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Original Research

Cavernosum smooth muscle relaxation induced by Schisandrol A via the NO-cGMP signaling pathway

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Abstract: To evaluate the effect of Schisandrol A on rabbit corpus cavernosum smooth muscle and elucidate the potential mechanism. Penises were obtained from healthy male New Zealand White rabbits (2.5-3.0 kg). The pre-contracted penis with phenylephrine (Phe, 10 μM) was treated with accumulative concentrations of Schisandrol A (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M). The change in intracavernosum pressure (ICP) and tension was recorded, cyclic nucleotides in the cavernosum tissue were measured by radioimmunoassay, mRNA level and expression of endothelial nitric oxide synthase (eNOS) and neuronal NOS (nNOS) were measured by real time PCR and western blot respectively. The corpus cavernosum smooth muscle relaxation induced by Schisandrol A was in a dose-dependent manner. Pre-treatment with NOS inhibitor (Nω nitro-L-arginine-methyl ester, L-NAME) or guanylyl cyclase inhibitor (1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one, ODQ) significantly diminished the relaxation. The cyclic guanosine monophosphate (cGMP) level was significantly increased in the cavernosum tissue. Real time PCR and western blot showed the mRNA level and expression of eNOS and nNOS was also upregulated. Schisandrol A relaxes the cavernosum smooth muscle by activating NO-cGMP signaling pathway. It may be a new promising treatment for erectile dysfunction and cardiovascular disease.

Key words: Schisandrol A, Cavernosum smooth muscle, Erectile dysfunction, NO-cGMP pathway.

Introduction

Penile engorgement is a dilatory vascular response to sexual stimulation/arousal that requires sufficient arterial blood inflow. Erectile dysfunction (ED) is the persistent inability to achieve or maintain an erection for satisfactory sexual performance (1). Epidemiologic studies suggest that ED affects more than 150 million men worldwide (2). ED shares common risk factors with cardiovascular disease (CVD), especially coronary artery disease (CAD), including age, lack of exercise, obesity, smoking, hypercholesterolemia, metabolic syndrome, diabetes, and hypertension (3-5). ED and CVD frequently coexist, men with known CVD have an ED prevalence of approximately 70% (6). Studies have confirmed that ED is an independent marker of increased CVD risk and commonly precedes clinical CAD (7-10).

Endothelial dysfunction is considered to be the etiologic link between CVD and vasculogenic ED. Nitric oxide (NO) released from endothelium possesses antiatherogenic, antithrombotic, and anti-inflammatory properties and promotes vasodilation of the vascular smooth muscle (11). Impaired endothelial function causes corpus cavernosum smooth muscle constriction, which induces decreased penile blood flow and failure of penile erection.

Schisandrol A, one of six major lignan constituents of *Schisandra chinensis* Baill fruit, has been demonstrated to have a significant relaxant effect in isolated rabbit cavernosum smooth muscle *in vitro* (12). However, the molecular mechanism remains unclear. The objective of the present study was to evaluate the effect of Schisandrol A on rabbit corpus cavernosum smooth muscle and elucidate the potential mechanism by using an *in vitro* penile perfusion model.

Materials and Methods

Chemicals and reagents

Nω nitro-L-arginine-methyl ester (L-NAME), 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ), and L-phenylephrine (Phe) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Schisandrol A was isolated and purified from the fruit of *Schisandra chinensis*, as described previously (12). All other chemicals were purchased from standard suppliers. Schisandrol A was dissolved in ethanol, and subsequently diluted in the buffer to the final concentration (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M). The other drugs were dissolved in distilled water except ODQ, which was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). The highest ethanol or DMSO concentration in the various test system was < 1%, (v/v).

