Sustained activation of renal N-methyl-d-aspartate receptors decreases vitamin D synthesis: a possible role for glutamate on the onset of secondary HPT

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the secretion of PTH (34). Thus, the presence of NMDAR in the renal tubular cells, which are responsible mainly for 1,25(OH)2D3 production, suggests that NMDAR activation can play a role in renal control of Ca homeostasis.

Thus, the aim of the present work was to determine the effect of the stimulation of renal NMDAR in Ca regulatory pathways in the kidney and its effects on vitamin D and PTH levels.

**MATERIALS AND METHODS**

**Animals and samples.** Experimental methods used on laboratory animals comply with Law 5 of June 21, 1995, by “Generalitat de Catalunya” of protection of animals used for experimentation and other scientific finalities and the Royal Decree 1201 of October 10, 2005, on the protection of animals used for experimentation and other scientific finalities. Moreover, the present work was approved by the Ethic Animal Experimentation Committee of the University of Lleida.

The study was performed in Sprague-Dawley rats (200–250 g, 50% male and 50% female) housed in groups and maintained under standard conditions. Food and water were available ad libitum. Room temperature was at 21°C with a 12:12-h dark-light cycle.

To assess the NMDAR function, we used NMDA, which activates specifically NMDAR and not the other glutamate receptors. For the in vivo experiments, blood and 24-h urine samples were obtained before and 4 days after daily NMDA intraperitoneal treatment (10 mg·kg−1·day−1). Some of the animals were also treated with calcitriol (10 ng/kg every other day) to determine the effect of replenishing active vitamin D levels on NMDA-treated animals.

At the end of the experiment, animals were euthanized with pentobarbital sodium (50 mg/kg), and kidney tissue was obtained after perfusion with PBS through abdominal aorta. Furthermore, tissue

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**Table 1. Primer sequences (5’-3’) for PCR for the different NMDAR subunits and GAPDH**

<table>
<thead>
<tr>
<th>Gene (Human)</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>NMDA R1A</td>
<td>Forward AGACGTGGGTTCGGTATCAG Reverse AGGCCCATCAGTGCTTG</td>
</tr>
<tr>
<td></td>
<td>NMDA R2A</td>
</tr>
<tr>
<td></td>
<td>NMDA R2B</td>
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<td>NMDA R2C</td>
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<td></td>
<td>NMDA R2D</td>
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<td></td>
<td>GAPDH</td>
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NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor.

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**Fig. 1.** A: PCR for N-methyl-D-aspartate (NMDA) receptor (NMDAR) subunits in human kidney 2 (HK-2) cells. Results show the presence of R1 and all R2 subunits. We used brain (Br) as a positive control. B: representative Western blot analysis showing NMDA R1 protein in HK-2 cells. C: fluo-4 determination of NMDAR activation. The entry of calcium caused by the activation of NMDAR can be visualized by the fluorochrome activation. Intracellular calcium levels are higher (measured as color intensity) after 1 min of NMDA treatment (500 μM).
from untreated animals was also obtained to use as normal controls for 1α-hydroxylase expression levels and renal glutamate content.

Animals with reduced renal mass were obtained by subtotal nephrectomy, following the method described by Perez-Ruiz et al. (36). Briefly, animals were anesthetized with isoflurane and underwent 2/3 nephrectomy in the left kidney by ligation of both poles. One week later, animals underwent contralateral uninephrectomy. After 8 wk, animals were euthanized and renal tissue was collected.

**Cell cultures.** For the in vitro experiments, we used the human kidney 2 (HK-2) cells (ATCC, no. CRL-2190), which are a cell line from normal human kidney. Cells were maintained in DMEM-Ham’s F-12 medium (1:1) containing 5% FBS, 100 U/ml penicillin-streptomycin, 20 mM HEPES, 1.12 g of D-glucose anhydride, 5 μg/ml transferrine, 5 μg/ml insulin, 5.10^-5 M dexametasone, 10^-9 M triiodothyronine, 10 nm EGF, and 60 nM selenium (Sigma). Cells were incubated at 37°C in a humidified 5% CO2 atmosphere.

Cells were treated with NMDA (500 μM) and glycine (500 μM) for 4 days, with the medium replaced every day. In some of the wells, U-O126 was also added (10 μM).

**Fluo-4 method.** Cells were cultivated in coverslips and after 2 days were washed with Krebs solution (145 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 11 mM glucose at pH 7.5) and incubated with 5 μM of fluo 4-AM (Molecular Probes, PoortGebouw, The Netherlands) during 30 min at 37°C. The slides were assembled in a adaptor specific for the confocal microscope Nikon TE-200, and treatment with NMDA (500 μM) was added. Analysis was performed at 485 nm of stimulation and 528 nm of emission in the Nikon TE-200 (Nikon, Tokyo, Japan) microscope. We took pictures every minute with a ×100 objective lens. Controls were 10 μM ionomicine for the maximum level of fluorescence and 20 μM EGTA for the minimum.

