Expression of adrenomedullin 2/intermedin, a possible reno-protective peptide, is decreased in the kidneys of rats with hypertension or renal failure

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Submitted 30 November 2009; accepted in final form 6 May 2010

Hirose T, Totsune K, Mori N, Mori T, Morimoto R, Metoki H, Asayama K, Kikuya M, Ohkubo T, Kohzuki M, Takahashi K, Imai Y. Expression of adrenomedullin 2/intermedin, a possible reno-protective peptide, is decreased in the kidneys of rats with hypertension or chronic renal impairment using quantitative RT-PCR, radioimmunoassay, and immunohistochemistry. Kidneys of 8-wk-old male spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were dissected into inner medulla, outer medulla, cortex, and glomerulus fractions. A rat renal impairment model was prepared by 5/6 nephrectomy in WKY rats. AM2/IMD mRNA levels were the highest in the cortex among four renal portions, and significantly lower in SHR than WKY rats in all renal portions. In the remnant kidneys of 5/6 nephrectomized rats, AM2/IMD mRNA levels were significantly decreased on days 3 and 56, whereas mRNA levels of calcitonin receptor-like receptor, receptor activity-modifying proteins-1 and -2, which form receptor for AM and AM2/IMD, were increased, compared with that in sham-operated rats. AM mRNA levels were decreased on day 3, but increased on day 56, after nephrectomy. Decreased immunoreactive AM2/IMD levels in the remnant kidneys of 5/6 nephrectomized rats on day 56 were confirmed by radioimmunoassay. The renal tubules were immunostained with anti-AM2/IMD antibody, with a decreased AM2/IMD immunostaining found in proximal tubular cells of 5/6 nephrectomized rats compared with sham-operated rats. In conclusion, intrarenal AM2/IMD expression is decreased in SHR and 5/6 nephrectomized rats. Given the organ-protective effects of AM2/IMD, the downregulation of AM2/IMD as an endogenous regulatory peptide may have a role in the progression of renal impairment.

immunohistochemistry; radioimmunoassay; renal impairment; reverse transcriptase polymerase chain reaction

ADRENOMEDULLIN 2/INTERMEDI (AM2/IMD) is a novel member of the calcitonin/calcitonin gene-related peptide (CGRP) family, which includes calcitonin, CGRP, amylin, and AM (3, 21, 29, 30). The CGRP family has multiple biological effects, including potent vasodilatation (CGRP and AM), reduced nutrient intake (amylin), and decreased bone resorption (calcitonin). Recently, two research groups discovered AM2/IMD almost simultaneously by searching the genome database, and named it intermedin (21) and adrenomedullin 2 (30), respectively. Reverse-transcriptase polymerase chain reaction (RT-PCR) has shown that AM2/IMD mRNA is widely distributed in various mouse tissues (30). Immunohistochemical investigations have revealed that AM2/IMD immunoreactivity was detectable in the heart and kidney of mice and humans (14, 29).

CGRP, AM, and AM2/IMD activate the complex of calcitonin receptor-like receptor (CRLR) and one of the three types of receptor activity-modifying proteins (RAMPs) to transduce their signals. The complex of CRLR and RAMP2 or RAMP3 forms the AM receptor, whereas the CRLR/RAMP1 complex forms the CGRP receptor (12). AM2/IMD binds nonspecifically to all three CRLR/RAMP complexes: CRLR/RAMP1, CRLR/RAMP2, and CRLR/RAMP3 (21). AM2/IMD stimulates cAMP production and has a potent vasodilator action, similar to AM and CGRP (21, 30). Intravenous injection of AM2/IMD decreases arterial blood pressure (16, 21, 30), and this effect is partially blocked by CGRP and AM receptor antagonists (21). Moreover, intrarenal infusion of AM2/IMD causes diuresis and natriuresis, without a significant decrease in systemic blood pressure in rats (4). There is an increasing body of evidence that shows that AM and CGRP are potent endogenous reno- and cardioprotective substances. Exogenous administration of AM and CGRP peptides or their gene delivery is a new preventive and therapeutic strategy for renal failure and cardiovascular diseases, such as hypertension, myocardial ischemia, and heart failure (5, 7, 11). Yang and coworkers recently demonstrated the cardioprotective effects of AM2/IMD in rat hearts both in vitro and in vivo (9, 20, 34, 35). Therefore, it is reasonable to suggest that AM2/IMD also has a reno-protective effect. Little information is available, however, regarding the pathophysiological role of the endogenous AM2/IMD in the diseased kidney, such as in chronic renal impairment and hypertension.

