

Rho/rho-kinase signalling in chronically alcohol-fed mice

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Background/aim: The effects of chronic ethanol consumption on male sexual function are debateable, and its effects on the Rho/Rho-kinase pathway are unknown. Therefore, we investigated smooth muscle reactivity and Rho/Rho-kinase signalling in the corpus cavernosum of ethanol-fed mice.

Materials and methods: Ethanol was added to drinking water at 5% concentration in the first 2 days with 5% increment every subsequent 2 days, up to a final concentration of 20% for 45 days. Corpora cavernosa were isolated and a cumulative dose-response curve to phenylephrine was obtained. Acetylcholine (10–6 M), electrical field stimulation (EFS, 40 V, 8–16 Hz) and Y-27632 (10⁻⁶–10⁻⁵ M)-induced relaxations were compared in the control and ethanol groups.

Results: Blood ethanol levels in the control and ethanol-treated mice were 1.5 ± 0.3 mg/dL and 37.4 ± 4.1 mg/dL (P < 0.001), respectively. Phenylephrine-induced contractile responses were potentiated in the ethanol group, with pD₂ values of 4.92 ± 0.18 and 5.71 ± 0.21 (P < 0.01) in the corpus cavernosum obtained from control and alcohol-fed mice, respectively. The relaxant responses to acetylcholine and EFS, but not Y-27632, were significantly augmented in the corpus cavernosum obtained from ethanol-treated mice. However, expression and activation of Rho-kinase remained unchanged in the alcohol group.

Conclusion: Ethanol drinking may increase sensitivity to both vasoconstrictors and vasodilators in the mouse corpus cavernosum without any alteration in Rho/Rho-kinase signalling.

Key words: Alcohol, corpus cavernosum, ethanol, erection, rho-kinase

1. Introduction

The effects of chronic alcohol consumption on male sexual function are debateable. It has been reported that chronic ethanol treatment leads to an increased responsiveness of the corpus cavernosum to both constrictor and relaxant agents (1). Moreover, ethanol consumption enhanced contractile responses of the vascular smooth muscles (2,3). Long-term ethanol intake caused a marked enhancement in noradrenergic nerve-mediated contractions and a reduction in the relaxant response to acetylcholine but no effects on α₁-adrenoceptor-mediated contraction of the rat corpus cavernosum (4). Attenuated or unchanged responses to α₁-adrenoceptor agonists have also been demonstrated (5,6). Moreover, it has been shown that ethanol consumption suppressed contractile responses and neurogenic relaxation; however, it enhanced ATP but not a nitric oxide donor, SNP-induced relaxation (7). Consequently, the net effect of chronic alcohol consumption on male erectile function is still a matter of much debate.

The small GTPase RhoA and its downstream effector, Rho-kinase (ROCK), play a crucial role in corpus

cavernosal contractions (8,9). Furthermore, this signalling may play an essential role in neurogenic-, myogenic-, and agonist-induced smooth muscle contractions in other urogenital tissues (10–12). Alteration in the activity of Rho/Rho-kinase signalling is of physiological importance in penile erection (9), and blockers of Rho-kinase have the potential to be used in the treatment of erectile dysfunction (8,9,13).

In the present study, we aimed to investigate the effects of chronic ethanol consumption on the reactivity of mouse corpus cavernosum. Moreover, we also examined possible alteration of Rho/ROCK signalling in the corpus cavernosum obtained from chronic alcohol-fed mice. For that purpose, we fed mice ethanol for 45 days and thereafter the responses to a-agonist phenylephrine, electrical field stimulation (for neurogenic stimulation), and acetylcholine as well as the selective Rho-kinase inhibitor, Y-27632, were evaluated in the isolated mouse corpus cavernosum. Furthermore, Rho-kinase (ROCK-2) expression and its activity (pMYPT1) were detected by western blotting.

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2. Material and methods

2.1. Animals, tissue preparation, and experimental design

The protocol of this study was approved by the Ethics Committee of Mersin University for Animal Research. Adult male Balb/c mice weighing 30–35 g were caged separately under a 12-h light/12-h dark photoperiod and a constant temperature (22 ± 1 °C). The mice received drinking water and standard mice chow ad libitum throughout the experiment.