**PCR and RT-PCR.** Total RNA was extracted from tissue using the Trizol method. Reverse transcription was performed with First Strand cDNA Synthesis Kit for RT-PCR (AMV, Roche) followed by a Tagman real-time PCR amplification also with gene-specific primers (Applied Biosystems, Branchburg, NJ), using human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as a reference. Forty cycles at 95°C for 15 s and 60°C for 1 min were performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Triplicate readings were taken, and the average was calculated. The relative RNA amount was calculated by standard formulae. The results referred to a randomly selected basal sample that we consider as value = 1.

In the case of NMDAR subunits in HK-2 cells, PCR amplification with gene-specific primers (Table 1) and human GAPDH as a reference was performed. Forty cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s were performed with an GeneAmp PCR System 2700 (Applied Biosystems).

**Western blot.** Protein extracts from renal and cell tissue were obtained as described previously (11). Briefly, 20 μg of protein was separated in an 8% polyacrylamide-SDS gel. After running and transfer to PVDF membranes (Immobilon-P; Millipore, Bedford, MA), blots were incubated in 5% nonfat milk in Tris-buffered saline-Tween 20 (TBST) for 1 h. Then, primary antibody for NMDAR1 (1:1,000; Affinity Bioreagents, Golden, CO), 1α-hydroxilase (1:1,000; The Binding Site, Birmingham, UK), pERK (1:1,000; Cell Signaling Technology, Boston, MA), total ERK (1:1,000; Cell Signaling Technology), or tubuline (1:30,000, Sigma) was added, and incubation was performed overnight at 4°C. After washing with TBST, HRP-conjugated secondary antibody (1:12,500; Amersham Biosciences) was added for an extra hour. Binding was detected with the ECL Advance Western Blotting Detection Kit (Amersham Biosciences) and the

**Fig. 2.** A: 1,25(OH)2D3 production by HK-2 cells after 4 days of NMDA treatment (500 μM/day). B: 1α-hydroxylase mRNA levels in HK-2 cells after 4 days of treatment with NMDA (500 μM/day; gray bars) with respect to the control ones (black bars). The inhibitory effect of NMDA treatment was abolished with the coinoculation with the MAPK/ERK inhibitor U-0126 (white bar). Values are means ± SE. **P < 0.05 vs. control. C:** representative Western blot for 1α-hydroxylase in HK-2 cells, control (Ct) or incubated with NMDA (500 μM). We used α-tubuline as load control. D: representative Western blot showing the activation of ERK1/2 on HK-2 cells after 60 and 72 h of NMDA (500 μM) incubation compared with the untreated cells or cells coincubated with U-0126 (10 μM). We used total ERK antibody as load control.
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RESULTS

To use HK-2 cells for the study of NMDAR activity in the kidney, first we wanted to assess whether the receptor is expressed in this cell line and its functionality. In Fig. 1A, we show PCR results with specific primers for all NMDAR sub-units, R1, R2A, R2B, R2C, and R2D, in the HK-2 cells compared with levels present in brain (taken as reference). Moreover, we detected the presence of the NMDAR1 protein by Western blot techniques (fig. 1B). To determine whether the receptor present in the HK-2 cells is functional, we performed fluo-4 analysis (Fig. 1C). When kidney cells were treated with NMDA (500 μM), intracellular calcium levels increased. This increment measured as fluorescence intensity indicates that the NMDAR channels present in the HK-2 cells are fully functional.

The activation of the NMDAR in this model induced modifications on the vitamin D synthesis pathway. Analyzing the synthesis of 1,25(OH)2D3 in the in vitro model, we observed that NMDA treatment decreased 1,25(OH)2D3 production (Fig. 2A). We analyzed 1α-hydroxylase levels on HK-2 (Fig. 2, B and C), and we detected that 1α-hydroxylase expression was decreased after 4 days of daily NMDA treatment (500 μM/ day), in parallel to an increase in ERK 1/2 phosphorylation (Fig. 2D). The decrease on 1α-hydroxylase was abolished when we added the ERK1/2 inhibitor U0126 (10 μM) to the medium (Fig. 2B), indicating a role of the MAPK/ERK pathway mediating the decrease in 1α-hydroxylase induced by NMDA.

