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EFFECTS OF AN INTRAVENOUS INJECTION OF NPY ON LEPTIN AND NPY-Y1 RECEPTOR MRNA EXPRESSION IN OVINE ADIPOSE TISSUE[1,2]

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Neuropeptide Y (NPY) is highly expressed in hypothalami of undernourished and genetically obese animals, and is a potent regulator of food intake and reproduction. Leptin, a protein expressed by adipocytes, has been reported to reduce hypothalamic NPY expression. We recently detected (by ribonuclease protection assay [RPA]) expression of the NPY receptor subtype Y1 (but not Y2) mRNA in adipose tissue. Based on these observations we hypothesized that NPY-Y1 receptors in adipose may represent a peripheral mechanism by which NPY can regulate leptin expression in a direct and rapid manner. To test this hypothesis, adipose samples were biopsied from the tailhead region of 48 ± 3 kg wether lambs immediately before and 30 min after a single intravenous injection of 50 μg porcine NPY ("treated" animals, $n = 5$), or vehicle ("control" animals, $n = 4$). Injection of NPY resulted in an increase in expression ($P = 0.013$; as measured by RPA) of both leptin and NPY-Y1 mRNA. In treated animals, negative correlations were found between response in leptin expression and body weight ($r = -0.82$, $P = 0.092$), and between leptin response and initial leptin mRNA levels ($r = -0.81$, $P = 0.097$). These data provide evidence of a peripheral mechanism by which NPY may regulate adipocyte expression of both leptin and NPY-Y1 receptor mRNA.

INTRODUCTION

Physiological regulation of food intake is a critical factor in both the rate at which an animal grows and its reproductive activity. Recently, progress has been made in elucidating a complex system in which insulin, leptin, and neuropeptide Y (NPY) play integral roles to monitor an animal's energy balance and regulate feed intake and fertility. NPY, produced in the hypothalamus, is a powerful stimulator of food intake (1,2), as well as a regulator of reproductive function (3,4). NPY mRNA is overexpressed in *ob/ob* mice, which do not produce a functional form of leptin (5); consequently, *ob/ob* mice are obese, hyperphagic, and infertile. Treatment of *ob/ob* mice with exogenous leptin decreased mRNA expression of NPY within the arcuate nucleus (6,7), restored these animals to relatively normal body weights (8), and improved several reproductive parameters (9). We (10) and others (11,12) have reported leptin receptor mRNA expression within the arcuate nucleus; concurrently, leptin receptor mRNA expression has been found colocalized with NPY mRNA-containing cell bodies in that region (13).

Sainsbury et al. (14) reported an increase in leptin expression in rats which received a chronic (6 d) intracerebroventricular (i.c.v.) infusion of NPY. These investigators hypothesized that the NPY infusion induced leptin expression via NPY-induced hyperinsulinamia. Recently we reported the detection of NPY-Y1 receptor mRNA expression within

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ovine adipose tissue (15). As both insulin (16) and leptin (6) have been shown to regulate NPY expression at the level of the hypothalamus, and NPY has been shown to regulate insulin production in the pancreas, leptin expression within adipocytes may also be regulated directly (positively or negatively) by NPY. Therefore, we tested the hypothesis that a single *intravenous* injection of NPY would exert rapid, direct effects on leptin and NPY-Y1 receptor mRNA expression in adipose tissue of wether lambs.

MATERIALS AND METHODS

Experimental Design. Nine yearling wethers (48 ± 3 kg body weight) were used and managed on autumnal pastures as approved by the University of Missouri Animal Care and Use Committee. Prior to treatment, a jugular blood sample was taken from each wether and body weight was recorded. A sample (approximately 2 g) of subcutaneous adipose tissue was collected through a small incision in one side of the tailhead region and immediately stored in crushed dry ice. The incision was immediately closed with degradable suture. Each wether then received either 0 (n = 4) or 50 μg (n = 5) porcine NPY (Peninsula Laboratories, Belmont, CA) in 1 ml saline as an i.v. injection into the jugular vein. At 30 min postinjection, blood and adipose tissue (taken contralaterally from the first sample) were again sampled from each wether. Adipose samples were stored at −80° C until used for total RNA isolation and ribonuclease protection assay (RPA) to quantitate expression of leptin and NPY-Y1 receptor mRNA. Serum samples were frozen at −20° C until assayed by radioimmunoassay for insulin and NPY.

