ADP-Ribosyl Cyclase in Rat Vascular Smooth Muscle Cells
Properties and Regulation

Frederico G.S. de Toledo, Jingfei Cheng, Mingyu Liang, Eduardo N. Chini, Thomas P. Dousa

Abstract—We investigated whether ADP-ribosyl cyclase (ADPR-cyclase) in rat vascular smooth muscle cells (VSMCs) has enzymatic properties that differ from the well-characterized CD38-antigen ADPR-cyclase, expressed in HL-60 cells. ADPR-cyclase from VSMCs, but not CD38 ADPR-cyclase from HL-60 cells, was inhibited by gangliosides (10 μmol/L) GT1b, GD1a, and GM3. Preincubation of membranes from CD38 HL-60 cells, but not from VSMCs, with anti-CD38 antibodies increased ADPR-cyclase activity; CD38 antigen was detected both in VSMCs and in HL-60 cells. ADPR-cyclase in VSMC membranes was more sensitive than CD38 HL-60 ADPR-cyclase to inactivation by N-endoglycosidase F and to thermal inactivation at 45°C. The specific activity of ADPR-cyclase in membranes from VSMCs was >20-fold higher than in membranes from CD38 HL-60 cells. Most importantly, VSMC ADPR-cyclase was inhibited by Zn2+ and Cu2+ ions; the inhibition by Zn2+ was dose dependent, noncompetitive, and reversible by EDTA. In contrast, Zn2+ stimulated the activity of CD38 HL-60 ADPR-cyclase and other known types of ADPR-cyclases. Retinoids act either via the nuclear receptor retinoic acid receptor or retinoid X receptor, including all-trans retinoic acid (atRA), and panagonist 9-cis-retinoic acid–upregulated VSMC ADPR-cyclase; the stimulatory effect of atRA was blocked by actinomycin D and cycloheximide. 1,25(OH)2–Vitamin D3 (calciferol) stimulated VSMC ADPR-cyclase dose dependently at subnanomolar concentrations (ED50 ≈ 56 pmol/L). Oral administration of atRA to rats resulted in an increase of ADPR-cyclase activity in aorta (≈+60%) and, to a lesser degree, in myocardium of left ventricle (+18%), but atRA had no effect on ADPR-cyclases in lungs, spleen, intestinal smooth muscle, skeletal muscle, liver, or testis. Administration of 3,5,3′-triiodothyronine (T3) to rats resulted in an increase of ADPR-cyclase activity in aorta (≈+89%), but not in liver or brain. We conclude the following: (1) ADPR-cyclase in VSMCs has enzymatic properties distinct from “classic” CD38 ADPR-cyclase, especially sensitivity to inhibition by Zn2+ and Cu2+; (2) ADPR-cyclase in VSMCs is upregulated by various retinoids, calcitriol, and T3 in vitro; and (3) administration of atRA and T3 increases ADPR-cyclase in aorta in vivo. We suggest that the cADPR signaling system plays an important role in the regulation of VSMC functions in response to steroid superfamily hormones. (Circ Res. 2000;86:1153-1159.)

Key Words: vascular smooth muscle cells ■ calciferols ■ antibodies ■ ADP-ribose ■ retinoids

It is well established that inositol 1,4,5-triphosphate (IP3) triggers Ca2+ release, via IP3 receptor/channel, from endocellular stores to cytoplasm.1 Recently,2,3 the nucleotide cyclic ADP-ribose (cADPR) was found to stimulate Ca2+ release from intracellular stores via ryanodine receptor/channel.1–3 Enzymatic synthesis and hydrolysis of cADPR are catalyzed by ADP-ribosyl cyclase (ADPR-cyclase) and cADPR-hydrolyase, respectively.2–4 ADPR-cyclase is regulated by hormones in vitro4–6 and in vivo.7 cADPR-ribose modulates Ca2+ fluxes in several types of contractile cells,8–10 including vascular smooth muscle cells (VSMCs).9,11–13 Furthermore, cADPR, alone and/or via interaction within the IP3 signaling system,14 regulates contractility8,10 and possibly other cellular functions.13 We recently observed that ADPR-cyclase present in rat VSMCs14 is upregulated by all-trans retinoic acid (atRA) and by 3,5,3′-triiodothyronine (T3).15 Our observations, and reports on regulatory action of cADPR in muscle cells,8–13 prompted us to investigate properties and regulatory responses of ADPR-cyclase of VSMCs to hormones.