We further investigated the effect of NMDAR activation on renal function, using an in vivo model where normal rats were treated during 4 days with NMDA (10 mg·kg−1·day−1). In these animals, treatment with NMDA induced a decrease of 1,25(OH)2D3 levels compared with the same animals before the treatment (Fig. 3A). In those rats, levels of renal 1α-hydroxylase were lower than in control animals both at mRNA

Fig. 3. A: 1,25(OH)2D3 levels in normal animals before (black bars) and after NMDA treatment (10 mg·kg−1·day−1 for 4 days; gray bars). Values are means ± SE. *P < 0.05 vs. basal. B: PTH levels in normal rats before (black bars) and after NMDA treatment (10 mg·kg−1·day−1 for 4 days; gray bars) or NMDA plus calcitriol (NMDA + CTR; 10 ng/kg every other day). Values are means ± SE. *P < 0.05 vs basal. C: 1α-hydroxylase mRNA levels in kidney tissue from normal animals (control) and from animals treated for 4 days with NMDA (10 mg·kg−1·day−1). Values are means ± SE. *P < 0.05 vs. control. D: representative Western blot of kidney tissue showing a decrease in 1α-hydroxylase protein levels in 3 different animals treated with NMDA (10 mg·kg−1·day−1 for 4 days). We used α-tubulin as load control.

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and at protein level (Fig. 3, C and D, respectively). NMDA-treated animals also showed higher levels of PTH with respect to control (Fig. 3B). When we replenished 1,25(OH)2D3 levels in those animals by administration of calcitriol (10 ng/kg every other day), PTH levels did not increase (Fig. 3B).

NMDA treatment induced several changes on renal function. Table 2 shows that NMDA treatment decreased the urinary excretion of calcium, whereas urinary excretion of phosphorus and sodium increased. Urine volume also was higher on NMDA-treated animals, and creatinine clearance decreased. Bone resorption (measured as amount of collagen type I fragments) and bone formation (estimated by osteocalcin levels) both increased. Furthermore, blood Ca levels were lower in NMDA-treated animals.

We wanted to analyze whether glutamate could have a role in the decrease in 1α-hydroxylase levels in animals with reduced renal mass. The levels of 1α-hydroxylase in kidney tissue of animals with CKD are lower than in the control ones (Fig. 4A). Moreover, levels of glutamate in the kidneys of these animals were higher than in control kidneys (Fig. 4B).

**DISCUSSION**

NMDAR is a potent calcium channel gated by the binding of glutamate and glycine (28). The role of NMDAR has been widely investigated in the central nervous system, but recent studies have shown the presence of NMDAR in kidney (13), where it plays a role in the maintenance of basal arterial tone; in bone, where it stimulates bone resorption (9, 35); and in the parathyroid gland (34), where it takes part in the regulation of PTH release. Thus, the presence and role of this receptor outside the nervous system is a new field of study.

We have determined the presence of different subunits of the receptor (NR1, NR2A, NR2B, NR2C, and NR2D) needed for its functionality in a cell line of human kidney cells. Furthermore, we have shown that the receptor is functional because its activation in vitro provokes an increase in free intracellular Ca. Intracellular calcium is an important secondary messenger and is essential for the normal physiological activity of all living cells (10).

In a previous study (34), we determined that the acute activation of the NMDAR in the PTG inhibits PTH release. In this study, we wanted to analyze the influence of the sustained activation of the NMDAR on 1,25(OH)2D3 synthesis and in the regulation of PTH secretion. Thus, NMDA treatment for 4 days causes downregulation of 1α-hydroxylase expression both in vivo and in vitro. This downregulation results in a drop in 1,25(OH)2D3 synthesis and in the blood levels of 1,25(OH)2D3. It is well known that low levels of 1,25(OH)2D3 strongly affect the PTG, stimulating the synthesis and release of PTH. Thus, the lack of inhibitory effect of vitamin D on the PTG in sustained treatments, together with the hypocalcemia induced by the same treatment, prevails over the direct effect of NMDAR activation on the parathyroid gland, provoking a net increase of PTH levels. Indeed, when we treated animals with both NMDA and 1,25(OH)2D3, PTH levels did not rise, confirming that a decrease in circulating 1,25(OH)2D3 levels is, at least in part, responsible for the increase in PTH induced by NMDA treatment.

