumulation (fig. S4A) and the adipogenic markers FAS and MEST in 3T3L1 cells (fig. S4, B and C), and this was significantly attenuated by concurrent treatment with pNaKtide. Treatment with pNaKtide also decreased protein carbonylation and blocked the Na/K-ATPase-regulated activation of Src and extracellular signal-regulated kinases 1 and 2 (ERK1/2) in 3T3L1 cells exposed to glucose oxidase (fig. S5, A to C, respectively).

Effect of pNaKtide on body weight and visceral and subcutaneous fat content in mice fed a high-fat diet

In our *in vivo* studies, C57Bl6 mice were exposed to a high-fat diet for 4 weeks and then given pNaKtide at different doses via intraperitoneal injection, and the high-fat diet was continued for another 8 weeks. In parallel experiments, rhodamine-labeled peptide was demonstrated to accumulate in adipose tissue (fig. S1B). Administration of pNaKtide at 25 mg/kg intraperitoneally every 8 days markedly reduced adiposity in these mice, with lower doses having less effects (Figs. 3 and 4).

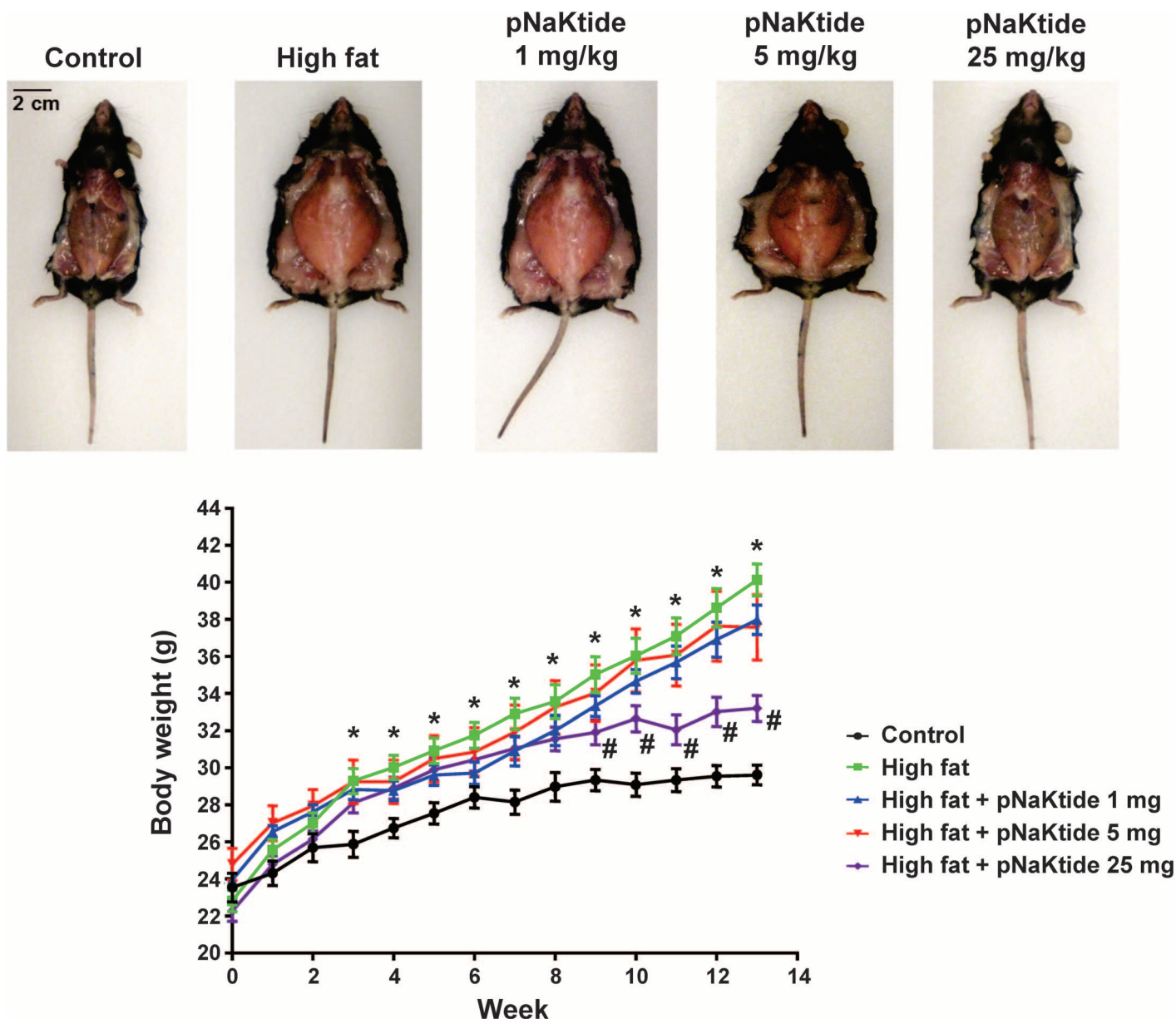


Fig. 3. Effect of increasing doses of pNaKtide on body weight in mice fed a high-fat diet. Treatment with pNaKtide at doses of 1 and 5 mg/kg did not significantly reduce body weight in C57Bl6 mice fed a high-fat diet. However, administration of pNaKtide at 25 mg/kg every 8 days in mice fed a high-fat diet for 8 weeks significantly reduced body weight as compared to high-fat diet-fed animals. There were no significant changes in food intake among the groups. Results are means \pm SE, $n = 7$ to 14 per group; * $P < 0.05$ versus control, # $P < 0.05$ versus high-fat diet.

However, more frequent administration of pNaKtide (25 mg/kg intraperitoneally every 2 days) induced some fluid retention (fig. S2). Therefore, we focused our subsequent studies on the effects of the administration of pNaKtide every 8 days on adipogenic markers and metabolic profile.

Effect of pNaKtide on adipogenic markers and adiponectin expression in mice fed a high-fat diet

Mice fed a high-fat diet exhibited an increase in FAS (Fig. 5A) and MEST (Fig. 5B) gene expression in visceral adipose tissue compared to the control group. Treatment with pNaKtide (25 mg/kg every 8 days)

significantly decreased FAS and MEST levels compared to mice fed a high-fat diet. Further, our results showed that pNaKtide treatment increased adiponectin levels in visceral adipose tissue compared to mice fed a high-fat diet (Fig. 5C). These observations support our hypothesis that pNaKtide not only reduces adipose tissue mass but also promotes the development of healthier adipocytes, with adiponectin levels comparable to those seen with regular diet.

Effect of pNaKtide on metabolic profile in mice fed a high-fat diet

The insulin resistance phenotype in mice fed a high-fat diet was reversed by the administration of pNaKtide. This was characterized by

