Marinobufagenin Stimulates Fibroblast Collagen Production and Causes Fibrosis in Experimental Uremic Cardiomyopathy


Hypertension 2007;49:215-224; originally published online Dec 4, 2006;
DOI: 10.1161/01.HYP.0000252409.36927.05
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Marinobufagenin Stimulates Fibroblast Collagen Production and Causes Fibrosis in Experimental Uremic Cardiomyopathy


Abstract—We have observed recently that experimental renal failure in the rat is accompanied by increases in circulating concentrations of the cardiotonic steroid, marinobufagenin (MBG), and substantial cardiac fibrosis. We performed the following studies to examine whether MBG might directly stimulate cardiac fibroblast collagen production. In vivo studies were performed using the 5/6th nephrectomy model of experimental renal failure (PNx), MBG infusion (MBG), PNx after immunization against MBG, and concomitant PNx and adrenalectomy. Physiological measurements with a Millar catheter and immunohistochemistry were performed. In vitro studies were then pursued with cultured isolated cardiac fibroblasts. We observed that PNx and MBG increased MBG levels, blood pressure, heart size, impaired diastolic function, and caused cardiac fibrosis. PNx after immunization against MBG and concomitant PNx and adrenalectomy had similar blood pressure as PNx but less cardiac hypertrophy, diastolic dysfunction, and cardiac fibrosis. MBG induced increases in procollagen-1 expression by cultured cardiac fibroblasts at 1 nM concentration. These increases in procollagen expression were accompanied by increases in collagen translation and increases in procollagen-1 mRNA without any demonstrable increase in procollagen-1 protein stability. The stimulation of fibroblasts with MBG could be prevented by administration of inhibitors of tyrosine phosphorylation, Src activation, epidermal growth factor receptor transactivation, and N-acetyl cysteine. Based on these findings, we propose that MBG directly induces increases in collagen expression by fibroblasts, and we suggest that this may be important in the cardiac fibrosis seen with experimental renal failure. (Hypertension. 2007;49:215-224.)

Key Words: cardiomyopathy ■ renal failure ■ transforming growth factor (TGF) β ■ cardiotonic steroids ■ reactive oxygen species ■ fibrosis

Cardiac disease is directly responsible for the extremely high morbidity and mortality seen in patients with end-stage renal disease. Clinically this cardiac disease of renal failure, also called uremic cardiomyopathy, is characterized by diastolic dysfunction and left ventricular hypertrophy in the setting of systemic oxidant stress. Although a number of potential mechanisms, such as elevations in parathyroid hormone, hypertension, and anemia, have been implicated as contributors to the cardiac disease seen in this setting, its pathogenesis is still a bit unclear.

On this background, we have demonstrated previously that the cardiotonic steroid marinobufagenin (MBG), signaling through the Na/K-ATPase, is directly responsible for many features of experimental uremic cardiomyopathy induced by partial nephrectomy (PNx) in the rat. Specifically, we noted that both rats subjected to PNx, as well as rats given MBG supplementation by minipump, developed considerable cardiac hypertrophy and fibrosis by 4 weeks, whereas rats immunized against MBG and subsequently subjected to PNx had attenuation of these changes. From these data, we formulated the hypothesis that MBG might directly induce cardiac fibroblasts to produce collagen, thus producing much of the cardiac fibrosis seen with experimental renal failure. To test this hypothesis and to determine the molecular basis by which this occurred, the following studies were performed.
Methods

Animals
Male, Sprague-Dawley rats were used for all of the studies. All of the animal experimentation described in the article was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals using protocols approved by the Medical University of Ohio Institutional Animal Use and Care Committee.

Experimental Groups
Briefly, Sprague-Dawley rats weighing ~250 g at the time of surgery were subjected to either sham surgery with no MBG infusion (Sham), sham surgery with placement of a minipump infusing MBG at 10 μg/kg per day (MBG), PNx, and PNx after immunization against MBG (PNx-IM). MBG of extremely high purity (>99%) was isolated from the venom of Bufo marinus by Kennedy et al.3 In addition to these maneuvers, a group of PNx animals was subjected to adrenalectomy as well (PNx-ADx).