Leptin and NPY-Y1 mRNA Quantification. Isolation of total RNA from adipose tissue samples was performed using Tri-reagent (Molecular Research Products, Cincinnati, OH). Reagents used in the in vitro transcription of riboprobes were purchased from Promega (Madison, Wisconsin). Quantification of leptin, NPY-Y1 and β-actin (a 264 bp internal control) mRNA was performed using the HybSpeed RPA kit® (Ambion, Austin, TX), as described elsewhere (15). The β-actin cRNA was radiolabeled at approximately one-tenth the specific activity of the leptin and NPY-Y1 cRNAs. Duplicate reactions using 20 μg adipose RNA were run for each animal and time of treatment. Sample lanes from RPAs are shown in Figure 1. Expression was quantified by densitometry and expressed as leptin (or NPY-Y1)/β-actin densitometric units. Densitometry was performed using a BioRad GS-700 densitometer and Molecular Analyst software (BioRad, Hercules, CA).

NPY Radioimmunoassay. Serum concentrations of NPY were determined as described by McShane et al. (3) with slight modifications. Briefly, duplicate serum samples (20 μl each, except for the post-treatment samples of the wethers receiving 50 μg NPY, where 2 μl were used) were incubated for 24 hr at 4° C with anti-NPY (final tube dilution 1:4000, provided by B. Noe (17), 10,000 DPM monoiodinated NPY (Amersham, Arlington Heights, IL), and assay buffer (0.05M PO_4, 0.3% BSA, 0.05M EDTA, 0.001% Na Azide, 0.1% Tween-20, pH 7.4). Preprecipitated sheep-anti-rabbit antibody was added and the antigen-antibody complex pelleted by centrifugation and counted on a LKB 1275 gamma counter (Gaithersburg, MD). Ovine serum samples (20–200 μl) displayed binding characteristics parallel to the standard curve (data not shown). Minimum detectable concentration of NPY was 1.0 pg, and the intra-assay coefficient of variation was less than 10%. Recovery was greater than 90%.

Insulin Radioimmunoassay. Serum concentrations of insulin were determined in triplicate aliquots of sample using a double-antibody immunoprecipitation RIA. This assay has been validated for use in our laboratory using the following procedures: to 12 × 75 mm polypropylene tubes, 300 μl of serum or standard concentrations ranging from 10 to 2000 pg of ovine insulin (Sigma Chemical Co., St. Louis, MO) were added to 100 μl of guinea pig anti-bovine insulin (kindly provided by T. Elsasser (18); final tube dilution

