Central Role for the Cardiotonic Steroid Marinobufagenin in the Pathogenesis of Experimental Uremic Cardiomyopathy

David J. Kennedy, Sandeep Vetteth, Sankaridrug M. Periyasamy, Mohamed Kanj, Larisa Fedorova, Samer Khouri, M. Bashar Kahaleh, Zijian Xie, Deepak Malhotra, Nikolai I. Kolodkin, Edward G. Lakatta, Olga V. Fedorova, Alexei Y. Bagrov, Joseph I. Shapiro

Abstract—Patients with chronic renal failure develop a “uremic” cardiomyopathy characterized by diastolic dysfunction, cardiac hypertrophy, and systemic oxidant stress. Patients with chronic renal failure are also known to have increases in the circulating concentrations of the cardiotonic steroid marinobufagenin (MBG). On this background, we hypothesized that elevations in circulating MBG may be involved in the cardiomyopathy. First, we observed that administration of MBG (10 μg/kg per day) for 4 weeks caused comparable increases in plasma MBG as partial nephrectomy at 4 weeks. MBG infusion caused increases in conscious blood pressure, cardiac weight, and the time constant for left ventricular relaxation similar to partial nephrectomy. Decreases in the expression of the cardiac sarcoplasmic reticulum ATPase, cardiac fibrosis, and systemic oxidant stress were observed with both MBG infusion and partial nephrectomy. Next, rats were actively immunized against a MBG-BSA conjugate or BSA control, and partial nephrectomy was subsequently performed. Immunization against MBG attenuated the cardiac hypertrophy, impairment of diastolic function, cardiac fibrosis, and systemic oxidant stress seen with partial nephrectomy without a significant effect on conscious blood pressure. These data suggest that the increased concentrations of MBG are important in the cardiac disease and oxidant stress state seen with renal failure. (Hypertension. 2006;47:488-495.)

Key Words: cardiomyopathy ▪ sarcoplasmic reticulum ▪ cardiotonic agents ▫ fibrosis ▪ renal failure ▪ reactive oxygen species

The care of patients with chronic renal failure is currently complicated by their propensity to develop cardiac disease. This cardiac disease is directly responsible for much of the extremely high morbidity and mortality seen in patients with end-stage renal disease.1 At present, the molecular basis of this “uremic cardiomyopathy,” which is characterized by a systemic oxidant stress state, marked cardiac hypertrophy, and diastolic dysfunction, is still poorly understood. Interestingly, even mild degrees of chronic renal failure appear to confer a significant increase in cardiovascular disease.2,3

The partial nephrectomy model in the rat has been used to simulate experimental uremia to study the cardiac abnormalities that accompany renal failure.4 A number of factors, including volume overload, have been implicated in the pathogenesis of the cardiac disease in this model (reviewed in Reference 5). We have observed that cardiac myocytes isolated from rats subjected to partial (ie, five-sixth) nephrectomy have diastolic dysfunction in vitro, which can be attributed to reduced sarcoplasmic reticulum calcium ATPase (SERCA) activity and, in turn, appears to be dependent on proportional decreases in SERCA2a protein and mRNA.6 It has been observed that steroid molecules, which bind to the plasmalemmal Na/K-ATPase and have structural similarity to the medication digitalis, accumulate in renal failure. These molecules have been referred to as digitalis-like substances or, more recently, cardiotonic steroids. Considerable effort has gone into the measurement of these molecules and the elucidation of their role in cardiac and renal physiology.7 Recent work has established that the cardiotonic steroid marinobufagenin (MBG) induces natriuresis and, in susceptible rat strains, increases blood pressure (BP).8,9 Elevations of circulating MBG have been clearly demonstrated in both clinical and experimental renal failure, whereas another cardiotonic steroid, endogenous ouabain, does not increase at 4 weeks in experimental renal failure.10,11
Interestingly, investigators postulated a role for endogenous natriuretic substances in the pathobiology of uremia decades before their identification. Our group and others have observed that cardiotonic steroids induce signaling through the plasmalemmal Na/K-ATPase, which resides in caveolae. This signaling requires the generation of reactive oxygen species, has genomic effects that can be attributable to the modulation of transcription factors, including SP-1, and induces hypertrophic changes in both neonatal and adult cardiac myocytes in vitro (Figure 1). Recently, our group has shown that passive administration of antibodies raised against MBG reduced Na/K-ATPase endocytosis and sodium excretion in Sprague-Dawley rats given a high-salt diet. On this background, we postulated that increases in sodium excretion in Sprague-Dawley rats given a high-salt diet against MBG reduced Na/K-ATPase endocytosis and caveolae. This signaling requires the generation of reactive oxygen species (ROS) and activation of ERK. This, in turn, leads to altered gene expression, including decreases in SERCA expression, as well as alterations in calcium cycling.

