

Evidence of Splanchnic-Brain Signaling in Inhibition of Ingestive Behavior by Middle Molecules

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Abstract. Anorexia, nausea, and vomiting are common symptoms of uremic intoxication. Fractions in the middle molecule weight range, isolated from normal urine and uremic plasma ultrafiltrate, inhibit ingestive behavior in the rat. To investigate their site of action and specificity, male rats were injected intraperitoneally, intravenously, or intracerebroventricularly with concentrated fractions of uremic plasma ultrafiltrate or normal urine (molecular weight range: 1.0 to 5.0 kD) and tested for ingestive and sexual behavior. An intraperitoneal injection of 0.5 ml of urine fraction (10:1) or 2.0 ml of uremic plasma ultrafiltrate fraction (25:1) inhibited carbohydrate intake by 76.3 and 45.9%, respectively, but an intravenous injection had no effect. However, intravenous injection of higher

doses inhibited carbohydrate ingestion. An intracerebroventricular injection of 5 or 10 μ l of urine (20:1) middle molecule fraction inhibited carbohydrate intake by 13.4 and 41.6%, respectively. An injection of 5 or 10 μ l of uremic plasma ultrafiltrate (125:1) middle molecule fraction inhibited carbohydrate intake by 22.6 and 49.5%, respectively. Injections of the corresponding fraction from normal plasma ultrafiltrate had no effect. Injection of urine or uremic plasma ultrafiltrate middle molecule fractions did not affect the display of sexual behavior. These results suggest that middle molecule fractions from uremic plasma ultrafiltrate or normal urine act in the splanchnic region and/or brain to inhibit food intake and that the effect is specific for ingestive behavior.

Protein-energy malnutrition is common in patients with advanced renal failure. The causes may include anorexia, nausea, and vomiting, which reduce nutrient intake in relation to requirements (1). The reduction of protein and energy intake that starts relatively early during the development of chronic renal failure is accompanied by a significant reduction in body weight and other nutritional parameters (2). Uremic patients with suppressed food intake regain appetite soon after starting dialysis treatment, presumably because of removal of one or more toxic factors that suppress appetite. Studies in patients treated with maintenance hemodialysis or continuous ambulatory peritoneal dialysis show a correlation between the dose of dialysis for small molecules and the intake of protein (3–6).

Quantitative methods have been developed during recent years for the study of appetite regulation, as influenced by various endocrine, metabolic, and nutritional factors (7,8). We have adapted an animal model in which rats were fed a carbohydrate, protein, or mixed diet solution via intraoral cannulas (9–11) as a bioassay for the study of uremic toxicity (12). This method showed that unprocessed uremic plasma ultrafiltrate injected intraperitoneally in rats inhibits the ingestion of nutrients, an effect not observed after injection of normal

ultrafiltrate (12). Concentrated subfractions of uremic plasma ultrafiltrate with molecular weight ranges of 1 to 5 kD and 5 to 10 kD, respectively, inhibit ingestion in a dose-dependent manner, whereas fractions with a molecular weight below 1 kD are inactive. An ultrafiltrate of normal urine also inhibited appetite, an effect that was confined to a fraction with a molecular weight of 1 to 5 kD (12).

In this study, we investigated the site of action of fractions in the middle molecule (MM) weight range (1 to 5 kD) isolated from urine and uremic plasma ultrafiltrate because this fraction had the strongest appetite-inhibiting effect in our previous study (12). The solutions of MM fractions were given intraperitoneally (ip), intravenously (iv), or intracerebroventricularly (icv) to intraorally fed rats. The effect of these fractions on sexual behavior was tested to investigate their behavioral specificity.

