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TRANSLATIONAL PHYSIOLOGY

Vitamin D deficiency is a potential risk factor for contrast-induced nephropathy

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Luchi WM, Shimizu MH, Canale D, Gois PH, de Bragança AC, Volpini RA, Girardi AC, Seguro AC. Vitamin D deficiency is a potential risk factor for contrast-induced nephropathy. *Am J Physiol Regul Integr Comp Physiol* 309: R215–R222, 2015. First published June 3, 2015; doi:10.1152/ajpregu.00526.2014.—Vitamin D deficiency (VDD) is widespread in the general population. Iodinated (IC) or gadolinium-based contrast media (Gd) may decrease renal function in high-risk patients. This study tested the hypothesis that VDD is a predisposing factor for IC- or Gd-induced nephrotoxicity. To this end, male Wistar rats were fed standard (SD) or vitamin D-free diet for 30 days. IC (diatrizoate), Gd (gadoterate meglumine), or 0.9% saline was then administered intravenously and six groups were obtained as the following: SD plus 0.9% saline (Sham-SD), SD plus IC (SD+IC), SD plus Gd (SD+Gd), vitamin D-free diet for 30 days plus 0.9% saline (Sham-VDD_{30}), vitamin D-free diet for 30 days plus IC (VDD_{30}+IC), and vitamin D-free diet for 30 days plus Gd (VDD_{30}+Gd). Renal hemodynamics, redox status, histological, and immunoblot analysis were evaluated 48 h after contrast media (CM) or vehicle infusion. VDD rats showed lower levels of total serum 25-hydroxyvitamin D [25(OH)D], similar plasma calcium and phosphorus concentration, and higher renal renin and angiotensinogen protein expression compared with rats fed SD. IC or Gd infusion did not affect inulin clearance-based estimated glomerular filtration rate (GFR) in rats fed SD but significantly decreased GFR in rats fed vitamin D-free diet. Both CM increased renal angiotensinogen, and the interaction between VDD and CM triggered lower renal endothelial nitric oxide synthase abundance and higher renal thiobarbituric acid reactive substances-to-glutathione ratio (an index of oxidative stress) on VDD_{30}+IC and VDD_{30}+Gd groups. Conversely, worsening of renal function was not accompanied by abnormalities on kidney structure. Additionally, rats on a VDD for 60 days displayed a greater fall in GFR after CM administration. Collectively, our findings suggest that VDD is a potential risk factor for IC- or Gd-induced nephrotoxicity most likely due to imbalance in intrarenal vasoactive substances and oxidative stress.

vitamin D deficiency; contrast-induced nephropathy; oxidative stress; renin-angiotensin system; gadolinium

CONTRAST-INDUCED NEPHROPATHY (CIN) has been considered the third leading cause of hospital-acquired acute kidney injury (AKI) related entirely to iodinated contrast media (IC). However, originally thought to be safe, gadolinium-based contrast medium (Gd) has recently been reported to induce a decrease in the glomerular filtration rate (GFR) in high-risk populations (9, 51). It is well known that CIN depends on the presence of risk factors. Frequently, those factors are associated with renal endothelial dysfunction and altered medullary microcirculation, resulting in medullary hypoxia and oxidative stress damage. Preexisting renal dysfunction, diabetes, congestive heart failure, volume depletion, hypercholesterolemia, and old age are the demonstrated major risk factors (18).

Apart from bone and mineral metabolism, a substantial amount of evidence demonstrates that vitamin D deficiency (VDD) is associated with endothelial dysfunction, oxidative stress, and upregulation of the renin-angiotensin system (RAS) (4, 42, 52). Studies have shown a widespread rate of hypovitaminosis D in the general population, even in tropical countries (44). In the United States, data from the third National Health and Nutrition Examination Survey (NHANES III) revealed that only 20–25% of the assayed population has serum 25-hydroxyvitamin D [25(OH)D] levels of at least 30 ng/ml, whereas 25–35% has definite VDD, i.e., 25(OH)D $<$ 20 ng/ml (1). In addition, VDD is highly prevalent and sometimes correlated with diseases that predispose CIN (47).

From the above, we hypothesized that VDD is a relevant condition that predisposes to renal impairment by contrast media (CM). To this end, renal function was assessed 48 h after IC or Gd administration in VDD rats and healthy control rats. Additionally, renal and systemic oxidative stress parameters, renal angiotensinogen, and endothelial nitric oxide synthase (eNOS) expression were evaluated to elucidate the possible pathogenesis of CIN in VDD rats.

MATERIALS AND METHODS

Animal protocols. All experimental procedures were approved by the local research ethics committee (CAPPesq, process no. 228/12) and were developed in strict conformity with local institutional guidelines and with well-established international standards for manipulation and care of laboratory animals. Experiments were designed to evaluate the potential nephrotoxic effect of IC and Gd in VDD rats. Male Wistar rats (3 wk old), provided by the University of São Paulo School of Medicine, were maintained on standard diet (SD) or vitamin D-free diet for 30 days (MP Biomedicals, Irvine, CA). Animals had ad libitum access to food and water. IC (76% diatrizoate, a high-osmolality agent, 6 ml/kg body wt) or Gd (gadoterate meglumine, osmolality 1,350 mosmol/kg H_2O, 1.5 mmol/kg body wt) were then given through the dorsal penile vein under isofluorane anesthesia. The use of these high doses is appropriate for studies in rats for three major

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reasons. First, the Federal Drug Administration recommends the use of a sixfold increase to obtain a human equivalent drug dose for the rat. Second, these contrasts are excreted primarily by glomerular filtration, and the rat has a higher GFR per unit body weight than humans (8 vs. 1.5 ml·min^{-1}·kg^{-1}) (11, 16). Besides, these CM doses have already been used on previous studies (3, 33). Control and VDD animals received the same volume of saline solution. We studied six groups (n = 12/group): SD plus 0.9% saline (Sham-SD), SD plus IC (SD+IC), SD plus Gd (SD+Gd), vitamin D-free diet for 30 days plus 0.9% saline (Sham-VDD$_{30}$), vitamin D-free diet for 30 days plus IC (VDD$_{30}$+IC), and vitamin D-free diet for 30 days plus Gd (VDD$_{30}$+Gd). After 24 h of CM infusion, animals were housed in metabolic cages to collect 24-h urine volume for measuring urinary excretion of sodium (UNaV) and potassium (UKV) and thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation.