The aim of the present study was to assess the hypothesis that intrarenal AM2/IMD is an endogenous regulatory peptide that responds to renal injury. AM2/IMD, AM, CRLR, RAMP1, RAMP2, and RAMP3 mRNA expression levels were studied in kidneys obtained from 5/6 nephrectomized (5/6N) rats by quantitative RT-PCR. Immunoreactive-AM2/IMD (IR-AM2/IMD) concentrations were also studied by a radioimmunoassay. Furthermore, intrarenal distribution of AM2/IMD, AM, CRLR, RAMP1, RAMP2, and RAMP3 mRNA was examined...
in rats with genetic hypertension. AM2/IMD in the rat kidneys was localized by immunohistochemistry.

**METHODS**

Animals. Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Tohoku University (Permission No. 21-Pharm-Animal-9). Male spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats (188–262 g; Charles River Japan, Tsukuba, Japan) were housed under standard conditions with free access to water and standard chow.

Renal portion. Eight-week-old male SHR and WKY rats (n = 6, each group) were anesthetized with pentobarbital sodium (50 mg/kg). After the abdominal aorta was cannulated, both kidneys were flushed with ice-cold saline and harvested. The kidneys were dissected into inner medulla, outer medulla, and cortex fractions, as previously described (8, 13). Moreover, the cortex portions were sliced, and most of them were used for isolation of glomeruli using a sieving technique, as previously described (10). In short, the cortex slices were forced through 180-, 100-, and 70-μm sieves, one at a time. The material remaining on the top surface of the 70-μm sieve was collected as glomeruli. Each part of the renal tissue (inner medulla, outer medulla, cortex, and glomeruli) was immediately stored at −80°C until RNA extraction.

5/6NPX. Renal mass ablation experiments were performed using male WKY rats, as previously reported (10, 13, 17). Briefly, 8-wk-old male WKY rats were subjected to 5/6NPX by two-thirds infarction of the right kidney and removal of the left kidney under ether anesthesia. In the first session, the right kidney was exposed via flank incision, and the two poles were encircled with loops of ligatures. After the loops were tightened, the incision was closed. Seven days later, the left kidney was exposed via flank incision and removed completely. The flank wound was then closed. Age-matched male WKY rats with sham operations underwent laparotomy with elimination of the connective tissue and closure of the abdomen only. The rats were killed under anesthesia 3, 14, or 56 days after the last operation (n = 6, all groups). The kidneys were harvested and stored at −80°C until RNA extraction. The remnant kidneys of 5/6NPX rats and the kidneys of sham-operated rats from day 56 were stored at −80°C for radioimmunoassay, or fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemistry.

RNA extraction and competitive and quantitative RT-PCR. Total RNA was extracted by the guanidinium isothiocyanate/cesium chloride method, and 4 μg of total RNA were reverse transcribed with 400 units of Moloney murine leukemia virus reverse transcriptase (RevertAse; TOYOBO, Osaka, Japan) using an oligo(dT) primer, as previously described (6, 31–33). Expressions of AM2/IMD, AM, CRLR, RAMP1, RAMP2, RAMP3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were determined using competitive, quantitative RT-PCR methods that have been previously reported (6, 31–33). In brief, to determine the equivalent concentration point, the competitive reference standard-DNA for AM2/IMD, AM, CRLR, RAMP1, RAMP2, RAMP3, and GAPDH was prepared, and a constant amount of wild-type cDNA and decreasing amounts of competitive reference standard-DNA (1:2 scheme) were added to each PCR tube. AM2/IMD, AM, CRLR, RAMP1, RAMP2, and RAMP3 mRNA concentrations were normalized by GAPDH mRNA expression levels. Every PCR reaction was repeated three times, and then the mean value was calculated.

