# Apolipoprotein A-IV interacts synergistically with melanocortins to reduce food intake

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# Apolipoprotein A-IV interacts synergistically with melanocortins to reduce food intake

**Koro Gotoh,[1] Min Liu,[2] Stephen C. Benoit,[1] Deborah J. Clegg,[1] W. Sean Davidson,[2] David D'Alessio,[3] Randy J. Seeley,[1] Patrick Tso,[2] and Stephen C. Woods[1]**

*Departments of [1]Psychiatry, [2]Pathology, and [3]Medicine, University of Cincinnati, Cincinnati, Ohio*

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**Gotoh, Koro, Min Liu, Stephen C. Benoit, Deborah J. Clegg, W. Sean Davidson, David D'Alessio, Randy J. Seeley, Patrick Tso, and Stephen C. Woods.** Apolipoprotein A-IV interacts synergistically with melanocortins to reduce food intake. *Am J Physiol Regul Integr Comp Physiol* 290: R202–R207, 2006. First published September 15, 2005; doi:10.1152/ajpregu.00502.2005.—Apolipoprotein (apo) A-IV is an anorexigenic gastrointestinal peptide that is also synthesized in the hypothalamus. The goal of these experiments was to determine whether apo A-IV interacts with the central melanocortin (MC) system in the control of feeding. The third ventricular (i3vt) administration of a subthreshold dose of apo A-IV (0.5 μg) potentiated i3vt MC-induced (metallothionein-II, 0.03 nmol) suppression of 30-min feeding in Long-Evans rats. A subthreshold dose of the MC antagonist (SHU9119, 0.1 nmol, i3vt) completely attenuated the anorectic effect of i3vt apo A-IV (1.5 μg). The i3vt apo A-IV significantly elevated the expression of c-Fos in neurons of the paraventricular nucleus of the hypothalamus, but not in the arcuate nucleus or median eminence. In addition, c-Fos expression was not colocalized with proopiomelanocortin-positive neurons. These data support a synergistic interaction between apo A-IV and melanocortins that reduces food intake by acting downstream of the arcuate.

melanocortin system; hypothalamus; proopiomelanocortin; c-Fos

FIRST DESCRIBED IN 1977 (31), apolipoprotein (apo) A-IV is a circulating glycoprotein secreted by the small intestine in humans (11). In rodents, although apo A-IV is secreted in small amounts from the liver, the small intestine is the major organ responsible for its presence in the circulation (1). In the intestine, apo A-IV is secreted in association with chylomicrons during the absorption of lipids, and it is displaced rapidly from the chylomicrons during their metabolism in the circulation (27, 35). Hence, apo A-IV has been considered a key peptide involved in the processing of ingested fat by the body. A possible signaling role of apo A-IV was suggested when it was found that systemic administration of exogenous apo A-IV dose dependently decreases food intake of rats (10) and that administration of apo A-IV antiserum increases food intake and body weight (9).

Apo A-IV is present in the cerebrospinal fluid, and its cerebrospinal levels increase when fat is absorbed (10). Moreover, the third ventricular (i3vt) administration of exogenous apo A-IV reduces food intake (9). Hence, one site of action of the anorexic effect of apo A-IV appears to be within the brain. Consistent with this, apo A-IV is synthesized in the ventrobasal hypothalamus (17, 18) in the general area in which other important feeding-related neuropeptides are produced (7, 28, 36), and hypothalamic apo A-IV mRNA levels fluctuate with metabolic state (20, 22) as well as time of day (21). All of these findings suggest that apo A-IV likely interacts with other signals involved in the regulation of energy homeostasis (see reviews in Refs. 33, 34).

The hypothalamic melanocortin system is important in the regulation of food intake and body weight (2, 5, 8, 16, 30). α-Melanocyte-stimulating hormone (α-MSH) derived from proopiomelanocortin (POMC) neurons in the hypothalamic arcuate nucleus (ARC) is the primary agonist for melanocortin type 3 and 4 receptors (MC3/4-R) in the brain. α-MSH is thought to exert a tonic inhibitory influence over feeding (5, 26). Agouti-related protein is a peptide produced in non-POMC cells in the ARC and antagonizes MC3/4-R (5, 23). Consistent with this, agouti-related protein elicits hyperphagia when it is centrally administered (6, 12, 13). The i3vt administration of metallothionein-II (MT-II), a synthetic MC3/4-R agonist, potently reduces feeding (8, 32), whereas i3vt administration of SHU9119, a synthetic MC3/4-R antagonist, blocks the anorectic effect of MT-II (30) and elicits feeding when administered alone at higher doses (12, 13).