Renal hemodynamics. Forty-eight hours after contrast infusion, inulin clearance (GFR, ml·min^{-1}·100 g body wt^{-1}), mean arterial blood pressure (BP, mmHg), and total renal blood flow (RBF, ml/min) were assessed and renal vascular resistance (RVR, mmHg·ml^{-1}·min^{-1}) was calculated. To this end, animals were anesthetized intraperitoneally with pentobarbital (50 mg/kg body wt). A tracheostomy was performed and the rats were maintained with breathing spontaneously. To measure BP and allow blood sampling, a PE-60 catheter was inserted into the right carotid artery. BP was assessed using Biopac Systems MP100 (Santa Barbara, CA). Jugular veins were cannulated with PE-60 for the administration of inulin and fluids. To collect urine samples, a small abdominal incision was made, and the urinary bladder was cannulated with PE-240 catheter. After the surgical procedure, inulin was injected as a loading dose (100 mg/kg body wt), followed by a continuous infusion of 0.27 mg/min. After a 30-min equilibration period, three urine collections and two blood samples were then obtained at the beginning and at the end. Blood and urine inulin were determined using the anthrone method (49). At the end of the experiment, a midline incision was performed, the left renal pedicle was carefully dissected, and the renal artery was isolated. Total RBF was monitored by a perivascular ultrasonic flowmeter (T402; Transonic Systems, Bethesda, MD). RVR was calculated by dividing the BP by total RBF. The blood sample was then collected from the catheter inserted in the carotid artery to perform the following analyzes: serum glutathione (GSH), plasma ionic calcium and creatinine (ABL800 Flex, Radiometer, Brønshøj, Denmark), and plasma phosphorus (Cobas C 111, Roche, Basel, Schweiz). Also, serum levels of total 25(OH)D [25(OH)D$_2$ and 25(OH)D$_3$] were measured by enzyme immunoassay using a commercial kit (Rat 25OH Vitamin D total ELISA Kit, ALPCO, Salem, NH). Finally, the distal abdominal aorta was cannulated with PE-60 and the kidneys were perfused with 0.1 M phosphate-buffered saline, pH 7.4. The right kidney was removed and frozen in liquid nitrogen and stored at −80°C. Left kidney sections were fixed in 10% neutral-buffered Formalin solution or methacarn solution for histological analyses.

To exclude the possibility that changes in GFR could be due to the high osmolality of the CM in VDD rats, an additional set (n = 5) of inulin clearance experiments was performed by infusing a mannitol solution with an equivalent osmolarity of the Gd contrast medium (1,350 mosmol/l). Moreover, to evaluate the effects of longer period of VDD on CIN, two additional groups (n = 5/group) were maintained on a vitamin D-free diet for 60 days and only GFR was assessed 48 h after contrast infusion (VDD$_{60}$+IC and VDD$_{60}$+Gd groups).

Preparation of total kidney homogenates. The right kidney was homogenized using a Teflon pestle glass homogenizer (Schmidt, Waldkraiburg, Germany) in an ice-cold buffer containing 200 mM mannitol, 80 mM HEPES, and 41 mM KOH, pH 7.5, and protease inhibitor cocktail (Sigma). The homogenates were centrifuged at a low speed (3,000 rpm) for 15 min at 4°C to remove nuclei and cell debris. Protein concentration was determined using the Bradford assay method (Bio-Rad Protein Assay kit, Bio-Rad Laboratories, Hercules, CA).

Electrophoresis and immunoblotting. Samples of kidney homogenates were run on 7.5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Milipore, Billerica, MA). Blots were blocked with 5% skim milk and 0.1% Tween 20 in phosphate-buffered saline for 1 h and then incubated overnight with primary antibodies directed against eNOS (BD Transduction Laboratories, San Jose, CA), angiotensinogen (AGT, Santa Cruz Biotechnology, Dallas, TX), renin (AnaSpec, Fremont, CA), or actin (Santa Cruz). Blots were then washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted at 1:2,000. After five consecutive washes with the blocking solution, the membranes were rinsed in phosphate-buffered saline and then incubated with the enhanced chemiluminescence detection system ECL (GE Healthcare, Buckinghamshire, UK). The images were obtained using the ImageQuant LAS 4000 mini (GE HealthCare, Piscataway, NJ). The quantification of the visualized bands was performed using IMAGEJ (NIH, Bethesda, MD) densitometry software.

Histological examination. Kidney tissue embedded in paraffin was sectioned at 4 μm, stained with hematoxylin and eosin, and examined under light microscopy.Tubular damage was scored at 40–60 fields (0.245 mm^2 each; magnification, ×400) by calculation of the percentage of tubules that displayed tubular epithelial swelling, vacuolar degeneration, necrosis, and desquamation, as follows: 0, <5%; I, 5–25%; II, 26–50%; II, 51–75%; and IV, >75%. To minimize bias, the observer was blinded to the treatment groups. Immunohistochemistry was performed to evaluate renal macrophage infiltration. Kidney paraffin sections of 4 μm in size were incubated with mouse monoclonal antibody to ED1 (1:100 overnight at 4°C; AbD Serotec, Oxford, UK). The reaction product was detected by avidin-biotin-peroxidase (Vector Laboratories, Burlingame, CA). The color reaction was developed in 3,3-diaminobenzidine (Sigma), in the presence of hydrogen peroxide, and the sections were counterstained with Harris' hematoxylin. The number of ED-positive cells was counted in 40–60 renal cortex fields (0.087-mm^2 each). Results were expressed as the average number of macrophage per squared millimeter (mm^2).