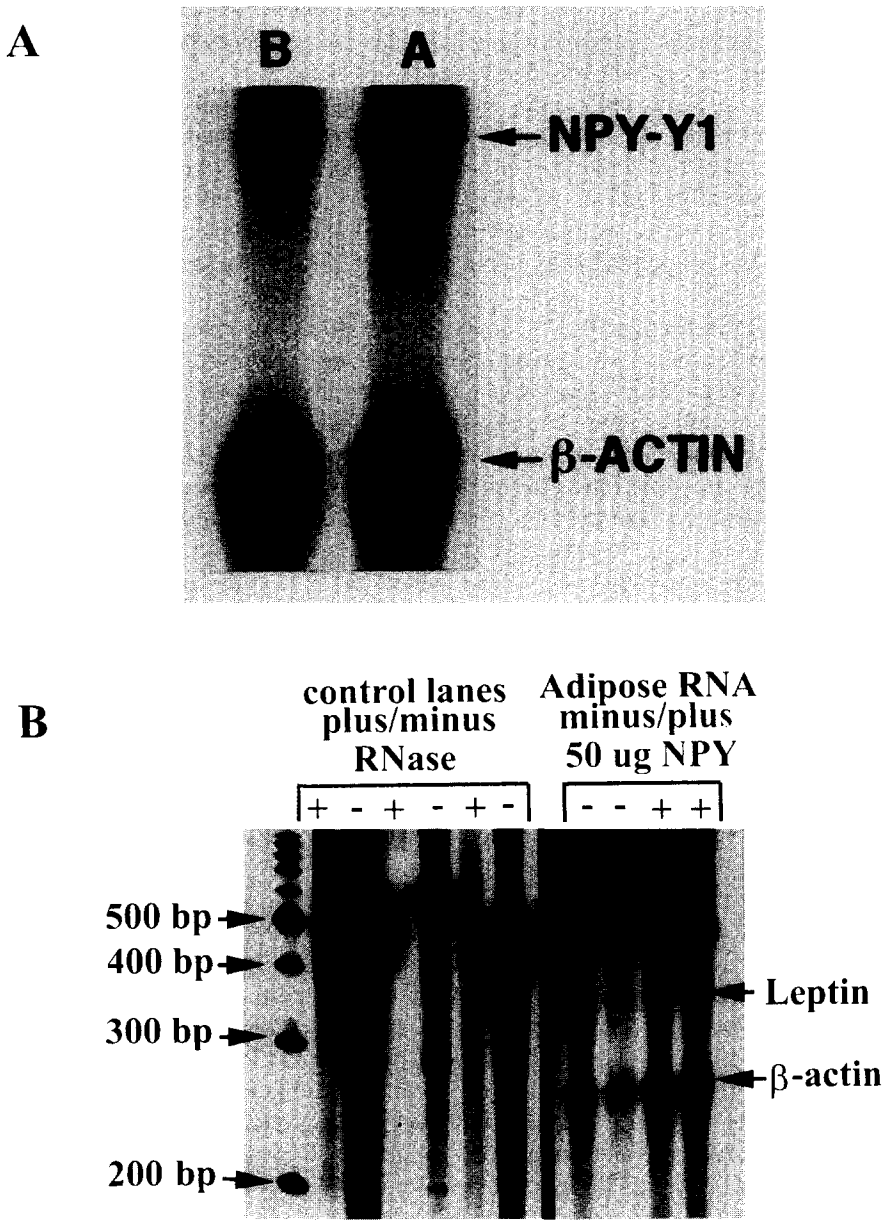

Figure 1. Representative ribonuclease protection assay results. (A) NPY-Y1 receptor and β-actin mRNA expression in adipose RNA isolated from a wether lamb before (B) and after (A) treatment with 50 μg NPY. (B) Leptin and β-actin mRNA expression in adipose RNA taken from a wether lamb before (−) and after (+) treatment with 50 μg NPY. Duplicate lanes using 20 μg adipose RNA are shown. Control lanes from left to right include 50 μg yeast RNA hybridized (plus or minus RNase treatment) to NPY-Y1, β-actin, or leptin probes, respectively.

1:60,000), and 100 μl of ^{125}I-porcine insulin (12,000 DPM; New England Nuclear, Wilmington, DE). The total assay volume was 500 μl with all reagents solubilized in buffer consisting of 0.1% gelatin, 0.1% Tween-20, 0.15 M NaCl, 0.01% NaN_3, 0.01 M PO_4 and 0.01 M EDTA, pH 7.2. Reagents were mixed and incubated at 4° C for 24 hr. A

preprecipitated goat anti-guinea pig second-antibody was added and incubated at 27° C for 15 min to precipitate the antigen-antibody complex. A precipitate was then pelleted by centrifugation, the supernatant was discarded and the pellet was counted for 1 min using an LKB 1275 gamma counter (Gaithersburg, MD). Serial dilutions of ovine serum were parallel to standard concentrations of insulin. Minimum detectable concentration of insulin was 33 pg/ml and inter- and intra-assay coefficients of variation were less than 10%. As a further measure of the reliability of this assay, identical values were obtained ($R = 0.999$; $P < 0.0001$) when a set of identical samples were assayed as described previously and when assayed using a Diagnostics Products Corporation (Los Angeles, CA) insulin RIA kit.

Statistical Analyses. All statistical analyses were performed using SAS (19). Because variances were determined to be heterogenous among treatments, responses in leptin expression, NPY-Y1 receptor expression, and serum insulin concentrations were compared nonparametrically between control (0 μg NPY) and treated (50 μg NPY) groups as follows: response values (defined as posttreatment values divided by pretreatment values and multiplied by 100) were ranked using the Proc Rank procedure, and the ranked values subsequently analyzed by the Proc GLM procedure in a one-way analysis of variance. A probability value ($P < 0.05$) was used to delineate statistically significant differences between treatments. Correlations between variables were determined using the Proc Reg procedure. Correlations in which $|R| < 0.8$, and $P < 0.10$ were considered statistically significant and are reported herein. Serum concentrations of NPY were analyzed by split-plot (repeated measures) analysis of variance.