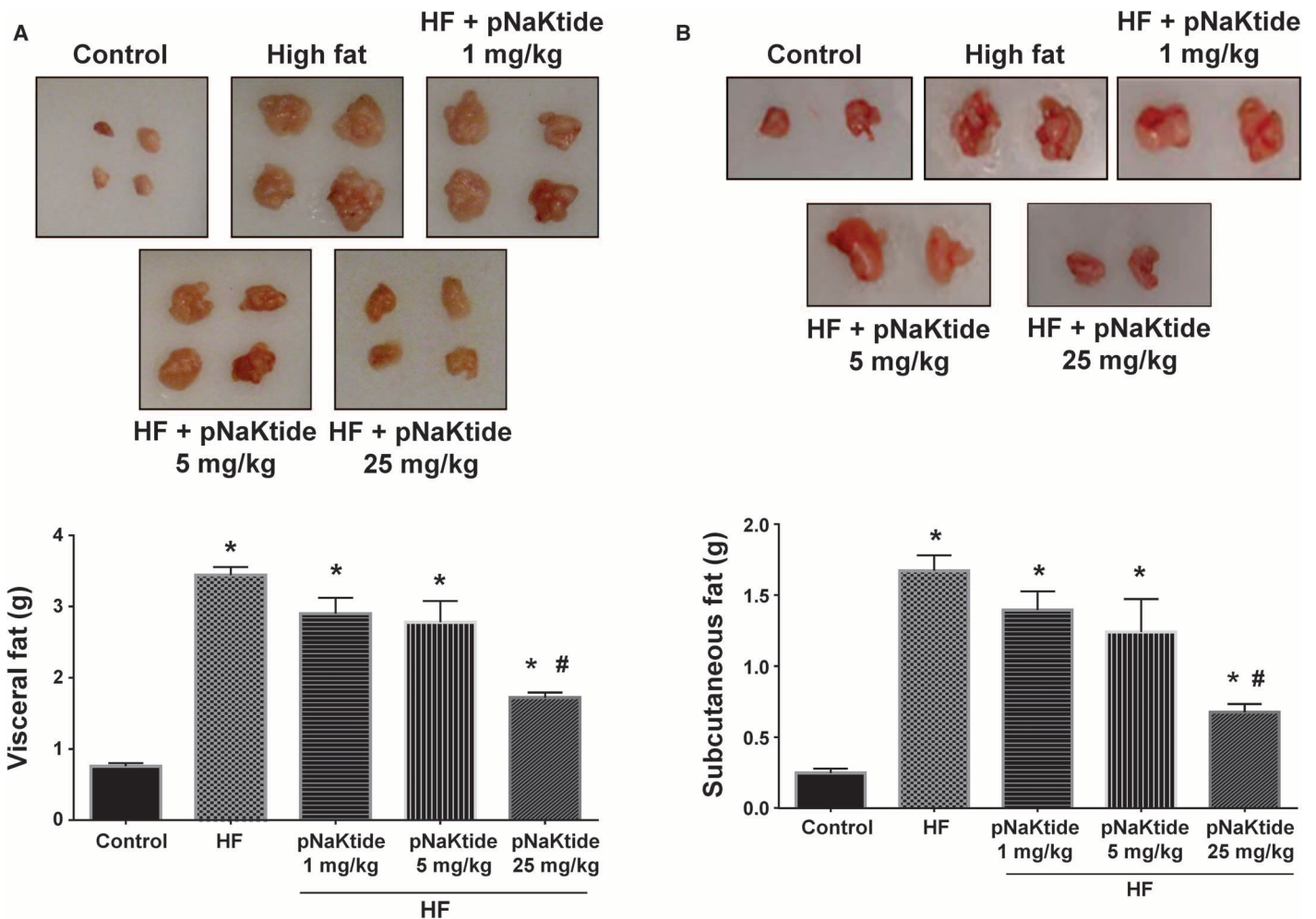


Fig. 4. Effect of increasing doses of pNaKtide on visceral and subcutaneous fat content in mice fed a high-fat diet. (A and B) C57Bl6 mice fed a high-fat diet (HF) for 8 weeks were injected with pNaKtide at doses of 1, 5, and 25 mg/kg every 8 days. Administration of pNaKtide at 25 mg/kg in mice fed a high-fat diet significantly reduced visceral (A) and subcutaneous (B) fat content as compared to high-fat diet–fed animals. Results are means \pm SE, $n = 7$ to 14 per group; * $P < 0.05$ versus control, # $P < 0.05$ versus high-fat diet.

significant improvements in the HOMA-IR score (Fig. 6A), improved glucose tolerance (Fig. 6B), attenuation of oxidative stress in visceral adipose tissue (Fig. 6C), and significantly elevated levels of circulating adiponectin in mice concurrently treated with the peptide (Fig. 6D). The administration of pNaKtide also significantly attenuated high-fat diet–induced protein carbonylation in visceral adipose tissue (Fig. 6E) and the activation of c-Src and ERK1/2 in mice fed a high-fat diet (Fig. 6, F and G).

DISCUSSION

This study characterizes the therapeutic potential for conditions associated with obesity and metabolic imbalance. Oxidative stress, a frequent accompaniment of chronic pathologies, has long been implicated in the pathogenesis of adiposity. A plethora of antioxidants have shown promise in reversing this effect in *in vitro* studies, with few *in vivo* successes. We have demonstrated in this report that pNaKtide

effectively reduces adiposity and restores metabolic homeostasis by antagonizing Na/K-ATPase–mediated amplification of ROS signaling.