Redox status measurements. TBARS was measured in urine and kidney homogenates. In brief, 0.4-ml urine or kidney samples were diluted in distilled water. Immediately, 1 ml of 17.5% trichloroacetic acid was added. After the addition of 1 ml of 0.6% thiobarbituric acid, pH 2.0, samples were placed in a boiling water bath for 15 min, after which it was allowed to cool. Subsequently, 1 ml of 70% trichloroacetic acid was added, and the mixture was incubated for 20 min at room temperature. Samples were then centrifuged for 15 min at 2,000 rpm. Optical density of the supernatant was read at 534 nm against a blank reagent using a spectrophotometer. Concentration of lipid peroxidation products was calculated as the malondialdeide (MDA) equivalent using a molar extinction coefficient for the MDA-thiobarbituric acid complex of 1.56 × 105 mol^{-1}/cm^{-1}. Urinary and kidney levels of TBARS were expressed as nanomoles per 24 h and nanomoles per microgram of protein, respectively. Reduced GSH, the major endogenous antioxidant in cells, was determined in blood and kidney homogenates. Whole blood was processed by addition of 4 volumes of ice-cold 5% metaphosphoric acid (Sigma) and centrifuged at 4,000 rpm for 10 min at 4°C. This assay consists of the reaction of supernatants of the total blood or kidney homogenates samples with Ellman's reagent to produce a yellow pigment measured spectrophotometrically at 412 nm. The serum and kidney levels of GSH were quantified by means of the standard curve (reported as μmol/ml and μmol/μg of protein, respectively.) Oxidative stress index was analyzed by renal TBARS-to-GSH ratio (expressed × 10^{-3}).

Statistical analysis. Data analysis was conducted to test the main effect of diet (two levels: vitamin D free or standard diet), the main effect of CM (three levels: 0.9% saline, IC or Gd), and the interaction between these two factors by two-way ANOVA followed by a post hoc Student-Newman-Keuls (SNK) test when appropriate. The anal-

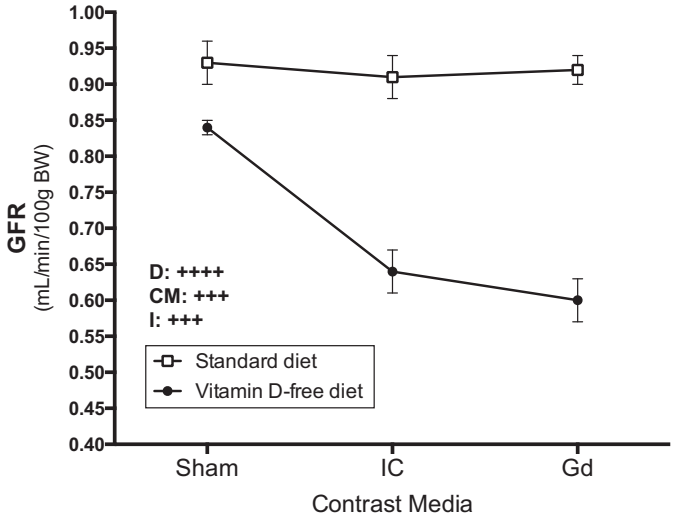

Fig. 1. Glomerular filtration rate (GFR), measured by inulin clearance, in rats fed standard or vitamin D-free diet subjected to infusion of 0.9% saline (Sham), iodinated (IC), or gadolinium (Gd) contrast media. D, diet factor; CM, contrast media factor; I, interaction between diet and CM factors. $++++P < 0.0001$ and $+++P < 0.001$ for the probability based on a two-way ANOVA. Data are expressed as means ± SE. $n = 12$/group.

ysis and the graphs were performed using GraphPad Prism software version Mac 6.0f and the table using MS-Word tables for two-way ANOVA software (5). When appropriate, unpaired *t*-test was conducted. Data are expressed as means ± SE. A P value < 0.05 was considered significant when considering the main effect of diet, the main effect of CM, the interaction between diet and CM, and the differences among groups.

RESULTS

No differences in food and water consumption (data not shown), as well as in body weight (278 ± 5 vs. 279 ± 4 g, $P = 0.92$), were observed between rats fed SD or vitamin D-free diet for 30 days. VDD rats had significantly lower levels of total serum 25(OH)D than rats fed SD (44.9 ± 1.0 vs. 4.0 ± 0.8 ng/ml, $P < 0.0001$), demonstrating the effectiveness of the model. On the other hand, plasma ionic calcium (4.0 ± 0.1 vs. 4.15 ± 0.1 mg/d, $P = 0.23$ vs. rats fed SD) and phosphorus (8.0 ± 0.4 vs. 7.7 ± 0.4 mg/dl, $P = 0.51$ vs. rats fed SD) were similar between rats receiving both types of diet.