Tissue preparation

This research was conducted in accordance with the internationally accepted principles for laboratory

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animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) or the guidelines (NIH publication #85-23, revised in 1985). The tissue was prepared as previously described (13). Healthy control male New Zealand White rabbits weighing 2.5–3.0 kg were anesthetized with ketamine (50 mg/kg intravenously) plus rompun (xylazine hydrochloride, 25 mg/kg), and exsanguinated. The entire penis, including the urethra was rapidly excised from the pubic bone. The urethra was dissected free from the penile body. During the preparation, each step was taken cautiously to prevent damage of functional endothelium or overstretching of the tissue.

The glans penis was cut out until the corpus cavernosum was exposed to air through a small opening with a diameter of 5 mm. Two small polyethylene tubes (inner diameter, 1.2 mm and outer diameter, 1.7 mm; Natsume, Tokyo, Japan), which contained small internal platinum electrodes, were inserted into the proximal opening of the crura for inflow, and ligated with a purse string silk suture to prevent leakage. The distal cut of the corpus cavernosum was opened to allow flow out of the penis. The distal end was sutured with a cotton thread to a holder at the bottom of the chamber. The cannulated penis was mounted vertically in a 50 mL fully humidified organ chamber without buffer outside of the penis. The penis was immediately perfused interstitially through the cannulae with HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid)) buffer using a peristaltic pump (0.5 mL/minute). The HEPES buffer contained the following (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; glucose, 10.0; and HEPES 10 with NaOH (pH 7.4). The perfusion solution was oxygenated with 100% O_2 and maintained at 36°C. The hollow organ chamber was covered with Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA) to maintain the temperature and humidity in the organ chamber at 36°C. ICP was measured using a pressure transducer (FT03, Grass Telefactor) connected to the inflow tube cannulated to the cruses and recorded using a PowerLab data 400 acquisition system (Software Chart, version 5, AD Instrument). After mounting, tissue was equilibrated for 100 minutes with several adjustments of length until a baseline force was stabilized at 10 g. The chamber for penile perfusion had a hole at the bottom to allow collection of the perfusate. Changes in tension were measured with a force transducer.

Evaluation of cumulative dose of Schisandrol A

Experiments were performed to investigate cumulative dose-dependent relaxation responses to Schisandrol A (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) in the Phe pre-contracted penile corpus cavernosum (PCC). The extracts were added to the medium in sequence, each for 10 min. To investigate the toxic effect of Schisandrol A, we repeated the procedure after tissue washed for 1 h and the PCC were collected after experiments. The tissue was pre-incubated with L-NAME (1 mM) for 30 min to block NOS, or pre-incubated with ODQ (10 μM) for 30 min to block guanylyl cyclase activity were also performed.

Radioimmunoassay (RIA) of cGMP concentration

For measurement of the cyclic guanosine monophosphate (cGMP) concentration in PCC, the samples were minced in 2 mL of ice-cold trichloroacetic acid and homogenized at 4 °C with three 30 sec bursts in a Polytron homogenizer. The homogenates were centrifuged at 1000 g for 10 min at 4 °C, and the supernatant was extracted with ether and dried. The pellet was treated with 500 μL NaOH (1N), ultrasonicated, and used for protein determination.

Levels of cGMP were measured with a specific RIA, as described previously (14). Briefly, standards or samples were taken up in a final volume of 100 μL of 50 mM sodium acetate buffer (pH 4.8) containing theophylline (8 mM), then 100 μL of diluted cGMP antiserum (Calbiochem-Novabiochem) and iodinated 2'-O-monosuccinyl-guanosine 3',5'-cyclic monophosphate tyrosyl methyl ester (125 I-ScGMP-TME; 10,000 counts/min (cpm) per 100 μL) were added for the measurement of cGMP and incubated for 24 h at 4 °C. For the acetylation reaction, 5 μL of a mixture of acetic anhydride and triethylamine (1:2 dilution) were added to the assay tube before antiserum and tracer were also added. The bound form was separated from the free form by charcoal suspension. The amount of cGMP were expressed as femtomol per milligram of PCC tissue.