The heart weight normalized to body weight, left ventricular hemodynamics (eg, τ value, slope of regression line fit to end diastolic pressure versus end diastolic volume generated by inferior vena cava occlusions, all determined with a Millar catheter), plasma [MBG] (determined after extraction on a C-18 column using DELPHIA as described previously4), aldosterone (determined with ELISA kit 10004377, Cayman Chemical,) and cardiac immunohistochemistry (vida infra) were assessed 4 weeks after surgery.

Isolated Cardiac Fibroblasts
Preparation of adult rat cardiac fibroblasts was performed as described previously by Brilla et al4 with modifications.

Western Blot Analysis
Western blot analysis was performed on protein isolated from tissue homogenates, cell culture whole cell lysates, or nuclear extracts as described previously.2

Collagen Synthesis
Collagen synthesis rates were determined by the method of Nishida et al5 with modifications.

Quantitative Measurement of Collagen-1 mRNA
Standardized RT-PCR was used to measure gene expression, with Quantitative Measurement of Collagen-1 mRNA et al5 with modifications. Collagen synthesis rates were determined by the method of Nishida with PNx-IM as compared with PNx. Using the Millar pressure/volume sensor catheter rather than echocardiography in our previous report,3 we observed that PNx induced decreases in end systolic volume and end diastolic volume, as well as increased ejection fraction compared with sham-operated controls. The end systolic volume and end diastolic volume were greater, and the ejection factor values were lower in PNx-IM as compared with PNx. Active relaxation assessed by τ was found to be impaired by both PNx and MBG compared with sham-operated controls, with PNx-IM showing lower values than PNx. Using pressure volume loops generated during vena cava occlusions (representative tracings shown in Figure 1a), we noted that the end diastolic pressure volume relationship (an inverse measurement of passive compliance) was increased in PNx- and MBG-treated animals compared with controls, whereas PNx rats had a lower end diastolic pressure volume relationship than PNx. Both PNx and MBG treatment increased the heart weight/body weight ratio compared with sham-operated controls, whereas PNx-IM animals had lower values than PNx (Table). Examining the ventricular myocyte cross-sectional area determined on trichrome images, we noted that PNx and MBG infusion both induced marked increases, whereas the myocyte cross-sectional area in

Statistical Analysis and Expanded Methods
Statistical analysis and details regarding experimental groups and specific measurements are provided in an online supplement (http://hyper.ahajournals.org).

Results
Effect of Experimental Renal Failure and MBG on Blood Pressure, Cardiac Hemodynamics, and Fibrosis
In the current in vivo studies (data summarized in the Table), we observed that MBG levels were increased in PNx- and MBG-treated rats compared with sham-operated controls. We also saw that both PNx and MBG rats had higher systolic blood pressure than controls and that PNx-IM rats had statistically similar systolic blood pressure values as seen with PNx. Using the Millar pressure/volume sensor catheter rather than echocardiography in our previous report,3 we observed that PNx induced decreases in end systolic volume and end diastolic volume, as well as increased ejection fraction compared with sham-operated controls. The end systolic volume and end diastolic volume were greater, and the ejection factor values were lower in PNx-IM as compared with PNx. Active relaxation assessed by τ was found to be impaired by both PNx and MBG compared with sham-operated controls, with PNx-IM showing lower values than PNx. Using pressure volume loops generated during vena cava occlusions (representative tracings shown in Figure 1a), we noted that the end diastolic pressure volume relationship (an inverse measurement of passive compliance) was increased in PNx- and MBG-treated animals compared with controls, whereas PNx rats had a lower end diastolic pressure volume relationship than PNx. Both PNx and MBG treatment increased the heart weight/body weight ratio compared with sham-operated controls, whereas PNx-IM animals had lower values than PNx (Table). Examining the ventricular myocyte cross-sectional area determined on trichrome images, we noted that PNx and MBG infusion both induced marked increases, whereas the myocyte cross-sectional area in

