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Accumulation of methylguanidine and changes in guanidino compound levels in plasma, urine, and kidneys of furosemide-treated rats

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Abstract

Antidiuresis and renal diseases alter the levels of guanidino compounds (GCs) in various tissues. Therefore, we hypothesized that diuresis could also disturb GC metabolism, storage, and elimination. In this study, rats were made diuretic to analyze GC levels in plasma, urine, and kidneys. Furosemide was chosen because of its wide use in various human pathologies. Rats were injected intraperitoneally 5 or 10 mg furosemide spread over a 24-hour cycle. Urine was collected over a period of 24 hours before and during furosemide treatment. Plasma was obtained from arterial blood. Renal zones were dissected. The GCs were determined by liquid chromatography. Five milligrams of furosemide provoked a significant increase in plasma and urine levels of GCs compared with those of the controls. The renal distribution and content of GCs were weakly modified by furosemide except for methylguanidine (MG). The level of MG was enhanced by 10 to 16 times in all renal zones. The MG level was 60% higher in renal zones of rats treated with 10 rather than 5 mg furosemide. The fractional excretion of MG was decreased by furosemide. Our data suggest that MG accumulation in kidney and plasma was caused by furosemide, which might induce MG synthesis, and that MG washout from tissue cells into urine by furosemide through the kidney may cause an increase in MG in the kidney.

1. Introduction

The family of guanidino compounds (GCs) includes all molecules containing a strong basic guanidinium radical. The GCs differ considerably in their structure, metabolism, tissue distribution, biological properties, and physiological functions [1]. Among the naturally occurring GCs, L-arginine (Arg), guanidinoacetic acid (GAA), creatine (CT), and few other GCs, using Arg as precursor, are synthesized in the mammalian kidneys. Furthermore, the male rat and mouse kidneys contain significant amounts of asymmetric N^G,N^G-dimethylarginine, symmetric N^G,N'^G-dimethylarginine, α-keto-δ-guanidinovaleric acid (α-keto-δ-GVA), guanidinosuccinic acid (GSA), creatinine (CTN), α-*N*-acetylarginine (α-*N*-AA), γ-guanidinobutyric acid (γ-GBA), β-guanidinopropionic acid (β-GPA), homoarginine (HArg), guanidine (G), and methylguanidine (MG) [2,3].

Regarding their renal distribution, these GCs are unevenly distributed within the corticopapillary axis; and each of them exhibits a typical distribution pattern [2]. Although poorly documented, the presence and the level of each GC in the different nephron segments might be related to the specific biochemical pathways and physiological roles undertaken by these compounds. For example, the proximal convoluted tubules express not only the enzymes that metabolize L-citrulline into Arg but also the enzyme L-arginine-glycine amidinotransferase that produces GAA and L-ornithine from Arg and L-glycine [4,5]. In addition, the conversion of GAA into CT has been reported in isolated rat kidneys; but the precise nephron segment in which this metabolic step takes place remains to be identified [6-8].

Interestingly, in healthy rat kidneys, the concentrations of α-keto-δ-GVA, GSA, CTN, MG, α-*N*-AA, and γ-GBA increase steeply from the inner stripe of the outer medulla to the papillary tip and run in parallel to the urea and osmotic gradients. The gradient of these 2 solutes is deeply affected in response to antidiuresis and diuresis [2,9,10]. Recently, we

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established that water deprivation for 24 and 48 hours was associated with an increase of the renal content of GSA, GAA, CT, CTN, and β-GPA along the corticopapillary axis in parallel with these 2 gradients [11]. In contrast, the renal content of Arg, G, and MG was decreased after 1 and 2 days of water deprivation. At present, it remains completely unkown whether diuresis, the opposite physiological state of antidiuresis, provokes changes in renal GC levels and distribution.

It has been also well established that the levels of GCs are altered in blood, urine, and kidneys of uremic animals and patients who progressively lose the ability to concentrate their urine and become diuretic [12-16]. It is noteworthy that diuresis is also a physiological state observed during insipidus and mellitus diabetes. Altogether, we hypothesized that diuresis might disturb GC metabolism, storage, and elimination in biological fluids and tissues. Given that several GCs play a pivotal role in energy production, exert different physiological roles, or are depicted as toxins [17], we felt that it was essential to give light to the metabolism of GCs during diuresis. In addition, the repercussions of diuretic state provoked by osmotic molecules or drugs on the metabolism of GCs as well as the renal distribution and levels remain completely unknown. Among the different models of diuresis, we chose the diuretic drug furosemide that is widely used in various human pathologies. Moreover, furosemide has been extensively used in animal models to induce diuresis as described and validated earlier in animals [18-20].

The purpose of the present study was to determine whether furosemide might disturb GC metabolism and storage in rats. To get an overview of the impact of furosemide more especially on the renal levels of GCs, we analyzed the levels of 12 GCs in the plasma (input), urine (output), and kidney that are involved in the catabolism and anabolism of several GCs. Significant changes in GC levels in these biological fluids and tissue reflect either an accumulation or an elimination of these compounds and might be associated to an enhancement or a lowering of their synthesis.