Real time PCR for NOS mRNA level

To evaluate the effect of Schisandrol A on nitric oxide synthase (NOS) mRNA level and expression, penises were incubated with Schisandrol A (10^{-4} M) for 2 or 4 h, PCC smooth muscle was then carefully dissected from the surrounding tunica albuginea for real time PCR and western blot. PCC tissues without perfusion and incubated with HEPES buffer for 4 h were also collected.

Total RNA was extracted from rabbit PCC using TRIZOL reagent (Invitrogen, Carlsbad, CA) and reverse transcription was performed using Superscript II and 18-mers Oligo-dT (Takara, Japan). Specific primers were designed using primer express software (Applied Biosystems, Carlsbad, CA) and their primer sequences were as follows: rabbit eNOS, 5'-TCGTCCCTGTGGAAAGACAAG-3' (forward) and 5'-CTTTGGCGAGCTGGTAACTGT-3' (reverse); rabbit nNOS, 5'-GAGAAGCAACGCCTGTTGGT-3' (forward) and 5'-CCCCATTTCCACTCCTCGTA-3' (reverse); and rabbit actin, 5'-TGGCATCCTGACGCTCAA-3' (forward) and 5'-TCGTCCCAGTTGGTCACGAT-3' (reverse).

The real-time PCR reaction contained in a final volume of 10 μL, 10 ng of reverse transcribed total RNA, 200 nM of forward and reverse primers and 2× PCR master mix. PCR reaction was carried out in 384-well plates using the ABI Prism 7900HT Sequence etection System (Applied Biosystems). All reactions were done in triplicate.

Western blot for eNOS and nNOS expression

The removed PCC tissue was homogenized in ice-cold buffer containing 0.32 M sucrose, 0.2 M HEPES (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin, 10 μg/mL trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenized solution was placed on

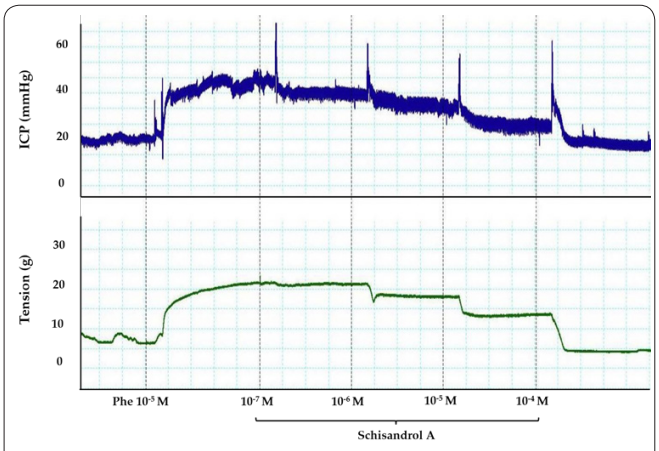

Figure 1. Representive figure of Schisandrol A induced relaxation in penile corpus cavernosum using *in vitro* penile perfusion model. ICP: intracavernosum pressure; Phe: phenylephrine.