The obtained PCR products were purified by agarose gel and sequenced by an autosequencer (model 3100; Applied Biosystems, Foster, CA), and their identities were confirmed 100% with the respective nucleotide sequences registered in the National Center for Biotechnology Information databases.

Peptide extraction and radioimmunoassay. The renal tissues of 5/6NPX rats and sham-operated rats from day 56 were extracted as previously reported (28). Briefly, the tissue (~500–1,000 mg) was boiled in 2 ml of 1 mol/l acetic acid for 10 min. Eight milliliters of 50% methanol in 0.5 mol/l acetic acid were added to each sample, and the tissue was homogenized. The homogenate was centrifuged at 15,000 g for 30 min. The supernatant was separated, dried by air, reconstituted in assay buffer, and assayed.

**Fig. 1.** Expression levels of adrenomedullin 2/intermedin (AM2/IMD) mRNA (A), adrenomedullin (AM) mRNA (B), calcitonin receptor-like receptor (CRLR) mRNA (C), receptor activity-modifying receptor 1 (RAMP1) mRNA (D), RAMP2 mRNA (E), and RAMP3 mRNA (F) in the inner medulla (IMD), outer medulla (OMD), cortex (Cx), and glomerulus (Glm) fractions of Wistar-Kyoto (WKY) rats (n = 6, open bars) and spontaneously hypertensive rats (SHR; n = 6, solid bars). Expression levels of each mRNA were normalized to the expression level of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are means ± SE. Statistical analysis was performed by one-way analysis of variance, followed by the Tukey honestly significant difference test for multiple comparison of differences among three portions (IMD, OMD, and Cx) of SHR and WKY rats, and the unpaired Student’s t-test for comparison of differences between WKY Glm and SHR Glm. *P < 0.05 and **P < 0.01, significantly different in the same portions of SHR and WKY rats; #P < 0.01, significantly higher in the other two portions of the same strain; ØP < 0.05, significantly different between WKY Glm and SHR Glm.
IR-AM2/IMD concentrations in the kidney tissues were measured by the radioimmunoassay using IMD/AM2 (rat) RIA kit (RK-010-52; Phoenix Pharmaceuticals, Burlingame, CA), following the manufacturer’s protocol. The assay could detect changes of 0.6 fmol/tube from zero at 95% confidence. The assay showed 100% cross-reaction with human AM2/IMD, but no significant cross-reaction with rat AM or rat CGRP.

Chromatographic characterization of the extract of remnant kidneys of 5/6NPX rats was performed by reverse-phase high performance liquid chromatography (HPLC) using a Bondapak C18 column (3.9 × 300 mm, Waters, Milford, MA). The tissue extract was reextracted with a Sep-Pak C18 cartridge (Waters, Milford, MA), reconstituted in 0.1% (vol/vol) trifluoroacetic acid, and loaded onto the column. The HPLC was performed with a linear gradient of acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid from 10–60% at a flow rate of 1 ml/min fraction over 50 min. Each fraction (1 ml) was collected, dried by air, reconstituted with assay buffer, and assayed.

Immunohistochemistry. Immunohistochemistry of AM2/IMD in the rat kidneys was performed using the ABC method with the Vector ABC kit (Vector Laboratories, Burlingame, CA), as previously reported (6, 14, 23, 27). The polyclonal antiserum against AM2/IMD (no. 0403-721) was raised in a rabbit, and the characteristics of this antiserum were examined in a radioimmunoassay that our laboratory previously developed and reported (14). This antiserum against AM2/IMD was used at dilutions of 1:1,000.

Normal rabbit serum (at a dilution of 1:10,000) was used as a negative control. The specificity of the AM2/IMD antiserum was examined by the absorption test. AM2/IMD antiserum (1:1,000) was preabsorbed with synthetic rat AM2/IMD, rat AM, human AM2/IMD, or human AM (all of these peptides were obtained from Peptide Institute, Minoh, Japan). The absorption test was performed using the antiserum incubated with an excess amount of peptide (50 nmol peptide/ml of the diluted antiserum) for 20 h at 4°C before use.