The present series of experiments assessed the hypothesis that central apo A-IV reduces food intake by potentiating the anorectic effect of central melanocortin agonists. To evaluate this possibility, a subthreshold dose of apo A-IV that had no effect on feeding when administered alone was first established. This subthreshold dose was then administered concomitantly with MT-II, and feeding and body weight were subsequently monitored. In complementary experiments, a similar paradigm was used to establish a subthreshold dose of centrally administered SHU9119, and this dose was then administered concomitantly with apo A-IV. Finally, c-Fos expression in the hypothalamus of rats pretreated with an anorectic dose of apo A-IV was assessed by using dual-labeled immunohistochemistry.

## METHODS

*Subjects.* Male Long-Evans rats (Harlan, Indianapolis, IN), weighing 340–400 g at the onset of the experiments, were housed in individual tub cages and maintained in an American Association for Accreditation of Laboratory Animal Care-accredited room illuminated from 0100 to 1300. All rats were individually handled for 5 min over 4 successive days to equilibrate their arousal levels before an experiment. Animals were anesthetized with ketamine/xylazine anesthesia (ketamine: 33 mg/rat, xylazine: 4.3 mg/rat; ip) and implanted with a cannula aimed at the i3vt. Coordinates were on the midline, 2.2 mm posterior to bregma, and 7.5 mm ventral to dura, as previously described (25). The guide cannula was cemented to anchor screws

Address for reprint requests and other correspondence: S. C. Woods, Dept. of Psychiatry, Univ. of Cincinnati, 2170 East Galbraith Road, Cincinnati, OH 45237 (e-mail: steve.woods@psychiatry.uc.edu).

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attached to the skull. After a 10-day recovery period during which body weight had returned to baseline, placement of the cannula was confirmed by i3vt infusion of 10 ng of angiotensin II (AT-II) in saline while the animals were water replete. Only animals drinking in excess of 5 ml over 1 h after AT-II infusion were included in the study. This protocol was approved by the University of Cincinnati Institutional Animal Care and Use Committee.

*Reagents.* Recombinant rat apo A-IV was expressed in *Escherichia coli* and purified in a bioactive form, as described by Liu et al. (19). Briefly, the protein was expressed in the pET expression system with an $NH_2$-terminal histidine tag sequence. The protein was isolated from a sonicated cell extract by passage down a nickel-chelating column. The His tag was removed by using the Igase protease, which cleaved a sequence engineered immediately at the $NH_2$-terminus of the mature protein (24). The mature protein was purified away from the cleaved tag by a second passage down the chelating column. The recombinant form of apoA-IV has been shown to be of similar molecular mass and formed similarly sized reconstituted lipoprotein particles as apo A-IV isolated from plasma. Most importantly, recombinant apo A-IV was demonstrated to be as functional as the native form with regard to suppression of food intake and did not induce a conditioned taste aversion (19). MT-II and SHU9119 were purchased from Phoenix Pharmaceuticals. All peptides were dissolved in physiological saline.

*Procedures.* One week following the AT-II tests, the animals were adapted to a regimen on which food was removed for the last 4 h of the light period each day (i.e., from 0900 to 1300). Rats were divided into four groups ($n = 10$ per group) matched on the basis of 4-h food intake for the previous 3 days. The same rats were used in *experiments 1–5*, with 7–10 days of nontesting occurring between experiments. For each subsequent experiment, the rats were reassigned to novel groups matched by baseline food intake. There was slight attrition over the course of the experiments as cannulas malfunctioned.