Role of VDD in CIN. As shown in Fig. 1, a two-way ANOVA yielded a significant main effect of diet, CM, and diet × CM interaction on GFR. Simple main effects analysis showed that inulin clearance does not differ between Sham rats fed both diets. However, after IC or Gd administration, significant decreases of GFR were observed in rats fed vitamin D-free diet. On the other hand, neither IC nor Gd affected GFR in rats fed SD. These results confirmed our hypothesis that VDD is a predispose condition to CIN. Moreover, an additional group of rats, mantained on vitamin D-free diet for 60 days (VDD_{60}), was studied. These animals displayed total levels of serum 25(OH)D of 3.48 ± 0.35 ng/ml. Infusion of IC or Gd on VDD_{60} rats induced a more pronounced fall in GFR than that of VDD_{30}+IC (0.48 ± 0.04 vs. 0.64 ± 0.03 ml·min^{-1}·100 g body wt^{-1}, $P < 0.01$) and VDD_{30}+Gd groups (0.46 ± 0.04 vs. 0.60 ± 0.03 ml·min^{-1}·100 g body wt^{-1}, $P < 0.01$), respectively.

As shown in Table 1, a two-way ANOVA and post hoc test revealed that VDD and IC or Gd significantly increased plasma creatinine and urinary TBARS and decreased serum GSH. Besides, these alterations were significantly more pronounced when these two independent main effects were combined, as revealed in VDD_{30}+IC or VDD_{30}+Gd groups. However, no diet × CM interaction was observed. Additionally, a two-way ANOVA revealed that rats fed vitamin D-free diet demonstrated significant higher BP and RVR than rats fed SD, but no significant main effect of CM and no diet × CM interaction were observed. Regarding RBF, no main effect of diet and CM and no diet × CM interaction were observed (Table 1). In addition, UNaV and UKV were affected by CM factor, but no significant main effect of diet and no diet × CM interaction were observed. Pairwise comparisons based on marginal means identified that IC or Gd significantly decreased UNaV and UKV compared with Sham. Regarding urine-24 h volume, no main effect of diet and CM and no diet × CM interaction were observed (Table 1).

As illustrated in Fig. 2*A*, a two-way ANOVA revealed that levels of renal TBARS were affected by the diet factor, indicating that rats fed vitamin D-free diet showed higher renal

Table 1. *Renal function, hemodynamics, serum, and urinary redox status of rats fed standard or vitamin D-free diet infused with 0.9% saline, iodinated, or gadolinium contrast media*

	Standard Diet			Vitamin D-Free Diet			Two-Way ANOVA[1] (*P* Value)		
Variable	Sham	IC	Gd	Sham	IC	Gd	Diet	CM	I
PCr, mg/dl	0.25 ± 0.02^{c}	0.30 ± 0.01^{b,c}	0.28 ± 0.02^{c}	0.29 ± 0.02^{c}	0.40 ± 0.03^{a}	0.36 ± 0.02^{a,b}	<0.001	<0.001	NS
BP, mmHg	114 ± 2^{b}	121 ± 2^{b}	116 ± 2^{b}	132 ± 2^{a}	131 ± 2^{a}	132 ± 2^{a}	<0.0001	NS	NS
Total RBF, ml/min	5.60 ± 0.03	5.60 ± 0.02	5.50 ± 0.01	5.60 ± 0.02	5.50 ± 0.02	5.50 ± 0.01	NS	NS	NS
RVR, mmHg·ml^{-1}·min^{-1}	20.5 ± 0.3^{b}	21.5 ± 0.4^{b}	21.1 ± 0.3^{b}	23.8 ± 0.3^{a}	23.8 ± 0.3^{a}	24.1 ± 0.3^{a}	<0.0001	NS	NS
24 h UV, ml	23 ± 3	22 ± 3	19 ± 2	22 ± 2	21 ± 2	21 ± 2	NS	NS	NS
UNaV, meq	0.73 ± 0.04	0.69 ± 0.03	0.60 ± 0.03	0.74 ± 0.08	0.63 ± 0.05	0.62 ± 0.04	NS	<0.05	NS
UKV, meq	2.30 ± 0.1	1.82 ± 0.07	1.80 ± 0.08	2.20 ± 0.20	1.80 ± 0.1	1.71 ± 0.07	NS	<0.01	NS
GSHs, μmol/ml	2.68 ± 0.14^{a}	2.33 ± 0.06^{b,c}	2.44 ± 0.13^{a,b}	2.28 ± 0.06^{b,c}	2.03 ± 0.05^{c}	2.04 ± 0.05^{c}	<0.0001	<0.01	NS
TBARSu, nmol/24 h	110 ± 11^{b}	152 ± 8^{b}	168 ± 24^{b}	184 ± 26^{b}	303 ± 52^{a}	310 ± 42^{a}	<0.0001	0.01	NS

.Values are means ± SE. $n = 10$–12/group. Sham, 0.9% saline; IC, iodinated; Gd, gadolinium; PCr, plasma creatinine; UV, urine over a 24-hperiod; UNaV, 24-h urinary sodium excretion; UKV, 24-h urinary potassium excretion; BP, mean blood pressure; RVR, renal vascular resistance; GSHs, serum glutathione; TBARSu, urinary thiobarbituric acid reactive substances. [1]Factor diet; CM, contrast media factor; I = Diet × CM interaction effect for the probability based on a two-way ANOVA. ns, nonsignificant. Different superscript letters (a, b, c) refer to the Student-Newman-Keuls post hoc analysis among groups. Means in a row without a common superscript letters differ ($P < 0.05$).