We observed that, in murine preadipocytes, exposure to pNaKtide attenuated oxidative stress as well as the accumulation of lipid and the manifestations of an obesity phenotype in a dose-dependent manner in response to either adipogenic media or exogenous administration of oxidative stress mediators, fructose or glucose oxidase. This decrease in adipogenesis in murine preadipocytes treated with pNaKtide was associated with significantly decreased expression of adipogenic regulators including PPAR γ and FAS along with increased production of adiponectin levels. Thus, the Na/K-ATPase inhibitor prevents dysfunctional adipogenesis, an effect that could be attributed to its effects on cellular redox. Moving to an *in vivo* model of obesity induced by a high-fat diet, we again observed that intraperitoneal administration of pNaKtide attenuated the development of obesity and the development of metabolic syndrome. pNaKtide treatment resulted in a significant reduction in weight gain complemented by attenuation of visceral

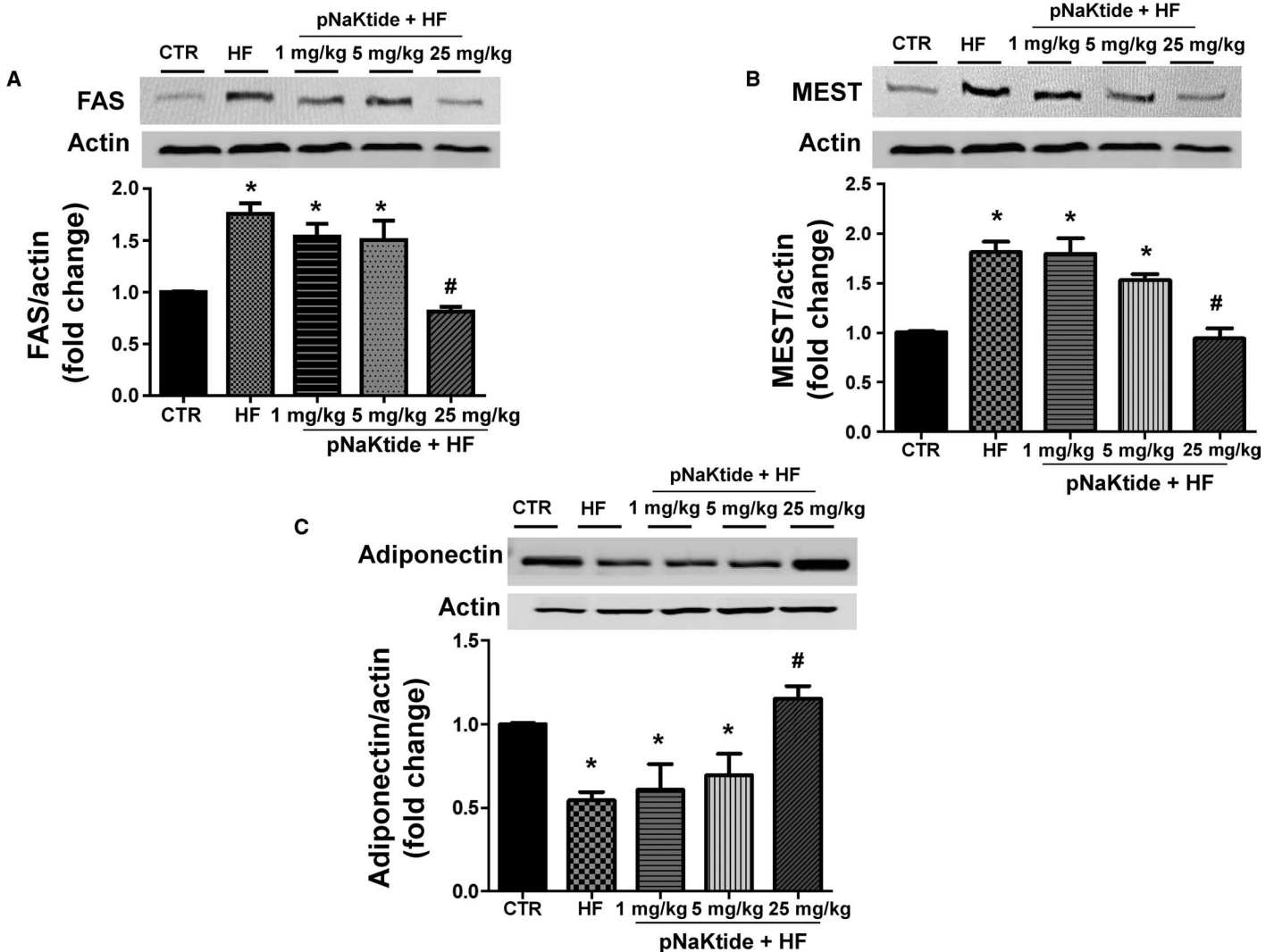


Fig. 5. Effect of increasing doses of pNaKtide on adipogenic markers and adiponectin expression in visceral adipose tissue in mice fed a high-fat diet. (A and B) Western blot and densitometric analysis of adipogenic markers in visceral adipose tissue, FAS (A) and MEST (B). Results are means \pm SE, $n = 7$ per group; * $P < 0.05$ versus control, # $P < 0.05$ versus high-fat diet. Data are shown as mean band density normalized to β -actin. (C) Western blot and densitometric analysis of adiponectin expression. Results are means \pm SE, $n = 7$ per group; * $P < 0.05$ versus control, # $P < 0.05$ versus high-fat diet. Data are shown as mean band density normalized to β -actin.