The mice were randomly divided into two groups; one contained the control mice and the other chronically ethanol-fed mice ($n = 15$ for each). In the alcohol group, the percent of ethanol in drinking water was 5% (v/v) in the initial 2 days and increased gradually 5% every other 2 days in order to reach a final concentration of 20% ethanol for 45 days. The control mice received only tap water. At the end of 45 days, the mice were anaesthetised by intraperitoneal injection of ketamine (500 mg/kg). The thorax was immediately opened and blood samples were collected from the heart. The blood samples were then centrifuged ($2000 \times g$ for 10 min) and supernatants were stored in -80 °C for ethanol analysis.

For the isolation of the corpus cavernosum, penises from the control and ethanol-treated mice were removed and placed in a petri dish containing Krebs solution (in mM, NaCl 118, KCl 4.8, CaCl_2 2.5, KH_2PO_4 1.2, NaHCO_3 24, glucose 11, MgSO_4 1.2, Na_2EDTA 0.01). Both the glans penis and urethra were excised and adherent tissues were carefully removed, keeping the tunica albuginea intact. The penises were dissected longitudinally in two strips. Subsequently, cavernosal strips were suspended through two platinum ring electrodes in organ baths (10 mL) filled with Krebs solution, gassed with 95% O_2 and 5% CO_2 under 0.3-g initial tension. Tension was recorded isometrically with a force transducer (COMMAT, Ankara, Turkey) and displayed on a Biopac acquisition system (Biopac Systems, CA, USA).

Following equilibration of 1 h, the cavernosal strips were maximally contracted by 50 mM KCl. After the tissue responses reached a steady state, the organ baths were replaced with fresh Krebs solution. Following incubation in Krebs solution for 45 min, phenylephrine was applied cumulatively (10^{-9} – 10^{-4} M) and dose-response curves were obtained. In the other set of experiments, following equilibration, cavernosal strips were precontracted with 5×10^{-5} M phenylephrine. After a steady state of contraction was obtained, the relaxant responses to electrical field stimulation (40 V, 0.5 ms, 15 s, 8 and 16 Hz at 2-min intervals), acetylcholine (10^{-6} M), or Y-27632 (10^{-6} – 10^{-5} M, cumulatively) were recorded. We chose 8 and 16 Hz for EFS and 10^{-6} M for acetylcholine because only at these frequencies and concentration we obtained relaxant

responses that allowed us to compare in both groups. The concentrations of Y-27632 were in the range to selectively inhibit Rho-kinase activity.

2.2. Measurement of blood ethanol levels

Blood ethanol levels were measured by an enzymatic assay using alcohol dehydrogenase (ADH) and NAD. The alcohol dehydrogenase method is based on oxidation of alcohol in the presence of ADH as enzyme and NAD^+ as coenzyme and formation of acetaldehyde and NADH that can be monitored by spectrophotometric measurement with the help of a Cobas Roche Integra 800 (F. Hoffmann-La Roche Ltd, Basel, Switzerland).

2.3. Western blotting

Corpus cavernosum samples were minced and homogenised on ice with a lysis buffer (Tris-HCl (pH 7.4) 50 mM, NaCl 400 mM, EGTA 2 mM, EDTA 1 mM, dithiothreitol 1 mM, phenylmethylsulphonyl fluoride 10 μM , leupeptin 10 $\mu\text{g}/\text{mL}$, pepstatin 1 $\mu\text{g}/\text{mL}$, NaF 1 mM, and benzamide 1 mM). The homogenate was centrifuged at $13,000 \times g$ for 10 min at 4 °C, and the supernatant was removed. Protein concentrations were estimated by Bradford assay using a protein assay kit (Bio-Rad, Munich, Germany). Equal amounts of proteins were loaded in wells, electrophoresed on polyacrylamide-sodium dodecyl sulphate (SDS) gels, and then transferred to a PVDF membrane overnight. The membrane was blocked with the blocking agent of the ECL-Plus enhanced chemiluminescence kit (Amersham Biosciences, Freiburg, Germany) in Tris-buffered solution containing 0.05% Tween-20 for 1 h. It was then probed with a primary antibody raised against ROCK-2 (polyclonal IgG, 1:200), pMYPT1 (1:200) or actin (1:1000) followed by horseradish peroxidase conjugated secondary antibodies (1:1000). Protein blots were then detected with a chemiluminescence detection kit (ECL plus, Amersham Biosciences, Freiburg, Germany) and visualised on commercial X-ray films. ROCK protein levels were normalised relative to actin, which is a constitutive protein.

