Physiological Impact of Increased Expression of the AT₁ Angiotensin Receptor

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Abstract—To test the effect of increased AT₁ receptor expression on blood pressure, we used gene targeting to generate mouse lines with a tandem duplication of the AT₁A receptor gene locus (Agtr1a) along with >10 kb of 5′ flanking DNA. By successive breeding, we generated mice with 3 and 4 copies of the Agtr1a gene locus on an inbred 129/Sv background. AT₁A mRNA expression and AT₁-specific binding of 125I-angiotensin II were increased in proportion to Agtr1a gene copy number. These animals survived in expected numbers, and their body, heart, and kidney weights were similar to wild-type, 2-copy control mice. Pressor responses to angiotensin II were blunted in the 4-copy mice compared with control mice. In male mice, there was no correlation between resting blood pressure and Agtr1a gene copy number or AT₁A mRNA levels. However, in female mice, there was a highly significant positive correlation between blood pressure and AT₁A receptor expression, paralleled by significant increases in aldosterone synthase expression with increase in gene copy number. Furthermore, in female but not male mice, there was a positive correlation between kallikrein and AT₁A receptor mRNA levels and an inverse correlation between renin mRNA and Agtr1a copy number. Thus, in female but not male mice, genetic variants that increase expression of AT₁ receptors affect blood pressure and gene expression programs. The impact of enhanced AT₁ receptor expression on blood pressure may be blunted by systemic compensatory responses and altered signal-effector coupling in the vasculature. (Hypertension. 2003;42:507-514.)

Key Words: hypertension, renal ─ renin ─ aldosterone ─ mice ─ gender

The renin-angiotensin system (RAS) plays a critical role in the regulation of blood pressure and sodium homeostasis.1 Enhanced activity of the RAS causes hypertension and end-organ injury.2–4 Variations in genes encoding components of the RAS have been associated with hypertension in human populations. For example, the M235T variant of the gene encoding angiotensinogen, the protein precursor of angiotensin II, has been associated with elevated plasma angiotensin II, has been associated with elevated plasma angiotensinogen levels and essential hypertension in white American and French populations.5 This allele is in linkage disequilibrium with a single nucleotide substitution in the promoter region of the angiotensinogen gene that appears to naturally occurring variants of the Agtr1a gene expression. Using genetically engineered mouse models,7 the major physiological actions of the RAS are mediated through the type 1 (AT₁) receptor, and clinical studies have demonstrated that pharmacological blockade of the AT₁ receptor effectively lowers blood pressure and protects against end-organ damage.6 Rodents have two AT₁ receptor isoforms, AT₁A and AT₁B, which are encoded by distinct genes. In most tissues, expression of the AT₁A receptor far exceeds that of the AT₁B receptor, and the AT₁A receptor is considered to be the murine homologue of the human AT₁ receptor. In previous studies, we found that incremental reductions in AT₁A receptor gene expression implemented through gene targeting caused a proportional lowering of resting blood pressure. For example, in heterozygous Agtr1a± mice, a 50% reduction in AT₁A receptor expression lowers systolic blood pressure by 12 mm Hg compared with control mice; the complete absence of AT₁A receptors in Agtr1a-/ mice is associated with a further lowering of blood pressure.9 This demonstration that reduced levels of AT₁A receptor gene expression affect blood pressure suggests that naturally occurring variants of the Agtr1a gene locus that increase the level of receptor expression could positively affect resting blood pressure.

To determine whether increased levels of AT₁A receptor expression would increase blood pressure, we generated mice with a targeted duplication of the Agtr1a gene locus and examined the physiological effects of the resulting quantitative variation of the AT₁A receptor gene expression. Using this model, we found that the impact of increased AT₁ receptor expression on blood pressure is complex and that...
Ligand Binding Assays

The density of AT₁ receptors in the kidney was determined by quantitative in vitro autoradiography as described previously.14 Twenty-micrometer kidney sections were incubated with [³²P]-[Sar²,Ile⁸] angiotensin II in the presence of the selective AT₁ antagonist candesartan (1 μmol/L, Astra Hässle) or the AT₂ antagonist PD123319 (10 μmol/L, Parke-Davis). The sections were then exposed to x-ray film (UM-MA HC medical x-ray film, Fuji Co). Radioactivity standards were exposed simultaneously, which then allowed the optical densities of the radiographic images to be converted into radioactivity levels (disintegrations per minute, dpm), using a computerized imaging system (MCID Imaging Research).