and subcutaneous fat content in mice fed a high-fat diet. Dysfunctional adipocytes are associated with the development of insulin resistance and hyperglycemia, and they favor a proinflammatory state (37). Severe insulin resistance, systemic inflammation, and dysregulation of protective adipokines characterize metabolic syndrome. Our results showed that pNaKtide decreased oxidative stress and insulin resistance with an increase in adiponectin levels. We further demonstrated that the decrease in obesity was associated with changes in adipogenic proteins. Mice fed a high-fat diet and treated with pNaKtide displayed decreased levels of MEST and FAS expression, further demonstrating the pNaKtide-mediated abrogation of adiposity and metabolic imbalance.

These observations are of potential interest for several reasons. First, we have clearly demonstrated the ability of the Na/K-ATPase signal

cascade to amplify ROS involved in adipogenesis, a process not previously linked to cardiotoxic steroids or the Na/K-ATPase signal cascade. Although a novel, well-tolerated antiobesity agent would be a welcome addition to our clinical armamentarium, much work is necessary to bridge the gap between rodents and humans. However, perhaps of greater importance, the concept that ROS amplification can occur through this pathway suggests that the Na/K-ATPase may serve as a potential therapeutic target in a number of conditions characterized by oxidative stress, which ultimately become maladaptive. Perhaps because pNaKtide targets the amplification of oxidants rather than a primary signal cascade, it could provide new approaches for the treatment of obesity and metabolic syndrome. If safety is demonstrated in human studies, the clinical use of pNaKtide might ultimately be possible.

