saRNA Guided iNOS Up-Regulation Improves Erectile Function of Diabetic Rats

Tao Wang, Mingchao Li, Huixin Yuan, Yin Zhan, Hua Xu, Shaogang Wang, Weiming Yang, Jihong Liu,* Zhangqun Ye and Long-Cheng Li

From the Department of Urology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, People’s Republic of China, and Department of Urology and Helen Diller Family Comprehensive Cancer Center, University of California-San Francisco (LCL), San Francisco, California

**Purpose:** Promoter targeted saRNAs mediate sequence specific up-regulation of gene expression. We explored the therapeutic effect of RNA activation mediated iNOS gene activation on improving erectile function in a rat model of diabetes mellitus.

**Materials and Methods:** An optimal saRNA sequence specific for iNOS promoter was cloned into an adenoviral vector, resulting in AdU6/shiNOS and AdU6/shControl. The corresponding viruses were used to transduce cultured rat cavernous smooth muscle cells. Streptozotocin induced diabetes models were established in rats and used to test the effects of intracavernous delivery of iNOS saRNA viruses on erectile function. iNOS expression in the cavernous smooth muscle cells or penile tissue of treated rats was assessed by reverse transcriptase-polymerase chain reaction and Western blot. Cyclic guanosine monophosphate was analyzed by enzyme-linked immunosorbent assay. Intracavernous pressure in response to cavernous nerve stimulation was measured using a data acquisition system on post-injection days 1, 3, 5, 7, 10 and 14.

**Results:** Adenovirus mediated expression of iNOS saRNA caused sustained up-regulation of iNOS in cavernous smooth muscle cells. Intracavernous injection of AdU6/shiNOS activated iNOS expression in vivo and significantly increased peak intracavernous pressure in streptozotocin induced diabetic rats via nitric oxide/intracellular cyclic guanosine monophosphate activation.

**Conclusions:** Results show that saRNA mediated iNOS over expression in the penis can restore erectile function in streptozocin diabetic rats via the nitric oxide-cyclic guanosine monophosphate pathway.

**Key Words:** penis; erectile dysfunction; RNA; diabetes mellitus, experimental; nitric oxide synthase type II

**Erectile dysfunction is a common problem affecting about 50% of men 40 to 70 years old.** It often develops as a complication of diabetes with a 27% to 75% prevalence in men with diabetes. Current pharmacological treatments for ED include oral, intracavernous and intraurethral administration of erekogenic drugs. The development of PDE5 inhibitors revolutionized ED management. Oraly administered PDE5 inhibitors are effective in most ED cases and less invasive than other treatment modes. Local intracavernous and intraurethral pharmacotherapy is a second line treatment in cases of failure of or contraindication to oral pharmacotherapy. Men with diabetes who have more severe forms of ED are less responsive to current pharmacological therapy. Moreover, the
Cellular carcinoma. Activating commonly dysregulation. The 3 NOS isoforms are eNOS, nNOS and muscle cell relaxation with subsequent penile erection.

5 days and were passaged every 5 to 6 days. Cells at passages 3 and 4 were used in this study.

In vivo and in vivo, and restored erectile function in diabetic rats. iNOS saRNA activated iNOS expression saRNA in cultured CSMCs and in the penis of diabetic rats. Recombinant adenovirus (AdU6/shiNOS) generation, amplification and titer were performed according to the method of Li et al. A control shRNA sequence was also cloned to generate control adenovirus (AdU6/shControl).

Adenovirus Infection

Cells were incubated with the corresponding virus (AdU6/shiNOS or AdU6/shControl) at an MOI of 25, 50 or 75 at 37C. After adsorption for 2 hours, 2 ml fresh growth medium were added and cells were incubated for 72 hours before harvest. Experiments were performed using virus at an MOI of 75 except as otherwise indicated.

Diabetes Induction in Rat

Procedures were approved by the animal care and use committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. A total of 117 male Sprague-Dawley® rats at age 8 weeks were randomly divided into 2 groups. The nonSTZ group of 13 rats received intraperitoneal injection of citrate buffer (50 mM sodium citrate, pH 4.5). The STZ group of 104 rats received intraperitoneal injection of 60 mg/kg STZ (Sigma® in citrate buffer (50 mM sodium citrate, pH 4.5). At 72 hours after STZ injection rats with a blood glucose of greater than 16.7 mmol/L were considered diabetic. Six rats were excluded from study because blood glucose was below 16.7 mmol/L. Two rats died of unknown reasons in the nonSTZ group and 19 in the STZ group died of complications associated with diabetes mellitus (tumor and/or infection).