Briefly, our results revealed that the renal distribution and content of GCs were weakly modified by furosemide treatment except for MG. Indeed, we discovered that rats injected with furosemide exhibited an impressive increase in MG levels in their plasma, urine, and kidneys, suggesting that MG synthesis was increased. Moreover, MG accumulation seemed to be furosemide dose dependent because rats injected 10 mg furosemide exhibited higher plasma and renal levels of MG than those receiving 5 mg.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (300-320 g body weight [BW]) from Iffa Credo (L'Arbresle sur Orge, France) were given free access to tap water and standard laboratory food (Souriffarat, 20% protein; Genthon, Chassieu, France) for an adaptive period of 5 days. The biscuits of food were pulverized to obtain a fine homogeneous powder. The rats were housed in a room maintained at 20°C with a 12-hour light-dark cycle. Rats were anesthetized by injecting intraperitoneally 0.1 mL/100 g BW sodium pentobarbital (Nembutal 6%; Clin Midy, Paris, France). Animal care complied with French regulations for the protection of animals used for experimental and other scientific purposes and with European Community regulations. The author is authorized (no. 69-33) to use animals for experiments.

2.2. Experimental protocols

The first part of the experiment (part I) was designed to quantify GCs in plasma and renal zones of untreated rats (controls). Arterial blood was collected in 36 rats, and the 2 kidneys of 3 groups of 10 rats were collected to dissect the 7 well-defined renal zones as previously published [2,11].

The second part of the experiment (part II) was performed to analyze the influence of a moderate dose of furosemide on the level of GC in plasma, urine, and the renal zones. Given that furosemide diuresis was supposed to decrease the concentration of GCs in fluids and tissues as known for urea [10], we enhanced the number of rats per group to collect more tissue in each renal zone. Forty-five rats were divided into 3 groups of 15 rats that were housed individually in metabolic cages for an adaptive period of 3 days before

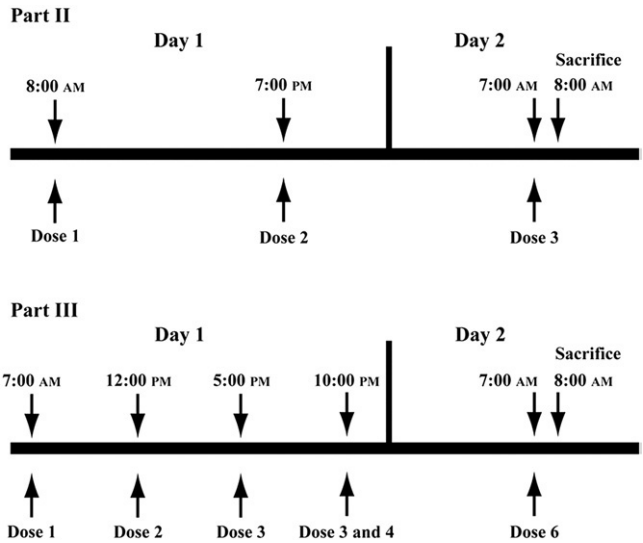

Fig. 1. Protocol for furosemide treatment in parts II and III. In part II, rats received 5 mg furosemide per day to diminish by 2 times urine osmolality compared with that of the control rats. In part III, rats received 10 mg furosemide per day to reduce by 4 times urine osmolality compared with that of the control rats. Furosemide was diluted by half in 0.9% NaCl solution (vol/vol). Rats were injected intraperitoneally 3 or 6 doses of furosemide spread over a 24-hour cycle. A dose of furosemide corresponds to about 1.61 mg furosemide per rat and per injection (0.5 mg furosemide per 100 g BW). In part III, rats received 2 doses of furosemide at 10:00 PM for the night (1.0 mg per 100 g BW or 3.22 mg furosemide per rat). During furosemide treatment, rats had free access to tap water containing 0.8% NaCl and 0.1% KCl. Rats were killed 1 hour after the last injection (ie, 8:00 AM).

inducing a diuresis with furosemide (Lasilix, injectable solution, 20 mg/2 mL; Aventis, Paris, France). Based on previous reports [18-20], we estimated that the injection of 5 mg furosemide per rat and per day may diminish by 2 times urine osmolality compared with that of the control rats. Furosemide was diluted by half in 0.9% NaCl solution (vol/vol). A dose of furosemide corresponds to about 1.61 mg furosemide per rat and per injection (0.5 mg furosemide per 100 g BW). Each rat was injected intraperitoneally 3 doses of furosemide spread over a 24-hour cycle as follows : 8.00 AM and 7:00 PM on the first day and 7:00 AM on the next day (Fig. 1). Rats were killed 1 hour after the last injection (ie, 8:00 AM). During furosemide treatment, rats were given free access to tap water containing 0.8% NaCl and 0.1% KCl as previously published [18,20].