Data supporting this schematic summarized from References.

Figure 1. Schematic depicting sodium pump signaling in cardiac myocytes. In the presence of a cardiotonic steroid, Na/K-ATPase is converted to a signal transducer, which complexes with Src and the epidermal growth factor receptor. A signal cascade is initiated, which depends on Ras and results in the generation of reactive oxygen species (ROS) and activation of ERK. This, in turn, leads to altered gene expression, including decreases in SERCA expression, as well as alterations in calcium cycling.

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
Rats that were subjected to sham surgery and no MBG infusion or partial nephrectomy are referred to as Sham (n=18). Rats subjected to partial nephrectomy (n=8), as well as those who received control immunization against BSA and partial nephrectomy (n=12), were very similar with respect to functional and biochemical analysis and were, therefore, pooled into one group and referred to as PNx (n=20). MBG-infused rats are referred to as MBG (n=20). Rats that were immunized against MBG-BSA conjugate before partial nephrectomy are referred to as PNx-IM (n=18).

MBG Infusion
MBG was isolated from toad (Bufo Marinus) venom as described previously. The isolated MBG was >99% pure based on high-performance liquid chromatography and mass spectroscopy analysis. MBG was infused for a period of 4 weeks at 10 μg/kg per day with an osmotic minipump (Alzet Model 2004, Durect Corp). The stability of MBG for 4 weeks at 37°C was confirmed by comparable inhibition of 86Rb uptake in LLCPK1 cells at 2.5×10^-7 and 1×10^-6 M concentrations as MBG prepared immediately, as well as by mass spectroscopy analysis.

Experimental Renal Failure
Partial nephrectomy (five-sixth nephrectomy) was induced by removal of the right kidney and selective infarction of two-thirds of the left kidney with silk ligatures as described previously.

Immunization Against MBG
Rats were immunized with an MBG-BSA conjugate and subjected to partial nephrectomy. The immunization schedule was 3 weekly injections (250 μg/kg per week SQ) in complete Freund’s adjuvant before the partial nephrectomy with a last boost at the time of surgery. This regimen, which has been used previously, induced high titers of antibodies (>1:10 000) to MBG. These antibodies had high affinity to MBG (4.7×10^6 to 5.5×10^6) and very little cross-reactivity (<1%) to aldosterone, ouabain, digoxin, bufalin, and progesterone.

Hemodynamics
BP was measured once a week by the tail-cuff method in conscious, restrained rats with equipment made by ITC, Inc (Amplifier model 229, Monitor model 31, Test chamber Model 306; IITC Life Science) as described previously. Before sacrifice at 4 weeks, animals had ventricular pressures determined by placement of a 2F Millar Microtip Catheter Transducer (Millar Instruments Inc) into the left ventricle through a carotid insertion. Hemodynamic data were acquired at 500 Hz and stored electronically using a BioPac MP110 acquisition system and AcqKnowledge 4.7.3 software (BIOPAC Systems, Inc). The values of left ventricular end-diastolic pressure (LVEDP), systolic pressure, developed pressure, maximal velocity of rise or fall in pressure (dP/dt) and the time constant for isovolumic relaxation were determined using standard methods.
**Rat Echocardiography**

At the end of 4 weeks, 2D and M-mode echocardiographic studies were performed using a Philips Sonos 5500 cardiovascular ultrasound imaging system (Philips Medical Systems) equipped with a 15 MHz linear transducer generously loaned to us by Dr Philip Binkley of the Ohio State University. Parasternal long-axis and short-axis views were obtained as described previously by Litwin et al. 25

**Measurement of MBG and Ouabain-Like Compound**

MBG and ouabain-like compound (OLC) in plasma and urine were determined at 4 weeks after extraction with C-18 columns as described previously. 11 An expanded Methods section is available online at http://hyper.ahajournals.org.