2.4. Drugs and chemicals

Phenylephrine, acetylcholine, and actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632) from Tocris Cookson Ltd. (Bristol, UK) and the primary antibody for ROCK-2, pMYPT1, and HRP-conjugated secondary antibody from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The ECL Plus kit was purchased from Amersham Biosciences (Freiburg, Germany). Y-27632, acetylcholine, and phenylephrine were dissolved in distilled water.

2.5. Statistical evaluations

All data were expressed as means \pm standard error of the mean (SEM). A computer program (GraphPad prism 3.0, San Diego, CA, USA) was used for analysing data. ROCK bands were measured relative to actin bands. One-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test or Student's t-test were used for statistical comparison. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of chronic ethanol consumption on blood ethanol, alanine transaminase, and total cholesterol levels as well as body weight alteration in mice

At the end of 45 days, the blood ethanol level of the alcoholic mice increased approximately 25-fold when compared with that of the control mice. Body weight decreased in the alcoholic mice; however, alanine transaminase and total cholesterol levels were not altered (Table).

3.2. Effect of chronic ethanol consumption on the responses to phenylephrine, acetylcholine, and electrical field stimulation

To investigate if chronic alcohol drinking could have any effects on the reactivity of mouse corpus cavernosum, we tested the most-used pharmacological tools and approaches such phenylephrine, acetylcholine, and electrical field stimulation. The contractile activity to the universally used α -adrenergic receptor activator, phenylephrine (10^{-9} – 10^{-4} M), was augmented in the ethanol group compared to the control group. pD_2 values for phenylephrine were 4.92 ± 0.18 and 5.71 ± 0.21 ($P < 0.01$) in the control and ethanol groups, respectively (Figure 1, upper panel). In contrast, the relaxant responses to acetylcholine (10^{-6} M, Figure 1, middle panel), a ubiquitously used tool to check endothelium-derived relaxation, and to electrical field stimulation (8 and 16 Hz, Figure 1, lower panel), to check neurogenic relaxation, were more pronounced in the corpus cavernosum obtained from the control group than they were in that from the ethanol group.

3.3. Effect of chronic ethanol consumption on the relaxant response to the Rho-kinase inhibitor, Y-27632

In order to examine whether relaxant response to the ROCK inhibitor, Y-27632, altered in the corpus cavernosum obtained from chronically alcohol drinking mice, we applied two concentrations of Y-27632 (10^{-6} – 10^{-5} M), at which the enzyme, ROCK, can be specifically inhibited. However, chronic ethanol drinking did not significantly change the relaxant responses to Y-27632 (Figure 2).

3.4. Effect chronic ethanol consumption on Rho-kinase (ROCK-2) expression and activity in the mouse corpus cavernosum

To understand whether chronic ethanol consumption has any effect on Rho-kinase (ROCK-2) expression and activity, we performed western blotting in the isolated corpus cavernosum obtained from chronically ethanol fed mice. Western blot analysis indicated that (ROCK-2) protein level (Figure 3) as well as its activity (Figure 4) did not change in the chronic alcohol group compared to the control group.

4. Discussion

The effects of chronic ethanol consumption on reactivity of smooth muscles are controversial. For instance, ethanol feeding suppressed (6,14) and enhanced (2,3) phenylephrine-induced contractions in the rat aorta. In addition, increased blood pressure and altered reactivity of the mesenteric bed were reported in chronic ethanol consumption via two possible mechanisms: an increased release of endothelium-derived vasoconstrictor prostanoids and a reduced modulatory action of endothelial NO (15). On the other hand, chronic ethanol consumption increased both relaxant responses to electrical field stimulation and contractile responses to the agonists such as phenylephrine and KCl in the rabbit corpus cavernosum (1). Therefore, the effects of ethanol on smooth muscle reactivity are debatable.