The third part of the experiment (part III) was performed to test the influence of a high dose of furosemide on the level of GC in plasma and the renal zones. As performed in part II, 6 rats were housed individually in metabolic cages for an adaptive period of 3 days before furosemide treatment. As explained above, we estimated that the injection of ≈10 mg furosemide per rat and per day might reduce by 4 times urine osmolality compared with that of the control rats. Each rat was injected intraperitoneally 6 doses of furosemide (0.5 mg furosemide per 100 g BW) subdivided as follows : 7:00 AM, 12:00 PM, and 5:00 PM on the first day and 7:00 AM on the next day (Fig. 1). At 10:00 PM on the first day, the rats were injected 2 doses of furosemide (1.0 mg per 100 g BW or 3.22 mg furosemide per rat) for the night. Rats were killed 1 hour after the last injection (ie, 8:00 AM).

2.3. Urine collection and analyses

Urines of rats housed individually in metabolic cages were regularly collected over a period of 24 hours to prevent bacteria growth and alteration of the GCs. In each group of furosemide-treated rats, urine was collected during the 48 hours preceding furosemide treatment to verify the stability of urine volume and osmolality for each rat. The values of each parameter obtained 24 hours before furosemide injection were used as their own control. For each rat, urine volume was measured, urine osmolality was determined by freezing point depression using an osmometer (Roebling, Berlin, Germany), and a sample of urine was frozen and maintained at −80°C until GC determination.

2.4. Dissection of the renal zones

After laparatomy, the renal pedicules were clamped and the 2 kidneys were rapidly removed and decapsuled. The blood contained in each kidney was removed with blotting paper (blood-free). Each kidney was sliced longitudinally with a razor blade and cut into 7 zones at 4°C under a stereomicroscope as follows : cortex (C); outer stripe of the outer medulla (OS); inner stripe of the outer medulla (IS); inner medulla (IM), which was subdivided into 2 equivalent portions (IM1 and IM2); and papilla, which was also subdivided into 2 equivalent portions (Pap1 and Pap2), as published earlier [11]. Pieces of C, OS, and IS were collected from each rat to determine the levels of each GC. In contrast, given that faint amounts of IM1, IM2, Pap1, and Pap2 were collected per kidney and per rat, it was essential to pool a given zone from 10 control rats, 15 rats treated with 5 mg furosemide, and 6 rats treated with 10 mg furosemide to determine the levels of each GC.

To avoid possible enzymatic and nonenzymatic synthesis and/or degradation of the studied metabolites, the dissected tissue was frozen immediately on dry ice. Samples were weighed and maintained frozen in liquid nitrogen until GC determination.

2.5. Blood and plasma sampling

Given that the renal pedicles were clamped, arterial blood was collected in the abdominal aorta with a 23G needle (Terumo, Neolus, Guyancourt, France) mounted on a 5-mL syringe (Terumo, Neolus) heparinized (Heparin; Roche Diagnostics, Meylan, France), 1 day before the experiment and dried at room temperature. The blood was transferred into lithium heparinized Vacutainer tubes (VWR, Val-de-Fontenay, France), maintained at 4°C, and centrifuged at 4000*g* for 20 minutes at 4°C to collect the plasma, which was stored frozen in liquid nitrogen until GC determination.

In parts II and III of the experiments, blood was not sampled during the control period. Our decision was based on the important stress provoked by anesthesia to collect blood by cardiac or ocular punction and the consequence of anesthesia on food intake during the experimental period (furosemide treatment).

2.6. Determination of GCs and urea

Renal tissue was homogenized in 1 mL water with a Tissue-Tearor (model 985; Biospec Products, Bartlesville, OK). The homogenization tube was held in chilled ice water. The probe was washed immediately with 1 mL trichloroacetic acid (300 g/L) in a second homogenization tube chilled in ice water. This volume of trichloroacetic acid was added to the homogenate, which resulted in protein precipitation after vortex mixing. After centrifugation (100000*g* for 30 minutes at 4°C), the clear supernatant was used for analysis. The GC concentrations were determined using an amino acid analyzer (Biotronic LC 5001; Biotronik, Maintal, Germany) adapted for GC determination. The GCs were separated over a cation exchange column using sodium citrate buffers and were detected with the fluorescence ninhydrin method [21]. Urea nitrogen was determined with diacetylmonoxime [22].

2.7. Chemicals

Standard GCs and salts were purchased from Sigma Chemical (St Louis, MO); CT and CTN were from Merck (Darmstadt, Germany). α-Keto-δ-GVA was synthesized enzymatically [21]. All other chemicals used were obtained from Merck and were of analytical grade.

2.8. Statistical analyses

Data are given as mean ± SE. To test the influence of furosemide on the biological parameters and the levels of GCs in plasma, urine, and the different renal zones, statistical differences were assessed using the nonparametric Kruskal-Wallis test at the 95% level of significance; and when appropriate, this test was followed by the nonparametric Mann-Whitney *U* test at the 95% level of significance (StatView II SE + Gr, logilabo; Abacus Concepts, Brain Power, Calabasas, CA).