**Western Blot Analysis**

At the time of sacrifice, left ventricles were quickly dissected out, frozen in liquid nitrogen, and stored at −80 °C until further analysis. Western blot analysis was performed as described previously using SDS-PAGE gels (Ready Gel, BioRad).

**Measurements of Oxidant Stress**

Total protein carbonyl concentration of the plasma and left ventricular homogenate was determined by ELISA using the Zentech PC Test kit (Northwest Life Science Specialties). 26, 27 Total plasma malondialdehyde was measured spectrophotometrically using the Bioxtech MDA-586 kit (Oxirx Research).

**Measurement of SERCA2a Activity**

To measure sarcoplasmic reticulum calcium ATPase activity, which is predominantly SERCA2a activity in left ventricles of rat cardiac tissue, the method of Simonides and van Hardeveld was used with minor modifications as described previously. 5

**Miscellaneous Blood Analyses**

Hematocrit was measured from whole blood collected in heparinized capillary tubes and spun on a microhematocrit centrifuge (Fisher Scientific). Plasma creatinine was determined spectrophotometrically with a colorimetric end point assay (Teco Diagnostics). Plasma aldosterone levels were measured by ELISA (Cayman). Plasma parathyroid hormone was assayed using the Rat Intact PTH ELISA kit (Immutopics). All of the analyses were performed on blood collected upon sacrifice at 4 weeks.

**Histology and Fibrosis Scoring**

An expanded section is available online.

**Statistical Analysis**

Data presented are mean±SEM. Please see http://hyper.ahajournals.org for an expanded Methods section.

### Results

#### Changes in MBG and Other Hormones

Rats with partial nephrectomy had substantial increases in plasma [MBG] and urinary MBG excretion rates (U_{MBGV}) at 4 weeks after surgery compared with control rats (Table 1). Infusion of MBG alone induced comparable increases in plasma [MBG] and U_{MBGV} as partial nephrectomy. Immunization against MBG-BSA in partial nephrectomy animals was associated with a decrease in U_{MBGV} compared with partial nephrectomy alone. Neither plasma or urine OLC levels were different between sham and partial nephrectomy as we have reported previously nor did we see a significant effect of either MBG supplementation or immunization against MBG on plasma or urine OLC levels (Table 1).

Partial nephrectomy led to marked increases in plasma creatinine and decreases in hematocrit, which were not affected by immunization (Table 1). MBG infusion to sham-operated rats did not significantly alter either of these measurements. Partial nephrectomy induced considerable increases in plasma aldosterone and parathyroid hormone concentrations in the plasma compared with the sham-operated controls, whereas immunization against MBG did not alter these hormone concentrations in the plasma compared with the sham-operated controls, whereas immunization against MBG did not alter these hormone concentrations compared with partial nephrectomy alone. Rats with partial nephrectomy had systemic and cardiac oxidant stress as indicated by increases in both plasma and left ventricular tissue carbonylated proteins, as well as increases in plasma malondialdehyde compared with control rats, whereas MBG

### Table 1. Effects of MBG on Various Biochemical and Functional Parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>PNx</th>
<th>MBG</th>
<th>PNx-IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma [MBG], pmol/L</td>
<td>359±16</td>
<td>564±36†</td>
<td>546±34†</td>
<td>430±36‡</td>
</tr>
<tr>
<td>Urinary MBG excretion (U_{MBGV}), pmol/24 h</td>
<td>31.3±2.3</td>
<td>60.2±4.5†</td>
<td>49.1±3.5†</td>
<td>44.7±4.0*†</td>
</tr>
<tr>
<td>Plasma [OLC], pmol/L</td>
<td>428±53</td>
<td>437±43</td>
<td>493±41</td>
<td>528±38</td>
</tr>
<tr>
<td>Urinary OLC excretion (U_{OLCV}), pmol/24 h</td>
<td>11.7±1.5</td>
<td>11.4±1.9</td>
<td>10.6±0.9</td>
<td>11.1±1.1</td>
</tr>
<tr>
<td>Plasma creatinine, mg/dL</td>
<td>0.30±0.03</td>
<td>0.95±0.12†</td>
<td>0.52±0.07</td>
<td>0.95±0.13†</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>44.5±0.8</td>
<td>38.8±1.3†</td>
<td>44.6±0.9</td>
<td>41.1±0.7†</td>
</tr>
<tr>
<td>Plasma parathyroid hormone, pg/mL</td>
<td>41±6</td>
<td>126±17*</td>
<td>41±9</td>
<td>140±26*</td>
</tr>
<tr>
<td>Plasma aldosterone, pg/mL</td>
<td>191±55</td>
<td>1780±371†</td>
<td>322±38</td>
<td>2207±474†</td>
</tr>
</tbody>
</table>