*Experiment 1: Determination of a subthreshold dose of apo A-IV on food intake and body weight.* On the experimental day, the animals were administered an i3vt bolus injection of saline or 0.5, 1.0, or 1.5 μg of apo A-IV at 1100. The injection, therefore, occurred 2 h before food was returned, as preliminary experiments on other rats indicated that the variance of food intake following apo A-IV was considerably reduced after the 2-h delay. All injections were in a volume of 1 μl. Food was then returned, and intake was measured after 30 and 60 min and 24 h. Body weight was recorded after 24 h. Water was available at all times.

*Experiment 2: Effect of a subthreshold dose of apo A-IV on MT-II-elicited suppression of food intake and body weight.* Rats were administered two injections on the test day. The injections contained either saline + saline, apo A-IV (0.5 μg) + saline, MT-II (0.03 nmol) + saline, or apo A-IV (0.5 μg) + MT-II (0.03 nmol). This dose of apo A-IV had no effect on food intake or body weight of rats in *experiment 1*. The dose of MT-II elicits a reliable decrease in food intake in our laboratory (30). The first i3vt injection contained either saline or apo A-IV and occurred at 1100 and was followed 10 min later by the second i3vt injection that contained either saline or MT-II. Food intake and body weight were recorded as in *experiment 1*.

*Experiment 3: Effect of apo A-IV on food intake and body weight when infused 10 min before a subthreshold dose of MT-II.* Groups of rats ($n = 9$ per group) were administered either saline + saline, apo A-IV (0.5 μg) + saline, saline + MT-II (0.01 nmol), or apo A-IV (0.5 μg) + MT-II (0.01 nmol) using the same protocol as in *experiment 2*.

*Experiment 4: Determination of a subthreshold dose of SHU9119 on food intake and body weight.* Four groups of rats were i3vt administered either saline or 0.05, 0.1, or 0.2 nmol of SHU9119 at 1100. Procedures were as in *experiment 1*.

*Experiment 5: Effect of SHU9119 on apo A-IV-elicited suppression of food intake and body weight.* Four groups of rats ($n = 8$ per group) were administered either saline + saline or saline + apo A-IV (1.5 μg), SHU9119 (0.1 nmol) + saline, or SHU9119 (0.1 nmol) + apo A-IV (1.5 μg) by using the paradigm described for *experiment 2*.

*Experiment 6*: Expression of POMC and c-Fos with dual-labeled immunohistochemistry in the paraventricular nucleus (PVN), ARC, and median eminence (ME). A novel cohort of male rats was given i3vt injections of apo A-IV (1.5 μg/μl) ($n = 5$) or saline ($n = 6$). Sixty minutes after the injection, rats were anesthetized with pentobarbital (60 mg/kg) and perfused transcardially with 400 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) over 15 min. Brains were removed, postfixed (for 4 h at 4°C), and stored in 20% sucrose in PB. Brains were sectioned at 40 μm with the use of a microtome at −20°C and stored in cryoprotectant (30% sucrose and 30% ethylene glycol in PB) at −20°C until they were processed for dual-labeled immunohistochemistry. Available antibodies recognizing these antigens were all raised in rabbit. To eliminate the possibility of cross reactivity, a modification of a recently described protocol was used (15). Free-floating sections were first rinsed in PBS (3 × 15 min) and blocked in BSA (0.1%, 1 h). They were then incubated with an anti-c-Fos antibody (sheep polyclonal, 1:2,000 dilution, Santa Cruz Biotechnoloy, Santa Cruz, CA) overnight at room temperature in PBS containing 0.1% BSA and 0.1% Triton X-100. Sections were subsequently exposed to biotinylated donkey anti-sheep IgG (1:400 in incubation solution for 60 min; Jackson Immunoresearch, West Grove, PA), avidin-biotin-horseradish peroxidase (ABC Elite, 1:1,500 in PBS for 60 min; Vector Laboratories, Burlingame, CA), biotinylated tyramine (1:250 in PBS containing 3% $H_2O_2$ for 10 min; Perkin Elmer Life Science, Boston, MA), and CY3-conjugated streptavidin (1:200 in PBS for 30 min; Jackson Immunoresearch). Sections were then incubated with an anti-POMC antibody (rabbit polyclonal, 1:2,000 dilution; Phoenix Peptides, Belmont, CA) overnight at room temperature in PBS containing 0.1% BSA and 0.1% Triton X-100 and in Alexa-488-conjugated donkey anti-rabbit (1:200 for 30 min; Molecular Probes, Eugene, OR). Developed sections were mounted on slides and coverslipped with Gelvatol containing an anti-fading agent (1,4-diazabicyclo[2,2]octane). Immunohistochemical controls included omission of primary antibody or preabsorption of diluted antiserum with nanomolar concentrations of appropriate purified peptides at 4°C for 24 h. In addition, controls included omission of second primary antibody and application of Alexa 488-conjugated secondary antibody. Fluorescent-stained sections were examined with a Zeiss laser-scanning confocal microscope system (Zeiss LSM510). Alexa 488 fluorescence was imaged with a 505-nm emission filter and an Argon laser (488 nm) and visualized as red signal; CY3 fluorescence was imaged with a 567-nm emission filter and a HeNe laser (544 nm) and visualized as green signal. Images were imported into Adobe Photoshop 6.0 and Microsoft Word to comprise Fig. 7. Images were not adjusted or altered in any way, except for occasional adjustment