Materials and Methods

Isolation of Fractions from Plasma and Urine Ultrafiltrates

These fractions were collected from uremic patients with anorexia who came for hemodialysis treatment for the first time. The serum urea level was 34.4 ± 4.6 mmol/L and the serum creatinine level was 694.8 ± 48 μ mol/L (mean \pm SD) before dialysis started. The plasma ultrafiltrate was obtained by applying isolated ultrafiltration during the first 30 to 60 min of the treatment, using a hollow-fiber dialyzer with a cellulose acetate membrane (CA 170 G, Baxter) and a Gambro AK 10 dialysis monitor set in ultrafiltration mode, with no dialysis fluid passing through the dialyzer. The ultrafiltrate, usually 500 to 1000 ml depending on the degree of overhydration of the patient, was collected in a plastic bottle, which was then immediately frozen and

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stored at -20°C . After thawing, the ultrafiltrates from patients were pooled, frozen, and kept at -70°C , pending use in tests or further processing.

Plasma ultrafiltrate was also obtained from healthy volunteers. Heparinized venous blood (200 ml) was collected from each of them, and the plasma was separated by centrifugation and pooled. It was then ultrafiltered *in vitro* through the same type of dialyzer as used with the patient's ultrafiltrate. The pooled ultrafiltrate was frozen and kept at -70°C , pending use in the appetite test or further processing.

Morning midstream urine samples from volunteers with normal renal function were collected and pooled. The samples were filtered *in vitro* through the same type of dialyzer as used with the uremic plasma ultrafiltrate, then buffered with sodium hydroxide to a pH of 6.5. The samples were stored at -70°C before use.

The ultrafiltrates from normal and uremic plasma, as well as from normal urine, were fractionated according to molecular weight and size by the molecular filtration technique, and then concentrated, as described earlier (12), using a 400-ml stirred cell chamber and 76-mm disc membranes with molecular weight cutoff points of 10, 5, 1, 0.5, and 0.1 kD (Molecular/Por Type C, Spectrum, LA). The separations were performed in a cold room at 8°C to minimize bacterial growth.

The concentrated MM fractions from uremic and normal plasma and from normal urine, representing a molecular weight of 1 to 5 kD, were used in the experiments in this study. Precipitates, if any, were removed by centrifugation, pH was corrected to 6.5, and the fractions were stored at -70°C until use.

MM fractions from normal and uremic plasma ultrafiltrates were concentrated 25 times, and the corresponding fraction from normal urine ultrafiltrate was concentrated 10 times. The concentrations selected in the present study were based on dose–response curves in a previous study from our laboratory (12). These fractions were further concentrated 5 and 2 times, respectively, for use in the icv injection experiments, which required much smaller injection volumes.

Animals, Operations, and Injections

Male Wistar rats (Møllegaard Breeding Laboratories, Ejby, Denmark), weighing 350 to 380 g, were maintained in an air-conditioned, temperature-controlled colony room in which the lights were off between noon and midnight. The rats had continuous access to food and water.

All surgery was performed under pentobarbital anesthesia (60 mg/kg ip; Mebumal, Nordvacc, Stockholm, Sweden). The intraoral (io) cannulas were implanted as described by Grill *et al.* (7). The animals were allowed to recover for 4 wk after the operation.

Cannulation of the right lateral brain ventricle was done while the rat was in a stereotaxic frame, using the following coordinates: 1.5 cm caudally, 1.5 mm laterally, and 3.5 mm ventrally from bregma. The concentrated MM fractions were injected slowly through a 28-gauge cannula (Plastic Products[®], Inc., Roanoke), using a 25- μl Hamilton syringe.

Intravenous cannulas were prepared from silicone medical-grade tubing (inner diameter 0.51 mm; outer diameter 0.94 mm; Degania, Bethlehem, Israel) and implanted in the right jugular vein, as described by Remie *et al.* (13), except that the cannula was not anchored to the skull, but was exteriorized in the subcutaneous region of the neck, approximately 2 cm caudally from the ears. The cannulation was completed within 10 min and performed 10 h before behavioral testing. This cannulation procedure does not affect intake under the conditions of this study (10).

Depending on the MM volume used in each experiment, the iv injections were given slowly in the tail (1 and 2 ml) or external jugular

vein (4- and 6-ml injections). There were no behavioral differences when different intravenous routes were used.

Diet Solution

A carbohydrate solution containing the carbohydrate moiety of a mixed nutritional solution (Fortimel[®], Nutricia Nordica AB, Stockholm, Sweden) with 10.4 g of starch was used as the intraoral diet solution. The caloric density of the solution is 100 kcal/100 ml.