Analyses were performed 4 weeks after sham operation (Sham, n=16), partial nephrectomy (PNx, n=20), MBG infusion (MBG, n=14), or immunization against MBG prior to partial nephrectomy (PNx-IM, n=18).

*P<0.05 vs Sham; †P<0.01 vs Sham; ‡P<0.05 vs PNx; and §P<0.01 vs PNx.

#### Table 2. MBG Induces Oxidant Stress In Vivo

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>PNx</th>
<th>MBG</th>
<th>PNx-IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma carbonylated protein, pmol/mg protein</td>
<td>171±9</td>
<td>320±20†</td>
<td>378±15†</td>
<td>241±24‡</td>
</tr>
<tr>
<td>Left ventricular carbonylated protein, pmol/mg protein</td>
<td>387±23</td>
<td>541±41*</td>
<td>505±22</td>
<td>393±39§</td>
</tr>
<tr>
<td>Plasma total malondialdehyde, nM</td>
<td>399±17</td>
<td>571±49†</td>
<td>474±41</td>
<td>428±34</td>
</tr>
</tbody>
</table>

Analyses performed 4 weeks after sham operation (Sham, n=8), partial nephrectomy (PNx, n=12), MBG infusion (MBG, n=14), or immunization against MBG prior to partial nephrectomy (PNx-IM, n=18).

*P<0.05 vs Sham; †P<0.01 vs Sham; ‡P<0.05 vs PNx; §P<0.01 vs PNx.
infusion alone only produced statistically significant increases in plasma carbonylation (Table 2). Immunization against MBG in partial nephrectomy animals substantially reduced oxidant stress compared with partial nephrectomy alone (Table 2).

**Hemodynamic Studies**

Partial nephrectomy was associated with marked increases in systolic BP during the 4 weeks of observation. MBG infusion alone produced some increases in BP compared with control, but these increases were less than that observed with partial nephrectomy alone. Immunization against MBG did not significantly attenuate the increases in BP seen with partial nephrectomy. These data are summarized in Figure 2a. Echocardiographic imaging studies (see online supplemental video clips) demonstrated that partial nephrectomy animals had considerable increases in left ventricular wall thickness compared with controls (Figure 2b and 2c). These data were consistent with the heart weight data obtained (vida infra). Left ventricular end-diastolic (Figure 2d) and end-systolic volumes (Figure 2e) were markedly reduced in the partial nephrectomy animals, and the calculated fractional shortening (FS) was also substantially increased (Figure 2f). MBG infusion was not associated with significant changes in wall thickness, chamber size, or FS compared with sham-treated controls. Immunization against MBG ameliorated the echocardiographic changes noted with partial nephrectomy (Figure 2b through 2f).

After 4 weeks, the animals were anesthetized, and a Millar catheter was introduced into the left ventricle to measure left ventricular hemodynamics. Partial nephrectomy surgery induced substantial increases in maximal velocity of rise in pressure (dP/dt) compared with controls (Figure 3a). However, diastolic function was impaired as assessed by the ratio of maximal positive dP/dt to maximal negative dP/dt (Figure 3b), an increase in LVEDP (Figure 3c), as well as the time constant for left ventricular isovolumic relaxation (Figure 3d). A similar pattern was noted in the rats subjected to MBG infusion, but only the changes in LVEDP and time constant for isovolumic relaxation achieved statistical significance. Immunization against MBG in partial nephrectomy animals considerably attenuated the changes in maximal positive dP/dt, the ratio of maximal positive dP/dt to negative dP/dt, LVEDP, and the time constant for ventricular relaxation seen with partial nephrectomy (Figure 3a through 3d).