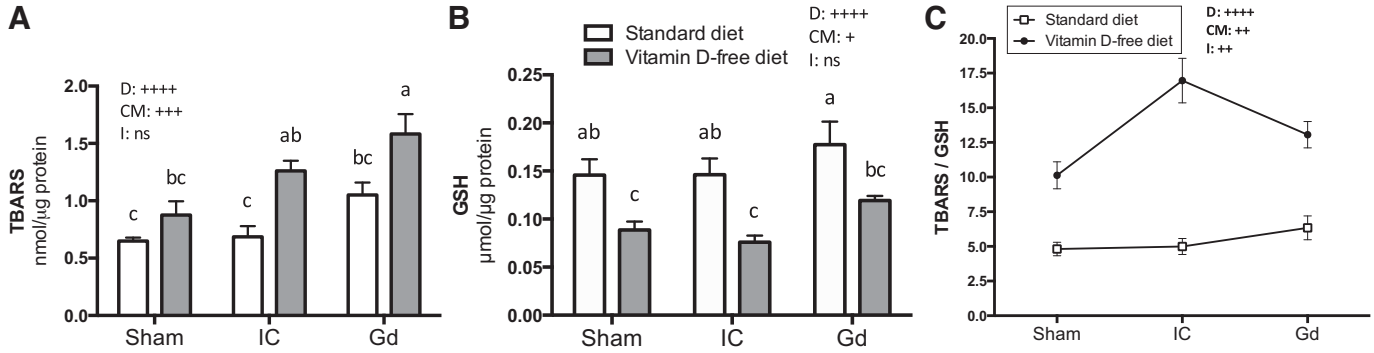

Fig. 2. Renal redox status in rats fed standard or vitamin D-free diet for 30 days infused with 0.9% saline (Sham), IC, or Gd contrast media. *A*: concentrations of thiobarbituric acid reactive substances (TBARS); *B*: reduced glutathione (GSH) in kidney homogenates. *C*: TBARS-to-GSH ratio, an oxidative stress index. Data are expressed as means ± SE. $n = 8$ rats/group. $++++P < 0.0001$, $+++P < 0.001$, $++P < 0.01$, $+P < 0.05$ and ns (nonsignificant) for the probability based on a two-way ANOVA. Bars with different lowercase letters are significantly different ($P < 0.05$) for the probability based on a Student-Newman-Keuls (SNK) multiple comparisons test.

TBARS levels than rats fed SD. Also, there was a significant main effect of CM factor, and post hoc analysis revealed that IC or Gd significantly increased renal TBARS compared with rats subjected to 0.9% saline infusion.In addition, Gd significantly increased renal TBARS compared with IC. No significant diet × CM interaction was observed with regarding renal TBARS.

As illustrated in Fig. 2*B*, a two-way ANOVA revealed a significant main effect of diet on renal GSH, indicating that VDD rats displayed lower levels of renal GSH compared with rats fed SD. The effect of CM was significant and post hoc analysis demonstrated that IC did not affect renal GSH, whereas Gd increased it compared with either with IC or 0.9% saline infusion. Interaction between diet and CM factors on renal GSH was not significant.

The oxidative stress index was analyzed by the TBARS-to-GSH ratio. As shown in Fig. 2*C*, a two-way ANOVA revealed a significant main effect of diet, CM, and diet × CM interaction. Simple main effects analysis showed that Sham-VDD_{30} rats exhibited significantly higher TBARS-to-GSH ratio compared with Sham-SD rats. After IC or Gd infusion, significant increases of TBARS-to-GSH ratio were observed in rats fed vitamin D-free diet, whereas this ratio did not change in rats fed SD. These results indicate that CM interacts with VDD, aggravating renal oxidative stress.

As depicted in Fig. 3, a two-way ANOVA revealed a significant main effect of diet on renal AGT protein expression, indicating that rats fed vitamin D-free diet display higher renal AGT protein expression than rats fed SD. Besides, a significant main effect of CM was observed and post hoc analysis revealed that IC or Gd infusion significantly increased renal AGT levels compared with rats that received 0.9% saline. The combination of VDD and CM independent effects gave a rise to a significantly more pronounced increase in AGT renal expression, as observed in VDD_{30}+IC and VDD_{30}+Gd groups, but there was no diet × CM interaction. Given that it has been previously demonstrated that vitamin D receptor represses renin mRNA transcription (54) and that renin is the first and rate-limiting step in intrarenal RAS activation, an additional experiment was performed to analyze the effect of 30-day vitamin D-free diet on renal renin expression. As shown in Fig. 4, Sham-VDD_{30} rats displayed higher levels of renal renin expression compared with Sham-SD rats (154 ± 10 vs. 100 ± 8%, $P < 0.01$).

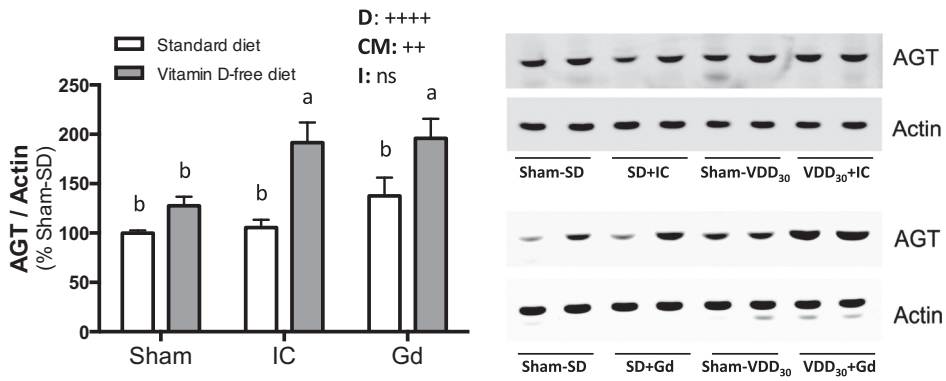

Fig. 3. Renal protein expression of angiotensinogen (AGT) in rats fed standard or vitamin D-free diet subjected to infusion of 0.9% saline (Sham), IC, or Gd contrast media. Immunoblotting and graphical representation of the relative rat renal expression levels of AGT in rats fed standard or vitamin D-free diet for 30 days infused with 0.9% saline (Sham), IC, or Gd contrast media. Equal amounts of kidney homogenates (100 μg for AGT and 5 μg for actin) were subjected to SDS-PAGE and transferred to a PVDF membrane. Blots were then incubated overnight with primary antibodies against AGT (1:1,000). Actin was used as loading control (1:50,000). Data are expressed as means ± SE. $n = 6$ rats/group. $++++P < 0.0001$, $++P < 0.01$, and ns (not significant) for the probability based on a two-way ANOVA. Bars with different lowercase letters are significantly different ($P < 0.05$) for the probability based on a SNK multiple comparisons test.