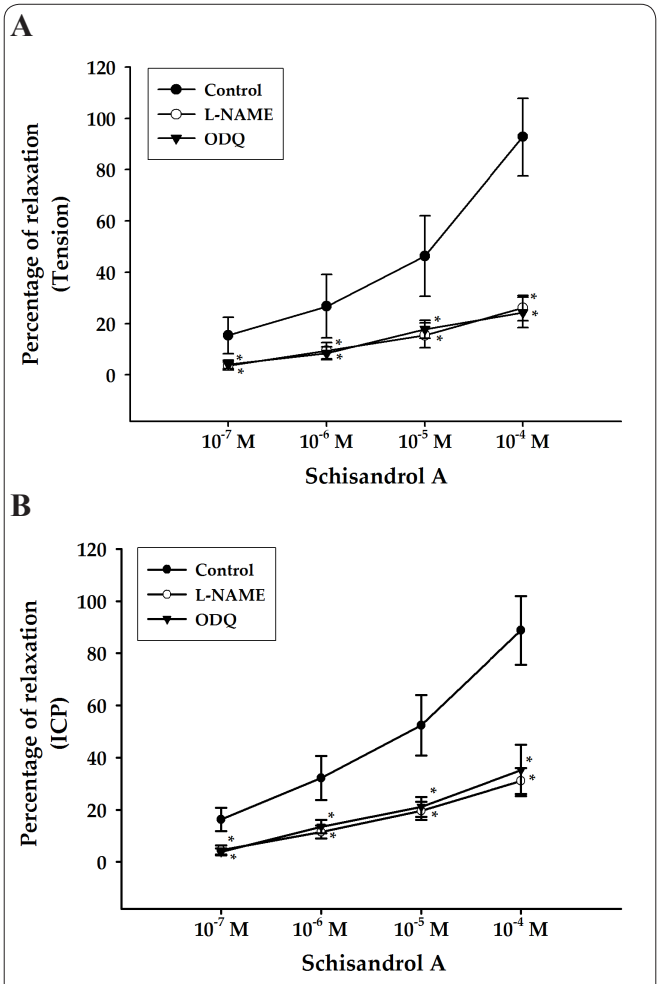

Figure 2. Percentage of relaxation induced by Schisandrol A (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M). A. Changes in penile tension; B. Changes in ICP. Each point represents the mean ± SD of percentages of maximal relaxation of the preceding submaximal contractile responses. L-NAME: Nω nitro-L-arginine-methyl ester; ODQ: 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one; ICP: intracavernosum pressure. * P < 0.01 *vs* control.

ice for 15 min, and then centrifuged at 4 °C for 13000 rpm for another 30 min, and the supernatant was separated. The separated solution used bovine serum albumin. Thirty μg of the quantitative protein was denatured at 95 °C for 5 min and electrophoresis was done on a 6% discontinuous sodium dodecylsulfate (SDSPAGE)-polyacrylamide gel. The proteins were then electroblotted onto a 0.2 μM polyvinylidenedifluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ, USA) for 150 min at 25 V. The membranes were reacted with blocking buffer (5% skim milk in TBS-T buffer) for 30 min at ambient temperature. eNOS antibody (Genscript, Piscataway, NJ, USA) and nNOS antibody (Abcam, Cambridge, MA, USA) were reacted for 12 h at 4 °C and the membrane was washed three times using TBST at intervals of 10 min. As the secondary antibodies, anti-mouse IgG-HRP and anti-goat IgG-HRP (1:2000 dilution; Zymed Laboratories, San Francisco, CA, USA) were reacted at ambient temperature for 1 h, and the membrane was washed again with TBST six times with an interval of 5 min between each washing. Chemiluminescence was detected using ECL western blotting detection reagents.

Statistical analysis

The submaximal penile contractile responses induced by Phe were taken as the 100% values, and all subsequent responses to Schisandrol A were expressed as a percentage of this value. The results are expressed as the mean ± standard deviation (SD), and n represents the number of tissues in each group. The statistical significance of differences was calculated by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. Concentration-dependent responses before and after the treatment with blockers were compared by Student's paired *t*-test. A probability value < 0.05 was considered significant.

Results

Evaluation of cumulative dose of the Schisandrol A

The relaxation induced by Schisandrol A (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) was in a dose-dependent manner (Figure 1). Penile tension was decreased by 15.53 ± 7.10, 26.82 ± 12.31, 46.36 ± 15.63, and 92.78 ± 15.16% in response to Schisandrol A 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M, respectively (Figure 2A). Similarly, ICP was also decreased by 16.35 ± 4.52, 32.15 ± 8.41, 52.36 ± 11.58, and 88.75 ± 13.22% in response to Schisandrol A 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M, respectively (Figure 2B). After washing, the tissues had the same response to Phe and Schisandrol A. Pre-treatment with L-NAME, or ODQ significantly inhibited the relaxation (n = 8, P <0.01, Figures 2A, B).