3. Results

3.1. Biological parameters

The administration of 5 mg furosemide to rats (part II) induced a 5-fold increase in urine volume and provoked a 2.4-fold decrease in urine osmolality (Table 1). Consequently, more solutes were excreted in urine during furosemide treatment compared with the control period (+229%). In an additional experiment (part III), rats received 10 mg furosemide to test whether the effect of furosemide on the plasma, urine, and renal levels of GCs would be more pronounced than in rats that received 5 mg furosemide. During the control period, urine volume, urine osmolality, and the amount of osmoles excreted did not differ between the rats treated with 5 or 10 mg furosemide (Table 1). In contrast, as expected, doubling furosemide load induced a 6.6-fold increase in urine volume, a 3.5-fold decrease in urine osmolality, and a 1.9-fold increase in the amount of osmoles excreted compared with the control period (Table 1). As expected, changes in urine volume and osmolality were more pronounced with 10 mg furosemide than with 5 mg. To reduce stress and prevent loss of urine out of the metabolic cages, rats were not weighted during the experiment. Food consumption could not be monitored because rats wasted food and powder of biscuits was found in the metabolic cage and with feces. Although we did not measure the volume of drinking water, we observed that water consumption was enhanced by furosemide treatment.

Table 1
Biological parameters in rats before (control period) and during furosemide treatment (diuresis)

Part of experiment	II		III	
Experimental period	Control	Diuresis	Control	Diuresis
Furosemide (mg)	None	5	None	10
No. of rats	45	45	6	6
Urine volume (mL)	10.2 ± 0.6	52.3 ± 2.1**	10.4 ± 0.7	68.7 ± 4.3*
Urine osmolality (mosm/kg H_2O)	2077 ± 106	858 ± 27**	2045 ± 100	583 ± 18*
Osmoles excreted per day	18.8 ± 0.5	43.1 ± 1.2**	21.0 ± 1.2	39.7 ± 1.6*

These biological parameters were measured in the same rats 24 hours before furosemide administration and during a period of 24 hours after the first injection of furosemide (see Fig. 1). Data are given as means ± SE. Diuresis was induced by injecting intraperitoneally 3 (part II) or 6 doses (part III) of furosemide (0.5 mg furosemide per 100 g BW). Data were statistically analyzed by using the Mann-Whitney *U* test (control period vs diuresis period for the same rat). Significant differences were considered when $P < .05$.

* $P < .004$.

** $P < .0001$ or less in all cases.

Table 2
Guanidino compound levels in plasma of control and furosemide-treated rats

Part of experiment	I	II	III
Experimental condition	Control	Furosemide	Furosemide
Furosemide injected (mg)	None	5	10
No. of rats	36	45	6
α-Keto-δ-GVA	0.306 ± 0.013	0.327 ± 0.015	0.386 ± 0.026
GSA	0.072 ± 0.003	0.086 ± 0.004 [a,c]	0.107 ± 0.008 [b]
CT	157 ± 6	126 ± 4 [a,c]	191 ± 6 [b]
GAA	2.61 ± 0.17	3.23 ± 0.16 [a]	3.63 ± 0.40
α-*N*-AA	0.144 ± 0.010	0.130 ± 0.013	0.059 ± 0.007 [b]
β-GPA	0.042 ± 0.002	0.056 ± 0.003 [a,c]	0.088 ± 0.007 [b]
CTN	38.5 ± 0.6	41.0 ± 1.4 [a,c]	49.1 ± 1.4 [b]
γ-GBA	0.513 ± 0.028	0.784 ± 0.035 [a]	0.827 ± 0.062 [b]
Arg	127 ± 5	123 ± 4 [c]	173 ± 12 [b]
HArg	1.86 ± 0.08	1.42 ± 0.08 [a]	1.71 ± 0.18
G	0.491 ± 0.056	0.198 ± 0.010 [a]	0.211 ± 0.022 [b]
MG	0.416 ± 0.047	0.941 ± 0.052 [a]	1.067 ± 0.093 [b]

Data are expressed as means ± SE (in micromoles per liter). The levels of argininic acid were less than the detection limit. Statistical differences were assessed using the nonparametric test of Kruskal-Wallis at a significance level of 95%; and when appropriate, this test was followed by the Mann-Whitney *U* test at a significance level of 95% with [a]I vs II, [b]I vs III, and [c]II vs III.

3.2. Plasma levels of GCs in control and furosemide-treated rats

A load of 5 or 10 mg furosemide over a period of 24 hours to rats induced changes in the plasma levels of most of the GCs except that of α-keto-δ-GVA (Table 2). The plasma levels of few GCs were diminished by furosemide (Kruskal-Wallis $P < .05$ followed by Mann-Whitney *U* test $P < .05$). In detail, the level of α-*N*-AA was decreased by 2.5-fold in rats injected with 10 mg furosemide. The level of G was reduced by 2.3- to 2.5-fold in both groups of treated rats. The plasma concentration of CT decreased in rats treated with 5 mg furosemide, whereas it increased in rats treated with 10 mg furosemide as compared with that of the control rats. Homoarginine decreased only in the plasma of rats that received 5 mg furosemide. The other GCs exhibited an enhancement in their plasma level (Table 2). An increase in the plasma GAA level occurred in rats treated with 5 mg furosemide and was at the limit of significance in rats receiving the high load of furosemide (Mann-Whitney *U* test $P = .0501$). Creatinine and Arg plasma concentrations were enhanced only in rats receiving 10 mg furosemide (Table 2). Concerning GSA, β-GPA, γ-GBA, and MG, their accumulation in the plasma seemed to be proportional with furosemide loading.