[³H]Angiotensin II Binding Studies

Kidneys from 2-copy or 3-copy mice (n=6) were removed and placed in ice-cold ×1 Dulbecco’s PBS buffer. Mouse glomeruli were isolated as described in detail previously.15 Equilibrium binding data were analyzed by Scatchard method to estimate Bmax and equilibrium Kd by fitting the data to a nonlinear model with the use of the ENZFITTER computer program (Elsevier-Biosoft).

Angiotensin II Infusions

We measured acute pressor response to 0.1, 1, and 10 μg/kg angiotensin II in (+/+), (2Agtr1a/+) and (2Agtr1a/2Agtr1a) animals as described.8 To inhibit endogenous angiotensin II production, ACE inhibitor enalapril (30 mg/kg per day orally) was administered for 2 days before study, and an additional 10 mg/kg IV was given at the start of the study.

Measurements of Resting Blood Pressure

Systolic blood pressures were measured in conscious mice with the use of a computerized tail-cuff system (Visitech Systems) that has been validated and described previously.16

Quantification of mRNA Expression by
Real-Time RT-PCR

Relative levels of mRNA for renin, the AT₁ receptor, aldosterone synthase, and kallikrein were determined by real-time RT-PCR with the ABI Prism 7700 Sequence Detection System as described, and the nucleotide sequences of the PCR primers and their fluorogenic probes have been published previously.17

Data Analysis

For comparisons between groups, statistical significance was assessed by nonparametric Mann-Whitney rank sum test. Linear regression analysis was used to correlate the relation between response variable and single predictor variable (Minitab Statistical Software).

Results

Normal Survival of Mice With 3 or 4 Copies of the Agtr1a Gene Locus

We assessed survival and general characteristics at weaning of 156 consecutive progeny from matings of 3-copy (2Agtr1a/+)×3-copy (2Agtr1a+/+) animals. The proportions of surviving 2- (+/+)/, 3- (2Agtr1a/+) and 4-copy (2Agtr1a/2Agtr1a) mice were 20.5%, 53.8%, and 25.6%, respectively. This distribution did not differ significantly from predicted (25%/50%/25%). Thus, an increased number of Agtr1a gene copies does not affect survival. Furthermore, growth, vigor, and fertility of 3- and 4-copy mice were indistinguishable from their (+/+) littersmates.

Methods

Gene Targeting Vector

Mice with a tandem duplication of the Agtr1a gene locus at its normal chromosomal location were generated with the use of gap-repair gene targeting strategy described previously.2 The targeting vector (Figure 1B) contains all of the known regulatory sequences in the Agtr1a promoter.2,11 ES cells of the E14TG2a line were transfected with the linearized targeting vector by electroporation, and selection was carried out with G418 and ganciclovir as positive and negative selection, respectively. Interrupted line gene and herpes simplex thymidine kinase (TK) gene were used to generate chimeras as described.9 To inhibit endogenous angiotensin II production, ACE inhibitor enalapril (30 mg/kg per day orally) was administered for 2 days before study, and an additional 10 mg/kg IV was given at the start of the study.

Figure 1. Duplication of Agtr1a gene by a gap-repair gene targeting. Agtr1a gene was cloned from a BAC genomic library made by strain 129 mouse DNA. A, Map of the endogenous Agtr1a locus with three exons of the gene shown as numbered white rectangles (5′ untranslated region) and black rectangle under the lines. Targeting vector used to duplicate the Agtr1a gene locus depicted in B is an O-type (insertional) targeting construct with a 10-kb 5′ homology region containing exon 1 along with ~8 kb 5′ upstream sequence, which includes all of the known regulatory sequences in the Agtr1a gene promoter. A 90-kb gap in the targeting construct relative to the targeted Agtr1a gene spans exons 2 and 3. A neomycin-resistance (neo) gene and herpes simplex thymidine kinase (TK) gene were used for positive and negative selection, respectively. Interrupted line represents no real length. Targeted locus after duplication of the Agtr1a gene is shown in C. Two horizontal square brackets indicate the duplicated region. Arrows 1 and 2 are primers for PCR (also indicated in A and B). Genomic DNA fragments that should hybridize to exon 3—specific probe after successful gap-repair targeting are indicated by double-headed horizontal arrows; their predicted sizes are given in kilobases. Restriction enzymes used are X (Kpn I) and K (Kpn I). Enzyme sites in parentheses were destroyed in the targeting construct relative to the targeted Agtr1a gene by a gap-repair gene targeting strategy described previously.7 The target-
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**Figure 2.** Effect of Agtr1a gene copy number on AT<sub>1A</sub> mRNA expression. AT<sub>1A</sub> mRNA levels were obtained from kidneys of 2-copy (n=25), 3-copy (n=26), and 4-copy mice (n=26). Mean is indicated by solid black circle ●. Median is indicated by horizontal line. Whiskers (vertical lines) extend to highest and lowest values. *P*<0.006 vs 2-copy mice; †*P*<0.007 vs 3-copy mice.