Ingestive Behavior

The pellets were removed at 7 a.m., and the animals were tested 6 h later, *i.e.*, 1 h after the lights were switched off. The rats were placed in circular (35-cm diameter) Plexiglas arenas and had their intraoral cannulas connected to a peristaltic pump (Alitea XV, Ventur Alitea, Stockholm, Sweden), which delivers a solution at 1 ml/min. This activates ingestive behavior that ends by the time the animal either actively rejects the solution or passively lets it drip from its mouth. At that time, the infusion is interrupted for 30 s and then continued. If the animal ingests the diet solution during at least 1 additional minute, the infusion is again stopped for 30 s by the time the animal rejects the solution or lets it drip from its mouth within 1 min after the start of the infusion. Usually, rats let the solution drip from their mouth within the first minute after the start of the second infusion period (9). Active rejection of the solution is less common. The pellets were replaced after testing. After recovery from the trauma of catheter implantation, a training and adaptation period is required, during which the intake of the orally infused solution gradually increases until a stable level of intake is attained. Then the rats were studied once a day on consecutive days. Under these conditions, rats commonly stabilize their carbohydrate and pellet intakes within 5 to 7 d (9,10).

In all experiments, the io infusion of the carbohydrate solution was started 10 min after the injections of the MM fractions. Only rats that had approximately equal intakes after the adaptation period were used in these studies.

Sexual Behavior

Male rats were tested individually for sexual behavior at the same time, *i.e.*, 1 h after the light was turned off and in the same arenas as used for ingestive behavior. A sexually receptive female (injected with 2 μg of estradiol benzoate [Sigma, St. Louis, MO], dissolved in 0.1 ml of sesame oil and injected subcutaneously [sc] 48 and 24 h before, and 0.5 mg of progesterone [Sigma], dissolved and injected in the same way as estradiol 6 h before testing) was placed with the male. Records were kept on the number of mounts with intromission (IF) before ejaculation and the latency from the first intromission to ejaculation, ejaculation latency (EL). The other parameters of sexual behavior, *i.e.*, latency to the first intromission (IL), the frequency of mounts without intromission (MF), and the time between ejaculation and the next intromission, the postejaculatory interval (PEI), were also recorded. Sexually experienced male rats intromit within a few seconds when a female is available, show very few mounts before ejaculation, and have very stable EL and PEI (14). The parameters most sensitive to manipulations are the number of intromissions and the EL and PEI. The males were used after showing a stable pattern of sexual behavior in five preliminary tests separated by 2 d.

Procedure

Effect of ip, iv, or icv Injection of MM Fractions on Carbohydrate Intake. A group of six rats was injected ip with 2 ml of concentrated uremic plasma ultrafiltrate MM fraction or normal plasma ultrafiltrate MM fraction in random order on 2 consecutive

days, and the amount of carbohydrate intake was determined. A similar experiment was done by ip injection of 0.5 ml of urine ultrafiltrate or saline in a separate group of rats ($n = 6$).

A group of eight rats was injected iv with 2 and 4 ml of uremic plasma ultrafiltrate MM fraction in random order on 3 consecutive days, and the amount of carbohydrate intake was determined. In a separate group ($n = 6$), 6 ml of the uremic MM fraction was given iv. In another group ($n = 8$), 2, 4, and 6 ml of normal plasma ultrafiltrate MM fraction was injected iv. Ingestion without any injection served as controls.

In eight rats, 0.5 and 1 ml of urine ultrafiltrate MM fraction or saline was injected iv, and the amount of carbohydrate intake was determined. In another group ($n = 6$), 2 ml of urine ultrafiltrate MM fraction or saline were given iv.

Eight male rats were injected icv with 5 or 10 μ l of uremic plasma ultrafiltrate MM fraction or the corresponding fraction of normal plasma ultrafiltrate in random order on 3 consecutive days, and the amount of carbohydrate intake was determined. The experiment was repeated using 5 or 10 μ l of urine ultrafiltrate or saline in another group of rats ($n = 8$).