Table 3
Urinary excretion of GCs before (control period) and during furosemide treatment (diuresis)

Part II	Control period	Furosemide treatment
	Day 1	Day 0
No. of rats	45	45
α-Keto-δ-GVA	1.269 ± 0.068	1.729 ± 0.081**
GSA	0.154 ± 0.005	0.220 ± 0.009**
CT	2.03 ± 0.15	2.10 ± 0.15
GAA	9.84 ± 0.80	9.89 ± 0.75
α-*N*-AA	0.717 ± 0.075	0.881 ± 0.097
Argininic acid	0.060 ± 0.002	0.076 ± 0.006
β-GPA	0.038 ± 0.005	0.045 ± 0.002*
CTN	106 ± 3	171 ± 6**
γ-GBA	6.26 ± 0.29	8.37 ± 0.31**
Arg	1.10 ± 0.05	2.76 ± 0.14**
HArg	0.060 ± 0.015	0.065 ± 0.003
G	0.911 ± 0.029	1.141 ± 0.031**
MG	9.48 ± 0.34	12.07 ± 0.39**
Sum GCs-CTN	31.84 ± 1.27	39.35 ± 1,35**

Each rat was maintained in an individual metabolic cage to collect its urine over a control period of 24 hours (before the treatment). The rat was then treated with 5 mg furosemide, and its urine was collected during the next period of 24 hours (during the treatment). Three groups of 15 rats were treated in the same manner. The GCs were analyzed in the urine of each rat. Results are expressed in micromoles per day and presented as means ± SE. Results were statistically analyzed by the nonparametric Mann-Whitney *U* test.

* $P < .001$.

** $P < .0001$.

3.3. Levels of GCs in urine of furosemide-treated rats

Each rat of the 3 groups of 15 rats was maintained in an individual metabolic cage to collect its urine over a control period of 24 hours (before the treatment). Each rat was injected 5 mg furosemide, and its urine was collected during the next period of 24 hours (during the treatment). The results indicate that furosemide significantly enhanced the urinary excretion of GCs compared with the control period (Table 3). In detail, urinary excretion of α-keto-δ-GVA, GSA, β-GPA, CTN, γ-GBA, G, and MG was increased by 20% or more; and that of Arg was enhanced 2.5-fold (Table 3, Mann-Whitney *U* test, $P < .001$ or less). In contrast, no modification in urine excretion of CT, GAA, α-*N*-AA, argininic acid (ArgA), and HArg was detected. The GCs were not analyzed in urine of rats treated with 10 mg furosemide because there was a single group of 6 rats.

3.4. Levels of GCs in the different renal zones from control and furosemide-treated rats

The effects of 5 or 10 mg furosemide on the level and the distribution of each GC in the rat kidney are presented in Figs. 2 and 3. Statistical analyses were performed to know whether the renal level of each GC was modified in rats treated with 5 mg furosemide compared with the control rats. Rats treated with 10 mg furosemide were excluded from these statistical analyses because there was only a single group composed of 6 rats.

A general view of the figures shows the heterogeneous and specific distribution of the GCs within the control rat kidney. Furosemide treatment neither altered this specific distribution nor affected the levels of few GCs but enhanced or reduced the levels of several other GCs. In detail, 5 mg furosemide significantly decreased the levels of Arg in IM1 and IM2 (Fig. 2). In contrast, 10 mg furosemide had a tendency to increase the level of Arg in OS as compared with the controls. In each renal zone, the level of GAA was not modified by 5 mg furosemide but tended to increase in C and OS of rats treated with 10 mg of the diuretic drug (Fig. 2). The levels of CT were significantly diminished in OS, IS, and IM2 in rats treated with 5 mg furosemide, whereas doubling the dose of furosemide had the opposite effect in most of the renal zones (Fig. 2). Five milligrams of furosemide significantly increased CTN content in OS (Fig. 2). In contrast, 10 mg of the diuretic drug tended to enhance CTN in C and OS, whereas it seemed to decrease in the inner medullary and papillary zones (Fig. 2). The renal content of GSA was almost stable in rats receiving 5 mg furosemide except in C where it decreased compared with the controls (Fig. 2). Ten milligrams of furosemide tended to reduce by 2-fold the renal content of GSA compared with the controls. In general, the renal content of α-keto-δ-GVA was affected neither by 5- nor 10-mg furosemide treatment except in IS where it was reduced by 5 mg furosemide (Fig. 2). The renal content of β-GPA and HArg was not modified in rats receiving 5 mg furosemide, whereas it tended to be enhanced in C and OS of rats treated with 10 mg furosemide (Fig. 3). The level of γ-GBA was significantly enhanced in C and OS of rats treated with 5 mg furosemide (Fig. 3). A similar result was observed when rats received 10 mg furosemide. In addition, the high dose of furosemide tended to enhance the mean level of γ-GBA in IS and diminish it in Pap2 (Fig. 3). The renal content of G was modified only in Pap1 of rats treated with 5 mg furosemide and seemed to increase in OS, IS, and the other medullary zones of rats treated with 10 mg furosemide (Fig. 3). The most interesting and unexpected result that might have important physiological consequences and clinical relevance was the dramatic increase in MG level in all renal zones provoked by furosemide treatment (Fig. 3). Five milligrams of furosemide induced a 16-fold increase in MG in OS and 10- to 12-fold increase in the other renal zones compared with that of the controls. Moreover, the accumulation of MG in the different renal zones seemed to be proportional to the dose of furosemide administrated because, in each zone, the levels of MG were ≈1.6-fold higher in rats receiving 10 mg than in those receiving 5 mg furosemide. Finally, urea was quantitated in the different renal zones to verify the diuretic effect of furosemide. Indeed, it is known that diuresis affects the urinary concentrating mechanism and urea distribution along the corticopapillary axis [23-25]. The results show that, within 24 hours, 5 mg furosemide had a tendency to reduce the level of urea in the medulla and papilla but did not reach statistical significance, whereas 10 mg furosemide