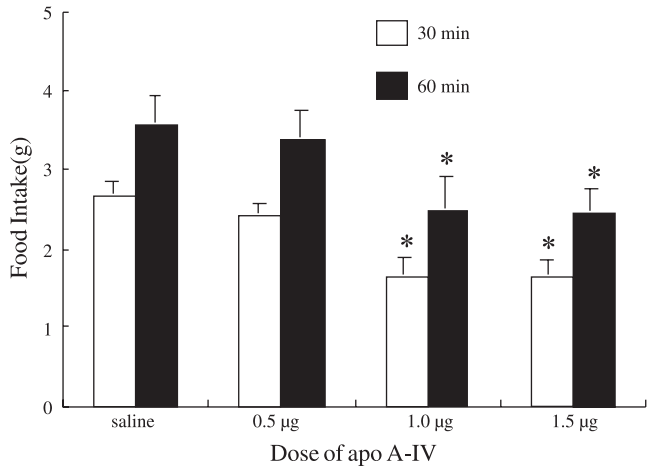


Fig. 1. Effect of third ventricular (i3vt) apolipoprotein (apo) A-IV (0.5, 1.0, or 1.5 μg) or saline on 30- and 60-min food intake. *$P < 0.05$ vs. saline and 0.5 μg of apo A-IV.

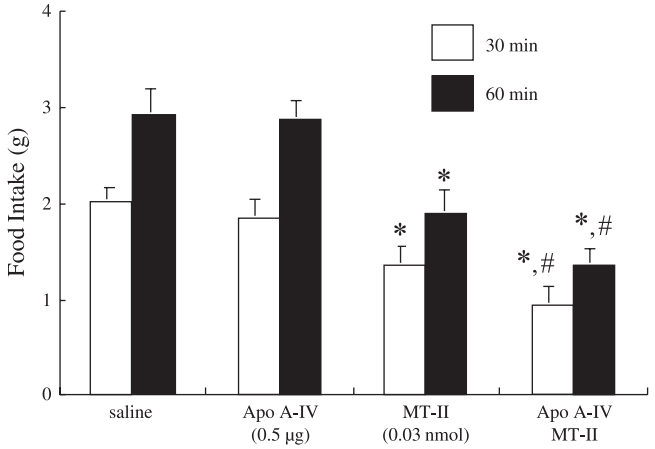


Fig. 2. Effect of i3vt saline or apo A-IV (0.5 μg), in combination with either saline or metallothionein-II (MT-II; 0.3 nmol), on 30- and 60-min food intake. *$P < 0.05$ vs. saline and apo A-IV; #$P < 0.05$ vs. MT-II.

of brightness. The number of c-Fos-positive cells, as well as POMC neurons labeled with c-Fos, was scored by observers blind to the identity of the subjects in approximately the same plane (3 sections per subject) of the PVN, ARC, and ME in both groups.

*Data analysis.* Data from each experiment were analyzed with one-way analysis of variance, followed by the Fisher protected least squares difference test. A $P$ value $\leq 0.05$ was considered significant. All data are expressed as means ± SE.