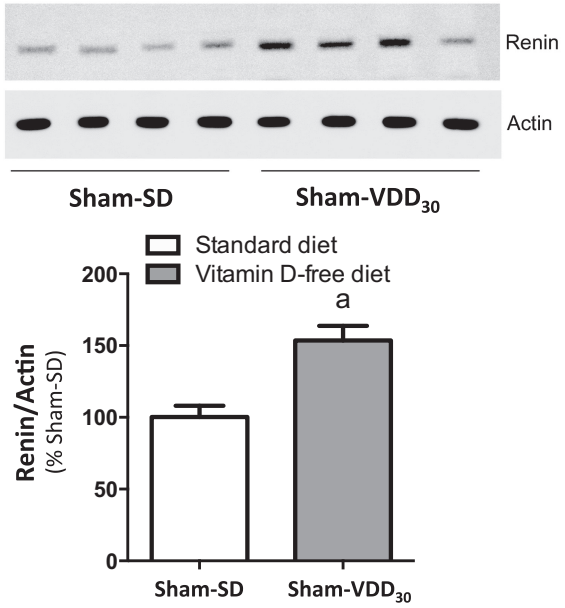

Fig. 4. Vitamin D deficiency increases rat renal renin expression. Immunoblotting and graphical representation of the relative expression levels of renin in kidney homogenates of rats fed standard (Sham-SD) or a vitamin-D free diet for 30 days (Sham-VDD_{30}). Equal amounts of kidney homogenates (150 μg for renin and 5 μg for actin) were subjected to SDS-PAGE and transferred to a PVDF membrane. Blots were then incubated overnight with primary antibodies against renin (1:1,000) or actin (1:50,000). Values are expressed as means ± SE. $n = 4$ rats/group. $^{a}P < 0.01$ vs. Sham-SD.

As shown in Fig. 5, a two-way ANOVA for eNOS protein expression revealed a significant main effect of diet, no significant main effect of CM, and a significant diet × CM interaction. Simple main effects analysis showed that Sham-VDD_{30} rats exhibited significant higher levels of renal eNOS protein expression compared with Sham-SD. Nevertheless, after IC or Gd administration, rats fed vitamin D-free diet significantly decreased renal eNOS protein expression. Conversely, rats fed SD increased renal eNOS after infusion of both CM. These results showed an opposite response to CM over renal eNOS in rats fed vitamin D-free diet compared with healthy animals.

The additional set of inulin clearance experiments, to analyze the impact of high osmolarity of CM on GFR in VDD rats, revealed that mannitol solution infusion did not alter GFR on VDD rats (0.83 ± 0.05 vs. 0.84 ± 0.01 $ml \cdot min^{-1} \cdot 100\ g\ body\ wt^{-1}$ in Sham-VDD_{30} group, $P = 0.83$).

As illustrated in Fig. 6*A* and taken from two-way ANOVA analysis, the percentage of tubular damage was low in all groups (<5% of tubular damage, i.e., score 0), and there was no significant main effect of diet and CM and no significant diet × CM interaction. Additionally, the number of ED1-positive cells was not affected by diet factor and CM factor and there was no significant diet × CM interaction (Fig. 6*B*).

DISCUSSION

In this study, we demonstrated for the first time that VDD is a risk factor for IC and Gd nephrotoxicity in rats. Our results indicate that reduction of GFR in VDD rats after CM infusion is associated with an intricate interplay among renal RAS, eNOS, and oxidative stress. These results have great relevance for clinical practice considering the frequent use of radiologic examinations employing CM, the current high prevalence of VDD in general population, and the safety attributed to Gd-based contrast.

The pathophysiology of CIN is not fully understood and it is speculated that the mechanisms underlying this injury involve an imbalance between vasodilator and vasoconstrictor factors, hypoxia, and generation of reactive oxygen species (ROS) in the medullary microcirculation, associated or not with direct cytotoxic effects on tubular cells (18). The outer medulla is a particularly vulnerable region to CIN since it is maintained at the verge of hypoxia due to relative high oxygen requirements for upholding the countercurrent mechanism. Contrast media increase oxygen demand and decrease oxygen supply in this nephron region. Higher oxygen consumption appears to be related to the activation of NKCC2 cotransporter, whereas lower oxygen supply may be attributed to reduction of medullary blood flow by descending vasa recta vasoconstriction. These alterations in CIN seem to be induced by endothelin, intrarenal RAS activation, nitric oxide synthase inhibition, and

