Losartan Preserves Erectile Function After Bilateral Cavernous Nerve Injury via Antifibrotic Mechanisms in Male Rats

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Purpose: Angiotensin II is a known mediator of smooth muscle vasoconstriction and fibrosis. It up-regulates thrombospondin-1, a major activator of latent transforming growth factor-β. Transforming growth factor-β induces vascular fibrosis via intracellular SMAD signaling pathways. We evaluated the effect of treatment with the angiotensin II type 1 receptor antagonist losartan on erectile function in the rat following bilateral cavernous nerve injury.

Materials and Methods: A total of 36 adult male rats were divided equally into 6 groups, including group 1—sham surgery with cavernous nerve exposure only plus vehicle, group 2—sham surgery plus oral low dose losartan (10 mg/kg per day), group 3—sham surgery plus high dose losartan (40 mg/kg per day), group 4—bilateral cavernous nerve injury (3-minute crush using a hemostat clamp) plus vehicle, group 5—bilateral cavernous nerve injury plus low dose losartan and group 6—bilateral cavernous nerve injury plus high dose losartan. Seven days following surgery erectile function was measured by electrically stimulating the cavernous nerves and monitoring intracavernous pressure. Penile tissue was collected for Western blot analysis of fibronectin, transforming growth factor-β, thrombospondin-1, α-actin, and phosphorylated and total SMAD2 and SMAD3 expression.

Results: Erectile function was significantly decreased after bilateral cavernous nerve injury compared with that after sham surgery (p < 0.01). Low and high dose losartan preserved erectile function after bilateral cavernous nerve injury compared to that in vehicle controls (p < 0.01 and < 0.05, respectively). Fibronectin, pSMAD2, pSMAD3, transforming growth factor-β-1, thrombospondin-1 and α-actin expression was up-regulated, and total SMAD2 and SMAD3 expression was down-regulated in the penis after bilateral cavernous nerve injury. Each dose of losartan after bilateral cavernous nerve injury significantly attenuated the up-regulated expression of fibronectin (p < 0.01), pSMAD2 (p < 0.05) and thrombospondin-1 (p < 0.05), and up-regulated total SMAD2 (p < 0.05).

Conclusions: These data suggest that fibrotic activators in the penis may cause decreased erectile function after bilateral cavernous nerve injury. Angiotensin II type 1 receptor antagonism may counteract this effect and promote erectile function preservation for conditions associated with penile fibrosis.

Key Words: penis, angiotensin II, erectile dysfunction, fibrosis, losartan

Erectile dysfunction is defined as the inability to attain or sustain erection satisfactory for sexual intercourse. Iatrogenic CN injury at some stage during radical pelvic surgery often leads to irreversible ED. CNs are involuntarily damaged even during nerve sparing RP, invariably causing...
ED to some degree. ED has emerged as an important quality of life issue after RP. The rate of complete ED after RP is 26% to 100%.1 EF may return, although this may require months or years and erectile capacity often remains poor.

Although the pathophysiology of ED associated with CN injury is not well-defined, the prevailing view is that the CN injury that occurs during surgery deprives the penis of its nerve supply at least temporarily, which leads to a loss of corporeal SM cells in the penis, and exaggerated deposition and disorganization of EMPs. This pathological effect has been documented in rat models of CN injury.5 It is conceivable that the progressive fibrotic process occurring in the corpus cavernosum after RP results from denervation and/or an ischemic process caused by the ligation of anomalous pudendal artery branches or venous plexus that drain to or from the corpora cavernosa.3,4

Ang-II, the principal vasoactive substance of the renin-angiotensin system, has various physiological actions, including vasoconstriction, aldosterone release and cell growth.5 Evidence has established that Ang-II is more than a hormone that exerts hemodynamic and renal actions. It is also a local, vasoprotective effects of nitric oxide.6 Ang-II not only induces endothelial dysfunction, but also activates expression of the proinflammatory phenotype of human vascular SM cells. In vascular SM cells Ang-II and TGFβ increase gene expression and the production of EMPs, such as fibronectin and collagen.8 Ang-II stimulates increased TSP-1 expression, resulting in enhanced TGFβ activation and increased EMP synthesis.9 The TGFβ isoforms TGFβ-1, TGFβ-2 and TGFβ-3 directly activate the SMAD signaling pathway, in addition to SMAD independent pathways.10 SMAD2 and SMAD3 proteins are the only known TGFβ-type I receptor substrates capable of signal transduction.10 Ang-II activates the SMAD signaling system in vascular cells in vivo and in vitro.11 SMAD proteins participate in Ang-II induced connective tissue growth factor over expression and EMP production.11

We investigated whether the Ang-II type 1 receptor antagonist losartan would promote EF recovery 7 days after BCNI in the rat. We also investigated whether the EF recovery effect of losartan treatment after BCNI involves changes in fibronectin, SMAD, TGFβ-1, TSP-1 and α-actin expression in rat penile tissue.