Effect of ip Injection of MM Fractions on Sexual Behavior. Male rats ($n = 6$) were injected ip with 2 ml of normal plasma MM fraction or 2 ml of uremic plasma ultrafiltrate MM fraction and tested for sexual behavior 10 min later. The injections were given in random order on 2 consecutive days.

The experiment was repeated on another group of rats ($n = 8$) injected ip with 0.5 ml of normal saline (0.9% NaCl) or 0.5 ml of normal urine ultrafiltrate (10:1) MM fraction.

Statistical Analyses

Data are presented as mean \pm SEM. A P value <0.05 was considered significant. Paired t tests were used for statistical analysis of differences. In the dose–response studies, the data were tested by ANOVA, which, when significant, was followed by Dunn’s test with Bonferroni correction for multiple comparison.

Results

Effect of MM Fractions on Carbohydrate Intake

The ingestion of the carbohydrate solution was reduced by 45.9% after ip injection of 2 ml of uremic plasma ultrafiltrate

MM fraction, compared with the ingestion after injection of the corresponding fraction isolated from normal plasma (Figure 1). Intravenous injection of 2 ml of uremic plasma ultrafiltrate MM fraction had no effect, injection of 4 ml inhibited ingestion by 10% (NS), and injection of 6 ml inhibited ingestion by 33% (Figure 1). Intravenous injections of up to 6 ml of MM fraction isolated from normal plasma had no effect on ingestion.

Intraperitoneal injection of 0.5 ml of MM fraction isolated from normal urine reduced carbohydrate intake by 76.3%. Intravenous injection of 0.5 ml of urine ultrafiltrate MM fraction had no effect, injection of 1 ml inhibited ingestion by 8% (NS), and injection of 2 ml inhibited ingestion by 32% (Figure 2).

Injections of 5- or 10- μ l MM fractions isolated from uremic plasma ultrafiltrate into the lateral ventricle of the brain reduced the ingestion of the carbohydrate solution by 22.6 and 49.5%, respectively, compared with injection of the corresponding fractions isolated from normal plasma ultrafiltrate (Figure 3). Intracerebroventricular injection of 5 or 10 μ l of 1 to 5 kD fraction isolated from normal urine reduced carbohydrate intake by 13.5 and 41.6%, respectively (Figure 4).

Effect of MM Fractions on Sexual Behavior

Intraperitoneal injection of MM fractions isolated from either uremic plasma ultrafiltrate or normal urine had no effect on sexual behavior (Table 1). Since ip injection of the MM had no effect on sexual behavior, we did not expect an effect of icv injection, and therefore this experiment was omitted.

Discussion

In the present study, ingestive behavior was assessed quantitatively by recording the consumption of an io infused carbohydrate solution by conscious, free-moving rats. This model has been used in neuropharmacologic studies on appetite regulation and has proved to be accurate and highly reproducible (7,9). An adaptation period is required, during which the intake