**Cardiac Morphology and Biochemistry**

Rats subjected to partial nephrectomy had marked increases in heart weight compared with control animals (Figure 4a). Although MBG infusion also resulted in significant increases in heart weight, these increases were less than that seen with partial...
nephrectomy. Partial nephrectomy was associated with activation of extracellular signal regulated kinase (ERK; Figure 4b) and Src (Figure 4c), upregulation of skACT (Figure 4d), as well as the downregulation of both the α1 and α2 isoform of the Na/K-ATPase (Figure 4e and 4f) and SERCA2a (Figure 4g). SERCA enzymatic activity was also decreased in partial nephrectomy-treated animals (Figure 4h). A similar pattern of changes in protein expression was noted in rats subjected to MBG infusion. Immunization against MBG prevented or attenuated the increases in cardiac size, activation of ERK and Src, Ras, reactive oxygen species, and ERK and oxidant stress.26,27 Oxidant stress is believed to be a major pathogenic factor in the cardiovascular disease seen in renal failure,31 as well as the general population.32

The concept that hormonal adaptations to decreases in renal function might participate in the pathogenesis of the uremic syndrome was elaborated in the 1960s. This concept is called “trade-off”; the idea is that body fluid and electrolyte homeostasis would be maintained despite renal insufficiency, but the elevated hormone levels might have deleterious consequences.12 The best characterized example of trade-off is the elevated parathyroid hormone levels, which maintain serum phosphate levels but have deleterious effects on bone and possibly other tissues.33 Interestingly, de Wardener,7 Bricker,33 and others specifically postulated that an inhibitor of the plasmalemmal Na/K-ATPase, which was natriuretic, would accumulate in the serum and cause organ dysfunction. However, our understanding of the cardiotonic steroids, previously referred to as digitalis-like substances, has undergone tremendous change. For one, specific chemicals have been identified and characterized. However, perhaps more importantly, focus has shifted from the pharmacological effect of these cardiotonic steroids on the enzymatic function of the Na/K-ATPase to the signaling that occurs through this system. Specifically, it has been clearly demonstrated that cardiotonic steroid signaling through the Na/K-ATPase causes endocytosis of the plasmalemmal Na/K-ATPase, which was natriuretic, to proceed.13,14 Cardiotonic steroid signaling through the sodium pump causes the Na/K-ATPase to be in caveolae to proceed.13,14 Car

Discussion

Patients with chronic renal failure develop cardiac disease with exceptional frequency.5 Even mild degrees of chronic renal insufficiency are associated with marked increases in cardiovascular mortality.3 Although historically the term uremic cardiomyopathy referred to patients with a dilated cardiomyopathy not attributable to other causes, the modern concept is that patients with renal failure develop diastolic dysfunction and cardiac hypertrophy, which are, at least in part, related to their renal disease.5 The pathogenesis of this uremic cardiomyopathy has been debated.5,20,30 Although it is believed that extracellular volume expansion plays a key role in the development of the left ventricular hypertrophy, other factors, including hyperparathyroidism, hypertension, and anemia, have been proposed to be important in the pathogenesis.5 It is interesting to note that patients with chronic renal failure also develop evidence for systemic inflammation and oxidant stress.26,27 Oxidant stress is believed to be a major pathogenic factor in the cardiovascular disease seen in renal failure,31 as well as the general population.32