Table. Blood ethanol, alanine transaminase, and total cholesterol levels as well as body weight alteration in control (n = 13) and ethanol-fed (n = 14) mice. Data were expressed as mean \pm SEM for n observations. Student's t-test was used for unpaired observation.

	Control	Ethanol
Ethanol	1.5 \pm 0.3 mg/dL	37.4 \pm 4.1 mg/dL ***
Weight	37.0 \pm 0.7 g	32.9 \pm 1.1 g **
Alanine transaminase	37.9 \pm 2.6 U/L	32.3 \pm 2.7 U/L
Total cholesterol levels	136.2 \pm 4.9 mg/dL	122.6 \pm 14.2 mg/dL

** $: P < 0.01$, *** $: P < 0.001$

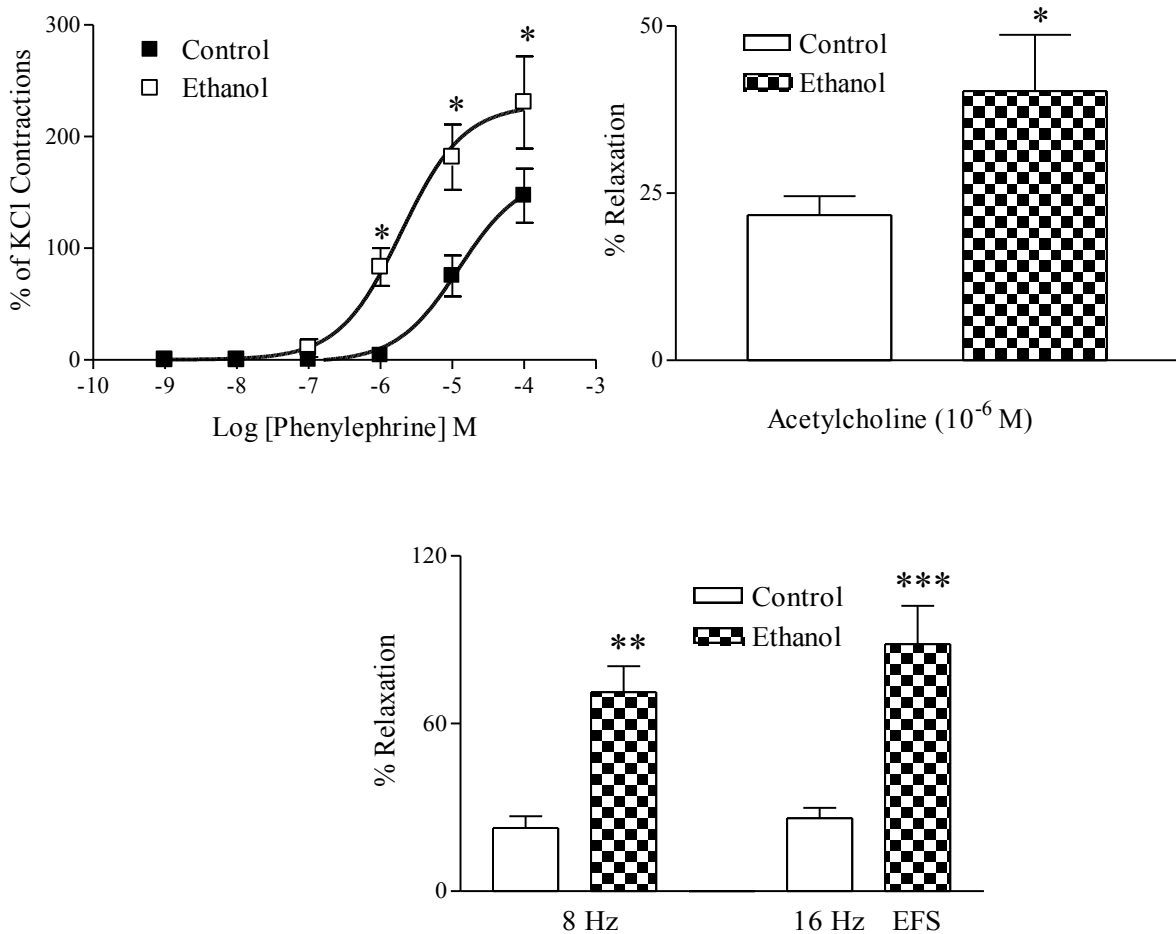


Figure 1. Effect of chronic ethanol consumption on the responses of mouse corpus cavernosum to phenylephrine (10^{-9} – 10^{-4} M, $n = 6$, upper panel), acetylcholine (10^{-6} M, $n = 4$ – 8 , middle panel), electrical field stimulation (40 V, 0.5 ms, 15 s, 8–16 Hz, $n = 10$ – 11 , lower panel). Phenylephrine-elicited contractions were expressed as percentage of the KCl (50 mM)-induced contractions. Relaxations were expressed as percentages of active tones induced by phenylephrine (5×10^{-5} M). Data were represented as mean \pm standard error of mean (SEM). One way ANOVA followed by Bonferroni post hoc test and Student's t-test were used for statistical analysis. (*: $P < 0.05$, **: $P < 0.01$; ***: $P < 0.001$).

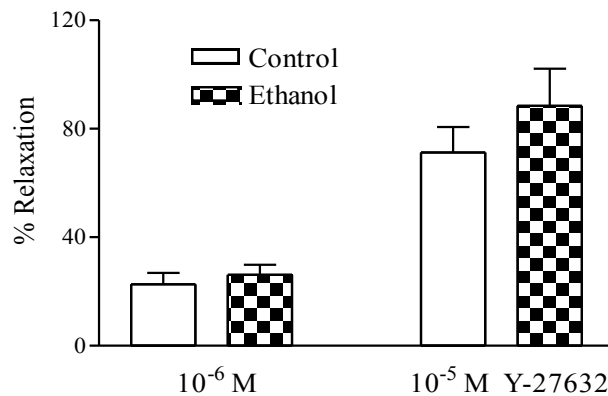