To date, most information is available about ADPR-cyclase that is identical with lymphocytic CD38 antigen, a molecule that has not only ADPR-cyclase but also cADPR-hydrolyase and NADase activities.2,3,16,17 This ADPR-cyclase (referred to hereafter as CD38 ADPR-cyclase) was found initially in hematopoietic cells,2,3 but was later also detected in several cell types and tissues.16–18 However, CD38 was not detected in vessels or in intestinal smooth muscle,18 suggesting that ADPR-cyclase in VSMCs may have properties different from CD38 ADPR-cyclase.3,16,17 Another well-described vertebrate ADPR-cyclase is the CD157 (BST-1/PB-3) antigen.4,17,19

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According to a recent report, knockout mice that lack CD38 are capable of living at least 8 months without abnormalities and suffer only nonlethal impairment of the immune system. Similarly, knockout mice that are deficient in BST-1 (CD157) antigen display only limited immune deficiency. From these observations, it can be inferred that, besides CD38 ADPR-cyclase and BST-1 ADPR-cyclase, other isozymes (or isoforms) of ADPR-cyclase must exist to sustain cADPR signaling system in diverse cell types, including VSMCs. These considerations prompted us to investigate some fundamental properties and regulation of ADPR-cyclase in rat VSMCs.

Materials and Methods

Cell Culture
VSMCs were isolated from rat abdominal aorta explant outgrowths, as previously described. Skin fibroblasts were obtained from dermis of rat abdominal skin as described previously. Mesangial cells were isolated from rat glomeruli and grown in primary culture as described previously. Cells were cultured in RPMI 1640 with 20% bovine FCS, 100 U/L penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B.

Preparation of Subcellular Fractions
The total membrane fraction for each type of cultured cell was prepared similarly as previously described for VSMCs. Cells were rinsed 3 times with ice-cold PBS and scraped into ice-cold homogenization buffer containing 40 mmol/L Tris-HCl (pH 7.2) and 0.25 mol/L sucrose (TSB). Cells were disrupted by and then centrifuged at 2000g for 10 minutes. The supernatant was centrifuged at 40 000g for 30 minutes. The pellet, resuspended in TSB, is referred to hereafter as the membrane fraction. Protein content in the homogenate was measured by the method of Lowry et al. Membrane fractions from rat tissues were prepared using a procedure similar to that used for cultured cells. Animal use in the present study was approved by the Mayo Clinic and Foundation Animal Care Committee.

In Vivo Studies
Studies in vivo were conducted on male Sprague-Dawley rats (200 to 250 g body weight). The rats were treated with alR A 3 mg (SC)/kg body weight per day for 3 days, and controls received vehicle (DMSO). Experiments examining the effects of T3 were done on surgically thyroparathyrectomized (TPTX) rats. Rats were treated 2 mg (IP) T3/kg body weight per day for 10 days before euthanization. Tissues were homogenized, and then membrane fraction was prepared as above.

ADPR-Cyclase Activity
Activity was measured using nicotinamide guanine dinucleotide (NGD) or nicotinamide hypoxanthine dinucleotide (NHD). Fluorescence was monitored with a 300-nm excitation wavelength and 410-nm emission wavelength using a Hitachi F-2000 spectrophotofluorometer. The Δ change in fluorescence was calibrated from standard curves generated from known concentrations of cGDP or cGDP.

Enzymatic Deglycosylation
Suspected membranes from VSMCs or HL-60 cells (1 to 2 mg/tube) were incubated without (controls) or with recombinant N-endoglycosidase F (16 U/mL) in a buffer containing final concentrations as follows: in mmol/L, EDTA 5, PMSF 0.1, and Tris-HCl (pH 7.4) 40; 0.5% Triton X-100; 5 μg/mL leupeptin; and 3 μg/mL pepstatin.