Effects of PNx, MBG, and PNx-IM on Hemodynamics and Plasma MBG

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sham</th>
<th>PNx</th>
<th>MBG</th>
<th>PNx-IM</th>
<th>PNx-ADx</th>
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</thead>
<tbody>
<tr>
<td>Plasma MBG, pmol/L</td>
<td>277±27</td>
<td>527±36†</td>
<td>484±47†</td>
<td>396±65‡</td>
<td>325±65§</td>
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<tr>
<td>Plasma aldosterone, pg/mL</td>
<td>184±32</td>
<td>2012±320†</td>
<td>295±355§</td>
<td>2492±493‡</td>
<td>228±65§</td>
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<td>Tail cuff measurements</td>
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<tr>
<td>Heart rate, bpm</td>
<td>367±7</td>
<td>388±9</td>
<td>367±9</td>
<td>380±6</td>
<td>365±7</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>102±2</td>
<td>197±6†</td>
<td>136±4†</td>
<td>180±9†</td>
<td>193±6†</td>
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<td>Ventricular hemodynamics</td>
<td></td>
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<tr>
<td>End systolic volume, μL</td>
<td>70±4</td>
<td>35±4†</td>
<td>60±6</td>
<td>68±9§</td>
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<td>End diastolic volume, μL</td>
<td>190±11</td>
<td>151±10*</td>
<td>162±14</td>
<td>188±14†</td>
<td>185±14</td>
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<td>Ejection fraction, %</td>
<td>73±1</td>
<td>79±2†</td>
<td>68±2*</td>
<td>71±1§</td>
<td>71±2§</td>
</tr>
<tr>
<td>τ, ms</td>
<td>10.0±0.3</td>
<td>14.5±0.9†</td>
<td>11.3±0.3*</td>
<td>10.6±0.3§</td>
<td>12.1±0.5¶†</td>
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<tr>
<td>EDV×1000, mm Hg/μL</td>
<td>24±2</td>
<td>52±4†</td>
<td>41±6†</td>
<td>31±3*§</td>
<td>38±4‡§</td>
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<tr>
<td>heart weight/body weight ratio, g/kg</td>
<td>2.5±0.1</td>
<td>3.6±0.2†</td>
<td>2.8±0.1*</td>
<td>2.9±0.11¶</td>
<td>3.3±0.11¶†</td>
</tr>
</tbody>
</table>

Analyses were performed 4 weeks after sham operation (Sham, n=20), partial nephrectomy (PNx, n=20), MBG infusion (MBG, n=20), or immunization against MBG prior to partial nephrectomy (PNx-IM, n=20). Results reported as mean±SEM.

*P<0.05 vs Sham; †P<0.01 vs Sham, comparing PNx-IM and PNx-ADx to PNx; ‡P<0.05; and §P<0.01.
PNx-IM was considerably smaller than that seen with PNx alone (Figure 1b).

Analyzing the immunohistochemistry results, heart tissues from rats subjected to MBG and PNx showed marked increases in collagen-1 and α smooth muscle actin staining. Immunization against MBG attenuated these increases (Figure 1c and 1d). Western blot analysis confirmed that PNx and MBG had 2 to 2.5 times the expression of procollagen-1 and α smooth muscle actin seen with sham-operated controls, whereas PNx-IM expression of both procollagen-1 and α smooth muscle actin was substantially less than that seen with PNx (Figure 1e and 1f).

To determine the molecular mechanism underlying this fibrosis, we examined the expression of several proteins important in fibroblast activation. Specifically, we examined tissue levels of transforming growth factor (TGF)-β, Smad 2/3, and Smad 4, as well as pSmad 2/3. We did not detect significant differences among the experimental
groups in the cardiac expression of these proteins (Figure 2a–2d).

A separate group of animals (N=11) was also subjected to PNx-ADx with physiological replacement of glucocorticoids and aldosterone. These animals developed a similar degree of hypertension compared with PNx but were noted to have much lower plasma MBG and aldosterone levels, as well as substantially lower heart/weight body weight ratio compared with PNx alone (Table). Moreover, these animals subjected to PNx-ADx had almost no evidence for cardiac fibrosis based on trichrome staining or immunohistochemistry staining for collagen-1 or α smooth muscle actin (see the online data supplement).