RESULTS

Serum concentrations of NPY were assessed before and 30 min after the delivery of a single iv injection of either 0 (control) or 50 μg (treated) porcine NPY to verify delivery of the NPY dose and to quantify serum concentrations of NPY in response to treatment (Figure 2).

Although serum concentrations of insulin did not differ with respect to treatment (Figure 2), a negative correlation existed between insulin response and initial insulin concentrations in treated wethers (see Figure 4).

Expression of leptin and NPY-Y1 mRNA increased in response to an i.v. injection of 50 μg NPY (Figure 3). Regression analysis revealed strong ($|R| > 0.8$; $P < 0.1$) correlations between leptin response and body weight, and leptin response and initial leptin mRNA expression in wethers given 50 μg NPY (Figure 4). No correlations were detected (using the parameters explained above) between NPY-Y1 response and body weight, NPY-Y1 response and initial NPY-Y1 mRNA expression, leptin response and NPY-Y1 response, leptin response and insulin response, or NPY-Y1 response and insulin response in treated or control groups (data not shown).

DISCUSSION

Sainsbury et al. (14) reported an increase in leptin mRNA expression after a chronic (6 d) intracerebroventricular infusion of NPY into rats. The design of that study did not allow for determination of whether the effects were attributable to some as yet unknown indirect pathway involving central tissues and/or insulin-secreting islet cells of the pancreas, or by a direct effect of NPY at the level of adipose tissue. A long period of infusion of NPY may have led to an effective amount of NPY diffusing from the ventricular and central nervous systems to the periphery. As we have previously detected NPY-Y1 receptors in ovine adipose tissue (15), we hypothesized that peripherally, NPY may act directly on adipose tissue to affect leptin and NPY-Y1 receptor mRNA expression. A

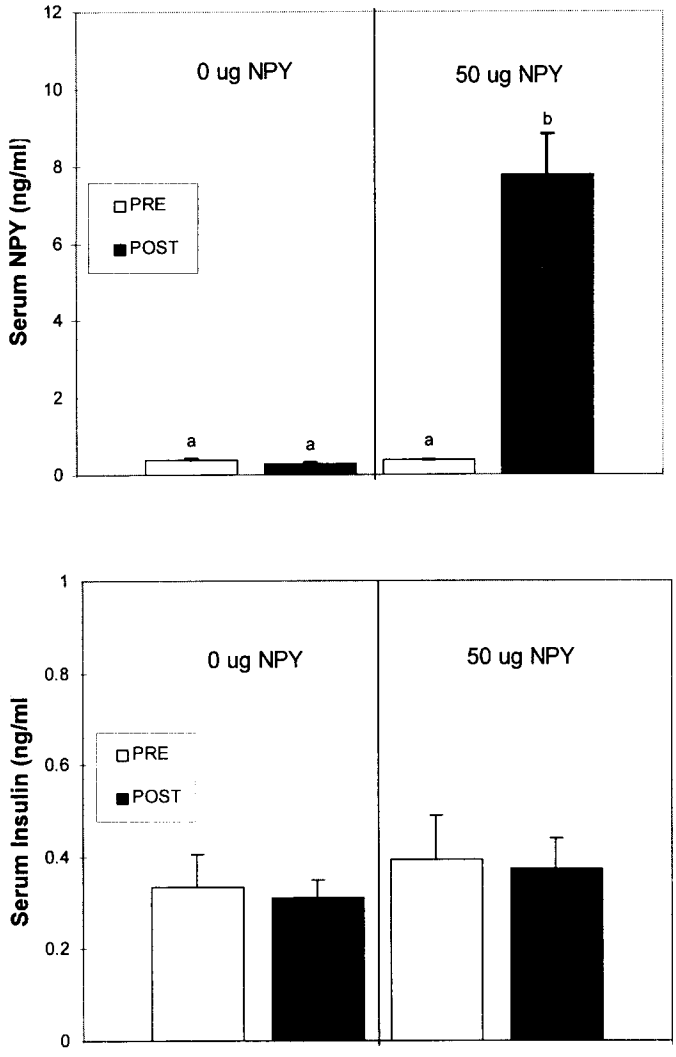

Figure 2. Serum concentrations of NPY (top) and insulin (bottom) in wethers treated with 0 or 50 μg NPY (post-treatment samples taken 30 min after injection). The letters "a" and "b" represent statistically different ($P < 0.0001$) mean values.