**AT<sub>1A</sub> Receptor mRNA Levels and Agtr1a Genotype**

To assess AT<sub>1A</sub> receptor mRNA expression, we isolated RNA from kidney tissues of male and female mice of all three genotypes and measured AT<sub>1A</sub> mRNA levels by real-time PCR. As shown in Figure 2, there was a stepwise increase in AT<sub>1A</sub> receptor mRNA expression with increasing Agtr1a gene copy number. AT<sub>1A</sub> receptor mRNA levels were significantly higher in 3-copy (2Agtr1a/+) mice (10.8±0.3 pg/μg total RNA) than in the 2-copy (+/+ mouse) (9.3±0.3 pg/μg total RNA; *P*<0.002), and there was a further increase in AT<sub>1A</sub> mRNA levels in the 4-copy (2Agtr1a/2Agtr1a) mice (12.3±0.4 pg/μg total RNA; *P*<0.007 versus the 3-copy (2Agtr1a/+ group). By linear regression analysis (not shown), there was a significant positive correlation between AT<sub>1A</sub> receptor mRNA levels correlated and the number of Agtr1a genes (*R<sup>2</sup>=0.313; *P*<0.0005). When expressed as percentages of the mean AT<sub>1A</sub> receptor mRNA level, assigning a value of 100% to the 2-copy (+/+ mouse), the AT<sub>1A</sub> mRNA expression in the 3-copy animals is 116% of normal and 133% in the 4-copy animals. As shown in the Table, the patterns of AT<sub>1A</sub> receptor mRNA expression in kidney did not differ significantly in females and males.

**Ligand Binding Assays**

To determine whether the Agtr1a gene duplication increases angiotensin binding sites, we compared 125I-angiotensin II binding assays with increasing gene copy number. The effects of increasing the number of Agtr1a gene copies. To quantify and compare the levels of AT<sub>1</sub>-specific binding between the groups, glomerular binding densities were measured as disintegrations per minute (dpm/mm<sup>2</sup>) (Figure 3b). This analysis was facilitated by the high levels of discrete binding in glomeruli. Similar to the patterns of AT<sub>1A</sub> mRNA expression, 125I-Ang II glomerular binding densities were significantly higher in the 3-copy (2Agtr1a/+) mice (870±27 dpm/mm<sup>2</sup>) than in 2-copy (+/+ mouse) (764±15 dpm/mm<sup>2</sup>; *P*<0.009) and were further increased in the 4-copy (2Agtr1a/2Agtr1a) mice (1056±38 dpm/mm<sup>2</sup>; *P*<0.006 versus the 3-copy group). The proportional percent increases in glomerular binding densities, 112% for the 3-copy group and 140% for the 4-copy group, paralleled the increases in AT<sub>1A</sub> receptor mRNA levels that we observed. Similar patterns were observed for binding densities in nonglomerular regions of the renal cortex (data not shown).