The effect of anti-CD38 antibodies (Abs) on ADPR-cyclase in membranes from VSMCs and HL-60 cells was determined with either goat anti-mouse CD38 Abs or goat anti-human CD38 Abs (Santa Cruz Biotechnology), diluted 1:1.

Western Blots for CD38
The cells were homogenized in 20 mmol/L Tris-HEPES buffer, pH 7.4, containing 1% Triton X-100. The sample of membranes (30 μg protein per lane) was subjected to 10% SDS-PAGE, and proteins were transferred onto nitrocellulose membrane and Western blot analysis using a standard technique. To determine thermal stability of ADPR-cyclase, membrane preparations were incubated at either 37°C or 45°C for various time periods indicated in the Results, and the ADPR-cyclase activity was measured in aliquots.

All values are expressed as mean±SEM. When appropriate, the results were statistically evaluated using the Student t test for paired or group comparisons. The values were considered statistically significant at P<0.05.
membranes from CD38 HL-60 cells with the same Abs significantly increased the ADPR-cyclase activity (Figure 2C). Incubation of CD38 HL-60 membranes with human anti-CD38 Abs (C-19) had a similar effect (data not shown). Western blot analysis shows that rat anti-CD38 Abs react well with VSMC membranes (45-kDa band), but only slightly with a 45-kDa band in HL-60 membranes (Figure 2E). Conversely, human anti-CD38 Abs reacted strongly with membranes from the HL-60 cell and, to a lesser degree, with VSMC membranes (Figure 2E).

Incubation with N-glycosidase F caused a minor reduction of ADPR-cyclase activity in membranes from CD38 HL-60 cells, but markedly diminished ADPR-cyclase in membranes from VSMCs (Figure 2D). Furthermore, ADPR-cyclase from VSMCs was more sensitive than the HL-60 cell enzyme to thermal inactivation. When incubated at 45°C, the rate of thermal inactivation. When incubated at 45°C, the rate of ADPR-cyclase activity in membranes from CD38 HL-60 cells was more sensitive than the HL-60 cell enzyme to thermal inactivation. When incubated at 45°C, the rate of ADPR-cyclase activity in membranes from CD38 HL-60 cells was more sensitive than the HL-60 cell enzyme to thermal inactivation. When incubated at 45°C, the rate of ADPR-cyclase activity in membranes from CD38 HL-60 cells was more sensitive than the HL-60 cell enzyme to thermal inactivation.

We examined the effect of Zn$^{2+}$ on ADPR-cyclase in rat fibroblasts and mesangial cells grown in cell culture (Table 1). ADPR-cyclase activities from rat mesangial cells and rat skin fibroblasts were inhibited by Zn$^{2+}$ in a way similar to that in VSMCs (Table 1).

### Effect of Hormones

In accordance with our previous study, incubation of VSMCs with atRA and T$_3$ enhanced ADPR-cyclase, and the upregulatory effect of atRA was blocked by actinomycin D and cycloheximide (Figure 3). We examined whether such an upregulating effect is also shared with retinoids that have a similar mechanism of action. The retinoid panagonist 9-cis-retinoic acid stimulated the activity on ADPR-cyclase (Table 2). A survey of the action of several retinoids that act through either of the nuclear receptors retinoic acid receptor (RAR) or retinoid X receptor (RXR), which are both present in VSMCs, indicates that all tested compounds can upregulate activity of VSMC ADPR-cyclase (Table 2). However, unlike in VSMCs, ADPR-cyclase in rat fibroblasts grown in primary culture was not stimulated by atRA (data not shown). Finally, we found that the 1,25(OH)$_2$–vitamin D$_3$ (calcitriol) does upregulate ADPR-cyclase in a dose-dependent manner (Figure 4); however, its precursor with low biologic activity, 25(OH)–vitamin D$_3$, was inactive (data not shown).