**MATERIALS AND METHODS**

**Animals and Treatment**

Adult male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Massachusetts) weighing 300 to 325 gm were used. All experiments were done in accordance with The Johns Hopkins University School of Medicine guidelines for animal care and use. The animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The prostate was exposed via a midline abdominal incision. The CN and major pelvic ganglion were identified postero-lateral to the prostate. Injury was induced by applying a No. 7 hemostat clamp (Fine Science Tools, Foster City, California) to the nerve 2 to 3 mm distal to the major pelvic ganglion. The hemostat clamp was held to closure for 3 minutes, causing moderate crush injury. Injuries were performed bilaterally. Sham surgery was completed by exposing the CN but not manipulating it. All experimental CN injuries were performed by the same trained investigator.

Losartan was prepared by dissolution in distilled water and administered by oral gavage at a low dose (10 mg/kg per day) and a high dose (40 mg/kg per day) starting the day of surgery. Rats were divided into 6 groups of 6 each, including group 1—sham surgery plus vehicle (distilled water), group 2—sham surgery plus low dose losartan, group 3—sham surgery plus high dose losartan, group 4—BCNI plus vehicle, group 5—BCNI plus low dose losartan and group 6—BCNI plus high dose losartan.

**In Vivo Erection Physiology Studies**

Seven days after surgery ICP and MAP were measured, as described previously.12 For electrically stimulated penile erections a bipolar electrode attached to an S48 stimulator (Grass Instruments, Quincy, Massachusetts) was placed around the previously injured or sham treated CN proximal to the injury site. Stimulation parameters were 4 V at a frequency of 16 Hz with a square wave duration of 5 milliseconds for 1 minute. EF was represented by the mean normalized mICP for maximal erection and by the mean normalized ICP AUC for total erection. Results were analyzed using MATLAB®. At the end of the experiment animals were sacrificed by a lethal intracardiac injection of saturated potassium chloride and the penes were removed.

**Western Blot Analysis**

To assess the expression levels of fibronectin, phosphorylated (activated) and total SMAD2 and SMAD3, TGFβ-1, TSP-1 and α-actin quantitatively Western blot analysis with densitometry was performed using penes from each treatment group. Whole penes were collected at day 7 after surgery and homogenized, as described previously.13 Samples (60 μg total protein) were loaded on 7.5%, 15% or 4% to 20% tris HCl gel (Bio-Rad®) and transferred to polyvinylidene fluoride membrane. Sample transferred membranes were incubated overnight at 4C with certain antibodies, including mouse monoclonal antibody for fibronectin (BD™ Biosciences) (1:500), TSP-1 (Thermo Fisher Scientific, Fremont, California (1:100), α-actin (1:1,000), β-actin (Sigma-Aldrich®) (1:10,000), and rabbit polyclonal antibody for pSMAD2 (Ser465/467) (1:
500), SMAD2 (Abcam®) (1:1,000), pSMAD3 (Ser423/425) (1:400), SMAD3 (Cell Signaling Technology®) (1:400) and TGFβ-1 (Santa Cruz Biotechnology, Santa Cruz, California) (1:500). Tissues used for positive controls were mouse uterus for TGFβ-1, rat spleen for TSP-1 and SMAD, and rat kidney for fibronectin. Densitometry results were normalized by total SMADs for pSMADs and by β-actin expression for the other proteins.

**Statistical Analysis**

Data are expressed as the mean ± SEM. Statistical analysis was performed using 1-way ANOVA, followed by Newman-Keuls multiple comparison test or Student’s t test when appropriate using GraphPad Prism® 4 with p ≤0.05 considered significant.

**RESULTS**

Maximal and total erectile responses, represented by mICP and ICP AUC, were significantly decreased 7 days after BCNI compared with sham group measurements (p <0.01). Low and high dose losartan treated rats had higher mICP values after BCNI than those in the BCNI plus vehicle treated group (p <0.01 and <0.05, respectively, fig. 1, A). Low and high dose losartan treatment also preserved ICP AUC compared with that in the BCNI plus vehicle treated group (p <0.01, fig. 1, B). Mean MAP values in vehicle, and low and high dose losartan treated rats were 96 ± 12, 92 ± 14 and 76 ± 27 mm Hg, respectively (p = 0.19). ICP responses in sham losartan treated rats were slightly higher than those in the nontreated group (p = 0.75).