Statistical analysis. Data are given as means ± SE. mRNA and protein concentrations were analyzed by unpaired Student’s t-test and one-way ANOVA, followed by the Tukey honestly significant difference test for multiple comparison of differences among the groups. Statistical significance was accepted at \( P < 0.05 \).

RESULTS

mRNA expression in renal portions of SHR and WKY rats. Among the renal portions, AM2/IMD mRNA levels were highest in the cortex (Fig. 1A). On the other hand, AM mRNA levels were markedly higher in the glomeruli, over fivefold of that in the other renal portions (Fig. 1B). AM2/IMD mRNA levels were ~50-fold higher than AM levels. SHR had significantly lower AM2/IMD mRNA levels in the inner medulla, outer medulla, cortex, and glomeruli than WKY rats (39, 27, 58, and 53%, respectively; Fig. 1A). In the glomeruli, SHR had significantly lower AM, CRLR, and RAMP2 mRNA levels than WKY rats (~60, 56, and 65%, respectively; Fig. 1, B, C, and E). There was no difference in RAMP1 and RAMP3 mRNA expression between SHR and WKY rats (Fig. 1, D and F).

mRNA expression in the remnant kidneys of 5/6NPX rats. AM2/IMD mRNA levels in 5/6NPX rats, compared with

Fig. 2. Expression levels of AM2/IMD mRNA (A), AM mRNA (B), CRLR mRNA (C), RAMP1 mRNA (D), RAMP2 mRNA (E), and RAMP3 mRNA (F) in the kidneys of sham-operated (SO) rats (open bars) and in the remnant kidneys of 5/6 nephrectomized (5/6NPX) rats (solid bars). The renal tissues were obtained 3, 14, and 56 days after the SO or 5/6NPX (n = 6, all groups). Expression levels of each mRNA were normalized to the expression level of the housekeeping gene, GAPDH. Data are means ± SE. Statistical analysis was performed by one-way analysis of variance, followed by the Tukey honestly significant difference test. *P < 0.05, **P < 0.01, significantly different between SO rats and 5/6NPX rats; ‡P < 0.01, significantly different from the 5/6NPX rats at 14 days.

Fig. 3. Immunoreactive-AM2/IMD (IR-AM2/IMD) concentrations in the kidneys of SO rats (open bar) and in the remnant kidneys of 5/6NPX rats (solid bar). IR-AM2/IMD concentrations were normalized to the tissues wet weight. *P < 0.05, significantly different between SO rats and 5/6NPX rats.
sham-operated rats, were significantly decreased on day 3 (~55% of day-matched, sham-operated rats), recovered to equal levels on day 14, and decreased again on day 56 (~27% of day-matched, sham-operated rats) (Fig. 2A). AM mRNA levels in 5/6NPX rats were significantly decreased on day 3 (~46%), were not changed on day 14, and were significantly increased on day 56 (~2.0-fold) compared with sham-operated rats (Fig. 2B). CRLR, RAMP1, and RAMP2 mRNA levels in 5/6NPX rats were significantly increased (about 1.6- to 26.4-fold) at the 3-day points, but RAMP3 mRNA levels were not changed (Fig. 2, C–F).

**AM2/IMD protein expression in the remnant kidneys of 5/6NPX rats.** IR-AM2/IMD concentrations in the remnant kidneys of 5/6NPX rats on day 56 were significantly decreased by ~46%, compared with those in the kidneys of sham-operated rats (Fig. 3). Reverse-phase HPLC in the remnant kidneys of 5/6NPX rats showed a single immunoreactive peak eluting in the position of synthetic rat AM2/IMD, with a shoulder of IR-AM2/IMD eluting later than the main peak (Fig. 4). Because the IMD/AM2 (rat) RIA kit used in this experiment showed no significant cross-reactivity with AM or CGRP, IR-AM2/IMD eluting in this shoulder may represent AM2/IMD peptide, with some modification or degradation generated during the extraction procedure.

**Immunohistochemistry of AM2/IMD in the rat kidney.** Immunohistochemistry of AM2/IMD showed positive immunostaining in the kidneys of sham-operated rats (Fig. 5A). The antiserum absorbed with synthetic rat AM2/IMD (Fig. 5B) or human AM2/IMD significantly attenuated the positive immunostaining, whereas the antiserum absorbed with synthetic rat AM or human AM (data not shown) did not affect the immunostaining. Normal rabbit serum showed no positive immunostaining (Fig. 5C).