Table 2. Effects of NMDA treatment (10 mg·kg−1·day−1) for 4 days in rats

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>NMDA (4 days)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine volume, ml/day</td>
<td>8 ± 1.5</td>
<td>30 ± 3.7</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Serum calcium, mg/dl</td>
<td>11.42 ± 0.07</td>
<td>9.87 ± 0.13</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Serum P, mg/dl</td>
<td>9.48 ± 0.39</td>
<td>9.41 ± 0.35</td>
<td>NS</td>
</tr>
<tr>
<td>Serum Na, mEq/l</td>
<td>138 ± 0.5</td>
<td>140 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>1.48 ± 0.12</td>
<td>1.11 ± 0.08</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Urinary excretion of Ca, mg/day</td>
<td>4.8 ± 1.57</td>
<td>1.2 ± 0.17</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Urinary excretion of P, mg/day</td>
<td>2.04 ± 0.91</td>
<td>19.6 ± 1.43</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Urinary excretion of Na, mEq/day</td>
<td>0.45 ± 0.11</td>
<td>1.2 ± 0.14</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Osmolarity, mOsm/kg</td>
<td>396 ± 40</td>
<td>8022 ± 156</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Collagen type I fragments, ng/day</td>
<td>3127 ± 786</td>
<td>178.2 ± 135</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Osteocalcin, ng/ml</td>
<td>98.4 ± 106</td>
<td>178.2 ± 135</td>
<td>P &lt; 0.05</td>
</tr>
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</table>

Values are means ± SE; n = 10. P, phosphorous; Na, sodium; NS, not significant.

**Fig. 4.** A: 1α-Hydroxylase protein levels in kidney tissue from healthy animals (black bars; control, n = 10) and from animals with 5/6 nephrectomy (white bars; n = 10). Values are means ± SE; *P < 0.05 vs. control. B: glutamate levels in kidney tissue from healthy rats (black bars) and from animals with 5/6 nephrectomy (white bars). Values are expressed as nmol of glutamate for μg of tissue protein. Values are means ± SE. *P < 0.05 vs. basal.
1,25(OH)2D3 is synthesized through 25(OH)D3 by the action of renal 1α-hydroxylase (14), the levels of which are tightly regulated. 1α-Hydroxylase promoter has response elements for different transcription factors, such as AP-1 and AP-2, Sp1, and NF-κB (20, 39), and for the MAPK activation pathway (37, 43). Periwad et al. (37) showed that ERK1/2 pathway activation caused a drop in the renal expression of 1α-hydroxylase in renal cells. The activation of the MAPK pathway by the NMDAR is a well-described process in the nervous system (16, 17, 30). Indeed, our results showed that NMDA treatment decreases 1α-hydroxylase levels by activating the MAPK/ERK pathway with U-O126 blunted both the increase in the activation of the MAPK/ERK pathway and the decrease in 1α-hydroxylase levels. This suggests that NMDAR activation decreases 1α-hydroxylase levels by activating the MAPK/ERK pathway.

One of the most important actions of vitamin D is the regulation of bone turnover (14); thus, when 1,25(OH)2D3 pathway.

decreases 1α-hydroxylase levels by activating the MAPK/ERK/H9251 hydroxylase levels. This suggests that NMDAR activation causes the activation of the MAPK/ERK pathway together with a decrease in 1α-hydroxylase levels. The inhibition of the pathway with U-O126 blunted both the increase in the activation of the MAPK/ERK pathway and the decrease in 1α-hydroxylase levels. The inhibition of the activation could activate the renal NMDAR and contribute to the suppression of the renal 1α-hydroxylase activity. In animals, acute metabolic acidosis was found to decrease 1,25(OH)2D3 synthesis through a decrease in 1α-hydroxylase activity (23, 24). However, chronic metabolic acidosis was repeatedly shown to increase 1,25(OH)2D3 and to concomitantly decrease PTH concentrations in humans (22). Indeed, chronic metabolic acidosis leads to a decrease in renal glomerulonephritis levels (15), which agrees again with a role of glutamate regulating 1α-hydroxylase levels.

In our study, we measured renal glomerulonephritis levels in kidneys from animals with CKD. We found that those levels were high, together with a decreased expression of 1α-hydroxylase levels. Although this fact does not imply causality, and both findings could be parallel and unrelated, our in vitro studies suggest that a link could be present. Further and more extensive studies, beyond the scope of the present work, are needed to prove an association between the increase in glomerulonephritis levels in kidneys of animals with CKD and the decrease in 1α-hydroxylase expression levels.

In conclusion, sustained NMDAR activation in the kidney causes an increase in PTH synthesis and release due to a decrease in 1α-hydroxylase synthesis and activity through the MAPK activation and the following decrease on 1,25(OH)2D3 synthesis. In CKD, an increase in renal glomerulonephritis levels could be involved in the decrease in renal 1α-hydroxylase activity and the related decrease in 1,25(OH)2D3 levels.

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GRANTS

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DISCLOSURES

B. Coll holds a contract from the Miguel Servet program.

REFERENCES

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