## RESULTS

*Experiment 1: Determination of a subthreshold dose of apo A-IV on food intake and body weight.* As depicted in Fig. 1, the i3vt administration of 0.5 μg of apo A-IV had no reliable effect on food intake, whereas the administration of apo A-IV at doses of 1.0 and 1.5 μg significantly reduced food intake measured at 30 and 60 min compared with saline ($P < 0.05$ in each case). Apo A-IV at these doses had no effect on 24-h food intake or body weight (data not depicted), and no adverse physiological reaction such as sedation or ataxia was observed after the i3vt infusion of apo A-IV. Thus we concluded that 0.5 μg of apo A-IV was subthreshold and that doses of 1.0 and 1.5 μg of apo A-IV were above threshold.

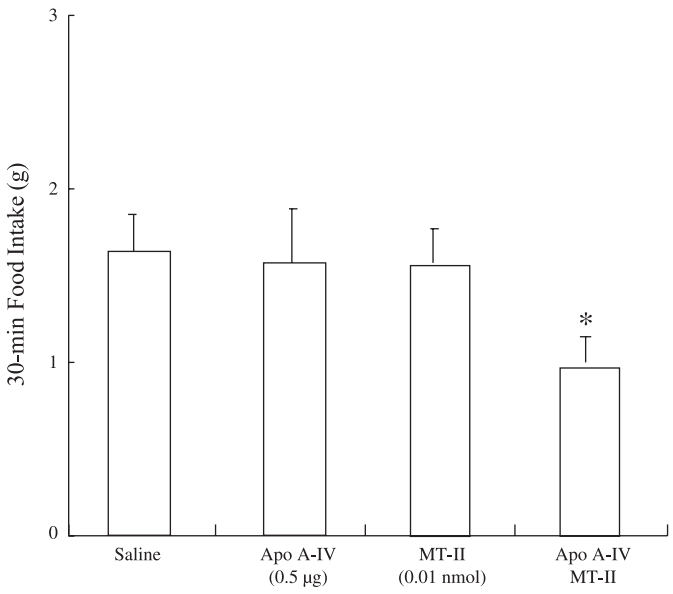


Fig. 3. Effect of i3vt saline or apo A-IV (0.5 μg), in combination with either saline or MT-II (0.01 nmol), on 30-min food intake. *$P < 0.05$ vs. each other group.

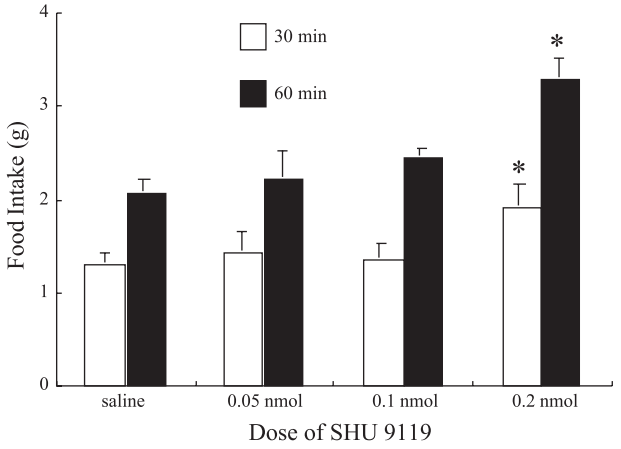


Fig. 4. Effect of SHU9119 (0.05, 0.1, or 0.2 nmol) or saline on 30- and 60-min food intake. *$P < 0.05$ vs. saline.

*Experiment 2: Effect of a subthreshold dose of apo A-IV on MT-II-elicited suppression of food intake and body weight.* As depicted in Fig. 2, saline + MT-II significantly suppressed food intake during the first 30 min (1.4 ± 0.2 g), compared with saline + saline (2.0 ± 0.1 g; $P < 0.05$) and saline + apo A-IV (1.9 ± 0.2 g; $P < 0.05$). Apo A-IV significantly increased the MT-II-induced suppression of feeding during the first 30 min (0.9 ± 0.2 vs. 1.4 ± 0.2 g; $P < 0.05$). Although there was no further enhancement of the reduction of food intake during the ensuing 30 min, there was also no apparent compensation in that the intakes of the saline + MT-II and apo A-IV + MT-II groups remained significantly below those of their controls.