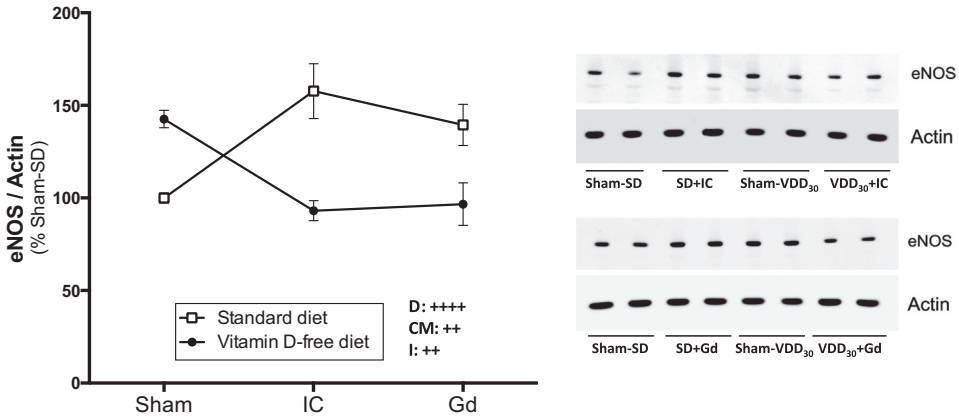

Fig. 5. Renal protein expression of endothelial NO synthase (eNOS) in rats fed standard or vitamin D-free diet. Effect subjected to infusion of 0.9% saline (Sham), IC, or Gd contrast media. Immunoblotting and graphical representation of the relative rat renal expression levels of eNOS in rats fed standard or vitamin D-free diet for 30 days infused with 0.9% saline, IC, or Gd. Equal amounts of kidney homogenates (150 μg for eNOS and 5 μg for actin) were subjected to SDS-PAGE and transferred to a PVDF membrane. Blots were then incubated overnight with primary antibodies against eNOS (1:500). Actin was used as loading control (1:50,000). Data are expressed as means ± SE. $n = 6$ rats/group. $++++P < 0.0001$, $++P < 0.01$, and ns (nonsignificant) for the probability based on a two-way ANOVA.

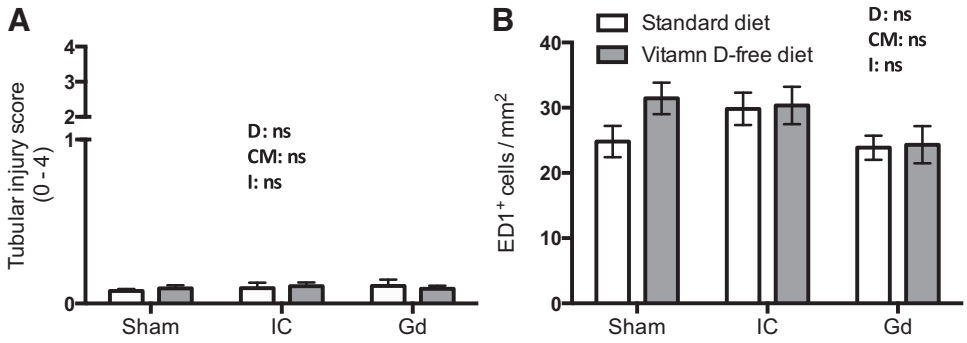

Fig. 6. Tubular injury and quantitation of renal macrophage infiltration in rats fed standard or vitamin D-free diet for 30 days subjected to infusion of 0.9% saline (Sham), IC, or Gd, contrast media. *A*: tubular injury score. *B*: number of infiltrating macrophages in to the kidneys is expressed as ED1-positive cells per mm^2. Data are expressed as means ± SE. n = 5–7 rats/group. ns (nonsignificant) for the probability based on a two-way ANOVA.

oxidative stress. Thus endothelium dysfunction associated with changes in renal microcirculation is key to CIN (8, 10, 18, 22, 31, 37). In this scenario, for clinical or experimental CIN development will be necessary the presence of predispose conditions capable of aggravating the insult on medullary microcirculation triggered for CM, disrupting the compensatory mechanism that preserves GFR. Likewise, healthy experimental animals do not develop CIN unless preconditioning is undertaken, i.e., uninephrectomy, salt-depletion, and/or administration of drugs that induce renal vasoconstriction or inhibit vasodilation(3, 18, 51). VDD has never been described as a risk factor for CIN before. In the present study, preconditioning was not carried out, underscoring the potential role of VDD as a risk factor for CIN in rats.

Several studies report a linkage between VDD and upregulation of RAS, oxidative stress, and endothelial dysfunction (4, 12, 24, 43). By means of flow-mediated dilation and serum TBARS in asymptomatic subjects, Tarcin et al. (42) have shown that VDD is associated with endothelial dysfunction and increased lipid peroxidation. In cultured endothelial cells, 1,25-dihydroxycholecalciferol induces NO production by increasing eNOS activity and mRNA expression (28, 40). Likewise, experimental studies have demonstrated that vitamin D reduces the expression of the NADPH oxidase subunit p22phox and decreases oxidative stress (12, 20). Moreover, vitamin D upregulates the expression of enzymes involved in GSH metabolism, namely glutamate cysteine ligase and gluthatione reductase, and stimulates the thioredoxin system, therefore catalyzing the biosynthesis of a major endogenous antioxidant (4, 21).

Our data show that VDD leads to changes in the health status of the animals. VDD rats display higher blood pressure, renal vascular resistance, and greater renal renin and AGT protein expression compared with rats fed SD. Plasma levels of calcium and phosphorus are similar between rats fed both diets, strengthening the notion that activation of intrarenal RAS by VDD is independent of calcium levels (55). Also, VDD rats have lower kidney and serum GSH, in line with the well-documented antioxidant effects of vitamin D. In the Sham-VDD_{30} group, elevation of eNOS protein expression probably represents a compensatory mechanism that is observed in endothelial dysfunction induced by hypertension and oxidative stress, to counterbalance the reduction in NO bioavailability (46). It is important to emphasize that under these conditions, oxidation of the eNOS cofactor BH4 by peroxynitrite may uncouple oxygen reduction from NO synthesis and convert eNOS to a superoxide-producing enzyme, the so-called "eNOS uncoupling," then, increasing superoxide (13, 46).