The expression of fibronectin (p <0.01), pSMAD2 (p <0.05) and TSP-1 (p <0.05) was significantly up-regulated and total SMAD2 expression (p <0.05) was significantly down-regulated in the penis after BCNI (figs. 2 to 5). Although it was not significant, the expression of pSMAD3 (p = 0.08), TGFβ-1 (p = 0.09) and α-actin (p = 0.19) was up-regulated and total SMAD3 expression (p = 0.20) was down-regulated in the penis after BCNI (data not shown).

The high and low doses of losartan after BCNI significantly attenuated the increased expression of fibronectin, pSMAD2 and TSP-1 compared to that in the BCNI plus vehicle group (p <0.01, <0.05 and <0.05, respectively, figs. 2 to 4). Each losartan dose also significantly attenuated the decreased expression of total SMAD2 after BCNI (p <0.05, fig. 5). Although results were not statistically significant, each dose of losartan treatment after BCNI decreased the expression of pSMAD3 (p = 0.08), TGFβ-1 (p = 0.07) and α-actin (p = 0.25), and increased the expression of total SMAD3 (p = 0.25) compared with vehicle treatment measurements after BCNI. There was also a slight but not statistically significant decrease in pSMAD2 expression in the penis in sham losartan treated rats (p = 0.24, data not shown).

**DISCUSSION**

This study extends current knowledge in the study of ED associated with CN injury by further exploring the molecular mechanisms in the penis that are associated with CN injury and examining the consequence of a plausible therapy that targets these mechanisms. We observed that BCNI caused a decreased erectile response, while low and high doses of losartan preserved EF. We also noted that TSP-1, pSMAD2 and fibronectin expression was significantly up-regulated and total SMAD2 expression was down-regulated in the penis after BCNI. These changes were significantly attenuated by losartan treatment. These findings confirm that fibrotic changes develop in the penis after CN injury and they suggest an ameliorative effect by losartan in this situation.

![Figure 1](image_url)

**Figure 1.** EF 7 days after BCNI and losartan treatment. A, mICP/MAP was decreased after BCNI vs that in sham operated group. Low (LL) and high (HL) dose losartan treatment of 10 and 40 mg/kg per day, respectively, increased mICP/MAP after BCNI. B, ICP AUC/MAP was decreased after BCNI vs that in sham operated group. Low and high dose losartan treatment increased ICP AUC/MAP vs that in BCNI group treated with vehicle. Dagger indicates p <0.01 vs sham operation. Single asterisk indicates p <0.05 vs BCNI plus vehicle. Double asterisks indicate p <0.01 vs BCNI plus vehicle.
Our results carry important clinical implications. In particular they suggest that losartan treatment may be clinically useful to facilitate improved erectile recovery following RP.

The more impressive functional results for low dose than high dose losartan may be explained by the greater antihypertensive effect of the latter treatment, which conceivably decreases pelvic blood perfusion to a greater extent. We do not know whether our treatment doses represent the optimal dosing for this drug. Western blot analysis findings were consistent with the beneficial effects shown functionally and they provided

Figure 2. Western blot analysis of fibronectin expression in penis 7 days after BCNI and losartan treatment. A, representative bands in sham operated, BCNI plus vehicle, BCNI plus low dose losartan and BCNI plus high dose losartan groups, respectively. B, densitometry data. Fibronectin protein expression was increased after BCNI vs expression in sham operated group. Low (LL) and high (HL) dose losartan decreased fibronectin expression vs that in BCNI group treated with vehicle. Dagger indicates \( p < 0.01 \) vs sham operation. Asterisk indicates \( p < 0.01 \) vs BCNI plus vehicle.

Figure 3. Western blot analysis of pSMAD2 expression in penis 7 days after BCNI and losartan treatment. A, representative bands in sham operated, BCNI plus vehicle, BCNI plus low dose losartan and BCNI plus high dose losartan groups, respectively. B, densitometry data. pSMAD2 protein expression was increased after BCNI vs that in sham operated group. Low (LL) and high (HL) dose losartan decreased pSMAD2 expression vs that in BCNI group treated with vehicle. Dagger indicates \( p < 0.05 \) vs sham operation. Asterisk indicates \( p < 0.05 \) vs BCNI plus vehicle.
additional evidence to support the losartan effect in penile tissue after BCNI.

Functional changes after BCNI were accompanied by molecular evidence of fibrotic changes in the penis. To evaluate fibrosis we measured changes in fibronectin expression. Increases in the amount of hydroxyproline, collagen and fibronectin, which are specific biochemical markers for fibrosis, have been well described in the extracellular matrix after tissue trauma. We chose a 7-day interval for study, rather than an extended time frame. We surmised that our results would have been confounded by a spontaneous

Figure 4. Western blot analysis of TSP-1 expression in penis 7 days after BCNI and losartan treatment. A, representative bands in sham operated, BCNI plus vehicle, BCNI plus low dose losartan and BCNI plus high dose losartan groups, respectively. B, densitometry data. TSP-1 protein expression was increased after BCNI compared to expression in sham operated group. Low (LL) and high (HL) dose losartan treatment decreased TSP-1 expression vs that in BCNI group treated with vehicle. Dagger indicates p <0.05 vs sham operation. Asterisk indicates p <0.05 vs BCNI plus vehicle.