At 2 months the remaining 79 rats in the STZ group were randomly divided into 8 subgroups of 9 to 11 per group, including the STZ sham treated, STZ shControl, and STZ shiNOS day 1, 3, 5, 7, 10 and 14 groups. The STZ sham group received sham treatment. The STZ shControl group received 1 intracavernous injection of AdU6/shControl virus and were analyzed on day 1 after injection. The STZ shiNOS day 1, 3, 5, 7, 10 and 14 groups received 1 intracavernous injection of AdU6/shiNOS virus and were analyzed on post-injection day 1, 3, 5, 7, 10 and 14, respectively. Body weight and blood glucose were determined before intraperitoneal injection of STZ and before sacrifice.

In Vivo Gene Delivery to Corpus Cavernosum

The penis was exposed after the rat was anesthetized with sodium pentobarbital (35 mg/kg intraperitoneally). A dose of 15 μl AdU6/shControl (7.94 × 10⁹ IU/ml), AdU6/shiNOS (7.94 × 10⁹ IU/ml) or saline was injected into the right side of the corpora with a 30 gauge needle.

Erectile Response Measurement

Erectile function was assessed by cavernous nerve stimulation, as described previously. Each penis was cut transversely into 3 segments and each segment was sub-

**MATERIALS AND METHODS**

**CSMC Isolation and Culture**

Rat CSMCs were isolated from 3-month-old rats and cultured as previously described. Cells reached confluence in approximately 5 days and were passaged every 5 to 6 days. Cells at passages 3 and 4 were used in this study.

**Construction of iNOS Specific saRNA Adenovirus Vector**

The iNOS promoter sequence was obtained from Ensembl genome databases (http://useast.ensembl.org/index.html) and analyzed against several genome and sequence data-bases, including dbTSS (http://dbtss.hgc.jp/), AceView (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi? db=mouse&q=Ngfb) and the UCSC Genome Browser (http://genome.ucsc.edu/), to determine the TSS. The program assigned weighted scores to all possible targets and returned a list of targets ranked by sum scores. A candidate target sequence was ACTATGCTGCCCAAACTAA at a location between −612 and −594 bp relative to the TSS of the iNOS gene. Recombinant adenovirus (AdU6/shiNOS) generation, amplification and titer were performed according to the method of Li et al. A control shRNA sequence was also cloned to generate control adenovirus (AdU6/shControl).
divided into 3 slices. One slice was randomly selected from each segment to form a new group. The 3 groups were randomly assigned for RNA, protein isolation and enzyme-linked immunosorbent assay, respectively.20

Real-Time Quantitative RT-PCR
Total RNA was isolated from penile tissue and CSMCs using TRIzol® reagent according to manufacturer instructions. Real-time PCR reactions were performed using SYBR® Green PCR Master Mix. The final PCR reaction volume was 25 μl. The table lists the sequences of the RT-PCR primers used.

Western Blot
For protein detection cells and penile tissue lysates were harvested. Equal amounts of total protein were separated and incubated with primary antibodies, including anti-p-eNOS (Ser-1177, 1:1,000), anti-eNOS (1:1,000), anti-nNOS (1:1,000), anti-iNOS (1:1,000) (Abcam®) and anti-β-actin (1:50,000) (Sigma-Aldrich®). Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (1:5,000 or 1:10,000) (ProMab Biotechnologies, Richmond, California) were used. Immunoreactive protein was detected using the enhanced chemiluminescence method (Amersham, Aylesbury, United Kingdom), as described by the manufacturer.

Cavernous cGMP Measurement
Penile tissue was homogenized, centrifuged and extracted with H₂O saturated diethyl ether. Samples were assayed for cGMP using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, Michigan).

Statistical Analysis
All experiments were repeated at least 3 times. Results are presented as the mean ± SD. Statistical analysis was performed with 1-way ANOVA, followed by the Bonferroni post test for multiple comparisons and the LSD t test. The paired t test was used to compare parameters of the same group at different time points. Differences were considered statistically significant at p < 0.05.