### In Vivo Studies

To determine whether ADPR-cyclase in VSMCs is upregulated by hormones in vivo, rats were treated with atRA 3 mg (SC)/kg body weight per 24 hours for 3 days, and ADPR-cyclase activity was determined in homogenates and in membrane fractions from harvested tissues. There was no significant difference between control rats and atRA-administered rats in ADPR-cyclase activities from spleen, lung, intestinal smooth muscle, hind-limb skeletal muscle, or liver (Table 3). In atRA-treated rats, ADPR-cyclase activity was higher in myocardium of left ventricle ($\Delta+18\%$; $P<0.05$) compared with controls (Table 3). ADPR-cyclase activity, both in homogenates and membrane fractions, from aorta was higher ($\Delta%\approx60\%$; $P<0.05$) than in controls (Figure 5). In another experiment, TPTX rats were treated with 2 mg (IP) T$_3$/kg body weight per day for 10 days.

### Table 1. Effects of Zn$^{2+}$ (0.1 or 1 mmol/L) Upon ADPR-Cyclase Activity in Cells and Tissues From Rat and Other Species

<table>
<thead>
<tr>
<th>Cell/Tissue</th>
<th>Control</th>
<th>0.1 mol/L Zn</th>
<th>1 mmol/L Zn</th>
<th>n</th>
<th>$\Delta$% Change</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat VSMCs (NGD)</td>
<td>56.8±0.6</td>
<td>2.1</td>
<td>5</td>
<td></td>
<td>96±1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Rat VSMCs† (NHD)</td>
<td>4.6±1.2</td>
<td>...</td>
<td>0.025</td>
<td>6</td>
<td>−94±1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Rat fibroblast</td>
<td>7.9±1.2</td>
<td>2.8±0.6</td>
<td>3</td>
<td></td>
<td>−65±3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Rat mesangial cells</td>
<td>9.7±0.34</td>
<td>...</td>
<td>0.7±0.08</td>
<td>4</td>
<td>−93±1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HL-60 cells</td>
<td>1.7±0.27</td>
<td>2.45±0.36</td>
<td>4</td>
<td></td>
<td>+44±8</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>HL-60 cells† (NHD)</td>
<td>0.21±0.02</td>
<td>1.31±0.12</td>
<td>4</td>
<td></td>
<td>+538±48</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Aplysia californica†</td>
<td>62.3±19.0</td>
<td>91.5±29.0</td>
<td>3</td>
<td></td>
<td>+40±7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Sea urchin eggs§</td>
<td>41.0±12.0</td>
<td>118.0±3.0</td>
<td>2</td>
<td></td>
<td>+191±12</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

| Membranes from cultured cells and rat tissues were prepared as described in Materials and Methods; sea urchin egg homogenate was prepared as described previously. 10,33 ADPR-cyclase activity was determined with 0.4 mmol/L NGD$^-$ substrate both without (control) and with addition of 0.1 mmol/L or 1 mmol/L ZnCl$_2$.

| $^*$Significance of difference by t test.

†Determined with 0.4 mol/L NHD substrate. Data are mean±SEM of n independent experiments.

‡Specific activity of Aplysia ADPR-cyclase is expressed in $\mu$mol cGDPR/min per mg protein.

§Specific activity of sea urchin egg ADPR-cyclase is expressed in pmol cGDPR/min per mg protein.

| $\dagger$Intestinal wall with mucosa and submucosa scraped off.
Kinin HL-60 cells by Zn$^{2+}$ addition of 1 mmol/L ZnCl$_2$, CuCl$_2$, MnCl$_2$, or MgCl$_2$. Data are from VSMCs (left) and HL-60 cells (right). Results are expressed as SEM (n = 6 mean ± SEM). IC$_{50}$ (mM) was 0.1 mmol/L ZnCl$_2$.

Enhancement of CD38 ADPR-cyclase in HL-60 cells by Cu$^{2+}$ was 0.1 mmol/L ZnCl$_2$.

Enhancement by Zn$^{2+}$ was 0.1 mmol/L ZnCl$_2$.

Enhancement of 2.5 mmol/L EDTA (all final concentrations), which caused partial inhibition of VSMC ADPR-cyclase. b, Conditions are similar to those in panel a, except that the concentration of added Zn$^{2+}$ (arrow) was 0.1 mmol/L ZnCl$_2$.

Figure 1. A, Dose dependence of Zn$^{2+}$ effects on ADPR-cyclase in VSMCs (top) and in CD38 HL-60 cells (bottom). ADPR-cyclase activity was determined with NHD as substrate. Enzyme activity was determined without (control) or with addition of various concentrations of ZnCl$_2$ (abscissa), and changes are expressed as Δ%.