Figure 5. Western blot analysis of total SMAD2 expression in penis 7 days after BCNI and losartan treatment. A, representative bands in sham operated, BCNI plus vehicle, BCNI plus low dose losartan and BCNI plus high dose losartan groups, respectively. B, densitometry data. Total SMAD2 protein expression was decreased after BCNI vs expression in sham operated group. Low (LL) and high (HL) dose losartan treatment increased total SMAD2 expression vs that in BCNI group treated with vehicle. Dagger indicates p <0.05 vs sham operation. Asterisk indicates p <0.05 vs BCNI plus vehicle.
level of erection recovery, which has been observed to occur after 7 days in our model of only moderate crush CN injury. More severe CN injury such as bilateral CN resection certainly leads to a profound increase in EMP synthesis in rat cavernous SM, resulting in fibrosis. Histological evaluation of human cavernous tissue after RP has also shown a time related, quantitative and qualitative decrease in elastic and SM fibers, and a progressive increase in EMPs.

It has been demonstrated that the corpus cavernosum produces and secretes physiologically significant amounts of Ang-II. To block the profibrotic/anti-erectile effects of Ang-II we used 2 doses of losartan, which have been reported in the literature.

In the anesthetized dog intracavernous injection of Ang-II causes cavernous SM contraction and terminates spontaneous erection, while losartan administration results in SM relaxation and, thus, erection. Previous studies of vascular SM cells in vitro and mouse aorta in vivo have shown that losartan treatment reversed and normalized interstitial fibrosis, and decreased the expression of EMPs and TGFβ-1.

Recent discoveries of the tissue actions of Ang-II have revolutionized our understanding of the role of this peptide in cardiovascular disease. Belmadani et al reported that TSP-1 is a significant mediator of the fibrotic complications of diabetes mellitus associated with stimulation of the renin-angiotensin system. Previous studies have demonstrated that CN injury leads to increased expression of TGFβ-1, which promotes collagen synthesis. TGFβ induces vascular fibrosis via intracellular SMAD signaling pathways. In our study after BCNI penile expression of TGFβ-1 was slightly increased, although to a nonsignificant degree compared with vehicle treatment. This discrepancy can be explained by acknowledging that SMAD proteins are also involved in the over expression of Ang-II induced EMPs independently of TGFβ and they may have been more demonstrably affected by losartan treatment. In support of this view we noted that fibronectin expression significantly increased after BCNI, which was prevented by losartan treatment.

We observed that SMAD2 phosphorylation was significantly up-regulated after BCNI, although this effect was also seen to a nonsignificant degree with SMAD3 phosphorylation. The SMAD signaling cascade is initiated by C-terminal phosphorylation of SMAD2 and/or SMAD3 by activated TGFβ type I receptor. However, for SMAD2 and SMAD3 to be phosphorylated by TGFβ type I receptor they must be recruited to the activated receptor complex. Adaptor proteins such as SARA (SMAD anchor for receptor activation) bind and present SMAD2 and SMAD3 to TGFβ type I receptors. Interestingly it has been observed that SMAD2 and to a lesser extent SMAD3 bind to SARA in human mesangial cells and this difference in binding may be responsible for the divergence between SMAD2 and SMAD3 activation, and/or the TGFβ response in different cell types. Further investigation in penile tissue may yield additional information with regard to the activation of different types of SMADs.

In our study we evaluated fibrotic protein expression and erection physiology studies a relatively short time after BCNI. The long-term (eg 30 to 45 days) effect of losartan treatment may be investigated in additional studies, which may also require more significant CN injury. Such long-term evaluation may also be useful to study whether losartan decreases observable morphological changes, such as tissue fibrosis in the penis of BCNI rats.

CONCLUSIONS

Using a rat model of BCNI we noted that CN injury causes a decreased erectile response in association with up-regulation of fibrotic activators in the penis. Ang-II induced activation of SMAD signaling by Ang-II type 1 receptors may be a mechanism contributing to penile fibrosis after CN injury. Furthermore, we found that losartan treatment effectively preserved EF in this setting, evidently through anti-fibrotic mechanisms. These data may have ramifications for preserving EF in men undergoing RP. However, recommendations for using losartan at the clinical level await further support from clinical investigations.

REFERENCES