The purpose of the current study was to examine whether this signaling by cardiotonic steroids through the Na/K-
Figure 4. MBG produces changes in cardiac morphology and protein expression consistent with experimental uremia. (a) Heart weight/body weight (HW/BW) ratio 4 weeks after sham operation (Sham, n=18), partial nephrectomy (PNx, n=20), MBG infusion (MBG, n=20), or immunization against MBG before partial nephrectomy (PNx-IM, n=18). (b) Extracellular signal-related kinase (ERK-1, p44) activation in the left ventricular cardiac homogenate 4 weeks after Sham (n=15), PNx (n=14), MBG (n=7), or PNx-IM (n=7). Gels were loaded with 50-μg left ventricle homogenate protein. Representative active and total ERK blots shown. (c) Src (Src p418) activation in the left ventricular cardiac homogenate 4 weeks after Sham (n=15), PNx (n=13), MBG (n=10), or PNx-IM (n=6). Gels were loaded with 75-μg left ventricle homogenate protein. Representative active and total Src blots shown. (d) Skeletal muscle actin (skACT), (e) Na/K-ATPase α1, (f) Na/K-ATPase α2, and (g) SERCA2a expression 4 weeks after Sham (n=15), PNx (n=13), MBG (n=10), or PNx-IM (n=6). Gels for d through g were loaded with 20 μg left ventricle homogenate protein. (h) SERCA2a enzymatic activity in the left ventricular cardiac homogenate 4 weeks after Sham (n=8), PNx (n=6), MBG (n=8), or PNx-IM (n=8). Bar graphs for Western blot data summarize densitometry analysis of the blots. **P<0.01 vs Sham, *P<0.05 vs Sham, #P<0.05 vs PNx, ##P<0.01 vs PNx.
Figure 5. MBG induces cardiac fibrosis. (a) Representative Masson’s trichrome sections of left ventricular cardiac tissue 4 weeks after sham operation (Sham), partial nephrectomy (PNx), MBG infusion (MBG), or immunization against MBG before partial nephrectomy (PNx-IM). (b) Semiquantitative grade and (c) quantitative morphometric fibrosis scoring for trichrome slides of left ventricular cardiac sections 4 weeks after Sham (n=8), PNx (n=10), MBG (n=10), or PNx-IM (n=10). (d) Fibronectin expression and quantified data from Sham (n=9), PNx (n=9), MBG (n=9), and PNx-IM (n=9). Gels were loaded with 50μg of left ventricle homogenate protein. *P<0.05, **P<0.01 vs Sham, #P<0.05, ##P<0.01 vs PNx.

Although plasma aldosterone concentrations were quite high in the partial nephrectomy model, we found that neither MBG infusion nor immunization against MBG appeared to alter these concentrations. Moreover, the antibody formed in rats in response to immunization against MBG did not react with aldosterone. It is interesting to note that aldosterone-induced fibrosis only appears to develop in settings where salt loading and volume expansion also occur,38 and these settings are likely to have increased circulating concentrations of MBG.39,40 To be sure, we noted considerable cardiac fibrosis in our renal failure model as demonstrated by histological analysis and fibronectin expression. Fourth and perhaps most important, we observed that active immunization against MBG was associated with very substantial attenuation of cardiac hypertrophy, cardiac fibrosis, and the oxidant stress state. The decreases in cardiac expression of SERCA2a, as well as SERCA enzymatic activity seen with partial nephrectomy, were also markedly attenuated by the active immunization. Although MBG infusions were associated with increases in BP, it is noteworthy that active immunization against MBG did not substantially attenuate the hypertension.

We expect that this underscores the greater importance of other factors (eg, renin–angiotensin–aldosterone activation) in the pathogenesis of hypertension in our model. Ferrandi et al41 reported that longer-term infusions of ouabain (15μg/kg per day for 18 weeks) produced hypertension and cardiac hypertrophy in rats. They also found that these effects of ouabain could be prevented by concomitant administration of an experimental molecular antagonist to ouabain, PST 2238. This is particularly interesting in light of the recent report demonstrating that increases in brain ouabain trigger increases in peripheral MBG concentrations in the setting of salt loading.8

Perspectives

Taken together, our data strongly support an important role for MBG in the pathogenesis of experimental uremic cardiomyop-
nthy in the rat. This is extremely interesting in the light that volume expansion appears to play a key role in cardiac hypertrophy seen with renal failure, and MBG concentrations are increased with volume expansion. Our finding that MBG is also associated with fibrosis in our model allows one to speculate that some cross-talk between MBG and aldosterone in the pathogenesis of cardiac fibrosis may be possible. If MBG is also found to be important in clinical uremic cardiomyopathy, therapy targeted against MBG signaling could potentially have clinical application.

Acknowledgments

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References


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