RESULTS
Design and Cloning of iNOS Specific saRNA Expressing shRNA
To deliver RNAs to corpus cavernosum tissue in vivo, we used an adenovirus to express saRNA as

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**Figure 1.** Oligonucleotide sequence and predicted RNA structure of specific iNOS sequences. A, annealed ds oligonucleotides were inserted in pDC316-EGFP-U6 between BamHI and HindIII sites. B, after packaging into adenovirus and transduction into CSMCs, oligonucleotides were transcribed into hairpin RNA. C, hairpin RNA was processed into functional saRNA. D, saRNA targeted position of iNOS promoter at position –612 relative to transcription start site.
shRNA. Based on published saRNA design rules, we selected a target on the iNOS promoter at a location between −612 and −594 bp relative to the TSS of the iNOS gene (fig. 1).

iNOS Up-Regulation by iNOS Promoter Targeted saRNA in Cultured CSMCs
We infected cultured rat CSMCs with 25, 50 and 75 MOI of AdU6/shiNOS with a mean transduction efficiency of 82.2% ± 2.7%, 82.5% ± 3.3% and 83.3% ± 3.5%, respectively. Cells infected with mock or 75 MOI AdU6/shControl served as controls. No viral toxicity in cells was observed at any MOI during transduction. Compared to AdU6/shControl infected cells, cells infected with AdU6/shiNOS showed significant higher iNOS mRNA levels (p < 0.05) and protein expression in a dose dependent manner (fig. 2). A 9.2-fold increase in iNOS mRNA expression was detected when AdU6/shiNOS was transduced at 75 MOI (fig. 2, A). Since AdU6/shiNOS at a MOI of 75 resulted in the strongest iNOS activation in CSMCs, all subsequent experiments were done at a MOI of 75.

iNOS saRNA Intracavernous Delivery
Improved erectile function. The final mean blood glucose concentration in STZ rats was significantly increased compared to that in nonSTZ controls and the final mean body weight of STZ rats decreased significantly (each p < 0.05). Increased iNOS slightly reduced blood pressure but the effect was not statistically significant (p > 0.05). The total ICP area on cavernous nerve stimulation in AdU6/shiNOS treated rats was significantly higher at all time points than in the STZ shControl and STZ sham groups (p < 0.05, fig. 3). The maximal response to intracavernous injection of AdU6/shiNOS was observed when ICP area was measured on day 5 after virus injection with ICP restored to levels close to those of the normal control nonSTZ group (fig. 3). It was particularly significant that the increased ICP area in the STZ shiNOS day 1, 3, 5, 7, 10 and 14 groups remained detectable 14 days after virus injection (fig. 3). Results suggest that intracavernous delivery of iNOS saRNA leads to sustained improvement of erectile function in diabetic rats.
Significantly higher induction of iNOS mRNA and protein levels was observed in diabetic rats that received intracavernous injection of AdU6/shiNOS virus, as analyzed at all time points (figs. 4, A and 5, B, \( p < 0.05 \)). iNOS mRNA and protein expression followed a trend similar to that of ICP area (fig. 3), suggesting that improved erectile function was due to iNOS induction mediated by its saRNA.

To further assess whether iNOS induction by its saRNA was target specific, we evaluated the expression of eNOS and nNOS. Diabetic rats had significantly lower eNOS and nNOS expression than nondiabetic rats (figs. 4, B and 5, B). Injection of AdU6/shiNOS virus did not alter eNOS or nNOS mRNA or protein expression compared to that in diabetic rats that received AdU6/shControl virus injection. This suggests that iNOS saRNA caused specific iNOS activation in penile tissue.

Diabetic rats transfected with AdU6/shControl and AdU6/shiNOS showed no significant change in p-eNOS (Ser-1177) expression compared with diabetic rats without treatment (fig. 5, A and C).

**Increased penile cGMP.** A significant increase in cGMP in the penile tissue of STZ rats treated with AdU6/shiNOS virus was observed compared to AdU6/shControl rats and diabetic rats without treatment (\( p < 0.05 \), fig. 6, A). Peak cGMP occurred at day 5 after treatment. This suggests that iNOS induction by saRNA leads to activation of the intracellular cyclic GMP pathway.

**DISCUSSION**

We constructed an iNOS specific saRNA expression adenovirus and used it to transduce rat CSMCs and the corpus cavernosum of diabetic rats. Results revealed that iNOS levels in CSMCs and the corpus cavernosum of STZ rats were up-regulated by saRNA, leading to restored erectile function in STZ diabetic rats via the NO-cGMP pathway.

RNA interference is a promising therapy for many diseases due to the high efficacy and high specificity of gene knockdown, and the low toxicity of small RNA molecules.\(^\text{21}\) However, many conditions are caused by aberrant inactivation of genes and there is a lack of safe methods to restore expression. Using the same therapeutic advantages of small RNA molecules, RNAa may provide expanded drug targets for small RNA based therapy for various conditions, including ED.