We have found that healthy rats exposed to IC or Gd do not display an imbalance in renal redox status, have an increase on eNOS expression in the kidney, and unaltered inulin clearance. These data strongly suggest that an additional insult or risk factor is necessary to induce CIN. As opposed to healthy animals, VDD rats develop AKI after infusion of both CM. IC and Gd increased renal AGT expression and the interaction of VDD with CM significantly reduced renal eNOS expression in VDD_{30}+IC and VDD_{30}+Gd rats. In this context, Goodman et al. (14) have demonstrated that eNOS expression is reduced in experimental AKI in uninephrectomized rats exposed to sodium iothalamate. Noteworthy, hypoxia is a known mechanism of inhibition of eNOS expression (27). Thereby, the reduction of renal eNOS expression supports the hypothesis of medullary hypoxia and renal injury generated by CM in a vitamin D deficiency state.

Renal generation of ROS favors vasoconstriction of outer medullary vasa recta by stimulating RAS and decreasing NO and may alter directly and/or indirectly glomerular and tubuloglomerular feedback functions (6, 26, 29, 50). Indeed, VDD rats showed lower levels of renal and systemic GSH and higher renal and urinary TBARS compared with rats with normal 25(OH)D levels. Conversely, to rats fed SD, VDD rats aggravated redox status imbalance after interaction with IC or Gd, demonstrated by remarkable increased of renal TBARS-to-GSH ratio in VDD_{30}+IC and VDD_{30}+Gd groups, an index of oxidative stress.

We have found that the reduction in GFR of rats with VDD infused with IC or Gd was not accompanied by changes on total RBF. Of note, experimental studies have inconsistently found either transient increases or decreases in total RBF that return to baseline levels after few minutes (38, 41). Additionally, IC infusion in high-risk patients, undergoing coronary angiography, was not associated with a fall in total RBF and there appears to be no relationship between any changes in total RBF and CIN (48). Indeed, cumulative evidence indicate that rather than changes in total RBF, it is the corticomedullary redistribution of RBF that may mostly contribute to the genesis of CIN and AKI induced by others causes (17, 19, 25, 30, 34, 39). Several patterns of rerouting of blood flow in the renal cortex and medulla have been demonstrated in healthy animals, depending on the type, volume, and route of administration of the CM (18). Interestingly, Agmon et al. (2) and Palm et al. (32) have shown an increase in outer medullary flow in healthy animals exposed to CM, which is paradoxically reduced after induction of endothelial dysfunction or diabetes. All together, these studies strongly suggest that reduction of the outer

medullary blood flow plays a crucial role in the development of CIN. Therefore, one may speculate that the dissociation between the total RBF and the reduction in GFR observed in our study may be attributed, at least in part, to renal microcirculatory hemodynamic changes associated with endothelial dysfunction in VDD rats.

It is important to emphasize that reduction of the GFR was observed in rats with VDD that received Gd, a CM that is originally thought to be safe in terms of nephrotoxicity. In this regard, Gd-based contrast agents have been reported to induce a decrease of the GFR in a high-risk population group, especially in patients with altered baseline renal function (9). These findings are in line with a previous study from our laboratory, in which we have demonstrated that five of six nephrectomized rats develop CIN after Gd contrast infusion (33). The results of the present study suggest that VDD might be a novel risk factor for Gd nephrotoxicity.

The existence of abnormalities on kidney morphology in CIN is variable and controversial. Most investigators have not found changes in renal morphology, whereas others only encountered vacuolization of the proximal tubular cells that were not correlated with kidney dysfunction (7, 18, 23, 45). Our experimental models exhibit low tubular injury score after IC or Gd contrast infusion, underscoring that intrarenal hemodynamics is the primary alteration that leads to GFR fall. Additionally, macrophage infiltration was similar in all groups, suggesting that inflammation does not contribute to renal impairment under these experimental conditions.

It is worth mentioning that the effect of CM on GFR in VDD rats occurred independently of the osmotic load of these agents, since no difference in inulin clearance was observed in response to a mannitol infusion, suggesting the chemotoxicity effects of CM, as observed in other studies (15, 35, 53). Furthermore, a more pronounced reduction of inulin clearance was detected in VDD_{60}+IC and VDD_{60}+Gd groups. These data suggest that the longer the period of the VDD is, the worst the impact of CM is on inulin clearance.

Perspectives and Significance

In summary, this study demonstrates for the first time that VDD is a potential condition that predisposes to both IC and Gd nephrotoxicity in rats. Lower GFR in VDD rats subjected to CM infusion was linked with an imbalance of intrarenal vasoactive substances and oxidative stress, suggesting that these mechanisms may partially account for the CIN associated with VDD. Of note, a recent longitudinal study has shown that low vitamin D levels measured before iodinated contrast agents exposure were independently associated with a high incidence of CIN development in patients undergoing coronary angiography (36). From these results, it would be advisable to maintain adequate levels of vitamin D in patients who will undergo radiologic examinations employing CM, inclusive Gd-based contrast.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: W.M.L. and A.C.S. conception and design of research; W.M.L., M.H.M.S., D.C., P.H.F.G., A.C.d.B., R.A.V., and A.C.C.G. performed experiments; W.M.L., M.H.M.S., D.C., P.H.F.G., A.C.C.G., and A.C.S. analyzed data; W.M.L., P.H.F.G., A.C.C.G., and A.C.S. interpreted results of experiments; W.M.L., D.C., and A.C.C.G. prepared figures; W.M.L. and A.C.C.G. drafted manuscript; W.M.L., P.H.F.G., A.C.C.G., and A.C.S. edited and revised manuscript; W.M.L., M.H.M.S., D.C., P.H.F.G., A.C.C.G., and A.C.S. approved final version of manuscript.

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