Effect of the Schisandrol A on cGMP in PCC

The cGMP level significantly increased in PCC tissue perfused by Schisandrol A in a concentration-dependent manner. The yield of cGMP peaked at a concentration of 10^{-4} M (n = 8, Figure 3).

Effect of the Schisandrol A on mRNA level of eNOS and nNOS

The mRNA levels of eNOS and nNOS in PCC were significantly up-regulated by the incubation with Schisandrol A (10^{-4} M), HEPES buffer had no significant effect on NOS mRNA level (n = 8, Figure 4, P <0.01).

Effect of the Schisandrol A on the expression of nNOS and eNOS

After incubated with Schisandrol A (10^{-4} M) for 4 h,

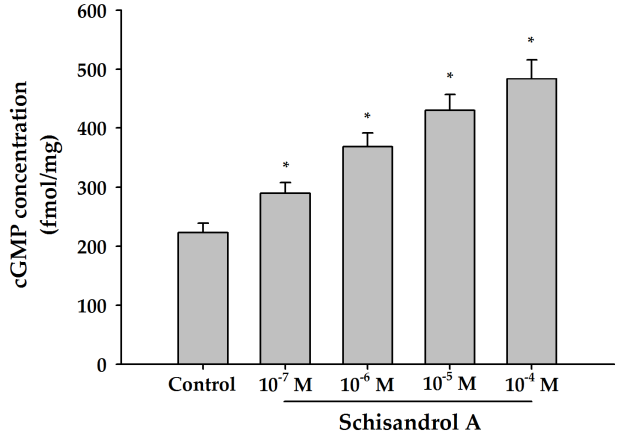

Figure 3. Effect of Schisandrol A (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) on cGMP level in penile corpus cavernosum. * $P < 0.01$ *vs* control.

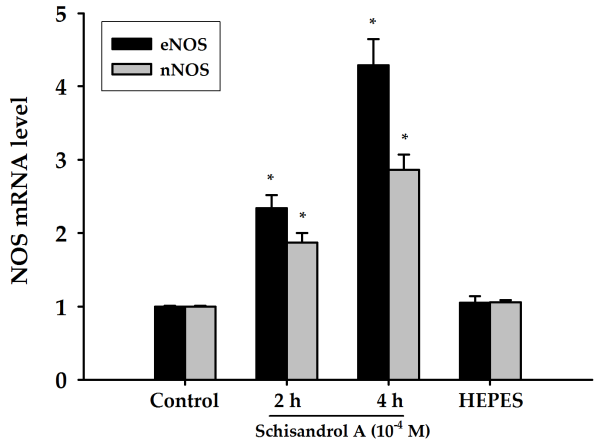

Figure 4. The mRNA level of eNOS and nNOS in penile corpus cavernosum after incubation with Schisandrol A (10^{-4} M) for 2 h and 4 h. Control: without perfusion; HEPES: perfused with HEPES buffer for 4 h. * $P < 0.01$ *vs* control.

the eNOS and nNOS levels in PCC were significantly increased approximately 3-fold and 1.8-fold, respectively. HEPES buffer had no significant effect on NOS expression (n = 8, Figure 5, $P < 0.01$).

Discussion

Penile erection is a complex neurovascular process that involves relaxation of the corpus cavernosum smooth muscle. Many studies have shown that NO released from non-adrenergic, noncholinergic (NANC) nerves and endothelium is a key factor necessary for penile erection (15). Released NO activates soluble guanylyl cyclase and increases the production of cGMP (16). Through a protein kinase cascade, hyperpolarization, and intracellular calcium sequestration, cavernosal smooth muscle relaxation and arteriolar vasodilation occur. As results showed (Figure 1-3) in this study, Schisandrol A significantly relaxed the PCC smooth muscle and increased the cGMP concentration in PCC tissue. Moreover, the relaxation was attenuated by NOS inhibitor or guanylyl cyclase inhibitor. These results suggested the relaxation induced by Schisandrol A involved NO-cGMP signaling pathway.