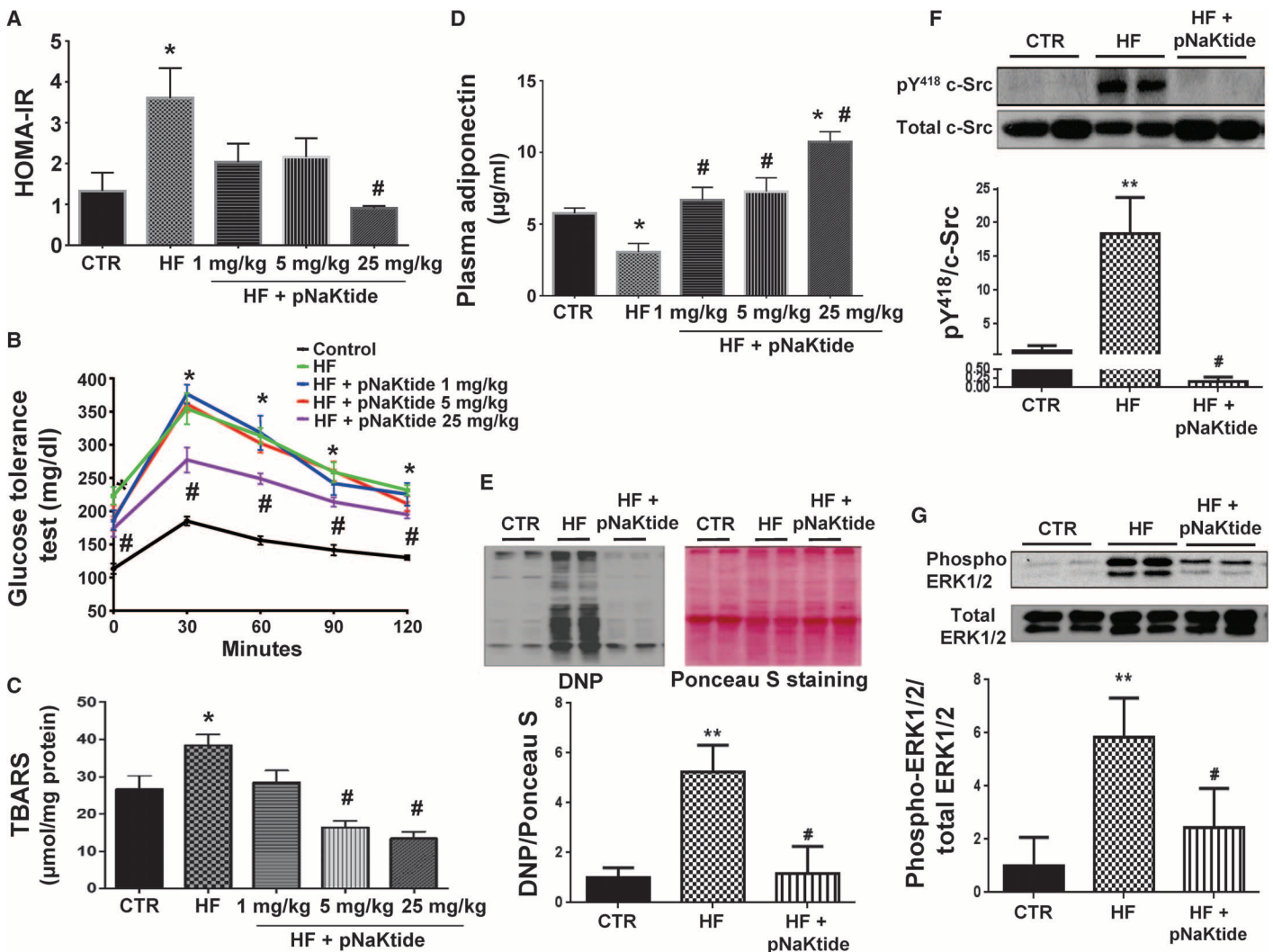


Fig. 6. Effect of increasing doses of pNaKtide on metabolic profile in mice fed a high-fat diet. (A) HOMA-IR. **(B)** Glucose tolerance test. **(C)** Thiobarbituric acid-reactive substance (TBARS), a marker of oxidative injury, was measured in visceral adipose tissue. **(D)** Plasma adiponectin levels. Results are means \pm SE, $n = 7$ to 14 per group; * $P < 0.05$ versus control, # $P < 0.05$ versus high-fat diet. **(E to G)** Effect of pNaKtide on protein carbonylation and phosphorylation of c-Src and ERK1/2 in fat tissue in mice fed a high-fat diet. **(E)** Protein carbonylation. **(F)** Phosphorylation of c-Src. **(G)** Phosphorylation of ERK1/2. Results are means \pm SE, $n = 4$ to 7 per group; ** $P < 0.01$ versus control, # $P < 0.01$ versus high-fat diet.