B, Kinetics of the inhibition of ADPR-cyclase from VSMCs by Zn$^{2+}$. The parameters ($K_m$, $V_{max}$) were determined by Eadie-Hofstee plot. ADPR-cyclase was assayed, with NHD substrate, without (control, dashed line) ($K_m$=39±12 μmol/L) 1 mmol/L ZnCl$_2$. $V_{max}$ with 1 mmol/L Zn$^{2+}$ was ~50% lower than $V_{max}$ of control. Data are mean ± SEM of 2 independent experiments. C, Effect of divalent metals (Zn$^{2+}$, Cu$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$) on ADPR-cyclase in membranes from VSMCs (left) and HL-60 cells (right). Results are expressed as Δ% change of ADPR-cyclase activity (assayed with NHD) from controls (no additions), as compared with activity with 1 mmol/L ZnCl$_2$, CuCl$_2$, MnCl$_2$, or MgCl$_2$. Data are mean ± SEM (n = 3 to 4).

D, Reversibility of Zn$^{2+}$ effects on the activities of ADPR-cyclases in membranes from VSMCs and membranes from HL-60 cells assayed with NHD as substrate. Ordinates: fluorescence of product cIDPR; abscissae: elapsed time (minutes); for details see Materials and Methods. Both VSMCs and HL-60 membranes were probed with anti-CD38 Abs (for details see Materials and Methods). Both VSMCs and HL-60 membranes were probed with anti-human CD38 Ab (C-19) (VSMC band density = 44±3%, n = 3, compared with HL-60 bands taken as 100%).
The ADPR-cyclase in homogenates of aorta from T₃-treated rats was higher (Δ±SEM; P<0.05) than in controls, whereas ADPR-cyclase activities in homogenates from brain and in liver were not different between control and T₃-treated rats (Figure 5).

**Discussion**

We demonstrate that ADPR-cyclase in rat VSMCs has distinct properties and is sensitive to regulation by several hormones both in vitro and in vivo. According to a recent report of an immunohistochemical study, the commonly occurring CD38 antigen that has ADPR-cyclase activity was not found in vasculature or other smooth muscles, thus suggesting that ADPR-cyclase in VSMCs differs from the well-characterized CD38 ADPR-cyclase.

Indeed, specific activity of ADPR-cyclase in membranes of VSMCs is many times (20) higher than CD38 ADPR-cyclase in membranes of HL-60 cells (Table 1) and was more sensitive to inactivation by N-glycosidase F (Figure 2D), to thermal inactivation, and to inhibitory effects of gangliosides (Figures 2A and 2B). Although the molecular basis of these differences remains to be elucidated, the findings clearly show that VSMC ADPR-cyclase differs in structure of enzyme-glycoprotein and/or its insertion into the membrane. Western blot analysis showed the presence of CD38 both in membranes from HL-60 cells and in membranes from VSMCs (Figure 2E). Interestingly, unlike CD38 HL-60 ADPR-cyclase, the VSMC ADPR-cyclase was not influenced by interaction with anti-CD38 Abs directed against C-terminal part of CD38 molecule (Figure 2C) that contains ADPR-cyclase activity.

Conceivably, because VSMC ADPR-cyclase is 20-fold more active than CD38 ADPR-cyclase (Table 1), CD38 ADPR-cyclase may well coexist within rat VSMC membranes with another, distinct VSMC ADPR-cyclase, but probably constitutes only a minute portion of overall ADPR-cyclase activity. The most prominent unique property of VSMC ADPR-cyclase is its sensitivity to inhibition by Zn²⁺, a property that is in contrast to all hitherto-described ADPR-cyclases, including CD38 ADPR-cyclase, BST-1 ADPR-cyclase, and ADPR-cyclase from sea urchin eggs (Table 2, Figure 4); all of these enzymes are stimulated, rather than inhibited, by Zn²⁺.