**Figure 3.** A. Representative picture of 125I-[Sar<sup>1</sup>,Ile<sup>8</sup>] angiotensin II binding assays in kidney tissues. Kidney sections were from Agtr1a knockout (negative control) (A) and 2-copy (B), 3-copy (C), and 4-copy (D) mice. B. Effect of Agtr1a gene copy number on angiotensin binding sites, measured by 125I-angiotensin II binding. Ligand binding density is expressed as disintegrations per minute (dpm/mm<sup>2</sup>). Mean is indicated by solid black circle ●. Median is indicated by horizontal line. Whiskers (vertical lines) extend to highest and lowest values. *P*<0.006 vs 2-copy, †*P*<0.007 vs 3-copy.
To determine whether increased levels of AT1A receptor expression affect angiotensin II–dependent physiological responses, we compared acute pressor responses to angiotensin II in 2- (+/+), 3- (2Agtr1a/2Agtr1a), and 4-copy mice (2Agtr1a/2Agtr1a). Because of known gender differences in the regulation of components of the renin-angiotensin system and in the epidemiology of hypertension, we also examined correlations between blood pressure and Agtr1a genotypes in the entire cohort and separately in male and female mice. In addition, because there were a range of AT1A mRNA expression levels among individual animals within the same genotype, we also examined correlations between blood pressure and individual AT1A mRNA levels. In the combined group, there was a general correlation between the mean level of blood pressure and gene copy number (102 for 2-, 104 for 3-, and 106 for 4-copy mice, respectively), but these differences were not statistically significant. When blood pressure was compared in the male mice, there was no apparent association between the mean blood pressure and Agtr1a gene copy number (107 for 2-copy mice compared with 104 for 3-copy mice, respectively) and no significant correlation between individual values of blood pressure and genotype or AT1A mRNA levels (data not shown). In contrast, blood pressure differences between genotypes were significantly correlated in female mice (99 for 2-copy mice compared with 104 for 3-copy mice, respectively; P=0.03 for 2-copy versus 3-copy mice, respectively; P=0.02 for 4-copy versus 3-copy mice; Figure 6b). Moreover, as shown in Figure 6b, there was a highly significant positive correlation
between blood pressure and AT\textsubscript{1}\textalpha{} receptor expression ($P=0.003$) in the female animals.

**Effects of Agtr1a Genotype on Gene Expression Programs**

To determine whether changes in Agtr1a gene copy number would lead to changes in the expression of other genes related to blood pressure regulation, we examined correlations between genotype, AT\textsubscript{1}\textalpha{} mRNA levels, and mRNA levels for three representative genes that are involved in blood pressure homeostasis: aldosterone synthase, renin, and kallikrein. We chose to specifically examine these three genes because of our previous observations of significant gender effects and interactions between their expressions and angiotensinogen genotype in mice.\(^{17}\) In male animals, we found no correlation between Agtr1a copy number or AT\textsubscript{1}\textalpha{} receptor mRNA level and mRNA levels of aldosterone synthase, renin, or kallikrein. In contrast, in female animals (Figure 6a), there were significant stepwise increases in aldosterone synthase mRNA expression with increase in Agtr1a copy number ($571\pm71$ [n=8], $592\pm34$ [n=5], and $760\pm23$ pg/\mu g total mRNA [n=7] for the 2-, 3-, and 4-copy groups, respectively, $P<0.04$ for 2- versus 4-copy), and there were significant stepwise reductions in renin mRNA levels between the Agtr1a genotypes ($27\pm3$ [n=8], $25\pm3$ [n=5], and $18\pm2$ [n=7] pg/\mu g total mRNA for the 2-, 3-, and 4-copy groups, respectively; $P=0.006$ for 2- versus 4-copy and $P<0.04$ for 3- versus 4-copy; Figure 7b). Moreover, in female mice, there was a highly significant correlation between kallikrein mRNA levels and AT\textsubscript{1}\textalpha{} receptor mRNA levels ($R=0.504$; $P=0.001$; Figure 7c).

**Discussion**

To understand the possible physiological consequences of genetic variants that increase AT\textsubscript{1} receptor expression, we generated mouse lines with 3 and 4 copies of the Agtr1a gene locus on an inbred genetic background. In these animals, AT\textsubscript{1}\textalpha{} gene expression is under the control of the natural Agtr1a promoter. However, the consequences of increasing Agtr1a gene copies on AT\textsubscript{1}\textalpha{} mRNA expression varied in different tissues. In the vasculature and the heart, increasing the number of Agtr1a gene copies from 2 to 4 caused a proportional doubling of AT\textsubscript{1}\textalpha{} mRNA expression levels to 200% of control levels. In contrast, AT\textsubscript{1}\textalpha{} mRNA expression in kidneys of 4-copy mice was only 133% of wild-type. This suggests that transcriptional regulation of the Agtr1a gene is...
complex and varies significantly between tissues. In view of our previous studies in mice showing that duplication of the angiotensigen gene (Agt) locus causes elevations in blood pressure, we anticipated that increasing expression of the AT\textsubscript{1A} receptor, the major murine AT\textsubscript{1} receptor isoform, would likewise increase blood pressure. However, the magnitude of the blood pressure effect of the Agt\textsubscript{1A} gene duplication was relatively modest, and blood pressure elevation could only be detected in female mice.