Figure 2. Effect of chronic ethanol consumption on the relaxant responses of mouse corpus cavernosum to the selective Rho-kinase inhibitor, Y-27632 (10^{-6} – 10^{-5} M, $n = 10$ – 11). Y-27632-elicited relaxation was expressed as percentage of phenylephrine (5×10^{-5} M)-induced contractions. Data were represented as mean \pm standard error of mean (SEM). One way ANOVA followed by Bonferroni post hoc test was used for statistical analysis.

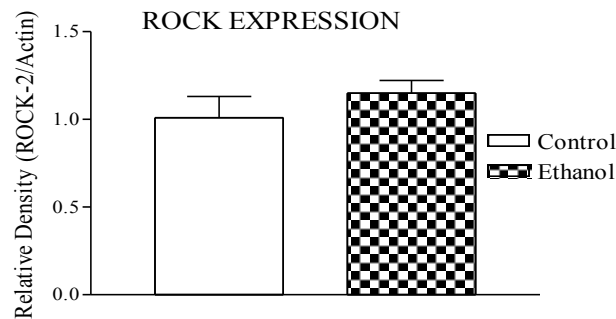


Figure 3. The expression of Rho-kinase in the corpus cavernosum obtained from control and chronic alcoholic mice. The corpora cavernosa were homogenised with a lysis buffer. Thereafter, the tissue homogenates were submitted to sodium dodecyl sulphate (SDS)-PAGE with 8%–10% polyacrylamide and then transferred to a nitrocellulose membrane. After blocking, the membrane was probed with a primary antibody raised against ROCK-2 (Santa Cruz, 1:200) or pMYPT1 (shows the activity of Rho-kinase, Santa Cruz, 1:200), followed by horseradish peroxidase-conjugated secondary antibody (donkey antioat, 1:1000). Blots were then assayed with an Enhanced Chemiluminescence Detection Kit (ECL, Amersham Bioscience) and visualised on commercially available X-ray films. C: Control, E: Chronic ethanol group (n = 4–5, duplicate). Data were represented as mean ± standard error of mean (SEM). Student's t-test was used for statistical analysis.