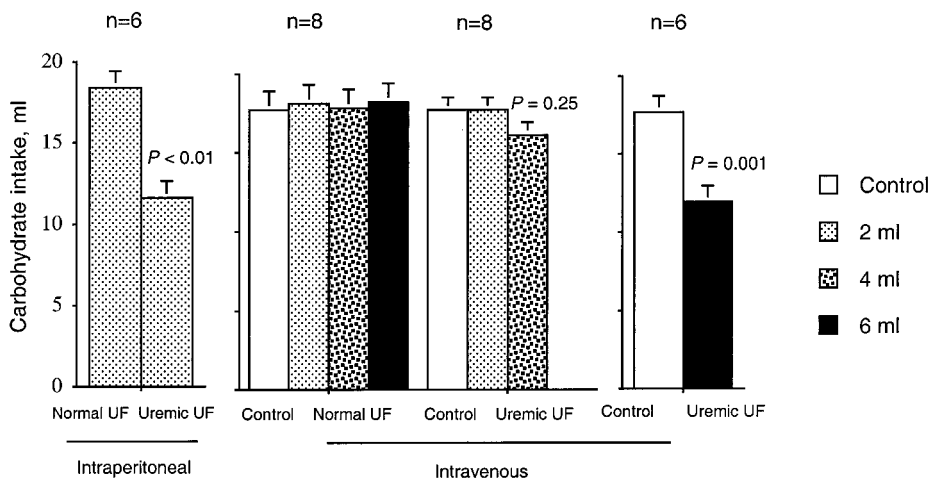


Figure 1. Effect of intraperitoneal (ip) or intravenous (iv) injections of uremic plasma ultrafiltrate middle molecule (MM) fractions on ingestion of carbohydrate solution in each group of male rats. The ingestion after ip injection of uremic ultrafiltrate (left) was compared with the ingestion after injection of the same volume of normal plasma ultrafiltrate. The ingestion after iv injections of the various doses (right) was compared with the ingestion without injection. Data are means \pm SEM.

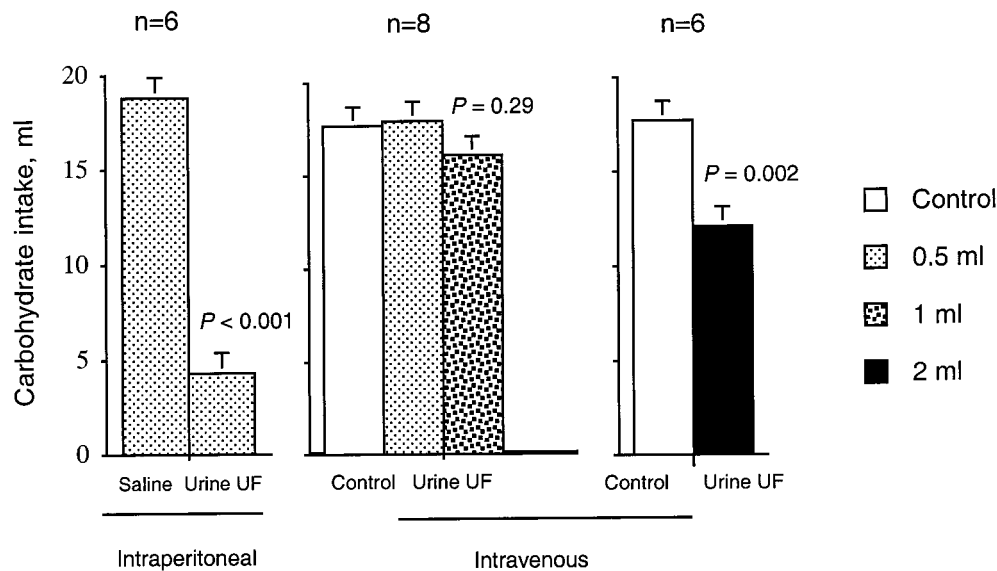


Figure 2. Effect of ip or iv injections of normal urine ultrafiltrate MM fraction on ingestion of carbohydrate solution in male rats. In each group, the ingestion after injection of 0.5 ml of saline served as control. Data are means \pm SEM.

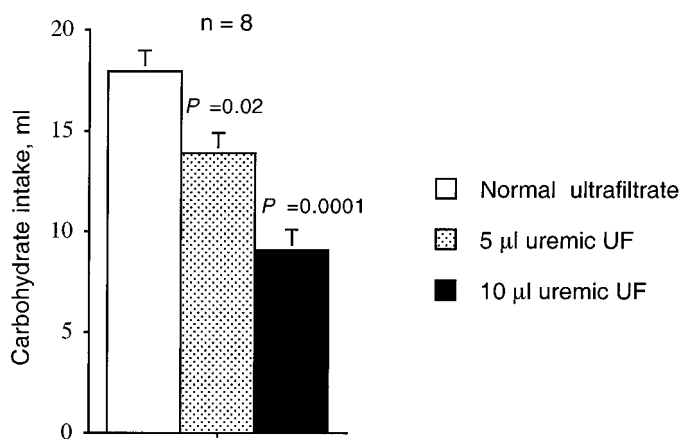


Figure 3. Effect of intracerebroventricular (icv) injections of concentrated uremic ultrafiltrate (125:1) MM fractions on ingestion of a carbohydrate solution in male rats. The concentrated MM fraction elicited strong inhibition of the ingestion of the carbohydrate solution (two-way ANOVA), which was dose-dependent. Data are means \pm SEM. At each concentration, the ingestion after ultrafiltrate injection was compared with the ingestion after injection of normal plasma ultrafiltrate.