*Experiment 3: Effect of apo A-IV on food intake and body weight when infused 10 min before a subthreshold dose of MT-II.* Apo A-IV + MT-II decreased food intake significantly more during the first 30 min than all other treatments (all $P <$

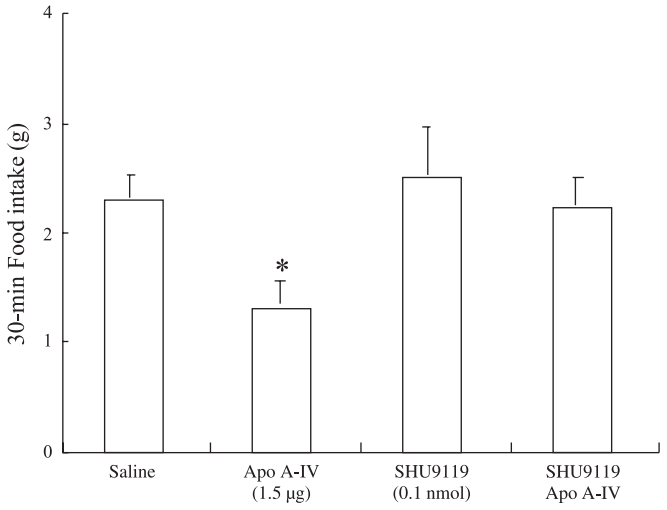


Fig. 5. Effect of i3vt saline or apo A-IV (1.5 μg), in combination with either saline or SHU9119 (0.1 nmol), on 30-min food intake. *$P < 0.05$ vs. each other group.

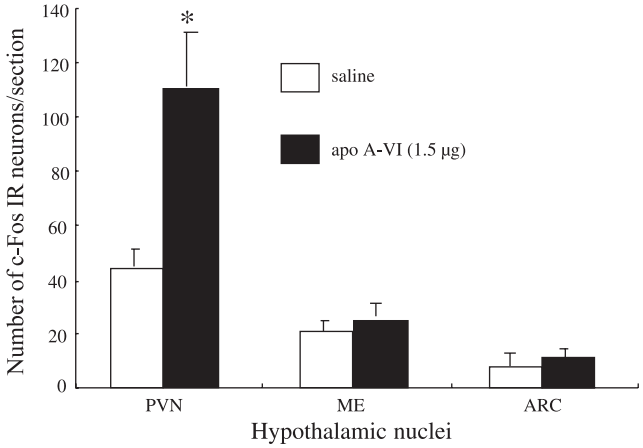


Fig. 6. Effect of i3vt apo A-IV (1.5 μg) on the expression of c-Fos in the paraventricular nucleus (PVN), arcuate nucleus (ARC), and median eminence (ME). IR, immunoreactive. *$P < 0.05$ vs. saline.

0.05, Fig. 3). The doses of 0.01 nmol MT-II and 0.5 μg apo A-IV by themselves did not reliably alter 30-min food intake. There were no significant differences of food intake during the following 30 min or after 24 h, as well as changes of body weight after 24 h, for any group.

*Experiment 4: Determination of a subthreshold dose of SHU9119 on food intake and body weight.* The i3vt administration of 0.05 and 0.1 nmol of SHU9119 had no effect on food intake, whereas a dose of 0.2 nmol SHU9119 stimulated food intake significantly during the first 30 min of feeding as well as during the following 30 min compared with saline (Fig. 4, $P < 0.05$). Moreover, the dose of 0.2 nmol SHU9119 produced a significant increase in food intake and body weight over 24 h ($4.1 \pm 0.5$ vs. $-1.0 \pm 0.5\%$, $P < 0.05$). Thus we defined the subthreshold dose of SHU9119 to elicit food intake as 0.1 nmol.