In the present study, the expression of eNOS and

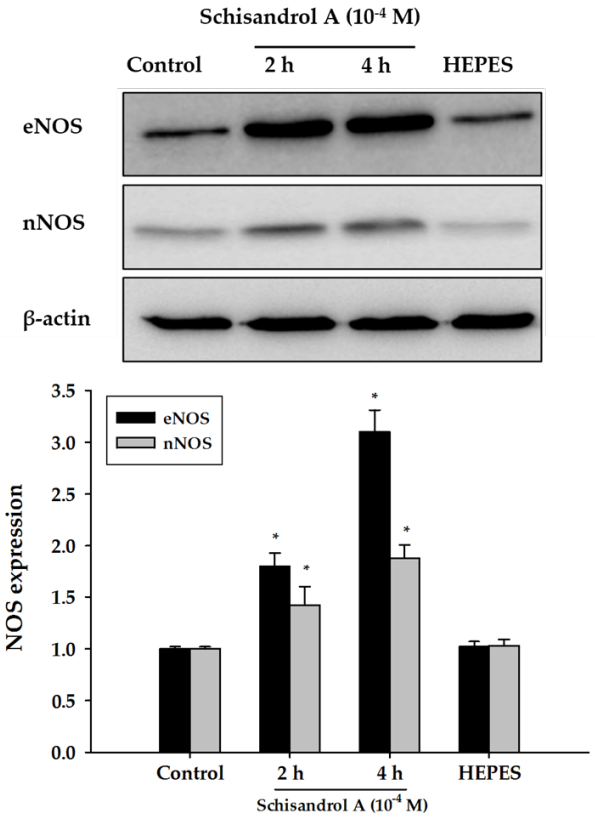

Figure 5. Western blot shows expression of eNOS and nNOS in penile corpus cavernosum after incubation with Schisandrol A (10^{-4} M) for 2 h and 4 h. Control: without perfusion; HEPES: perfused with HEPES buffer for 4 h. * $P < 0.01$ *vs* control.

nNOS in PCC was enhanced by Schisandrol A (Figure 4). NO is produced as the enzymatic byproduct of molecular oxygen (O_2) and L-arginine under the control of NOS. So far, three distinct isoforms of NOS have been identified: nNOS, eNOS, and inducible NOS (iNOS). All three isoforms are present in corpora cavernosa, although with a different cellular localization. NANC neurones express nNOS, while endothelial and smooth muscle cells express the other isoforms (17). Studies have shown that ED is caused by inadequate relaxation of the corpus cavernosum with defects in NO production (18). Up-regulating eNOS and nNOS expression may restore the production of NO in ED patients.

Results from the Massachusetts Male Aging Study confirmed the association of ED with CAD, finding for patients with heart disease a 39% probability of complete ED, and subsequent studies have shown ED rates in patients with CAD as high as 75% (19-21). The artery-size hypothesis may help explain why patients with CAD frequently report ED before CAD detection. Because the lumen of the penile arteries (1-2 mm) is considerably smaller than that of the coronary (3-4 mm), carotid (5-7 mm), and femoral (6-8 mm) arteries, endothelial dysfunction or plaque burden that significantly impairs circulation in the penile arteries may be associated with subclinical plaque disease of the larger vessels (22). Multiple studies have documented the occurrence of ED 2-5 years prior to CAD presentation (9, 23). Vascular endothelial dysfunction is the probable pathophysiologic link between the two disorders. Enhance the expression and activity of eNOS, increase the production of NO may restore the impaired endothelium function. Therefore, Schisandrol A may also have beneficial

effects on CVD. Further study is needed to confirm the hypothesis.

In conclusion, Schisandrol A significantly relaxed the cavernosum smooth muscle by activating NO-cGMP signaling pathway. It may be a new promising treatment for erectile dysfunction and cardiovascular disease.

Acknowledgements

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