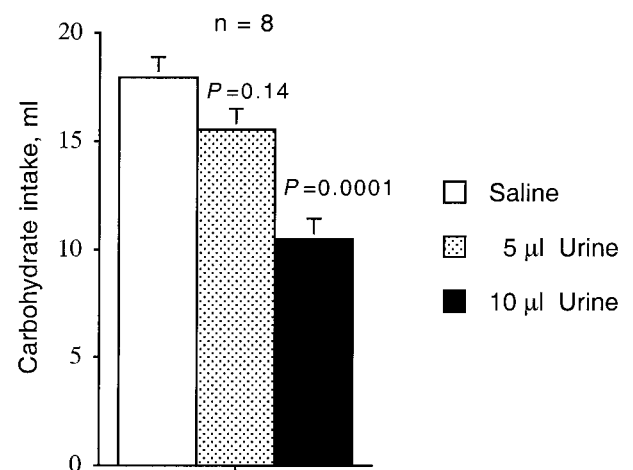


Figure 4. Effect of icv injections of concentrated normal urine ultrafiltrate (20:1) MM fractions on ingestion of a carbohydrate solution in male rats. The concentrated MM fraction elicited strong inhibition of the ingestion of the carbohydrate solution (two-way ANOVA), which was dose-dependent. Data are means \pm SEM. At each concentration, the ingestion after ultrafiltrate injection was compared with the ingestion after injection of saline.

of the solution increases until a stable level of intake is attained (11).

We previously demonstrated that ip injection of 20 ml of unprocessed uremic plasma ultrafiltrate or 10 ml of normal urine significantly inhibited food intake, but plasma ultrafiltrate from healthy subjects had no effect (12). We also showed that the effect was obtained only with fractions having a molecular weight range between 1 to 10 kD, the strongest effect being obtained with fractions in the range of 1 to 5 kD.

The first experiment in this series confirms our previous findings that ip injection of fractions in the MM weight range of 1 to 5 kD, isolated from uremic plasma ultrafiltrate and

normal urine, inhibits ingestive behavior (12). The absence of an effect on ingestive behavior of the normal plasma ultrafiltrate MM fraction supports the conclusion that the anorexic effect of the uremic plasma ultrafiltrate is a feature of uremic intoxication and that the testing system is reliable (12). The ingestion of the carbohydrate solution was also reduced after the injection of MM fraction of normal urine ultrafiltrate, an effect that was greater than after injection of uremic plasma ultrafiltrate as we reported before (12).

The concentrated MM subfractions from uremic plasma ultrafiltrate or normal urine inhibited appetite when given ip but had a less marked effect when given iv. This suggests that

Table 1. Effect of intraperitoneal injection of uremic plasma ultrafiltrate or urine middle molecule fractions on sexual behavior^a

Parameter	Uremic UF	Normal UF	Urine UF	Saline
MF	7.0 ± 1.7	6.0 ± 0.5	11.8 ± 3.6	10.3 ± 2.3
IF	9.5 ± 1.4	11.3 ± 1.2	12.8 ± 2.2	11.3 ± 1.5
IL	0.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.8	0.4 ± 0.2
EL	4.4 ± 0.8	4.9 ± 0.7	5.6 ± 0.8	4.3 ± 0.6
PEI	5.9 ± 0.5	5.4 ± 0.5	5.7 ± 0.3	5.1 ± 0.4

^a UF, ultrafiltrate; MF, mount frequency; IF, intromission frequency; IL, intromission latency; EL, ejaculation latency; PEI, postejaculatory interval. Each parameter was compared with rats after injection of the same volume of normal plasma ultrafiltrate ($n = 6$) or saline ($n = 8$), using the paired t test. Data are mean ± SEM.

the MM fractions act on the splanchnic region to suppress ingestive behavior. To be effective *iv*, these MM fractions must be given in higher doses to reach their site(s) of action because they are diluted in the general circulation.