single intravenous injection of 50 μg porcine NPY in wether lambs resulted in positive responses in leptin and NPY-Y1 receptor mRNA expression within adipose tissue 30 min after treatment, thus supporting the hypothesis that peripheral NPY may directly regulate leptin and NPY receptor expression at that site.

Insulin has been reported to increase leptin mRNA expression (20,21). Although it is possible that the positive response in leptin mRNA expression in the treated wethers may have been attributable to an effect of NPY on insulin, it is unlikely because: 1) there was not a significant response in serum insulin levels in these animals, and 2) there is evidence that NPY can act at the level of pancreatic islets to *decrease* insulin release (22,23).

The positive response in adipose NPY-Y1 receptor mRNA expression in response to NPY may be indicative of direct up-regulation of receptor expression by its ligand. Physiologically, this may function as an additional level of control in an attempt by the

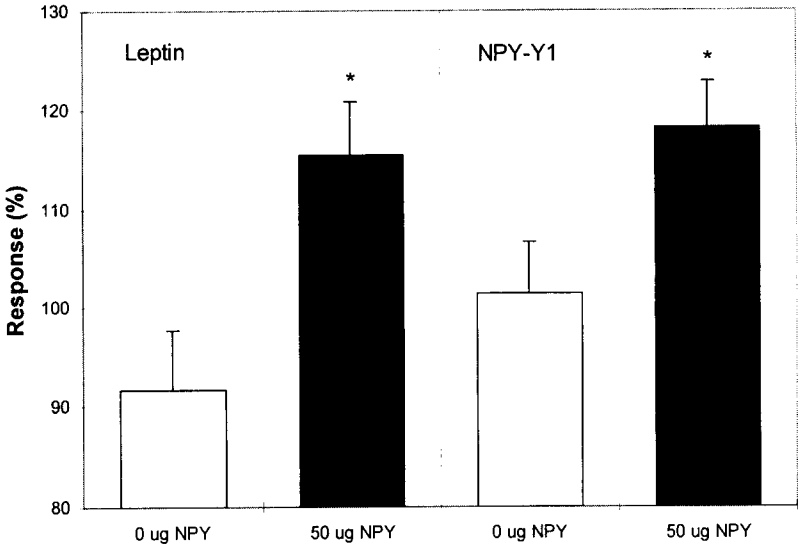

Figure 3. Adipose expression of leptin mRNA and NPY-Y1 receptor mRNA in wethers treated with a single i.v. injection of 0 μg (n = 4) or 50 μg NPY (n = 5). "Response" is the post-treatment value divided by pretreatment value multiplied by 100. *P < 0.01 The P value listed for each variable was calculated by first ranking the responses in each treatment, then comparing them by analysis of variance (post-treatment samples taken 30 min after injection).

animal to maintain homeostatic levels of both leptin and NPY. This is the first known report of any kind of regulation by NPY of its various receptor subtypes. Further studies are underway to determine if up-regulation of NPY-Y1 mRNA expression by NPY also occurs at other locations. Of particular interest are the cell bodies within the arcuate nucleus of the hypothalamus, as both NPY (24) and NPY-Y1 mRNA (25) are expressed within this area (although colocalization of these transcripts has not yet been reported at a cellular level).

There was a significant amount of animal-to-animal variation in most of the variables measured in this experiment, thus necessitating nonparametric comparison of response variables in control and treated animals. We hypothesized that some of this variation may be a result of other factors, such as body weight or initial (set-point) levels. For instance, serum leptin levels have been found to be closely correlated with adiposity in humans (26). Strong correlations were observed in treated wethers between leptin response and body weight, leptin response and initial leptin mRNA expression, and insulin response and initial serum insulin levels. Because the wethers were all within 3 wk of age, body weight variations may be a result of variations in *composition*, and particularly of *adiposity*. These correlations, particularly between leptin response and body weight, are intriguing, and are consistent with the "set-point" theory of adiposity and weight loss (27).