*Experiment 5: Effect of SHU9119 on apo A-IV-elicited suppression of food intake and body weight.* Saline + apo A-IV (1.5 μg) reduced food intake significantly during the first 30 min compared with saline + saline, SHU9119 (0.1 nmol) + saline, and SHU9119 (0.1 nmol) + apo A-IV (1.5 μg) group (Fig. 5, $P < 0.05$ for each). Hence the subthreshold dose of SHU9119 completely attenuated the anorectic effect of apo A-IV over the same time that the SHU9119 by itself had no effect on feeding. These effects were gone in the following 30 min. There was no significant difference in food intake after 24 h, as well as no change in body weight for 24 h for any group (data not depicted).

*Experiment 6: Expression of POMC and c-Fos with dual-labeled immunohistochemistry in the PVN, ARC, and ME.* The mean numbers of c-Fos immunoreactive cell neurons in the PVN, ARC, and ME are depicted in Fig. 6. The group pretreated with the central infusion of an anorectic dose of apo A-IV had significantly more c-Fos-positive neurons than did saline-treated rats in the PVN ($P < 0.05$). However, there was no significant difference in the number of c-Fos-positive neurons between apo A-IV-treated rats and control rats in the ARC or the ME. Moreover, no colocalization of POMC and c-Fos was observed in the ARC or ME of apo A-IV-treated rats, as depicted in Fig. 7.

## DISCUSSION

These experiments tested the hypothesis that an interaction occurs between apo A-IV and the melanocortin system with regard to the control of feeding by the brain. Our data indicate that there is synergism between apo A-IV and MC3/4-R signaling that causes anorexia. Moreover, it appears that the site of this interaction is downstream of the arcuate. It could be in the PVN, a central site for the integration of signals regulating ingestive behavior, although this cannot be ascertained from the present data. Thus these findings extend previous

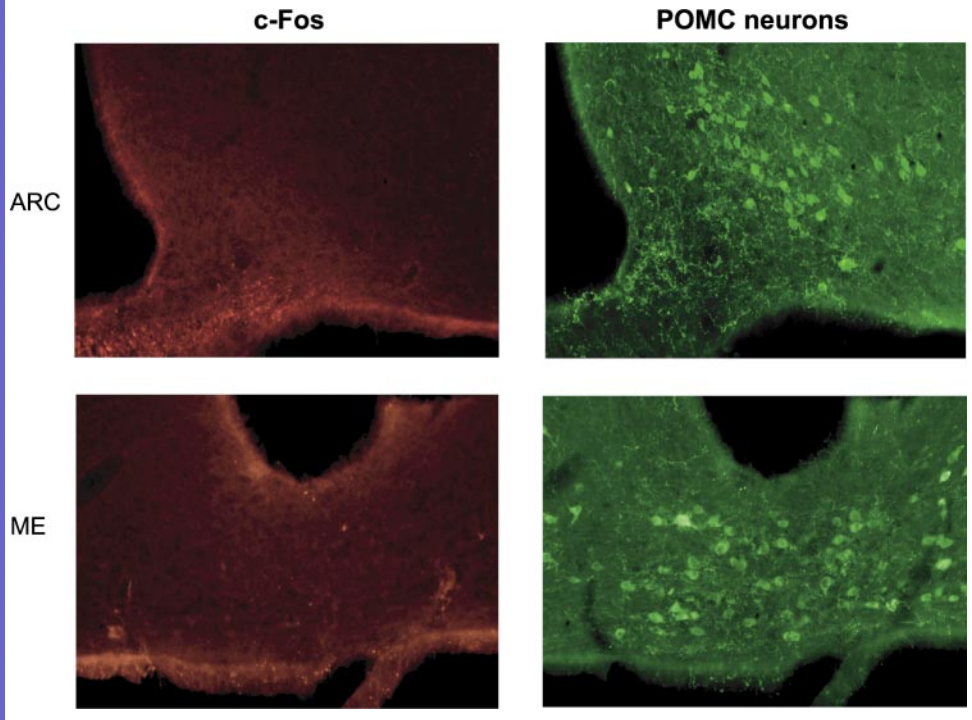


Fig. 7. Representative photomicrographs illustrating dual-labeled immunohistochemistry for c-Fos and proopiomelanocortin (POMC) neurons in the ARC and the ME 60 min after i3vt administration of apo A-IV (1.5 μg). Scale bar: 50 μm.

work, suggesting a role for apo A-IV in the control of feeding (9, 10), and lay the groundwork for determining the mechanism by which this brain-gut peptide acts.