**Effect of Cardiotonic Steroids on Fibroblast Collagen Expression**

To further examine the molecular basis of this cardiac fibrosis, isolated cardiac fibroblasts were subjected to increasing doses of MBG (10⁻¹⁰, 10⁻⁹, and 10⁻⁸ M). After 24 hours of exposure to 10⁻⁹ and 10⁻⁸ M MBG, procollagen content determined by Western blot was increased 2-fold (both P<0.01; Figure 3a). This phenomenon was not specific for MBG; other cardiotonic steroids also induced similar increases in procollagen content (Figure 3b). Of interest, the threshold for effect for MBG seemed to be between 10⁻¹⁰ and 10⁻⁹ M, whereas for ouabain, which circulates at similar concentrations in uremic rats, the threshold was ≈10 times higher (ie, between 10⁻⁸ and 10⁻⁷ M). For both MBG and ouabain, the threshold for inducing collagen expression was log units below the doses necessary for detectable effects on ⁸⁶Rb uptake in these cells (Figure 3c). In parallel studies examining radiolabeled proline incorporation into collagen, we observed that 10⁻⁹ and 10⁻⁸ M MBG induced significant increases in both proline incorporation into total protein, both matrix and supernatant. Using collagenase digestion, we observed that the vast majority of the proline incorporation was into collagen (Figure 3d). Using standardized RT-PCR, we observed a doubling of mRNA for collagen-1 at 24 hours in response to 10 nM of MBG (Figure 3e). However, we did not detect any increases in procollagen stability (determined by examining procollagen-1 expression after...
exposure to cycloheximide) in response to this concentration of MBG (Figure 3f).

Effect of Inhibition of Na/K-ATPase Signaling on MBG-Stimulated Collagen Expression

To examine whether cardiotonic steroids induced collagen synthesis by signaling through the Na/K-ATPase, we performed the following studies. First, we used pharmacological antagonism at several steps in the Na/K-ATPase cascade. Specifically, we used pharmacological antagonism of Src activation with PP2, nonspecific tyrosine kinase inhibition with herbimycin, inhibition of EGFR transactivation with AG1478, and nonspecific antioxidant administration with N-acetyl cysteine. Each of these maneuvers prevented MBG stimulation of collagen synthesis (Figure 4a). To confirm these data, we also examined radiolabeled proline incorporation in response to MBG in the presence and absence of either PP2 or N-acetyl cysteine. As was the case for procollagen expression, both PP2 and N-acetyl cysteine prevented increases in proline incorporation into collagen in the primary fibroblast cultures (Figure 4b). Next we performed studies in the SYF and SYF/H11001 cells (details available in the online supplement). SYF+ cells responded to MBG and ouabain in a very similar way as the primary cardiac fibroblast cultures with respect to upregulation of procollagen expression, whereas the SYF cells had essentially no response to either MBG or ouabain (Figure 4c).

Relationship Between TGF-β and MBG-Stimulated Collagen Production

To further examine the molecular mechanisms by which cardiotonic steroids induce collagen production in fibroblasts, we examined the effects of MBG on TGF-β expression, as well as the expression of Smad 2/3, Smad 4, and pSmad 2/3. As was the case for the in vivo experiments described earlier, we did not

Figure 2. Representative Western blot for and quantitative densitometric data shown for (a) TGF-β1, (b) Smad 2/3, (c) Smad 4, and (d) pSmad 2/3. Data derived from N=6 experiments in each group and shown as mean±SEM. Note similar expression of these proteins in all of the 4 experimental groups.
observe significant changes in TGF-β, Smad 2/3, Smad 4, or pSmad 2/3 expression in vitro (Figure 5a). Next, we examined whether TGF-β induced collagen production and whether there was synergism between TGF-β and MBG. In the primary cultured cells, we saw similar effects of TGF-β (5 ng/mL) on procollagen expression as observed with cardiotonic steroids; however, we did not note any synergism between TGF-β (5 ng/mL) and MBG (10 nM). However, it is important to point out that we never completely serum starve the primary cultures, and because serum is always present, some TGF-β is always present.
To address this further, we also examined the effect of the TGF-β receptor antagonist, SB431542, on MBG stimulated collagen production. Interestingly, SB431542 at 100-μmol/L concentration did not reduce procollagen expression below baseline on our Western blots (Figure 5b) but did decrease radiolabeled proline incorporation below that seen with control cells (Figure 5c). The SB431542 completely blocked both TGF-β and MBG (10 nM) stimulation of collagen expression and radiolabeled proline incorporation (Figure 5b and 5c).