A previous study showed that lentiviral based delivery of saRNAs could activate vascular endothelial growth factor expression by targeting promoter sequences in an ischemic mouse model.\(^\text{22}\) To our knowledge we established a new saRNA delivery system using adenovirus as the saRNA delivery vector. Since adenovirus provides high transduction efficiency and does not integrate into the host genome, it is safer.\(^\text{23}\) Our results show that the AdU6/shiNOS adenovirus is highly infective to various cell lines and host types, and it can effectively activate iNOS expression in CSMCs in vitro and in penile tissue in vivo.

NO synthesis is catalyzed by NOS and, thus, it is an attractive target for ED gene therapy. Of the 3
NOS isoymes iNOS synthesizes much more NO in a sustained manner and functions independently of intracellular calcium flux. Therefore, even if iNOS is expressed in relatively few cells, a wide area of cells could be exposed to NO. A recent study showed that iNOS has an important role in protecting the penile corpus cavernosum from the profibrotic effects of hyperglycemia. In recent years researchers have used different methods to deliver iNOS into the corpus cavernosum and found that gene therapy targeting iNOS is feasible for treating ED. For example, iNOS cDNA could be used as a potential antifibrotic agent to reverse fibrotic changes that impair cavernous function. Another group used plasmid and adenovirus to deliver an exogenous iNOS gene into the corpus cavernosum. They achieved a significant increase in erectile response by enhancing NO production.

However, the unbalanced production of NO and its derivatives, collectively termed reactive nitrogen species, leads to pathological changes. Excessive NO production by iNOS may generate nitrosative stress and significant amounts of NO can lead to surrounding tissue damage due to peroxynitrite formation, inducing cell apoptosis.

In our study enhanced NO production did not decrease eNOS or nNOS expression, or damage endothelial cells or neurons. Thus, at certain levels NO may have profibrotic effects and may not injure tissue, while excess NO production would damage tissue due to reactive nitrogen species. We used RNAs to restore endogenous iNOS gene expression. Adenovirus mediated RNAs successfully enhanced iNOS gene expression in CSMCs and in the penis of diabetic rats, resulting in prolonged improvement in erectile function in the rats. RNAs may provide unmatched advantages over conventional ectopic

Figure 4. iNOS saRNA activated iNOS expression in vivo. NonSTZ and STZ rats received 1 intracavernous injection dose of indicated virus or were sham injected. Penile tissue was harvested for RNA isolation after measuring erectile function. cDNA derived from isolated RNA by reverse transcription reaction was amplified by real-time RT-PCR. Result are shown as mean ± SD of mRNA expression normalized to β-actin in all rats in each treatment group and plotted as fold change vs nonSTZ group. A, iNOS mRNA. B, NOS mRNA. Single asterisk indicates p < 0.05 vs nonSTZ group. Double asterisks indicate p < 0.05 vs STZ sham and STZ shControl groups. Pound sign indicates p < 0.05 vs STZ sham group.
Figure 5. iNOS saRNA activated iNOS protein and impacted nNOS, eNOS and p-eNOS proteins in diabetic rat penile tissue. NonSTZ and STZ rats received 1 intracavernous injection dose of indicated virus or were sham injected. Penile tissue was harvested for protein isolation after measuring erectile function. A, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. iNOS, eNOS, nNOS and p-eNOS proteins were detected by Western blot using iNOS, eNOS, nNOS and p-eNOS antibodies, respectively. β-actin protein was also detected as protein loading control using β-actin antibody. B and C, mean ± SD protein was determined by quantifying Western blot membrane band intensity (A) in all rats in each group. B, relative iNOS, eNOS and nNOS. C, relative P-eNOS and proteins. Single asterisk indicates p < 0.05 vs nonSTZ group. Double asterisks indicate p < 0.05 vs STZ sham and STZ shControl groups. Pound sign indicates p < 0.05 vs STZ sham group.
expression of exogenous genes due to its unique ability to restore endogenous gene expression.

CONCLUSIONS

Taken together, our results reveal adenovirus-mediated RNAa activated iNOS gene expression in vitro and in vivo, and improved erectile function in diabetic rats for a prolonged period via activation of the NO-intracellular cGMP pathway. These findings provide proof of concept that RNAa-mediated overexpression of iNOS can be used to restore erectile function in diabetic rats.

REFERENCES