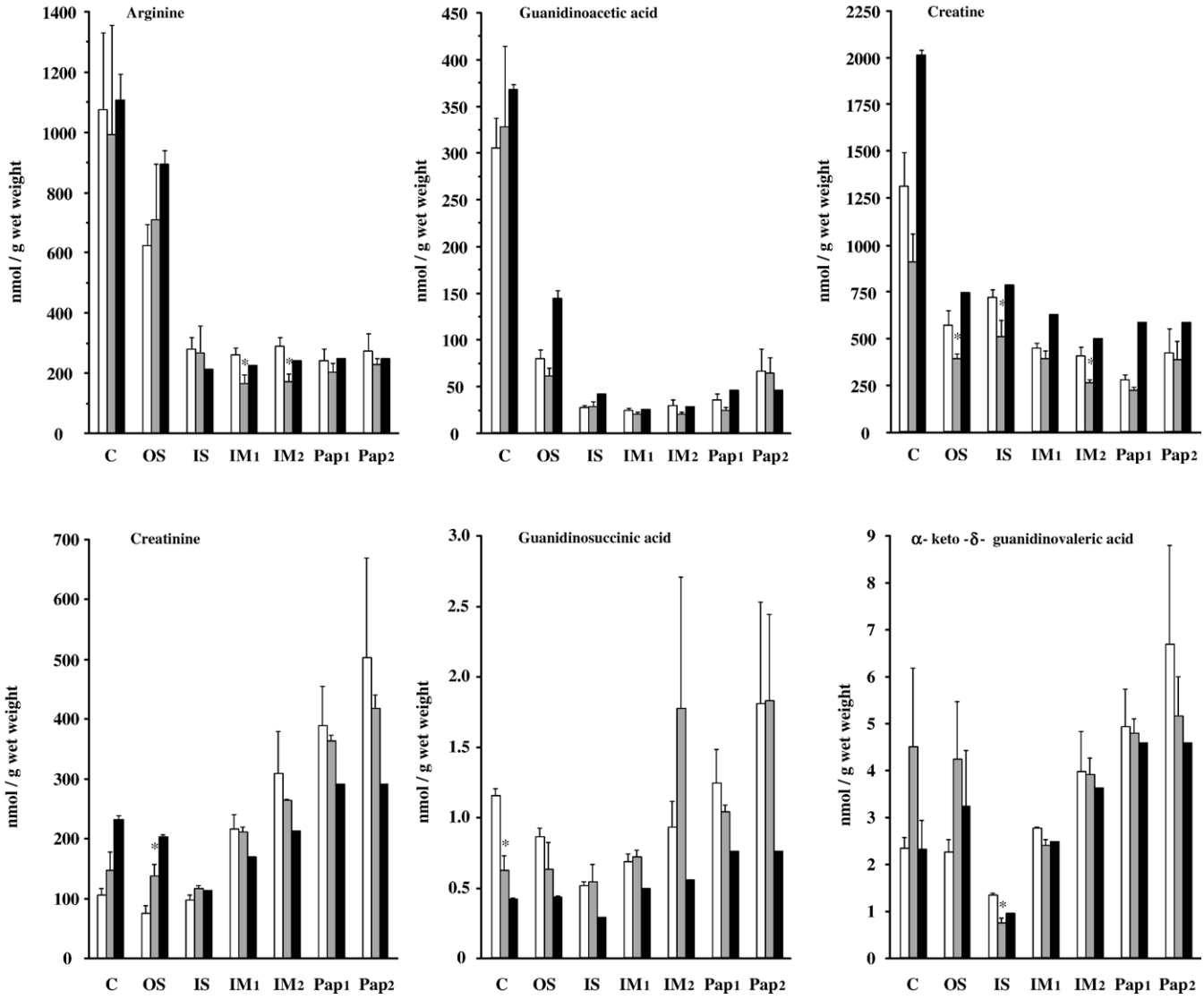

Fig. 2. Distribution of Arg, GAA, CT, CTN, GSA, and α-keto-δ-GVA within the kidney of control and furosemide-treated male rats. The values (in nanomoles per gram wet weight) are given as means ± SE; n = 3 experiments for control rats (part I: open bars) and rats receiving 5 mg furosemide (part II: dotted bars). In each experiment, 10 and 15 rats were used, respectively. The results of the rats treated with 10 mg furosemide (part III, closed bars, n = 6 rats) are expressed as means ± SE for C and OS and as means for IS, IM1, IM2, Pap1, and Pap2. To test the influence of furosemide on the renal level of GCs (part I vs part II), the data were analyzed using the nonparametric Mann-Whitney *U* test; and a $P < .05$ (*) was accepted as significant. IM1 indicates outer stripe of the IM; IM2, inner stripe of the IM; Pap1, outer stripe of the papilla; Pap2, papillary tip.

clearly decreased the level of urea in the different medullary zones (Fig. 3).