**Discussion**

Cardiac fibrosis is an important component of many cardiomyopathies, and it is a very characteristic component of uremic cardiomyopathy.\(^8,9\) Our group and others have observed that MBG and other cardiotonic steroids induce a signal transduction cascade through the plasmalemmal Na/K-ATPase residing in caveolae, which results in activation of Src, transactivation of the EGFR, generation of reactive oxygen species, and, ultimately, activation of p42/44 mitogen-activated protein kinase.\(^10-16\) Interestingly, a number of clinical situations associated with cardiac fibrosis other than renal failure are associated with increased circulating concentrations of cardiotonic steroids (eg, hypertension, primary hyperaldosteronism, and congestive heart failure).\(^17,18\)

Although it is preliminary to discuss the possible relevance of our findings to cardiomypathies other than renal failure, we should point out that Ferrandi et al\(^19\) have observed that antagonism of endogenous cardiotonic steroids with PST 2238 ameliorates hypertension, as well as cardiac hypertrophy in Milan hypertensive rats.

In the current study, we confirmed that PNx and MBG treatment induce similar but not identical phenotypic changes in hemodynamics and cardiac morphology. It is quite likely that some factors other than MBG contribute to the phenotypic changes seen in PNx. That said, both PNx-IM and PNx-ADx, which reduce circulating MBG, substantially attenuate the cardiac functional and morphological changes without significantly affecting blood pressure. We should...
point out that experiments in the PNx-ADx model were performed because we reasoned that as adrenal cells grown in culture seem to make MBG,\(^{20,21}\) it was likely that this procedure would lower the circulating levels of this hormone. However, whereas our data in the PNx-ADx animals support the concept that the adrenal gland is the major (but not the only) site of MBG production in vivo, it is also possible that other hormones made in the adrenal gland modulate MBG production elsewhere. Further work will be necessary to clarify exactly where MBG is produced under normal and pathological conditions.

With these findings implicating MBG in the pathogenesis of cardiac fibrosis, we were particularly interested in the molecular mechanisms underlying this hormone. Interestingly, evidence for increases in TGF-\(\beta\) or signaling through the Smad proteins was not evident. We stress that these data do not exclude a role for TGF-\(\beta\) in this process, because earlier increases in these proteins, translocation of the Smads, and/or a permissive role for signaling through this pathway (vida infra) could certainly be present.

Based on these in vivo data, we pursued studies in isolated cardiac fibroblasts. We observed that MBG in physiological concentrations directly stimulated the fibroblasts to produce more collagen. This increase in collagen production was also observed with other cardiotonic steroids, although the threshold concentration seemed to be \(-1\) log unit lower for MBG than for ouabain. We emphasize that the concentration of both MBG and ouabain necessary to stimulate collagen expression was lower for both substances than that needed to appreciably inhibit \(^{38}\)Rb uptake. Further evidence for this phenomenon being dependent on signaling through the Na/K-ATPase was that this increase was prevented by reactive oxygen species scavenging, antagonism, or knockout of Src, as well as prevention of EGFR transactivation, maneuvers that we have demonstrated previously to block signal transduction through the Na/K-ATPase signalosome.\(^{13,14,22-24}\) We also observed that the increases in collagen production were associated with increases in proline incorporation, as well as increases in mRNA for collagen-1. No increase in procollagen-1 stability could be demonstrated in response to MBG.