The prominent positive AM2/IMD immunostaining was observed in the renal tubular cells of the kidneys of both 5/6NPX rats (Fig. 6, B, D, and F) and sham-operated rats (Fig. 6, A, C, and E). The proximal, thick ascending limb, distal, and collecting tubular cells were diffusely immunostained in sham-operated rats (Fig. 6, A, C, and E). While the thick ascending limb, distal, and collecting tubular cells were diffusely immunostained in 5/6NPX rats similarly to sham-operated rats, AM2/IMD immunostaining in the proximal tubular cells was weaker in 5/6NPX rats than in sham-operated rats (Fig. 6, B, D, and F). Vasa recta was not immunostained in 5/6NPX rats and sham-operated rats. Vascular smooth muscle cells of renal arterioles were positively immunostained in 5/6NPX rats (Fig. 6H) and sham-operated rats (Fig. 6G). The glomerular tufts were positively immunostained in 5/6NPX rats (Fig. 6J), and sporadically immunostained in sham-operated rats (Fig. 6I).

**DISCUSSION**

The present study has shown for the first time that AM2/IMD expression is regulated in the diseased kidney in a different manner from AM expression. AM2/IMD expression was decreased in the kidneys of rats with renal impairment and hypertension. By contrast, AM mRNA expression was decreased only in the glomerular fraction of the kidneys of hypertensive rats and increased in the remnant kidneys of 5/6NPX rats on day 56. CRLR, RAMP1, and RAMP2 mRNA expression was increased in the remnant kidneys of 5/6NPX rats, whereas CRLR mRNA expression was decreased in the glomerular fraction of the kidneys of hypertensive rats. These findings suggest that 1) the activity of both AM and AM2/IMD may be decreased in the kidney of hypertensive rats; 2) the activity of AM may be increased in the remnant kidneys of 5/6NPX rats at day 56; 3) the activity of AM2/IMD may be limited due to its decreased expression, despite the upregulation of CRLR, RAMP1, and RAMP2 expression in the remnant kidneys.
Fig. 6. Immunohistochemistry of AM2/IMD in the rat kidney. All panels represent at least three samples. 
A and B: the renal cortex of SO and 5/6NPX rats, respectively. C and D: the renal medulla of SO and 
5/6NPX rats, respectively. E and F: the renal papilla of SO and 5/6NPX rats, respectively. G and H: the 
vessels of SO and 5/6NPX rats, respectively. I and J: the glomeruli of SO and 5/6NPX rats, respectively. 
Bar = 50 μm. Arrows indicate proximal tubular (PT) cells.
kidneys of 5/6NPX rats. The regulation of AM2/IMD expression in the diseased kidneys may, therefore, be different from that of AM or its receptor components (CRLR and RAMP1-3). The expression of AM is induced in various types of cells, including vascular endothelial and smooth muscle cells by certain stresses, such as hypoxia, inflammatory cytokines, and oxidative stress (2, 15, 25). It has recently been reported that hypoxia induced AM2/IMD expression in the murine lung and cultured murine pulmonary microvascular endothelial cells (18). However, there have been no other reports on the induction of AM2/IMD expression by other types of stresses. Therefore, AM2/IMD expression may not be so likely to be induced by various stresses, as observed in AM expression.

RT-PCR and immunohistochemistry revealed prominent AM2/IMD expression in the renal cortex. Fujisawa et al. (4) have reported that AM2/IMD increased urine flow and urinary sodium excretion, when infused continuously into the renal artery of rats. Therefore, the present results suggest that AM2/IMD may act as an autocrine or paracrine regulator of water and electrolyte transport in the renal tubular cells via its natriuretic action. AM2/IMD mRNA was also expressed in the glomerulus, and, particularly, glomerular tufts were more immunostained in 5/6NPX rats than in sham-operated rats. Further studies are required to identify the type of AM2/IMD-positive cells in the glomerulus and the role of AM2/IMD in the glomerular function, such as the control of the glomerular capillary tone.