Whereas NPY has been studied extensively as a classical neuropeptide (i.e., a protein released by neurons to act centrally on other neurons) within the hypothalamus, the role(s) of *peripheral* NPY in the regulation of feed intake and reproduction are largely unknown. NPY may reach adipocytes or pancreatic islet cells through the general circulation and/or through innervation of those tissues, and may contribute to the high degree of regulation needed to maintain a homeostatic level of food intake by modulating insulin release and leptin expression. The data presented here are the first known to be reported in a domestic animal given a peripheral (circulating) injection of NPY, and offer exciting insights as to the inter-relationships existing between leptin and NPY in livestock.

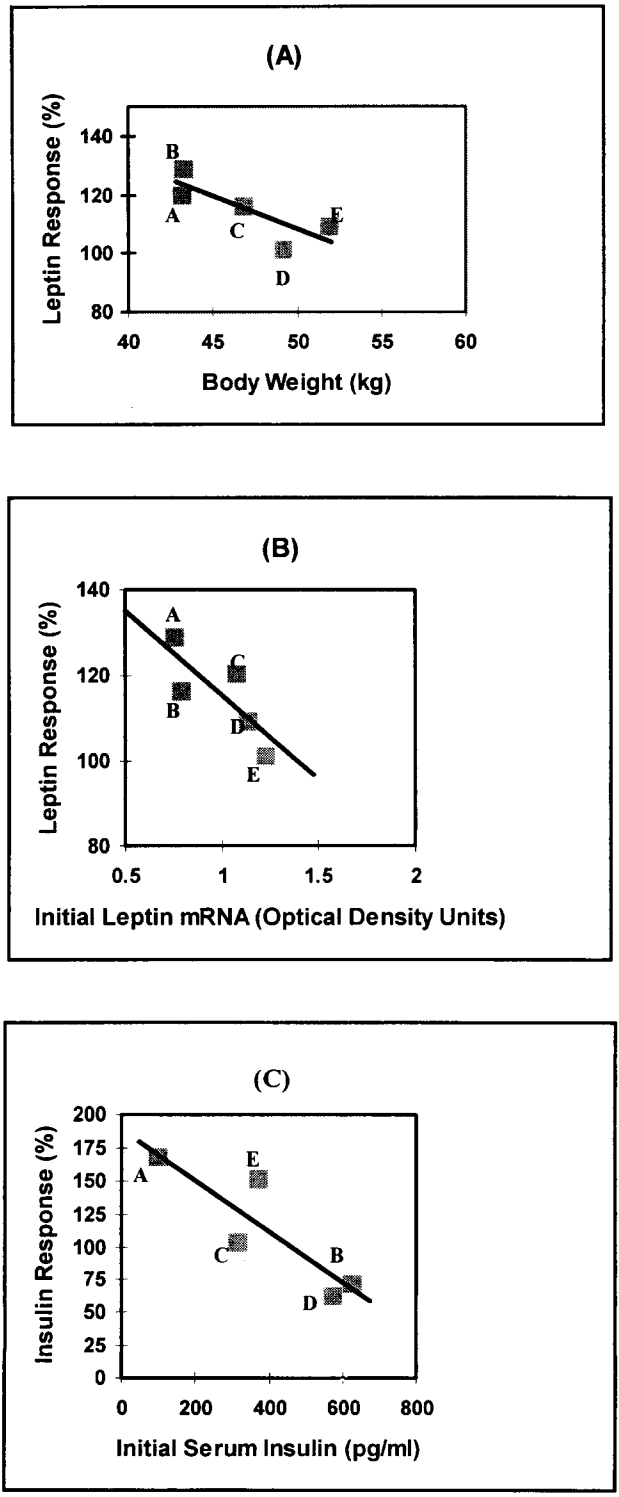

Figure 4. Graphic illustration of correlations between (A) leptin mRNA response and body weight, (B) leptin mRNA response and initial leptin mRNA expression, and (C) insulin response and initial serum insulin in wether lambs treated with 50 μg NPY (n = 5). Individual animals are labeled "**A**" through "**E**."

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