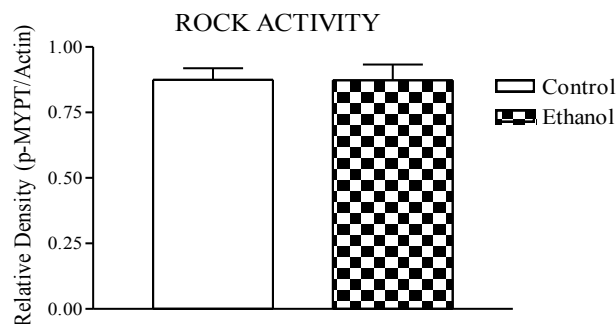


Figure 4. The activity of Rho-kinase in the corpus cavernosum obtained from control and chronic alcoholic mice. The corpora cavernosa were homogenised with a lysis buffer. Thereafter, the tissue homogenates were submitted to sodium dodecyl sulphate (SDS)-PAGE with 8%–10% polyacrylamide and then transferred to a nitrocellulose membrane. After blocking, the membrane was probed with a primary antibody raised against pMYPT1 (shows the activity of Rho-kinase, Santa Cruz, 1:200), followed by horseradish peroxidase-conjugated secondary antibody (donkey antioat, 1:1000). Blots were then assayed with an Enhanced Chemiluminescence Detection Kit (ECL, Amersham Bioscience) and visualised on commercially available X-ray films. C: Control, E: Chronic ethanol group (n = 4–5, duplicate). Data were represented as mean ± standard error of mean (SEM). Student's t-test was used for statistical analysis.

In the present study, chronic alcohol drinking did not alter either alanine transaminase (ALT) or total cholesterol level at least at the dose and the duration of exposure we used. Furthermore, we showed that chronic ethanol consumption augmented the contractile responses of the isolated mouse corpus cavernosum to phenylephrine, giving support to the work by Saito et al (1). It has been previously demonstrated that the α -agonist phenylephrine recruits Rho/Rho-kinase pathway, at least in part, to induce contraction in urogenital tissues (10–12,15). This signalling pathway is of fundamental importance for corpus cavernosum reactivity, and the inhibition of this signalling results in penile erection (8,9). However, chronic

alcohol feeding did not alter either ROCK expression or its activation in this study. This may implicate that phenylephrine comprises cellular signalling cascades other than the Rho/Rho-kinase pathway such as protein kinase C and intracellular calcium release (16). This may also be supported by the finding that ethanol consumption did not change Y-27632-elicited relaxation at concentrations at which it selectively inhibits Rho-kinase (10^{-6} – 10^{-5} M).

With regard to the relaxations induced by the stimuli that caused nitric oxide production/release such as electrical field stimulation (EFS) and acetylcholine (8,17), interestingly, ethanol feeding augmented EFS and acetylcholine-induced relaxation. In support, it has been

suggested that ethanol could increase the bioavailability of nitric oxide (18,19) and expression of nitric oxide synthase (20). In addition, ethanol treatment induced NO synthesis in cultured human umbilical vein endothelial cells (21). Moreover, ethanol increased basal and flow-induced endothelial NOS activity in cultured aortic endothelial cells (22). Furthermore, chronic alcohol consumption results in the induction of inducible nitric oxide synthase (iNOS) (23), which may also mediate ethanol-elicited liver damage together with increased superoxide and peroxynitrite production (24,25). Therefore, it seems plausible that the underlying mechanism of enhanced relaxation of mouse corpus cavernosum could involve nitric oxide signalling.

Now that ethanol consumption could enhance the responses to both vasoconstrictors and vasodilators, there must be a common pathway in endothelial, neuronal, and smooth muscle cells within the cavernosal tissue to facilitate both vasodilatation and vasoconstriction.

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One plausible explanation might be the activation of calcium channels under the influence of chronic ethanol consumption because calcium influx triggers smooth muscle contraction (26,27) and the release of vasodilator substances from the endothelium (28,29) as well as nitrergic nerves (30,31). Nevertheless, more detailed studies are required to elucidate ethanol-evoked enhancement to vasodilators and vasoconstrictors.

In conclusion, the present study showed that chronic ethanol consumption could lead to hyperreactivity of the mouse corpus cavernosum to both vasoconstrictor and vasorelaxant agents. However, the underlying mechanism may not involve Rho/Rho-kinase signalling.

Acknowledgements

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