Ingestive behavior is controlled by signals generated in the gastrointestinal tract and in the postabsorptive metabolism of energy-yielding substrates (15). Several studies have suggested a physiologic role of the liver in regulating food intake (16–18). Thus, Del Prete and Scharrer (19) suggested that an intact hepatic vagus branch is a prerequisite for feeding and that hepatic vagal glucoreceptors participate in the control of feeding. A recent study by Friedman (20) indicates that an event common to the metabolism of glucose and fats gives such a signal, and that ingestive behavior is triggered by a signal tied to the concentration of ATP, which is carried from the liver to the brain via vagal afferents. However, it is not known whether the effects reported here are related to an alteration in hepatic glucoreceptors.

Because of the limited brain intraventricular fluid and location at the vicinity of appetite-regulating brain areas, the MM subfraction was concentrated 125 times and injected in much smaller volumes into the lateral brain ventricle. Using these procedures, we observed that fractions in the MM weight range, isolated from uremic plasma ultrafiltrate and normal urine, inhibited ingestive behavior in a dose-dependent manner. However, the effect of MM on the brain is unknown, and as yet we know little about their exact molecular weight and availability to the brain. Nevertheless, our results suggest that, in addition to acting in the splanchnic region, MM may also act on the brain, possibly by modifying neurotransmitters that control feeding.

Numerous neurotransmitters influence ingestive behavior (21). For example, dopamine, serotonin, noradrenaline, and adrenaline apparently act via forebrain and brainstem receptor mechanisms (21). We recently observed (22) that the levels of tryptophan, a precursor of serotonin, and 5-hydroxyindoleacetic acid, a metabolite of serotonin, were elevated in the cerebrospinal fluid of uremic rats, suggesting increased serotonin synthesis in the brain in uremia. The dopamine metabolites

noradrenaline and 3,4 dihydroxyphenolacetic acid showed an increase, reflecting an increase in dopamine turnover in uremia. Whether these effects of experimental uremia on cerebral monoamines are related to effects of the MM observed here, however, is an open question.

These results suggest that the MM may act as satiety signals in uremia. For example, they may act in a paracrine manner, *i.e.*, a sustained high plasma level of MM may stimulate/inhibit receptor sites in the splanchnic region, which then send information via the vagus nerve to the brain. It could also be that the inhibition of ingestive behavior by *iv* or *icv* injection of the MM reflects a direct interference with neurotransmission. Yet another possibility is that the effect of the MM is mediated via the endocrine system. Obviously, further investigations will be necessary before this issue can be resolved.

The finding of an inhibitory effect on behavior, such as the effects in this study, raises the issue of behavioral specificity. Here we found that doses of MM fractions that effectively inhibited ingestive behavior when injected *ip*, *icv*, or *iv* had no effect on sexual behavior if given *ip*. This suggests that the behavior inhibitory effects of the MM were to some degree specific for ingestive behavior. However, it seems likely that higher doses of the MM fractions will inhibit behaviors other than those related to ingestion of nutrients, particularly since ingestive and sexual behavior share some neuroendocrine control mechanisms (9).

There is a decline in the sexual activity of patients on both peritoneal dialysis and hemodialysis treatments. Despite the improvement in ingestive behavior after starting dialysis therapy, Auer *et al.* (23) showed a significant reduction in sexual activity on dialysis, compared to 12 mo before therapy. This applied equally to peritoneal dialysis and hemodialysis patients; only 31% of patients had satisfactory sexual activity, compared with nearly 60% before dialysis, whereas the non-existent sexual activity category increased from 22% predialysis to nearly 50% on treatment. This contrasts with the present study's finding that the MM had no effect on sexual behavior.

These results, therefore, suggest that the anorexic effect of the MM fractions is produced in the splanchnic region and/or in the brain to inhibit food intake and that the effect of these fractions is specific for ingestive behavior.

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