4. Discussion

The present study examines whether diuresis, the opposite physiological state of antidiuresis, induced by the diuretic drug furosemide might disturb GC metabolism, storage, and elimination in rats. In our working hypothesis, changes in GSA, GAA, CT, CTN, and β-GPA metabolism and decreases in their renal concentrations were expected. However, furosemide treatment provoked a sharp accumulation of MG in the kidneys and in a lesser extent in the plasma, but only weak changes in the plasma levels of other GCs.

Given that furosemide treatment led to a storage of MG in the kidneys and the plasma, it can be assumed that MG synthesis was dramatically enhanced and/or that the urinary excretion of MG was not adjusted. The absolute urinary excretion of MG was enhanced by 28% by furosemide as compared with the control period, whereas CTN urinary excretion was enhanced by 61% in the presence of furosemide. This calculation suggests that the relative urinary excretion of MG was diminished during furosemide diuresis. To support this statement, we calculated the fractional excretion of MG defined as the ratio (MG clearance to CTN clearance) during the control and experimental periods.

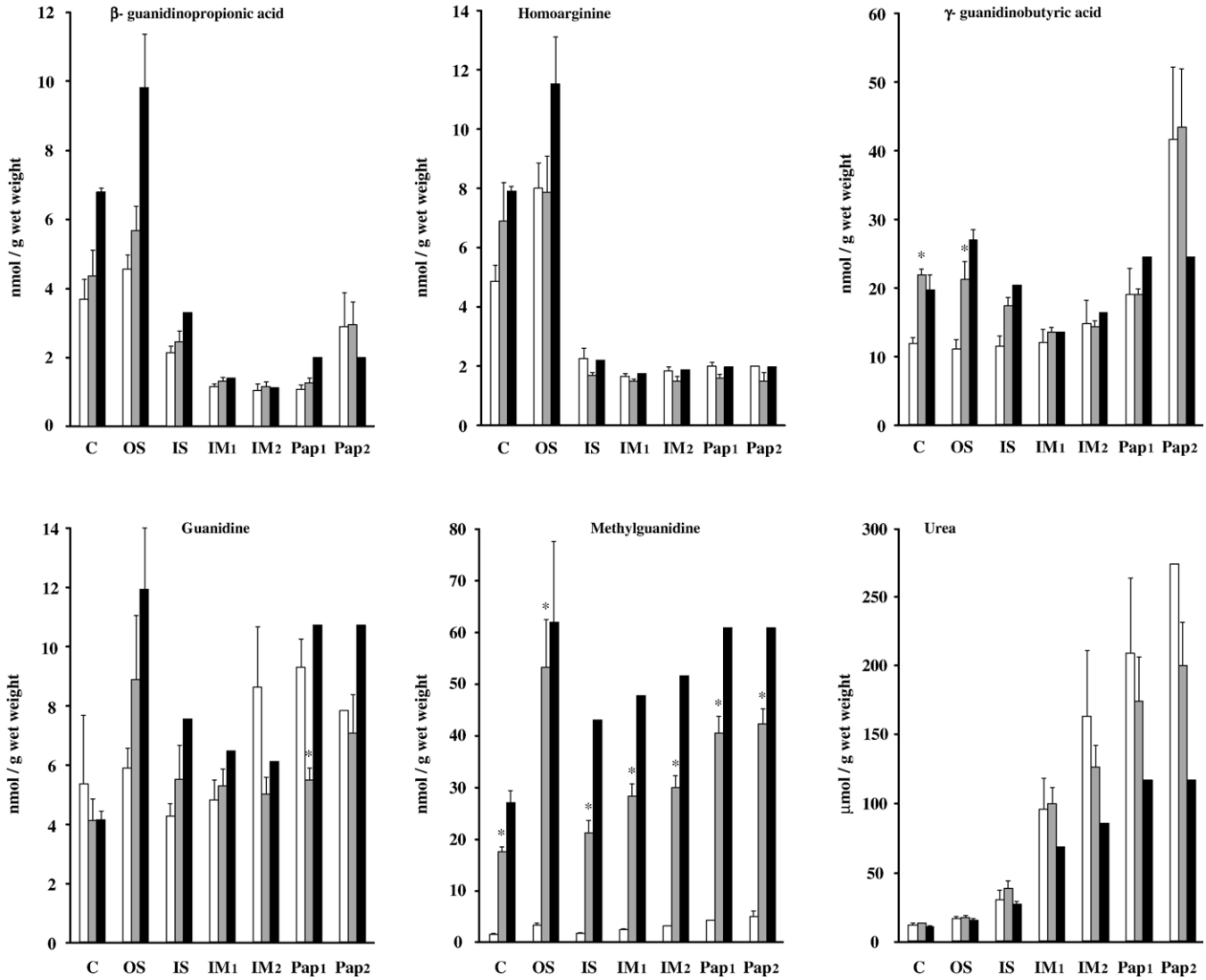

Fig. 3. Distribution of *β*-GPA, HArg, γ-GBA, G, MG, and urea within the kidney of control and furosemide-treated male rats. The values (in nanomoles per gram wet weight) are given as means ± SE; n = 3 experiments for control rats (part I: open bars) and rats receiving 5 mg furosemide (part II: dotted bars). In each experiment, 10 and 15 rats were used, respectively. The results of the rats treated with 10 mg furosemide (part III, closed bars, n = 6 rats) are expressed as means ± SE for C and OS and as means for IS, IM1, IM2, Pap1, and Pap2. To test the influence of furosemide on the renal level of GCs (part I vs part II), the data were analyzed using the nonparametric Mann-Whitney *U* test; and a $P < .05$ (*) was accepted as significant.

The data of the control rats presented in Table 2 were used because we did not collect blood during the control period of part II. On this basis, CTN clearance and MG clearance were 1.91 and 15.82 mL per minute, respectively, in the control period and 2.90 and 8.9 mL per minute during furosemide treatment. Given that the fractional excretion of MG was 8.28 and 3.08 in the control and experimental periods, respectively, we propose that MG washout from tissue cells into urine by furosemide through the kidney may cause an increase in MG in the kidney. Changes in MG levels seem to be furosemide specific because in rats subjected to 72% nephrectomy, urine volume and urinary excretion of MG increased without any modifications of MG concentration in the plasma, liver, skeletal muscle, and kidneys [26]. Methylguanidine accumulation during furosemide treatment led us to hypothesize that MG level might be also enhanced in other organs; however, because such a result was unforeseeable, none of the other tissues were collected in furosemide-treated rats to test this point.