This finding that gender modifies the phenotype in these mouse lines is consistent with previously described gender differences in the incidence and character of hypertension. For example, the incidence of hypertension is lower in premenopausal women than age-matched men. In several animal models of hypertension, blood pressure elevation develops sooner and is more severe in male than female animals. Likewise, the impact of genetic variation in the renin-angiotensin system is also modified by gender. For example, in several human populations, the M235T polymorphism in the \textit{AGT} gene is associated with hypertension and elevated circulating levels of angiotensigen. Linkage of the 235T allele with high blood pressure was seen in male but not in female subjects. Similarly, increasing angiotensigen levels in mice by increasing Agt gene copy caused a highly significant increase in blood pressure in male mice. In individual female mice, the increase in blood pressure was strongly correlated with their Agt expression in the liver, but the correlation between gene copy number and blood pressure failed to reach significance. Thus, at least for variants in the angiotensigen gene, the influence of gender on blood pressure is very similar in humans and mice. In the present study, we found clear evidence of sexual dimorphism in our mutant animals with increased AT\textsubscript{1A} receptor expression. However, unlike variants of the angiotensigen gene, the effect on blood pressure of increasing expression of AT\textsubscript{1A} receptors was only seen in female mice. This sexual dimorphism is not due to differences in the levels of AT\textsubscript{1A} mRNA expression per se. Thus, although gender effects on RAS gene functions are pervasive, these influences are complex and may vary dramatically between different RAS genes.

A major objective of our study was to model the capacity for increased AT\textsubscript{1}-receptor expression to affect blood pressure. Polymorphisms of the human AT\textsubscript{1} receptor gene (\textit{AGTR1}) have been identified and one variant, A1166C, has been associated with hypertension, changes in aortic distensibility, and increased left ventricular mass. Careful hemodynamic studies in nonhypertensive individuals have suggested that this polymorphism may affect baseline renal function and physiological responses to angiotensin II. The A1166C nucleotide substitution is located outside the coding region within the 3′ untranslated region of the AT\textsubscript{1} receptor mRNA, and it is not clear how this substitution could affect AT\textsubscript{1} receptor expression or function, unless it alters mRNA stability or is in linkage disequilibrium with other mutations in the \textit{AGTR1} gene. To date, no alterations in levels of AT\textsubscript{1} receptor expression have yet been documented between individuals with the different \textit{AGTR1} alleles, with the caveat that changes in AT\textsubscript{1} receptor expression would be difficult to measure in humans because of the relative inaccessibility of structures with high levels of expression such as glomeruli. Nonetheless, our studies would predict that the consequences of any mutations that modestly increase AT\textsubscript{1} receptor expression will be affected by gender.

It is noteworthy that the magnitude of the impact of alterations of AT\textsubscript{1A} receptor expression on blood pressure changes varies markedly, depending on whether expression is increased or decreased. We previously demonstrated that incremental reductions in AT\textsubscript{1A} receptor levels caused relatively large reductions in blood pressure that were proportional to the gene copy number. By contrast, in the current study, increasing AT\textsubscript{1A} receptor levels up to 2-fold had only a modest effect on blood pressure that was gender-dependent. These findings suggest that the capacity to compensate for reduced expression of AT\textsubscript{1} receptors is limited, whereas the capacity to compensate for enhanced AT\textsubscript{1} receptor levels is more robust, perhaps because of the pathological consequences of increasing AT\textsubscript{1} receptor activity.