Although increases in TGF-\(\beta\) or the Smad proteins were also absent in the fibroblasts treated with MBG, it is important to note that the fibroblasts that we studied were never truly serum starved. In fact, based on a Hyclone web page (http://www.hyclone.com/pdf/atsv19n3.pdf), we would estimate that the fibroblasts were exposed to \(-0.12\) ng/mL of TGF-\(\beta\) even when cultured in the serum-depleted (1% FBS)
medium. This may, in part, explain why SB431542 was so effective in preventing MBG-stimulated collagen production. Working with a similar preparation, Lijnens and Petrov\textsuperscript{25} noted that long incubations (48 hours) and high concentrations of TGF-\(\beta\) (15 ng/mL) were necessary to induce maximal (2 times) increases in collagen production. We should also note that TGF-\(\beta\) blockade with SB431542 actually decreased proline incorporation below baseline, even in the setting of MBG synthesis, although this same pharmacological maneuver only reduced procollagen expression to baseline when measured with Western blot. We suspect that other mechanisms of regulation of collagen synthesis (eg, procollagen stability) might come into play when the TGF-\(\beta\) pathway is interrupted, although we did not explore this point further in the current studies. On balance, our data argue, albeit preliminarily, against a major role for TGF-\(\beta\) or upregulation of Smad proteins in cardiotonic steroid–induced increases in fibroblast collagen production.

Our data suggest that, in our experimental rodent model, MBG is implicated in the pathogenesis of the cardiac fibrosis, and the concentrations of MBG that develop in this setting, as well as other cardiotonic steroids, have in vitro effects that are consistent with this observation. One issue that immediately comes to mind is whether the clinical use of digitals might have similar effects. To this question, we would suggest the following possibilities. First, it may be that the free concentrations of digoxin that occur in vivo are not sufficient to induce substantial cardiac fibrosis. Total digoxin levels are typically maintained <2 ng/mL in patients treated with digoxin, a concentration that corresponds with \(\approx\)2.5-nM concentration. However, only 70\% to 80\% of the plasma digoxin is free, and the free concentration might fall below the threshold level of digoxin necessary to stimulate human cardiac (or other tissue) fibroblasts. Perhaps more relevant, we observed a fairly flat dose–response curve to MBG and ouabain with respect to stimulation of fibroblast collagen production once the threshold for an effect was reached. We suggest that in the setting of heart failure, a condition known to have associated increases in MBG and other cardiotonic steroids, the threshold level of digoxin necessary to stimulate human cardiac (or other tissue) fibroblasts. Perhaps more relevant, we observed a fairly flat dose–response curve to MBG and ouabain with respect to stimulation of fibroblast collagen production once the threshold for an effect was reached. We suggest that in the setting of heart failure, a condition known to have associated increases in MBG and other cardiotonic steroids, the addition of digoxin at therapeutic doses might not have a detectable effect. Finally, we would point out that a systemic examination of whether digoxin induces or influences cardiac fibrosis in humans has not been thoroughly investigated, although the clinical efficacy of this agent in treating congestive heart failure has been extensively examined.\textsuperscript{26–28} It is important to note that the rate at which humans develop cardiac fibrosis seems to be considerably slower than that seen with rodents,\textsuperscript{29} which might further obfuscate whether digoxin has profibrotic effects in clinical subjects.

In summary, we observed that concentrations of MBG similar to that which develop in experimental renal failure produced increased synthesis of collagen in primary cardiac fibroblasts grown in culture in a manner dependent on volume expansion states, the implications of our observations may extend to other situations complicated by cardiac fibrosis.

Perspectives
Cardiac fibrosis is an important component of cardiac diseases seen in a variety of disease states. Our data in the experimental renal failure model suggest that cardiotonic steroids, such as MBG, may contribute in a very substantial role in the cardiac fibrosis seen in this setting. Because increases in MBG are likely to accompany a variety of volume expansion states, the implications of our observations may extend to other situations complicated by cardiac fibrosis.

Acknowledgment
We thank Carol Woods for her excellent secretarial assistance.

Sources of Funding
Portions of this study were supported by the American Heart Association (D.J.K., fellowship award from the Ohio Valley Affiliate) and the National Institutes of Health (HL67963). This work has also been supported in part by the Intramural Research Program, National Institute on Aging, National Institutes of Health.

Disclosures
None.

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