We propose the presence of a novel variety of ADPR-cyclase in VSMCs that is distinct from enzymes described to date. The results presented herein show that VSMC ADPR-cyclase is upregulated by hormones. The mechanism by which studied hormones/agents upregulated ADPR-cyclase in VSMCs was not analyzed in the present study. However, it

**TABLE 3. Upregulation of ADPR-Cyclase in VSMCs by Various Retinoids**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Nuclear Receptor Affinity</th>
<th>Concentration (µmol/L)</th>
<th>ADPR-Cyclase Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>atRA</td>
<td>RAR&gt;RXR</td>
<td>24%*</td>
<td>&lt;</td>
</tr>
<tr>
<td>9-cis RA</td>
<td>RAR=RAR</td>
<td>108%</td>
<td>=</td>
</tr>
<tr>
<td>AM580</td>
<td>RARc</td>
<td>42%</td>
<td>&lt;</td>
</tr>
<tr>
<td>TTNPB</td>
<td>RAR</td>
<td>39%</td>
<td>&lt;</td>
</tr>
<tr>
<td>Methoprene acid</td>
<td>RXR</td>
<td>69%</td>
<td>≈</td>
</tr>
</tbody>
</table>

*Activities of ADPR-cyclase in membrane fractions from homogenates of tissues from control rats and rats treated with 3 mg aTRA· kg⁻¹· d⁻¹ for 3 days (see Materials and Methods); the activity of ADPR-cyclase is expressed in nmol cGMP/min per mg protein. Values are means±SEM; number of observations in parentheses.

*For significance of difference between aTRA-treated and controls (by t test for group comparison). No activity of ADPR-cyclase was detected in membrane or supernatant fractions from homogenates of testicular tissue.
might be at least assumed that the studied hormones, ie, retinoids, T₃, and vitamin D₃, also exert their upregulatory effect on ADPR-cyclase by binding to requisite nuclear receptors and via transcriptional control mechanisms that are typical for hormones of steroid superfamily. Inhibition of the upregulatory effect of atRA on ADPR-cyclase by actinomycin D and cycloheximide indeed suggests that regulation of ADPR-cyclase requires ongoing DNA and protein synthesis (Figure 3).

In conclusion, we report that VSMCs are endowed with highly active ADPR-cyclase and differ in a number of properties from the widespread CD38-antigen ADPR-cyclase. The VSMC ADPR-cyclase activity is most likely upregulated by retinoids that act through either RAR or RXR, or via heterodimers, and by 2 other hormones, calcitriol and T₃. We found the presence of a unique ADPR-cyclase in VSMCs derived from aorta, liver, and brain of control TPTX rats (C,  loophole) and rats treated with atRA 3 mg/kg body weight per 3 days (atRA, □). Data are mean±SEM of 6 to 9 rats. *Significant activity (P<0.01, t test) higher than corresponding value of homogenate. Bottom, Activity of ADPR-cyclase in homogenates from aorta, liver, and brain of control TPTX rats (C,  loophole) and TPTX rats treated for 10 days with 2 mg (IP) T₃/kg body weight per day (T₃, □ loophole). Data are mean±SEM of 3 to 5 rats.

Figure 5. Effect of in vivo administration of atRA or T₃ on ADPR-cyclase (assayed with NGD substrate) in aorta; for details see Materials and Methods. Top, Activity of ADPR-cyclase in homogenates and in membrane fraction of aortic tissue of control rats (C,  loophole) and rats treated with atRA 3 mg/kg body weight per 3 days (atRA, □). Data are mean±SEM of 6 to 9 rats. *Significant activity (P<0.01, t test) higher than corresponding value of homogenate. Bottom, Activity of ADPR-cyclase in homogenates from aorta, liver, and brain of control TPTX rats (C,  loophole) and TPTX rats treated for 10 days with 2 mg (IP) T₃/kg body weight per day (T₃, □ loophole). Data are mean±SEM of 3 to 5 rats.

Acknowledgments

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-30759 and by the Mayo Foundation. F.G.S.d.T., J.C., and E.N.C. were postdoctoral research fellows supported by the Mayo Foundation. E.N.C. was also supported by the National Kidney Foundation. We acknowledge Michael A. Thompson and John Walker for providing excellent technical assistance.

References


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Circ Res. 2000;86:1153-1159
doi: 10.1161/01.RES.86.11.1153

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