We initially determined that doses of 1.0-μg apo A-IV and higher given i3vt induce a hypophagic response within 30 min, and that a dose of 0.5 μg of apo A-IV does not affect food intake. These findings are compatible with a previous report that the i3vt infusion of 1.0 μg or higher inhibited food intake (9). Based on these results, a dose of 0.5-μg apo A-IV was defined as subthreshold and used in subsequent experiments. We next determined that apo A-IV, in combination with an anorectic dose of MT-II (0.03 nmol), produced a greater reduction of 30-min food intake than MT-II alone. These results indicate that a subthreshold dose of apo A-IV can potentiate the anorectic effect of MT-II. However, it cannot be determined from this observation whether there is an additive or a synergistic interaction, as the MT-II was effective by itself. We, therefore, repeated the experiment, but used a smaller (i.e., subthreshold) dose of MT-II (0.01 nmol). In this instance, the combination of subthreshold doses of apo A-IV and MT-II elicited a significant reduction in food intake. The observation that this dose of MT-II is below the threshold to affect feeding when administered by itself is consistent with other reports (3, 4, 6). The present results, therefore, indicate that the interaction of MT-II and apo A-IV meets the criteria for a synergistic interaction, leading to the reduction in food intake (29). The data do not reveal how apo A-IV exerts its anorectic action. Apo A-IV could, for example, interact with neurons that are activated through MC3/4-R, or it could regulate α-MSH release at the axon level, or it could interact more directly with MC3/4-R, or it could interact at some point downstream of melanocortin receptors.

The interaction of apo A-IV with the melanocortin system suggested that the anorectic effect of apo A-IV may be blocked by pretreatment with a subthreshold dose of SHU9119. *Experiments 4* and *5* assessed this hypothesis. We first determined that 0.1 nmol (and lower) doses of SHU9119 have no effect on feeding or body weight over 24 h, an observation consistent with a previous report (14). This dose of SHU9119, when administered in combination with apo A-IV, attenuated the reduction of food intake induced by apo A-IV over 30 min. SHU9119, when administered i3vt at higher doses, elicits hyperphagia that lasts several days (12, 13). However, neither apo A-IV nor SHU9119 alone had any effect on 24-h intake at the doses used; likewise, neither MT-II nor the combination of apo A-IV plus MT-II reduced 24-h intake.

All of the behavioral data are, therefore, consistent and indicate that apo A-IV exerts its anorectic effect by interacting with signals that stimulate MC3/4-R. One possible mechanism to explain these data is that apo A-IV stimulates the release of POMC-derived α-MSH originating in the ARC and projecting to the PVN, as α-MSH binds and activates MC3/4-R and as the PVN expresses MC3/4-R. This possibility would be consistent with the observation that a subthreshold dose of apo A-IV increased the MT-II-induced suppression of feeding and that a subthreshold dose of SHU9119 attenuated the anorectic effect of apo A-IV. However, the results of the dual-labeled immunohistochemistry do not easily support an effect of apo A-IV to increase α-MSH release, as there was no change of c-Fos activity in the ARC and no colocalization with POMC. Although it is possible that apo A-IV activates POMC neurons without stimulating c-Fos, there is no precedent for this in previous studies of the melanocortin system. It seems more likely that apo A-IV acts at feeding-related neurons downstream of the arcuate, for example in the PVN on neurons that either express the MC3/4-R or else converge on neurons that do. MC3/4-R are expressed in a number of hypothalamic sites, including the PVN (5), such that the precise site of interaction cannot be determined from the present data. However, the recent demonstration that apo A-IV mRNA is present in the ventral hypothalamus, near but ventral to the PVN (17), suggests that an endogenous circuit may exist within the hypothalamus in which apo A-IV contributes to anorexia by interacting with MC3/4-R-containing neurons. In conclusion, the present experiments indicate that there is a synergistic interaction between the melanocortin system and apo A-IV in the brain. This interaction causes a short-term reduction of food intake at some point downstream of POMC neurons.

## GRANTS

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