Increased expression of certain vasoactive peptides and cytokines, such as atrial natriuretic peptide (31), endothelin-1 (22), transforming growth factor-β (1), and urotensin II-related peptide (13), has been reported in the injured kidney. Indeed, in the present study, AM mRNA expression was also upregulated on day 56 after 5/6NPX. These upregulations are considered compensatory counterregulation for renal injury. In contrast, AM2/IMD mRNA and protein expression was decreased in the kidneys of renal impairment and genetic hypertensive rats. It is noteworthy that AM2/IMD mRNA expression levels in the kidney were ~50-fold higher than AM mRNA expression levels, as shown in Figs. 1 and 2. Therefore, AM2/IMD may be a major player in the renal AM2/IMD system. Given the possible renoprotective effect of AM2/IMD, the decreased expression of AM2/IMD may result in failure to maintain the renal function, and perhaps promote the progression of renal injury.

AM2/IMD and AM mRNA expression levels were decreased 3 days after 5/6NPX. The decrease may be due to the unstable state of wound healing with acute renal injury. On day 14, when compensatory renal growth reached maximal hypertrophy, AM2/IMD and AM expressions recovered to the levels equal to those of sham-operated rats. These findings suggest that the AM2/AM system produced an increase in water-electrolyte excretion to compensate for the volume overload. On the other hand, renal hypertrophy, which occurred around on day 14 itself, might result in increased renal AM2/IMD expression. On day 56, when the hypertrophied glomeruli were exhausted and functioning nephrons were decreased in numbers, AM2/IMD expression levels were decreased. By contrast, AM expression levels were increased on day 56. This increased AM expression may be induced by some stresses, such as oxidative stress and/or stimuli of some cytokines in the remnant kidney, and AM may support the renal function at this stage of the remnant kidneys of 5/6NPX rats. AM2/IMD and AM share the CRLR/RAMP receptor system and may, therefore, have similar biological actions, such as a vasodilator action, diuretic and natriuretic actions, antioxidative stress action, and modulatory actions on cell proliferation (12, 21, 26). However, AM2/IMD and AM may play distinct (patho) physiological roles in the kidney impairment, due to their different regulation of expression.

Immunohistochemistry showed AM2/IMD immunostaining in the proximal tubular cells were weaker in 5/6NPX rats than in sham-operated rats. Decreased expression of AM2/IMD mRNA and peptide in the kidney of 5/6NPX rats may be due to its decreased expression in proximal tubular cells and/or decreased number of functioning renal tubular cells expressing AM2/IMD. The decreased expression of AM2/IMD in proximal tubular cells may be caused by certain cellular dysfunction due to volume overload, pressure overload, or other noxious stimuli, accompanied with renal impairment or hypertension. We recently reported that AM2/IMD mRNA expression was upregulated in the heart, but not changed in the kidney of rats with congestive heart failure (6). Therefore, it is unlikely that volume overload caused the decrease in renal AM2/IMD expression.

In conclusion, AM2/IMD mRNA expression is downregulated in the remnant kidney of rats with renal mass ablation and in the kidney of genetic hypertensive rats. With a decreased expression in proximal renal tubular cells and a decrease in the number of functioning nephrons, the intrarenal AM2/IMD may fail to compensate for renal dysfunction and may not be able to prevent renal injury progression, despite the upregulation of AM and their receptor system (CRLR, RAMP1, and RAMP2). The present study also suggests that the renal AM2/AM system is a clear target for the prevention and treatment of chronic kidney diseases and hypertension.

ACKNOWLEDGEMENTS

The authors are grateful to Hironobu Sasano and the staff members of the Department of Pathology, Tohoku University Graduate School of Medicine for technical assistance, to Kumi Kikuchi for technical assistance, and to the Biomedical Research Core of Tohoku University Graduate School of Medicine for usage of equipment.

GRANTS

This study was supported in part by Grants-in-aid for Scientific Research (C) from the Ministry of Education, Science, Sports and Culture of Japan, by a Research Grant from the Takeda Science Foundation (2005), by Grant-in-Aid for Japan Society for the Promotion of Science fellows, and by the Tohoku University 21st Century Center of Excellence Program, Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation.

DISCLOSURES

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

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