The alternative or concomitant cause that contributes to explain the accumulation of MG in rat tissues might be an enhancement of MG synthesis. The metabolic pathway required for MG biosynthesis has been ignored for a long time; but CTN, a molecule believed to be metabolically inert, has been identified as the precursor of MG. The use of ^{14}C CTN allowed the detection of labeled MG [27]. Control rats intraperitoneally injected with CTN exhibited high levels of MG in the liver, kidneys, lung, and muscles [28]. Isolated rat hepatocytes were first identified to metabolize CTN into MG in a time- and concentration-

dependent manner [28]. Oxidation of CTN with active oxygen (eg, hydrogen peroxide) was proven to generate MG, whereas scavengers of active oxygen (eg, glutathione, sorbitol) considerably reduced MG synthesis [29]. The use of [^{13}C-methyl] CTN demonstrated that CTN was oxidized to produce creatol (2-amino-5-hydroxy-1-methyl-4[5H]-imidazolone), the precursor of MG [30]. Methylguanidine synthesis requires integrity of hepatocytes, occurs in the microsomal fraction, and involves the presence of NADPH (reduced nicotinamide-adenine dinucleotide phosphate) or NADH (reduced nicotinamide-adenine dinucleotide), CTN, 2 oxidases (the FAD [flavin adenine dinucleotide]-containing monooxygenase and the P-450–dependent oxidase system), active oxygen such as superoxide radical, hydrogen peroxide, and hydroxyl radical [31]. More interestingly, isolated rat hepatocytes produce creatol proportionally with the increase of CTN concentration. Creatol synthesis decreased in the presence of the hydroxyl radical scavenger dimethyl sulfoxide and stimulated by puromycin aminonucleoside known to enhance the generation of hydroxyl radicals in cells [32]. Finally, although most of the experiments were performed in the liver, a 37-kDa enzyme containing a flavoprotein that oxidizes creatol into MG has been isolated from the rat kidney. The analysis of homology revealed that the complementary DNA cloned from this enzyme perfectly matched with the rat kidney long-chain L-2-hydroxy acid oxidase (EC 1.1.3.15) [33]. These studies reveal that the liver and the kidneys oxidize CTN into creatol that is converted into MG. Although our data do not allow the identification of the molecular mechanisms by which furosemide enhanced MG production, nonspecific effects might be attributed to furosemide as recently reviewed [34].

Nevertheless, it must be underlined that several reports pointed that an increase in MG concentration modifies several physiological functions [35-37]. We estimated that MG concentration might reach 50 to 75 μmol/L in renal cells in rats treated with 5 or 10 mg furosemide. It should be interesting to test whether this abrupt accumulation of MG in the kidney is capable of disturbing physiological and biochemical events. Another point raised by our results is whether or not furosemide increased MG accumulation in patients treated with furosemide.

In conclusion, we found that furosemide treatment provoked an abrupt accumulation of MG in plasma, urine, and kidneys of male rats, which might be explained by an overproduction of MG and a decrease of the fractional excretion of MG.

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