Two previous studies found that transgenic mice with enhanced expression of AT\textsubscript{1} receptors in cardiac myocytes have massive cardiac enlargement, congestive heart failure, and early death. This is in obvious contrast to the relatively benign phenotype of our animals with enhanced AT\textsubscript{1}-receptor expression from duplication of the \textit{Agr1a} locus. However, there are fundamental differences in the studies that probably explain the different phenotypes. In our experiments, the increase in expression of the AT\textsubscript{1} receptors was modest and was under the control of the natural regulatory elements present in the \textit{Agr1a} gene. By contrast, the cardiac-specific expression of AT\textsubscript{1} receptors was generated in the transgens by using the heterologous \textalpha-MHC promotor, and the level of expression was very high. It is likely that aberrant levels, pattern, and dynamics of AT\textsubscript{1} expression are responsible for their dramatic phenotype. Differences in the genetic backgrounds of the mice may also account for the different outcomes of these experiments.

Along with its effects on blood pressure, duplication of the \textit{Agr1a} gene significantly affected expression of other genes that regulate cardiovascular functions. As with blood pressure, the effects on gene expression differed between male and female mice. In female mice, increasing \textit{Agr1a} gene copies caused a series of apparently interrelated changes in gene expression that probably affected blood pressure regulation. Previous studies have demonstrated that AT\textsubscript{1}-receptor activation in adrenal glomerulosa cells stimulates expression of aldosterone synthase. Accordingly, increasing the number of AT\textsubscript{1A} receptors in female mice was sufficient to enhance expression of aldosterone synthase mRNA, and this may have contributed directly to blood pressure elevation. In contrast, expression of aldosterone synthase was not affected in male mice with increased \textit{Agr1a} gene copies, paralleling the lack of a blood pressure effect. In the female 3-and 4-copy mice, the elevation in blood pressure may have triggered a compensatory inhibition of renin mRNA expression, attenuating the degree of blood pressure increase. Kallikrein expression was also enhanced in the female mice, and this change may also blunt the rise in blood pressure. In the male mice, there were no alterations in renin and kallikrein expression, indicating that increased blood pressure may have
been the trigger for these compensatory responses. Because the level of blood pressure seen in the 3- and 4-copy female mice was not different from wild-type males, the blood pressure threshold required to affect these gene expression programs may differ between male and female mice. The gender-related differences in gene expression observed in the present study and our previous study\textsuperscript{17} are consistent with conserved patterns of response to genetic variants within the RAS.

We also found evidence for vascular compensations that would tend to countermand the effects of the \textit{Agtr1a} gene duplication on blood pressure. In the 4-copy mice, vasoconstrictor responses after infusion of relatively low concentrations of angiotensin II were blunted substantially, but at higher concentrations, the effects of these compensations were overcome. Thus, there appear to be natural mechanisms that oppose or ameliorate the potentially maladaptive consequences of exaggerated AT\textsubscript{1} receptor expression in vascular tissue. These compensatory mechanisms were overcome by high-dose angiotensin II. The nature of these inhibitory mechanisms is not clear. Since AT\textsubscript{1}-specific binding is increased in proportion to \textit{Agtr1a} gene copy number without any change in ligand affinities, it is likely that alterations in signaling or signal-effector coupling explain the impaired vascular responses in the 4-copy mice. This may occur through changes in the stoichiometries of receptor interactions with downstream signaling molecules such as G proteins or in receptor-receptor interactions. The formation of AT\textsubscript{1}-receptor homodimers or heterodimers with other GPCRs may modulate activation and receptor signaling.\textsuperscript{32,33} Further understanding and characterization of these pathways that negatively modulate AT\textsubscript{1}-receptor signaling will have obvious applications to therapeutics and to understanding disease pathogenesis.

**Perspectives**

We have generated a mouse model for testing the capacity of genetic variants that increase expression of AT\textsubscript{1} receptors to cause hypertension. Our studies suggest that the impact of increased \textit{AGTR1} gene expression on blood pressure will be modified by gender, by vascular compensations, and by altered expression of other genes involved in cardiovascular regulation. Thus, even for a key component of the renin-angiotensin system, the potential for incremental changes in expression of a single gene to affect blood pressure is buffered by compensations at a number of levels. Moreover, these compensations are also subject to genetic regulation that may vary between male and female subjects. Accordingly, more profound changes in blood pressure may be expected when there are concomitant mutations in genes within compensatory pathways. This is consistent with current views suggesting a polygenic basis for human hypertension.

**Acknowledgments**

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