MATERIALS AND METHODS

Experimental design for in vitro experiment

Frozen mouse preadipocytes (3T3L1) were resuspended in a α -minimal essential medium (α -MEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic solution. The cultures were maintained at 37°C in a 5% CO₂ incubator, and the medium was changed after 48 hours and every 3 to 4 days thereafter. When the 3T3L1 cells were confluent, the cells were recovered by the addition of trypsin. 3T3L1 cells (passages 2 to 3) were plated in 96- and 24-well plates at a density of 10,000 cells/cm² and cultured in α -MEM until 80% confluence was achieved. The medium was replaced with adipogenic medium, and the cells were cultured for an additional 7 days. Cells were treated everyday with 0.1, 0.3, 0.5, 0.7, 1, 2, and 4 μ M pNaKtide. After 7 days, the cells were stained with Oil Red O solution to analyze adipogenesis as previously described by Sodhi *et al.* (34).

Measurement of lipid droplet size

Cell size was measured using ImagePro Analyzer. The classification of the size of lipid droplets was based on size by area (pixels) as described previously by Burgess *et al.* (38).

Measurement of superoxide levels for in vitro experiment

3T3L1 adipocytes were cultured on 96-well plates until they achieved about 70% confluence. After treatment with 0.1, 0.3, 0.5, 0.7, 1, 2, and 4 μ M pNaKtide for 72 hours, the cells were incubated with 10 μ M dihydroethidium for 30 min at 37°C. Fluorescence intensity was measured using a Perkin-Elmer luminescence spectrometer at excitation/emission filters of 530/620 nm (39).

Experimental design for in vivo experiment

All animal studies were approved by the Marshall University Animal Care and Use Committee in accordance with the National Institutes of

Health (NIH) *Guide for the Care and Use of Laboratory Animals*. C57Bl6 mice (6 to 8 weeks old, male) were purchased from The Jackson Laboratory. Upon arrival to the Robert C. Byrd Biotechnology Science Center Animal Resource Facility (ARF), the mice were placed in cages and were fed normal chow diet and had access to water ad libitum. The high-fat diet was commercially purchased from Bio-SERV. The high-fat diet contained 58% fat from lard, 25.6% carbohydrate, and 16.4% protein yielding 23.4 kJ/g. After 4 weeks of the high-fat diet, the animals were divided into four groups, and treatment was done for 8 weeks as follows: pNaKtide (dissolved in saline and injected intraperitoneally) at doses of 0, 1, 5, and 25 mg/kg every 8 days. Body weight was measured every week. At the end of the 12-week period, the mice were subjected to an 8-hour fast and then anesthetized with sodium pentobarbital (65 mg/kg, intraperitoneally); blood was obtained from the tail vein for measurement of glucose with a glucometer and of insulin with an enzyme-linked immunosorbent assay (ELISA) kit (Abcam). At the time of sacrifice, the body weight, visceral and subcutaneous fat content, and liver weight of all mice were measured. Blood samples were collected for determination of adiponectin levels. Visceral adipose tissue was flash-frozen in liquid nitrogen and maintained at -80°C until assayed.

An additional group in which pNaKtide was administered at 25 mg/kg intraperitoneally every 2 days ($n = 7$) was also studied. These animals had less attenuation of weight gain than the group administered with 25 mg/kg intraperitoneally every 8 days, but at the time of sacrifice, it was clear that these animals had significant fluid retention. Their subcutaneous (0.63 ± 0.06 g, $P < 0.01$ versus high-fat diet) and visceral fat content (1.60 ± 0.08 g, $P < 0.01$ versus high-fat diet) were virtually identical to those of mice injected with 25 mg/kg intraperitoneally every 8 days. The fluid retention was not surprising given the substantial sodium content of the vehicle given every 2 days along with the role of Na/K-ATPase signaling in the adaptive natriuresis with salt loading (21).

Blood measurements of adiponectin

The high molecular weight form of adiponectin was determined in serum with an ELISA assay according to the manufacturer's protocol.

Western blot analysis

Visceral fat was pulverized in liquid nitrogen and placed in homogenization buffer. Homogenates were centrifuged, the supernatant was isolated, and immunoblotting was performed. The supernatant was used for the determination of FAS, adiponectin, PPAR γ , and MEST. β -Actin was used to ensure adequate sample loading for all Western blots as previously described, Li *et al.* (3) and Sodhi *et al.* (40).

Measurement of c-Src and ERK1/2 phosphorylation

Visceral fat and whole-cell lysates obtained from 3T3L1 adipocytes were prepared with NP-40 buffer, and activation of c-Src and ERK1/2 was determined as previously described by Yan *et al.* (28). After immunoblotting for phospho-c-Src and phospho-ERK1/2, the same membrane was stripped and immunoblotted for total c-Src and total ERK1/2. Activation of c-Src and ERK1/2 was expressed as the ratios of phospho-c-Src/total Src and phospho-ERK1/2/total ERK1/2, respectively, with both measurements normalized to 1.0 for the control samples.

Assessment of protein carbonylation

Visceral fat and whole-cell lysates were prepared with NP-40 buffer, and Western blotting for protein carbonylation assay was done (24, 28).

The signal density values of control samples were normalized to 1.0 with Ponceau S staining as a loading control.

Glucose tolerance test

Glucose clearance was determined using an intraperitoneal glucose tolerance test before termination of the experiment. Mice were fasted for 8 hours, after which a glucose solution (2 g/kg, injected as a 10% solution) was injected into the peritoneal cavity. Samples were taken from the tail vein at 0, 30, 60, and 120 min after glucose injection. Blood glucose was measured using the Accutrend Sensor glucometer.

Determination of homeostasis model assessment of insulin resistance

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from mouse blood using glucose and insulin concentrations obtained after 8 hours of food withdrawal, using the following formula: $\text{HOMA-IR} = [\text{fasting insulin (ng/ml)} \times \text{fasting glucose (mM)}] / 22.5$.

Lipid peroxidation measurement. Visceral fat lipid peroxidation was measured as TBARS using an assay kit according to the manufacturer's protocol. Visceral fat samples were homogenized in a buffer solution containing 50 mM tris-HCl (pH 7.4) and 1.15% KCl and then centrifuged. The supernatant was used for the assay. Data were normalized to total protein and presented as micromoles per milligram of protein.

Distribution of rhodamine B-labeled pNaKtide. For the in vitro study, murine preadipocytes (3T3L1) were grown on glass coverslips and treated with or without rhodamine B-labeled pNaKtide (2 μM). At the indicated time points, the cells were fixed with cold absolute methanol and mounted with 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Labs). Images were taken (emission readings for DAPI and rhodamine B were 415 to 475 nm and 580 to 650 nm, respectively) with a Leica SP5 TCS II equipped with coherent chameleon multiphoton vision II (IR) laser and analyzed by Leica LAS/AF software. For the in vivo study, mice were injected intraperitoneally without (as control) or with rhodamine B-labeled pNaKtide (25 mg/kg). Three hours after injection, the mice were sacrificed, and the adipose tissues were imaged and analyzed as described earlier.

Statistical analyses

Statistical significance between experimental groups was determined by the Fisher method of analysis of multiple comparisons ($P < 0.05$). For comparisons among treatment groups, the null hypothesis was tested by a two-factor analysis of variance (ANOVA) for multiple groups or unpaired *t* test for two groups. Data are presented as means \pm SE.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/1/9/e1500781/DC1>

Fig. S1. pNaKtide distribution in 3T3L1 cells and adipose tissue.

Fig. S2. pNaKtide decreased large lipid droplets and oxidative stress and increased small lipid droplets in 3T3L1 adipocytes.

Fig. S3. pNaKtide decreased lipid accumulation and adipogenic markers in 3T3L1 adipocytes exposed to fructose.

Fig. S4. pNaKtide decreased lipid accumulation and adipogenic markers in 3T3L1 adipocytes exposed to glucose oxidase.

Fig. S5. pNaKtide decreased carbonylation and phosphorylation of Src and ERK in 3T3L1 adipocytes exposed to glucose oxidase.

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